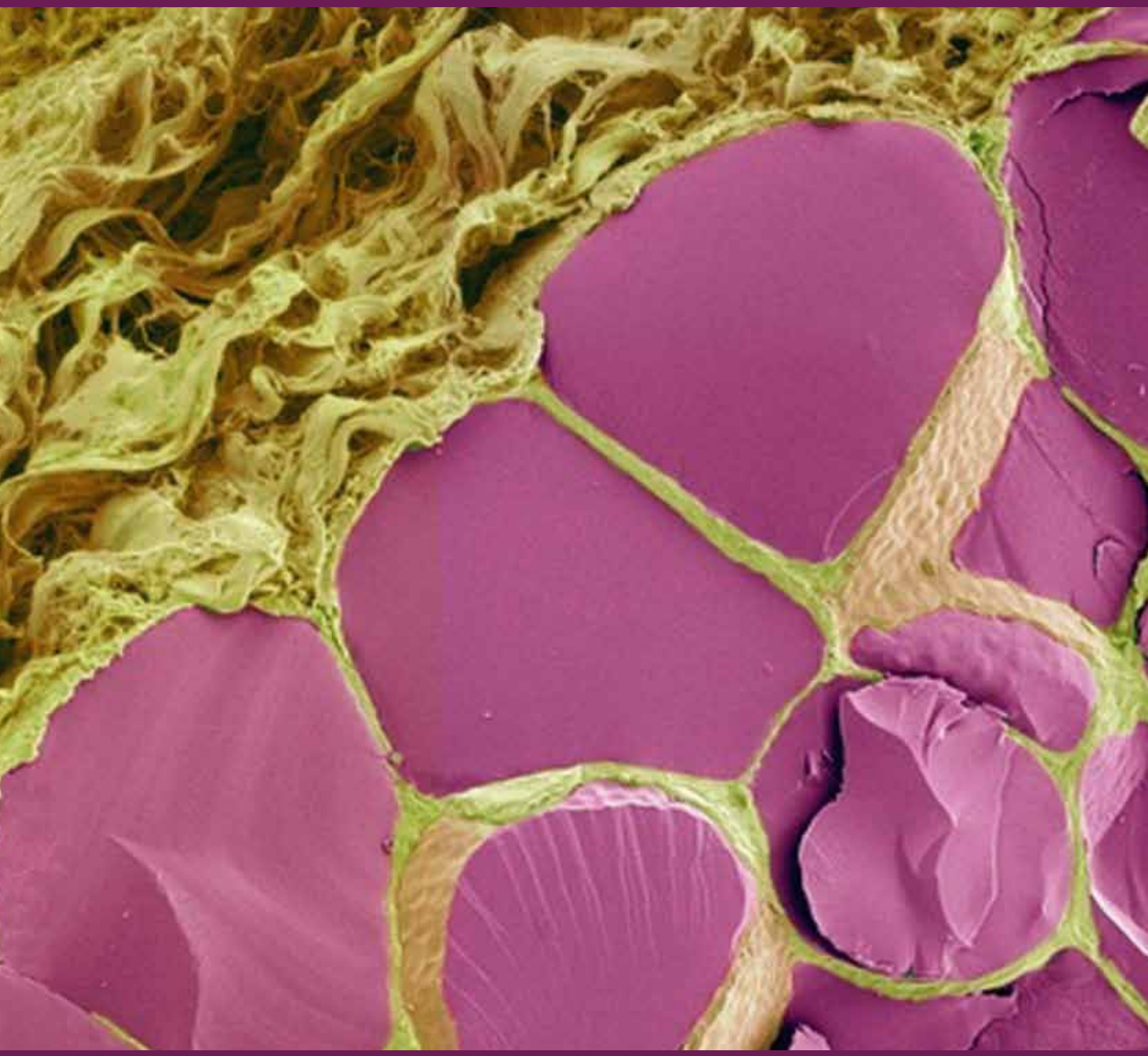
International Journal of Endocrinology

Osteoporosis

Guest Editors: Ling-Qing Yuan, Cory Xian, Mei Li, Peng-Fei Shan, and Guang-Da Xiang



Osteoporosis

International Journal of Endocrinology

Osteoporosis

Guest Editors: Ling-Qing Yuan, Cory Xian, Mei Li, Peng-Fei Shan, and Guang-Da Xiang

Editorial Board

Contents

Osteoporosis, Peng-Fei Shan, Cory J. Xian, Mei Li, Guang-Da Xiang, and Ling-Qing Yuan
Volume 2013, Article ID 952858, 2 pages

Related Biological Research in the Interface between Bone Cement and Bone after Percutaneous Vertebroplasty, ZhenMing Hu, Gang Zhao, LiJun Wang, Bo Pu, Jie Hao, HanChang Lao, XiaoJun Zhang, Qiang Gan, and Wei Jiang
Volume 2013, Article ID 109784, 7 pages

Metabolic and Clinical Consequences of Hyperthyroidism on Bone Density, Jagoda Gorka, Regina M. Taylor-Gjevre, and Terra Arnason
Volume 2013, Article ID 638727, 11 pages

A Study on Bone Mass in Elderly Chinese Foot-Binding Women, Yi Pan, Ling Qin, Mian Xu, Yin He, Juan Bao, Xian Guo, and Jun Shu
Volume 2013, Article ID 351670, 6 pages

Puerarin Suppress Apoptosis of Human Osteoblasts via ERK Signaling Pathway, Ling-juan Liu, Li-qun Liu, Tao Bo, Shi-jun Li, Zhen Zhu, Rong-rong Cui, and Ding-an Mao
Volume 2013, Article ID 786574, 6 pages

Relation of Visceral and Subcutaneous Adipose Tissue to Bone Mineral Density in Chinese Women, Ling Wang, Wei Wang, Li Xu, Xiaoguang Cheng, Yimin Ma, Dan Liu, Zhe Guo, Yongbin Su, and Qianqian Wang
Volume 2013, Article ID 378632, 5 pages

Silicon: A Review of Its Potential Role in the Prevention and Treatment of Postmenopausal Osteoporosis, Charles T. Price, Kenneth J. Koval, and Joshua R. Langford
Volume 2013, Article ID 316783, 6 pages

Glucocorticoid-Induced Bone Loss Is Associated with Abnormal Intravertebral Areal Bone Mineral Density Distribution, Louise I. Manning, Andrew M. Briggs, Sharon Van Doornum, Ashwini Kale, Susan Kantor, and John D. Wark
Volume 2013, Article ID 768579, 9 pages

Dietary Calcium Intake and Calcium Supplementation in Hungarian Patients with Osteoporosis, Gábor Speer, Pál Szamosujvári Jr., Péter Dombai, Katalin Csóré, Kinga Mikófalvi, Tímea Steindl, Ildikó Streicher, Júlia Tarsoly, Gergely Zajzon, Péter Somogyi, Pál Szamosújvári, and Péter Lakatos
Volume 2013, Article ID 754328, 8 pages

Serum β-Catenin Levels Associated with the Ratio of RANKL/OPG in Patients with Postmenopausal Osteoporosis, Xiao-Juan Xu, Lin Shen, Yan-Ping Yang, Rui Zhu, Bo Shuai, Cheng-Gang Li, and Man-Xiang Wu
Volume 2013, Article ID 534352, 7 pages

Osteoporosis Associated with Antipsychotic Treatment in Schizophrenia, Haishan Wu, Lu Deng, Lipin Zhao, Jingping Zhao, Lehua Li, and Jindong Chen
Volume 2013, Article ID 167138, 7 pages

Effects of Growth Hormone Replacement Therapy on Bone Mineral Density in Growth Hormone Deficient Adults: A Meta-Analysis, Peng Xue, Yan Wang, Jie Yang, and Yukun Li
Volume 2013, Article ID 216107, 13 pages

Bone Mineral Density and Osteoporosis after Preterm Birth: The Role of Early Life Factors and Nutrition, Claire L. Wood, Alexander M. Wood, Caroline Harker, and Nicholas D. Embleton
Volume 2013, Article ID 902513, 7 pages

Self-Repair of Rat Cortical Bone Microdamage after Fatigue Loading In Vivo, Bo Wu, Chan Zhang, Bo Chen, Ling Zhang, Ruchun Dai, Xianping Wu, Yebin Jiang, and Eryuan Liao
Volume 2013, Article ID 321074, 8 pages

Prevalence of Fracture Risk Factors in Postmenopausal Women Enrolled in the POSSIBLE US Treatment Cohort, Nicole Yurgin, Sally Wade, Sacha Satram-Hoang, David Macarios, and Marc Hochberg
Volume 2013, Article ID 715025, 9 pages

Omentin-1 Stimulates Human Osteoblast Proliferation through PI3K/Akt Signal Pathway, Shan-Shan Wu, Qiu-Hua Liang, Yuan Liu, Rong-Rong Cui, Ling-Qing Yuan, and Er-Yuan Liao
Volume 2013, Article ID 368970, 6 pages

Comparison of QCT and DXA: Osteoporosis Detection Rates in Postmenopausal Women, Na Li, Xin-min Li, Li Xu, Wei-jie Sun, Xiao-guang Cheng, and Wei Tian
Volume 2013, Article ID 895474, 5 pages

Establishment of OPG Transgenic Mice and the Effect of OPG on Bone Microarchitecture, Ying Wu, Jianghua Liu, Hui Guo, Qiong Luo, Ziying Yu, Eryuan Liao, and Xuyu Zu
Volume 2013, Article ID 125932, 7 pages

Selective Determinants of Low Bone Mineral Mass in Adult Women with Anorexia Nervosa, Andrea Trombetti, Laura Richert, François R. Herrmann, Thierry Chevalley, Jean-Daniel Graf, and René Rizzoli
Volume 2013, Article ID 897193, 9 pages

Dysfunction of Collagen Synthesis and Secretion in Chondrocytes Induced by *Wisp3* Mutation, Min Wang, Xiao-Fei Man, Ya-Qing Liu, Er-Yuan Liao, Zhi-Feng Shen, Xiang-Hang Luo, Li-Juan Guo, Xian-Ping Wu, and Hou-De Zhou
Volume 2013, Article ID 679763, 9 pages

Age-Related Changes in Trabecular and Cortical Bone Microstructure, Huayue Chen, Xiangrong Zhou, Hiroshi Fujita, Minoru Onozuka, and Kin-Ya Kubo
Volume 2013, Article ID 213234, 9 pages

The Role of Vitamin D Deficiency in the Incidence, Progression, and Complications of Type 1 Diabetes Mellitus, Marlene Chakhtoura and Sami T. Azar
Volume 2013, Article ID 148673, 10 pages

Effects of Low-Dose Testosterone Undecanoate Treatment on Bone Mineral Density and Bone Turnover Markers in Elderly Male Osteoporosis with Low Serum Testosterone, Yan-Jiao Wang, Jun-Kun Zhan, Wu Huang, Yi Wang, Yuan Liu, Sha Wang, Pan Tan, Zhi-Yong Tang, and You-Shuo Liu
Volume 2013, Article ID 570413, 6 pages

The Relationship between Serum Osteocalcin Concentration and Glucose Metabolism in Patients with Type 2 Diabetes Mellitus, Qingqing Wang, Beibei Zhang, Yulan Xu, Hongdi Xu, and Nan Zhang
Volume 2013, Article ID 842598, 7 pages

Establishing Reference Intervals for Bone Turnover Markers in the Healthy Shanghai Population and the Relationship with Bone Mineral Density in Postmenopausal Women, Wei-Wei Hu, Zeng Zhang, Jin-Wei He, Wen-Zhen Fu, Chun Wang, Hao Zhang, Hua Yue, Jie-Mei Gu, and Zhen-Lin Zhang
Volume 2013, Article ID 513925, 7 pages

Relationship between Serum Levels of OPG and TGF-β with Decreasing Rate of BMD in Native Chinese Women, Xi-Yu Wu, Yi-Qun Peng, Hong Zhang, Hui Xie, Zhi-Feng Sheng, Xiang-Hang Luo, Ru-Chun Dai, Hou-De Zhou, Xian-Ping Wu, and Er-Yuan Liao
Volume 2013, Article ID 727164, 8 pages

Novel Adipokines and Bone Metabolism, Yuan Liu, Cheng-Yuan Song, Shan-Shan Wu, Qiu-Hua Liang, Ling-Qing Yuan, and Er-Yuan Liao
Volume 2013, Article ID 895045, 9 pages

Comparison of Classification Algorithms with Wrapper-Based Feature Selection for Predicting Osteoporosis Outcome Based on Genetic Factors in a Taiwanese Women Population, Hsueh-Wei Chang, Yu-Hsien Chiu, Hao-Yun Kao, Cheng-Hong Yang, and Wen-Hsien Ho
Volume 2013, Article ID 850735, 8 pages

Sex Steroids and Bone Health Status in Men, Kok-Yong Chin and Soelaiman Ima-Nirwana
Volume 2012, Article ID 208719, 7 pages

Palm Tocotrienol Supplementation Enhanced Bone Formation in Oestrogen-Deficient Rats, Ima Nirwana Soelaiman, Wang Ming, Roshayati Abu Bakar, Nursyahrina Atiqah Hashnan, Hanif Mohd Ali, Norazlina Mohamed, Norliza Muhammad, and Ahmad Nazrun Shuid
Volume 2012, Article ID 532862, 7 pages

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 952858, 2 pages
http://dx.doi.org/10.1155/2013/952858

Editorial
Osteoporosis

Peng-Fei Shan,[1] Cory J. Xian,[2] Mei Li,[3] Guang-Da Xiang,[4] and Ling-Qing Yuan[5]

[1] *Department of Endocrinology and Metabolism, Second Affiliated Hospital, Zhejiang University College of Medicine, 88 Jiefang Road, Hangzhou, Zhejiang, China*
[2] *Sansom Institute for Health Research and School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5001, Australia*
[3] *Department of Endocrinology, Endocrine Key Laboratory of the Ministry of Health of China, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, 1 Shuaifu Road, Beijing, China*
[4] *Department of Endocrinology, Wuhan General Hospital of Guangzhou Command, Wuluo Road 627, Wuhan, Hubei, China*
[5] *Institute of Metabolism and Endocrinology, the Second Xiang-Ya Hospital, Central South University, 139 Middle Renmin Road, Changsha, Hunan, China*

Correspondence should be addressed to Ling-Qing Yuan; allenylq@hotmail.com

Received 1 August 2013; Accepted 1 August 2013

Osteoporosis, a major worldwide public health issue, is characterized by a progressive loss of bone mineral density, disruption of bone microarchitecture, and an increased risk of fractures. Osteoporosis-related fracture causes disability, mortality, and significant financial burden. With the aging of the world population and thus the increasing prevalence, osteoporosis is attracting more and more attention from patients, researchers, clinicians, and government agencies. In recent years, there have been significant advances in our knowledge in bone cell biology, bone metabolism, genetics and pathogenesis of osteoporosis, evaluation of fracture risk of osteoporotic patients, and management of osteoporosis. Our current special issue presents a series of original research and review papers on recent advances in the pathophysiology and clinic aspects of osteoporosis.

Several studies investigated the relationship between bone turnover biomarkers and bone mineral density (BMD). After examining 2799 individuals aged 20–79 years in Shanghai city of China, W.-W. Hu et al. established normal reference ranges for bone turnover markers (P1NP, OC, and β-CTX). They found that these bone turnover markers correlated with BMD. Wu et al. investigated the relationship between the BMD decrease rate and serum levels of OPG, TGF-β1, and TGF-β2 in 465 healthy Chinese women aged 35–80 years, which showed that TGF-β1 is a positive determinant of bone decrease rate. A. Trombetti and colleagues determined the relationship between amenorrhea, IGF-1, and BMD in patients who suffered from anorexia nervosa. Their results suggested that spine BMD is related to hypogonadism, whereas IGF-I predicts proximal femur BMD. Xu et al. found that serum β-catenin level is decreased in postmenopausal osteoporotic women compared with postmenopausal non-osteoporotic women. Meanwhile, β-catenin is negatively correlated with the expression ratio of RANKL/OPG in the bone.

BMD is the gold standard in osteoporosis diagnosis, and dual X-ray absorptiometry (DXA) is the most widely used tool for BMD measurement. Other methods include quantitative ultrasound, quantitative computed tomography (QCT), peripheral DXA, and other radiographic techniques. N. Li and colleagues compared the osteoporosis detection rates using DXA and QCT. They suggested that QCT may be more sensitive for detecting osteoporosis and may prevent overestimation of BMD by DXA caused by spinal degeneration, aortic calcification, or other sclerotic lesions.

FRAX is a very popular computer-based algorithm that estimates fracture probability. However, there is no information about the prevalence of fracture risk factors in postmenopausal women who are receiving antiosteoporosis treatment. N. Yurgin et al. demonstrated that multiple risk

factors (age >70 years; history of fracture since age 50; minimum reported hip or spine T-score ≤ -2.5 at diagnosis; body mass index <18.5 kg/m^2; rheumatoid arthritis; parental history of hip fracture; current cigarette smoking; and oral glucocorticoid use in the 6 months prior to study entry) are associated with a greater incidence of on-study fractures even through the women who have received antiosteoporosis therapies. Their results confirmed that the women with multiple risk factors still remain at an elevated fracture risk even after initiating therapy.

BMD is affected by several factors. In this special issue, some researchers reviewed the relationship between the effect of preterm birth and BMD as well as the impact of age-related changes in trabecular and cortical bone microstructure. To investigate whether foot binding, a historical social custom in China, had effect on BMD, Y. Pan and colleagues demonstrated that foot binding in Southwest China had no influence on lumbar vertebra and femoral neck BMD compared with the unbound control group.

Some drugs used to treat other diseases may have effect on bone metabolism. Y.-J. Wang et al. found that a low dose of testosterone undecanoate (20 mg/d) which is used in elderly male with low serum testosterone could effectively increase lumbar spine and femoral neck BMD. Meanwhile, growth-hormone replacement therapy used in growth hormone deficient adults might have beneficial influence on BMD. H. Wu et al. reviewed the relationship among antipsychotic medication, hyperprolactinaemia, and osteoporosis.

Previously, obesity was thought to have a protective effect on BMD. However, recent studies have suggested an obesity paradox of lower BMD in obese subjects. L. Wang et al. examined the effects of visceral adipose tissue (VAT) and subcutaneous adipose (SAT) tissue on vertebral trabecular bone density using quantitative computed tomography in 320 Chinese women. Their finding suggested that the VAT volume is negatively correlated with BMD in Chinese women aged <55 years old, whilst the SAT volume is not correlated with BMD. Meanwhile, the studies of S.-S. Wu and colleagues demonstrated that omentin, a novel adipokine secreted by visceral adipose tissue, can induce osteoblast proliferation via the PI3K/Akt signaling pathway. Y. Liu et al. reviewed the recent progress in adipokine and bone metabolism. While these studies have provided some clues to explain the relationship between adipose tissue and bone metabolism, further studies are needed to gain a greater appreciation of the mechanisms underlying the effects of obesity on BMD and fracture risk.

Several other basic studies were reported. L. Liu and colleagues reported that puerarin, an isoflavone contained in traditional Chinese herb, could inhibit osteoblast apoptosis through activating estrogen receptor/ERK signal pathway and decreasing Bax/Bcl-2 expression ratio. I. N. Soelaiman et al. revealed that palm tocotrienol, a potent antioxidant, could improve bone histomorphology parameters in overiectomy rats but had no effect on bone biomarkers. Wu et al. successfully established OPG transgenic mouse through microinjection of an OPG vector into fertilized zygotes. They also found that the cancellous and cortical bone volumes and 3D microarchitecture in OPG transgenic mice are significantly improved.

There are still other several interesting articles in this special issue regarding calcium, silico, sex hormone, vitamin D, and bone metabolism as well as bone health.

The story of osteoporosis is still going on, and our understanding of this disease is advancing. We hope our special issue could bring some latest progress in this filed to the readers.

Acknowledgments

We would like to express our special thanks and gratitude to all the authors for their excellent work submitted to this selected issue. We also thank all the reviewers for their hard work in reviewing and providing suggestions for improving these papers. Finally, we really appreciate the Editor Broad of International Journal of Endocrinology for giving us this opportunity to publish this special issue on this important disease.

Peng-Fei Shan
Cory J. Xian
Mei Li
Guang-Da Xiang
Ling-Qing Yuan

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 109784, 7 pages
http://dx.doi.org/10.1155/2013/109784

Research Article

Related Biological Research in the Interface between Bone Cement and Bone after Percutaneous Vertebroplasty

ZhenMing Hu,[1] Gang Zhao,[2] LiJun Wang,[3] Bo Pu,[2] Jie Hao,[1] HanChang Lao,[2] XiaoJun Zhang,[1] Qiang Gan,[1] and Wei Jiang[1]

[1] *Department of Spine Surgery, The First Affiliated Hospital of Chongqing Medical University, 1 Youyi Road, Chongqing 400016, China*
[2] *Department of Orthopaedic Surgery, The Second Affiliated Hospital of Kunming Medical College, 1 Ma Yuan, Kunming, Yunnan 650101, China*
[3] *Department of Orthopaedic Surgery, Dazu District Hospital of Chongqing, 138 West Longgang Road, Dazu District, Chongqing 402360, China*

Correspondence should be addressed to ZhenMing Hu; zhenminghu62@aliyun.com

Received 2 January 2013; Revised 1 June 2013; Accepted 24 June 2013

Academic Editor: Peng-Fei Shan

Percutaneous vertebroplasty (PVP) is widely used in the treatment of painful osteoporotic vertebral compression fractures with the injection of PMMA cement, and the controversy for PMMA damage to the osteoporotic bone tissue and to affect the fractures repairing never stops. 72 old female rabbits, each age 3.0~3.5 y, rabbits were assigned randomly to two groups of thirty-six each; PMMA cement were injected into vertebral body in rabbits via mimic PVP, sacrificed at 1 h, 24 h, 3 d, 7 d, 4 w, and 12 w. The expression VEGF and collagen type I, the tissue response, and repair reaction in the interface between PMMA and bone tissue were observed dynamically with RT-PCR and western blot technique; the osteocalcin expression were studied by immunohistochemistry. Compared with the control group, the expression of collagen I increased at 1 hour and was higher from 24 h to 3 d. From 4 weeks to 12 weeks after injection of PMMA. The expression of VEGF decreased at 1 hour and 24 hours, significantly increased at 3 days, decreased once again at 7 days, then increased significantly at 4–12 weeks. The osteocalcin expression continued to increase during 4 to 12 week. PMMA would not cause local bone permanent necrosis, and interface injury repairing cycle could be prolonged in a vertebroplasty.

1. Introduction

Percutaneous vertebroplasty (PVP), a persistent developing procedure, is nowadays widely used in the treatment of painful osteoporotic vertebral compression fractures with the injection of polymethylmethacrylate (PMMA) cement, which is accepted most in clinical surgery contributing for its effect of immediate pain relief, biomechanical function reconstruction, and low price, whereas PMMA was also denounced of goodish bone compatibility and combination [1–4]. Nevertheless, research is still being conducted to develop better injectable bone augmentation materials and biodegradable or bioactive bone "pastes" [5, 6]. Most researchers believe that pain relief is achieved through mechanical support and stability provided by the bone cement. The semisolid mixture of PMMA, an acrylic cement from monomer polymerization reaction, and exothermal reaction contributes to the cytoxicity and mutation toxicity in periapical tissue [7, 8]. Meanwhile, since vertebroplasty was first used in the treatment of painful osteoporotic vertebral compression fractures, the controversy for PMMA damage to the osteoporotic bone tissue and to effect the fractures repairing never stops [9, 10]; the postoperative biological influence and variation of the involved vertebrae has been less reported so far. The investigation was incorporated in an animal study that was performed to observe the biological changes on PMMA and bone interface after rabbit vertebroplasty in the sight of morphology and molecular biology. Evaluated contents consist of expression of vascular endothelial growth factor (VEGF) and collagen type I of bone

interface tissue by RT-PCR and Western Blot, the osteocalcin expression was studied by immunohistochemistry.

2. Material and Methods

2.1. Animal Randomization. Seventy-two old New Zealand female rabbits (provided by KunMing Medical College, China), each aged from 3.0 to 3.5 years, weighed between 3 and 4.2 kg, were used in this study. All the rabbits were demonstrated as osteoporosis by DXA (Lunar, USA). Preoperative frontal and lateral position X-ray film demonstrates that all these rabbits had normal lumbar vertebrae sequences and bone architectures. Rabbits were assigned randomly to two groups of thirty-six each. All groups were treated with the same surgical procedures and evaluations but were sacrificed at different postoperative times at 1 h, 24 h, 3 d, 7 d, 4 w, and 12 w. All animals received general anesthesia using 4% of pentobarbital sodium (30 mg/kg) followed by helix veinintravenous injection. Considering elimination of biological variance caused by different level of vertebrae, injection (powder and liquid mixed at 20 g : 5 mL) of modified PMMA (Tianjin Synthetic Material Research Institute, China) applied to randomly selected lumbar vertebrae in the experimental group. The control group received operation alone, without PMMA injection.

2.2. Animal Model Preparation and Material Implantation. A skin incision was made in the median of the back at the level of vertebral L_7 (top of hip); the subcutaneous tissue and masseter muscle were divided to expose the anterior half of L_4, L_5, and L_6. Referred to clinical PVP surgical procedures [3, 4], an epidural needle is advanced into the vertebral body via a transpedicular or parapedicular approach with injection depth 4 ~ 6 mm. The pasty PMMA is slowly injected into the corresponding vertebral body at dosage of 0.5 ~ 0.6 mL through an epidural needle connecting to a 1 mL volume syringe. The injection and extraction were performed insynchronism. All above procedures applied to 2 vertebrae (L_2, L_4) of each rabbit. Postoperatively, intramuscular injection of 1 million units penicillin per day was done until the 7th day, and all the rabbits were fed and monitored by the experimental animal center of our university. Rabbits were without death both before and after operation.

2.3. X-Ray Inspection. Each group of animals, respectively, took lateral X-ray film to inspect the presence, and morphologic change of PMMA before the animals was sacrificed at their predetermined time.

2.4. Sample Preparation. Each group of animals was sacrificed at their predetermined time; the vertebraes (L_2, L_4) were dissected and removed out the PMMA and cut into blocks containing the interface between bone cement and bone tissue, part of them for total tissues RNA extraction and the others for histology.

2.5. RT-PCR. Total RNA extraction of tissues [11, 12] TRIzol method (Invitrogen Company) was used to extract RNA out for agarose gel electrophoresis, detecting the completeness of RNA. RNA was subpackaged in store at −70°C (RT-PCR equipment, Bio-Rad).

Primer sequences are used in the experiment as follows:

VEGF-L 5′-GAC ATC TTC CAG GAG TAC CC-3′ 157 bp

VEGF-R 5′-TGA GGT TTG ATC CGC ATG AT-3′

actin-L TGG CTC TAA CAG TCC GCC TAG 295 bp.

2.6. Western Blot. Total protein extraction was manipulated on ice and preserved at −70°C following the instruction of BCA-100 protein quantitation kit (Shanghai Biocolor BioScience & Technology Company).

The first antibody for 18 h was incubated under 4°C after diluted to 1 : 100 (VEGF) and 1 : 1000 (collagen), subsequently went through TBST rinshing for 4 × 10 min. The second antibody was put under ambient temperature for 1 h and TBST rinshing for 4 × 15 min. The PVDF film which was prior incubated in ECL reagent for 5 min was placed into the chromogenic film cassette. Finally in dark room carried out the exposure and developing with X-ray film according to an optimal fixing time.

2.7. Immunohistochemistry. The immunohistochemical procedure has been described previously reports [13], the avitin-biotin-complex method was used with ABC Peroxidase Staining Kit. The sections were incubated with rabbit anti-rabbit osteocalcin polyclonal antibodies; the negative controls were the four different grafted maxillary sinuses, where normal mouse IgG was used instead of primary antibodies for staining.

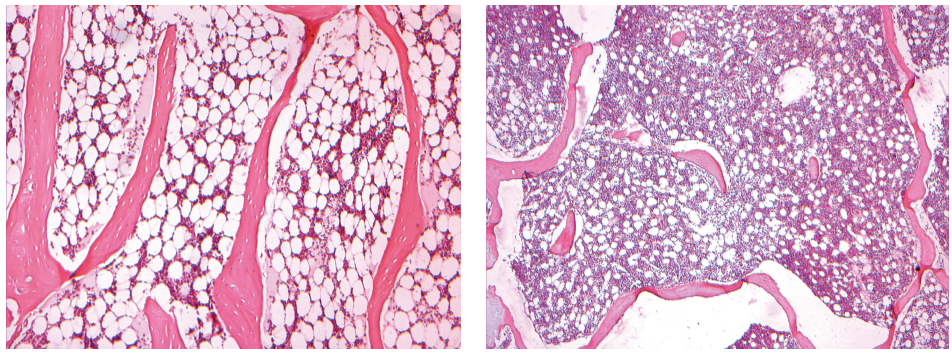
2.8. The Result Analysis. RT-PCR result computation: firstly, sample Ct-intraparameter Ct = ΔCt, to compute ΔΔCt we set a sample ΔCt (the control group as usual) as reference. Computing method was ΔCt (sample) − ΔCt (reference). Outcome illustrated the mathematics relation between samples was 2 − (−ΔΔCt)th power.

Protein relative amount computation: place the X-ray film into gel Image Processing System (Tanon) to detect intensity and area of the target band. Relative amount = intensity × area.

2.9. Statistical Treatment. The data were represented in the form of mean ± standard deviation (X ± S) and analyzed utilizing the one-way ANOVA. The means of sample from both groups were compared using t-test. All statistical analyses were done by SPSS 13.0.

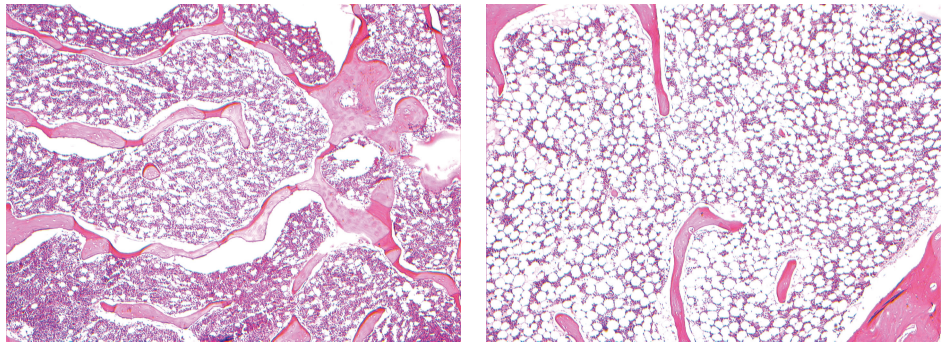
3. Results

3.1. General Observation after Spliting the Samples. Bone trabecula was full of PMMA cement that is tightly connecting to bone tissues and well distributed. PMMA was covered with considerable soft tissues and a few new born bone tissues in 12 w. In the control, soft tissues coverage and bone



(a) (b)

Figure 1: (a) Normal bone trabecula of vertebral in control, HE ×50. (b) Bone trabecula apparently reduced with inflammatory cells infiltration after PMMA injected 24 h, HE ×50.



(a) (b)

Figure 2: (a) Inflammatory cells infiltration after PMMA injected 3 d, HE ×50. (b) Inflammatory reaction lightened after PMMA injected 7 d, HE ×50.

defect about 0.5 cm were visible. The bone defect whose volume kept invariableness was filled with hematoma and some granulation tissues but without new osteogenesis.

3.2. X-Ray Investigation. All injected cement did not appear of any defluxion, crack, or loosening. The boundary between PMMA and bone became indistinct with new osteogenesis in subgroup 12 w.

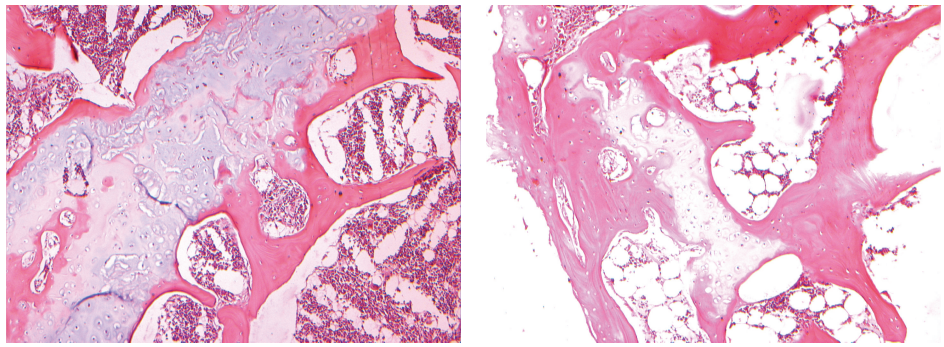
3.3. Histomorphological Observation. Compared with that of control, the samples were tightly combined with PMMA cement. The interface between bone tissues and PMMA was infiltrated with inflammatory cells and fibrous tissues. Inflammatory cell infiltration was obvious in 24 h, developed to a peak in 3 d, alleviated comparatively in 7 d. Chondrocytes were found growing in cluster and differentiating to woven bone at the interface without conspicuous inflammatory cells in 4 w; massive lamellar bone formed and occasional haematopoietic bone marrow could be observed without any inflammation in 12 w (Figures 1, 2, and 3).

3.4. VEGF RT-PCR Amplification and Protein Expression. VEGF was amplified to the target band of 157 bp molecular weight, taking actin as intraparameter. It confirmed the target band on coincidence to the gene fragment in the literature according to intraparameter.

3.5. VEGF RT-PCR Amplification Result. The VEGF mRNA expression was lower ($P < 0.05$) than that of control at 1 h and 24 h, whereas the expression was highly increased at 3 d. It was decreased and lower than the control again ($P < 0.05$) in 7 d. Then, it experienced a persistent augment from 4 w to 12 w; the difference was significant compared to the 7 d and control ($P < 0.001$) (Figure 4(a)).

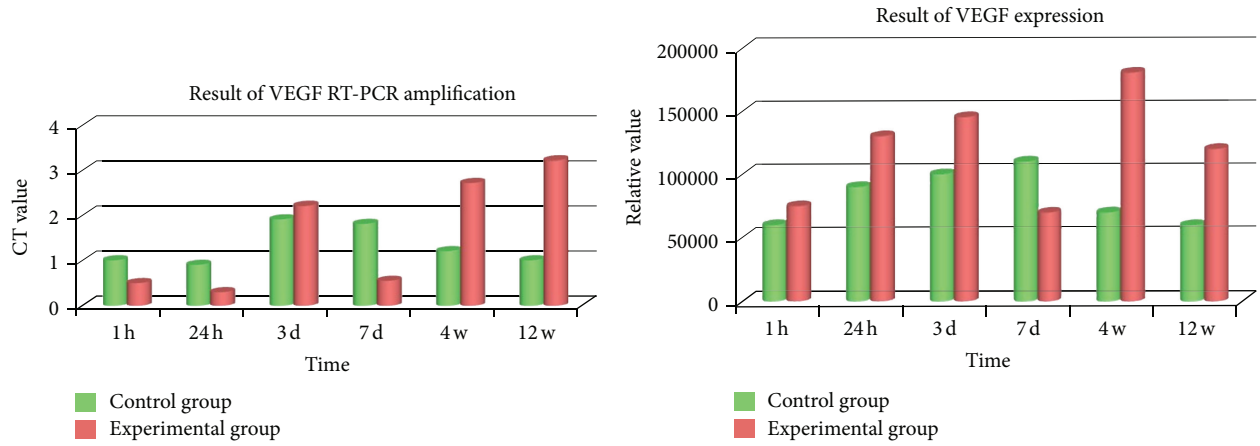
3.6. Detection Result of VEGF Protein. VEGF protein expression was increased a little at 1 h and 24 h. It was increased a lot in 3 d while decreased in 7 d. After that the expression was transparently elevated and higher than that of control ($P < 0.05$) (Figure 4(b)).

3.7. Collagen Type I RT-PCR Amplification and Protein Expression. Collagen type I mRNA expression was increased from 1 h. A persistent high level formed from postoperative 24 h to 3 d. Despite of being slightly descended in 7 d, it kept a comparative high expression from 4 w to 12 w which was remarkably higher than that of control ($P < 0.01$) (Figure 5(a)). High protein expression of Collagen type I was



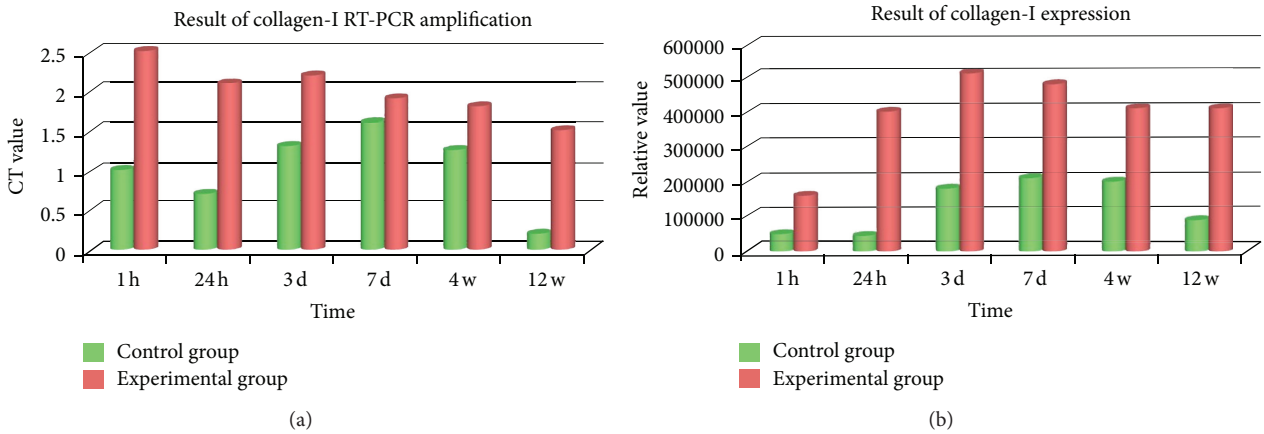
(a) (b)

Figure 3: (a) Large quantity of chondrocytes growing in cluster after PMMA injected 4 w, HE ×100. (b) Chondrocytes in cluster without conspicuous inflammation after PMMA injected 4 w, HE ×100.



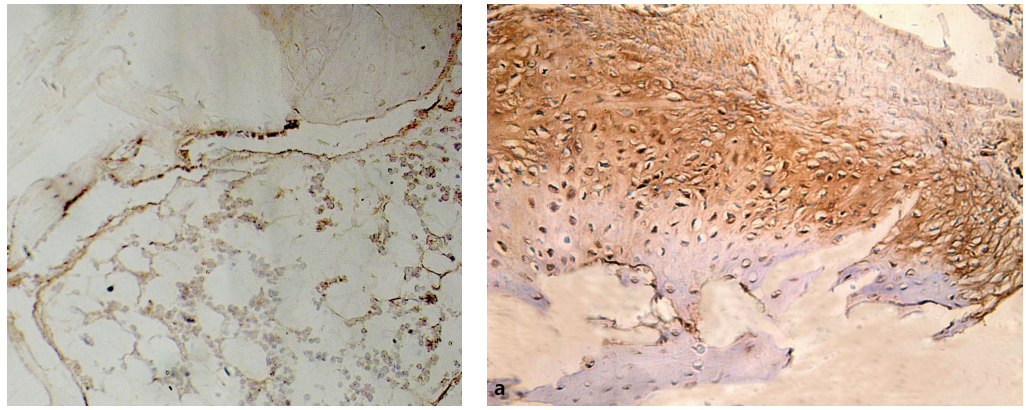
(a) (b)

Figure 4: (a) VEGF mRNA expression was lower in 1 h and 24 h ($P < 0.05$), whereas the expression was highly increased in 3 d but decreased in 7 d. Then, it experienced a persistent augment from 4 w to 12 w ($P < 0.001$). (b) VEGF protein expression was increased in 1 h, 24 h, and 3 d; also it was decreased in 7 d. After that the expression was transparently elevated and higher than that of control ($P < 0.05$).



(a) (b)

Figure 5: (a) Collagen type I mRNA expression was increased in 1 h. A persistent high level was formed in postoperative 24 h to 3 d. Despite being slightly descended in 7 d, it kept a comparative high expression from 4 w to 12 w which was remarkably higher than that of control ($P < 0.01$). (b) A high protein expression of Collagen type I was detected in 3 d, 7 d, and 4 w. The expression of 12 w descended considerably compared to 4 w with significant difference ($P < 0.01$).



(a) (b)

Figure 6: (a) OC expression slightly increased in PMMA-bone interface in 1 week, 20 × 10SP + HE. (b) OC expression in PMMA-bone interface in 4 weeks, when chondrocytes were found to be growing in cluster and differentiating to woven bone, 20 × 10SP + HE.

detected in 3 d, 7 d, and 4 w. The expression was descended at 12 w considerably compared to 4 w ($P < 0.01$) (Figure 5(b)).

3.8. Immunohistochemistry. Compared with normal bone tissue, after 1 h, 24 h, and 3 d, the OC expression had no significant increase in both groups ($P < 0.05$). At 7 days, the OC expression of control group increased slightly, the PMMA group had no significant change ($P > 0.05$). At 4 weeks, the OC expression increased in both groups, but the control group was more significant than the PMMA group ($P > 0.05$). The OC expression continued to increase during 4 to 12 weeks in experimental group. It is slightly decreased in the control group ($P > 0.05$) (Figures 6(a) and 6(b)).

4. Discussion

Several inherent advantages to PMMA include familiarity for orthopedic surgeons, ease of handling, good biomechanical strength and stiffness, and cost effectiveness [7–9, 14]. Several disadvantages, include no biologic potential to remodel or integrate into the surrounding bone, no direct bone apposition, excessive inherent stiffness, high polymerization temperature, and potential monomer toxicity [2, 3, 7, 10]. Although good clinical results have been reported in several series of both vertebroplasty and kyphoplasty procedures [15–21], it is still unclear whether some component of the pain relief is secondary to the mechanical stabilization, chemical toxicity, or thermal necrosis of surrounding tissues and nerve ends; in addition, the other surgery-related factors would affect the inflammatory progress. The concern regarding thermal bone necrosis is still theoretical, as to date, there has been no obvious evidence to support this [17, 22, 23]. In a baboon vertebral augmentation study, there were a few necrotic segment of bone present in both the vertebroplasty and kyphoplasty vertebrae. It was not, however, clear that the necrosis was caused by a PMMA polymerization process [22].

Lieberman et al. identified particles consistent with cement and/or barium sulfate in vascular spaces in human vertebrae obtained from surgical excision and autopsy cases [24, 25]. Our investigation of the histology and interfacial between cement and bone, PMMA induced somewhat inflammatory reaction, which subsided after 1 week post-operatively, totally disappeared at 4 weeks post-PVP. In which showed some component of the chemical toxicity, or thermal necrosis of interface tissues, the concern regarding thermal bone necrosis is still temporarily damage. Early research demonstrated that toxicity effect of PMMA induced osteoblast necrosis through peroxidation caused by hydroxy radical release. Free radical of PMMA monomer induces macrophages to release arachidonic acid in the interface of bone and cement [5–7]. Further, local tissues produce more lactate dehydrogenase resulting in granuloma like inflammation that aggravates tissue injury. Lately through improving compounding, PMMA has been proved high safety as the toxicity effect decreases [2]. In our investigation, we found that even though PMMA cement injected to stabilize or fix pathological fractures after vertebral augmentation, the painful osteoporotic compression fractures vertebral continued a healing and repairing processes [26].

The result of our research on VEGF illustrated in the 1 h and 24 h was lower than the corresponding of control ($P < 0.05$), whereas the expression of 3 d was highly increased; it was lower decreased at 7 d ($P < 0.05$). Then, it experienced a persistent augment from 4 w to 12 w. Descent of 24 h might ascribe to the exothermic reaction of PMMA solidification and oxidizing reaction of free radical monomer. The above two factors inhibit cytoactivity of osteoclast, contributing to lower VEGF expression [15, 27]. The first peak at 3 d results from an intensive inflammatory reaction which does favor to VEGF expression through inflammatory cells like macrophage and fibroblast; VEGF expression weakened along with extinction of inflammatory at 7 d. Accompanied with oxygen free radical reduction, repair of exothermic reaction injury, recovery of cellular microenvironment, and normalization of osteoblastic cytoactive, another high level of VEGF expression appeared. After osseous tissue repair is completed in 12 w, the VEGF expression was released mainly by osteoblasts [27, 28].

Meanwhile, in our studies, collagen type I at 1 h was multiple amplified, and a persistent high level formed from postoperative 24 h to 3 d. PMMA has the effect of promoting collagen information. Viateau et al. [29] discovered in an ovine study of padding bone defect that the membrane of PMMA concentrated BMP, bone stem cells, and other promotive osteoplastic components with vasiformation and collagen type I expression. Twa et al. [18] reported PMMA material implanted in vivo could activate keratocyte and collagen formation. Fibroblast is able to express collagen type I mRNA [30, 31]. The descendent at 7 d was probably caused by regression of inflammation. The high expression from 7 d to 12 w illustrated chondrocytes had been transforming to osteoblasts. HE staining revealed lamellar bone had been constructed 4 w postoperatively. Collagen type I is mainly produced by osteocytes, in our research it was expressed at a high level 12 w postoperatively which was distinct from conventional time (considerably decreased 8 w postoperatively). As same as VEGF and collagen type I, the OC showed high expression at the time point and delayed compared with control group. HE staining showed complete ossification accomplished at that time. However, the result of protein expression was similar to the literature.

In the process of fracture healing, collagen type I is the characteristic marker representing bone formation and molding. Its peak expression occurred between 3 w and 5 w after fracture which is the state from intracellular ossification of chondrocyte to bone information, being related to vasiformation. From the result of our study, PMMA cement does harm to bone trabecula at a certain degree in PVP. The major influence on osteoporotic fracture repair is time delay. Repair mechanism coincides with normal fracture healing, whereas the time is delayed for 4 weeks around. It is thus clear that, the PMMA injected in PVP influenced the function of local bone tissue and cell, but it did not induce irreversible damage.

In conclusion, interface injury caused by PVP injection of domestic advanced PMMA bone cement could be repaired and mineralized undergoing a process similar to normal fracture healing. However, the expression of VEGF, collagen type I, and OC delayed about 4 weeks. Bone cement would not cause local bone permanently necrosis; interface injury repairing cycle could be prolonged in a vertebroplasty.

Abbreviations

PVP: Percutaneous vertebroplasty
PMMA: Polymethylmethacrylate
VEGF: Vascular endothelial growth factor.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

Authors' Contribution

Dr. Gang Zhao and LiJun Wang also can be considered as the first authors.

Acknowledgments

This work was supported in part by the Scientific Research Foundation of Chongqing Government.

References

[1] R. Buchbinder, D. Kallmes, and P. Glasziou, "Vertebroplasty versus conservative treatment for vertebral fractures," *The Lancet*, vol. 376, no. 9758, pp. 2070–2071, 2010.

[2] A. W. Burton, L. D. Rhines, and E. Mendel, "Vertebroplasty and kyphoplasty: a comprehensive review," *Neurosurgical Focus*, vol. 18, no. 3, pp. 1–9, 2005.

[3] R. Rousing, M. O. Andersen, S. M. Jespersen, K. Thomsen, and J. Lauritsen, "Percutaneous vertebroplasty compared to conservative treatment in patients with painful acute or subacute osteoporotic vertebral fractures: three-months follow-up in a clinical randomized study," *Spine*, vol. 34, no. 13, pp. 1349–1354, 2009.

[4] J. Hao, Z.-M. Hu, and C.-Y. Meng, "Percutaneous Vertebroplasty for severely osteoporotic vertebral compression fractures," *Chinese Journal of Orthopedics*, vol. 17, no. 8, pp. 884–886, 2009.

[5] J. G. Kennedy, P. O'Grady, D. R. McCarthy et al., "An investigation into the role of oxygen free radical scavengers in preventing polymethylmethacrylate-induced necrosis in an osteoblast cell culture," *Orthopedics*, vol. 23, no. 5, pp. 481–485, 2000.

[6] S. M. Horowitz, T. L. Gautsch, C. G. Frondoza, and L. Riley Jr., "Macrophage exposure to polymethyl methacrylate leads to mediator release and injury," *Journal of Orthopaedic Research*, vol. 9, no. 3, pp. 406–413, 1991.

[7] D. San Millán Ruíz, K. Burkhardt, B. Jean et al., "Pathology findings with acrylic implants," *Bone*, vol. 25, no. 1, pp. 85S–90S, 1999.

[8] J. M. Lane, C. E. Johnson, S. N. Khan, F. P. Girardi, and F. P. Cammisa Jr., "Minimally invasive options for the treatment of osteoporotic vertebral compression fractures," *Orthopedic Clinics of North America*, vol. 33, no. 2, pp. 431–438, 2002.

[9] S. Mukherjee and Y.-P. Lee, "Current concepts in the management of vertebral compression fractures," *Operative Techniques in Orthopaedics*, vol. 21, no. 3, pp. 251–260, 2011.

[10] L.-J. Wang, Z.-M. Hu, and J. Hao, "The Biomaterials for percutaneous vertebroplasty," *Chinese Journal of Bone Tumor and Bone Disease*, vol. 9, no. 1, pp. 71–75, 2010.

[11] Z. Hu, S. A. F. Peel, S. K. C. Ho, G. K. B. Sándor, and C. M. L. Clokie, "Platelet-rich plasma induces mRNA expression of VEGF and PDGF in rat bone marrow stromal cell differentiation," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*, vol. 107, no. 1, pp. 43–48, 2009.

[12] Z.-M. Hu, S. A. F. Peel, S. K. C. Ho, G. K. B. Sándor, and C. M. L. Clokie, "Comparison of platelet-rich plasma, bovine BMP, and rhBMP-4 on bone matrix protein expression in vitro," *Growth Factors*, vol. 27, no. 5, pp. 280–288, 2009.

[13] Z. Hu, S. A. F. Peel, S. K. C. Ho, G. K. B. Śndor, Y. Su, and C. M. L. Clokie, "The expression of bone matrix proteins induced by different bioimplants in a rabbit sinus lift model," *Journal of Biomedical Materials Research A*, vol. 95, no. 4, pp. 1048–1054, 2010.

[14] L. Wood, M. Egger, L. L. Gluud et al., "Empirical evidence of bias in treatment effect estimates in controlled trials with different interventions and outcomes: meta-epidemiological

study," *British Medical Journal*, vol. 336, no. 7644, pp. 601–605, 2008.

[15] Z.-M. Hu, G. Zhao, B. Pu et al., "The related research for interface between PMMA and bone tissue after rabbits," *Bone*, vol. 43, no. 1, supplement, pp. S112–S112, 2008.

[16] B. S. Vladimirov and S. A. Dimitrov, "Growth factors—importance and possibilities for enhancement of the healing process in bone fractures," *Folia Medica*, vol. 46, no. 2, pp. 11–17, 2004.

[17] D. P. Beall, A. Datir, S. L. D'Souza et al., "Percutaneous treatment of insufficiency fractures: principles, technique and review of literature," *Skeletal Radiology*, vol. 39, no. 2, pp. 117–130, 2010.

[18] M. D. Twa, J. Ruckhofer, R. L. Kash, M. Costello, and D. J. Schanzlin, "Assessing intraocular lens calcification in an animal model," *Journal of Cataract & Refractive Surgery*, vol. 27, no. 8, pp. 1473–1484, 2001.

[19] P. Fransen and F. P. Collignon, "Direct anterolateral balloon kyphoplasty for a painful C-2 osteolytic malignant lesion," *Journal of Neurosurgery*, vol. 23, no. 6, article 374, 2007.

[20] H. G. Deen and T. P. Fox, "Balloon kyphoplasty for vertebral compression fractures secondary to polyostotic fibrous dysplasia. Case report," *Journal of Neurosurgery*, vol. 3, no. 3, pp. 234–237, 2005.

[21] S. A. Levine, L. A. Perin, D. Hayes, and W. S. Hayes, "An evidence-based evaluation of percutaneous vertebroplasty," *Managed Care*, vol. 9, no. 3, pp. 56–63, 2000.

[22] C. A. H. Klazen, P. N. M. Lohle, and J. DeVries, "Vertebroplasty versus conservative treatment in acute osteoporotic vertebral compression fractures (Vertos II): an open-label randomised trial," *The Lancet*, vol. 376, no. 9746, pp. 1085–1092, 2010.

[23] H. Deramond, N. T. Wright, and S. M. Belkoff, "Temperature elevation caused by bone cement polymerization during vertebroplasty," *Bone*, vol. 25, no. 1, supplement 2, pp. 17S–21S, 1999.

[24] I. Lieberman and M. K. Reinhardt, "Vertebroplasty and kyphoplasty for osteolytic vertebral collapse," *Clinical Orthopaedics and Related Research*, vol. 9, no. 415, supplement, pp. S176–S186, 2003.

[25] K. Goto, M. Hashimoto, H. Takadama et al., "Bioactive bone cements containing micron-sized titania particles," *Key Engineering Materials*, vol. 309–311, pp. 793–796, 2006.

[26] J. Urrutia, C. M. Bono, P. Mery, and C. Rojas, "Early histologic changes following polymethylmethacrylate injection (vertebroplasty) in rabbit lumbar vertebrae," *Spine*, vol. 33, no. 8, pp. 877–882, 2008.

[27] M. Sandberg, H. Aro, P. Multimaki, H. Aho, and E. Vuorio, "In situ localization of collagen production by chondrocytes and osteoblasts in fracture callus," *Journal of Bone and Joint Surgery A*, vol. 71, no. 1, pp. 69–77, 1989.

[28] T. Bodamyali, C. R. Stevens, M. E. J. Billingham, S. Ohta, and D. R. Blake, "Influence of hypoxia in inflammatory synovitis," *Annals of the Rheumatic Diseases*, vol. 57, no. 12, pp. 703–710, 1998.

[29] V. Viateau, G. Guillemin, Y. Calando et al., "Induction of a barrier membrane to facilitate reconstruction of massive segmental diaphyseal bone defects: an ovine model," *Veterinary Surgery*, vol. 35, no. 5, pp. 445–452, 2006.

[30] S. Kujala, T. Raatikainen, J. Ryhänen, O. Kaarela, and P. Jalovaara, "Composite implant of native bovine bone morphogenetic protein (BMP), collagen carrier and biocoral in the treatment of resistant ulnar nonunions: report of five preliminary cases," *Archives of Orthopaedic and Trauma Surgery*, vol. 124, no. 1, pp. 26–30, 2004.

[31] N. Kakudo, K. Kusumoto, Y. B. Wang, Y. Iguchi, and Y. Ogawa, "Immunolocalization of vascular endothelial growth factor on intramuscular ectopic osteoinduction by bone morphogenetic protein-2," *Life Sciences*, vol. 79, no. 19, pp. 1847–1855, 2006.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 638727, 11 pages
http://dx.doi.org/10.1155/2013/638727

Review Article

Metabolic and Clinical Consequences of Hyperthyroidism on Bone Density

Jagoda Gorka,[1] Regina M. Taylor-Gjevre,[2] and Terra Arnason[3]

[1] *Department of Medicine, University of Saskatchewan, Saskatoon, Canada S7N 0W8*
[2] *Division of Rheumatology, Department of Medicine, University of Saskatchewan, Saskatoon, Canada S7N 0W8*
[3] *Division of Endocrinology and Metabolism, Department of Medicine, University of Saskatchewan, Saskatoon, Canada S7N 0W8*

Correspondence should be addressed to Terra Arnason; terra.arnason@usask.ca

Received 3 January 2013; Accepted 25 June 2013

Academic Editor: Cory Xian

In 1891, Von Recklinghausen first established the association between the development of osteoporosis in the presence of overt hyperthyroidism. Subsequent reports have demonstrated that BMD loss is common in frank hyperthyroidism, and, to a lesser extent, in subclinical presentations. With the introduction of antithyroid medication in the 1940s to control biochemical hyperthyroidism, the accompanying bone disease became less clinically apparent as hyperthyroidism was more successfully treated medically. Consequently, the impact of the above normal thyroid hormones in the pathogenesis of osteoporosis may be presently underrecognized due to the widespread effective treatments. This review aims to present the current knowledge of the consequences of hyperthyroidism on bone metabolism. The vast number of recent papers touching on this topic highlights the recognized impact of this common medical condition on bone health. Our focus in this review was to search for answers to the following questions. What is the mechanisms of action of thyroid hormones on bone metabolism? What are the clinical consequences of hyperthyroidism on BMD and fracture risk? What differences are there between men and women with thyroid disease and how does menopause change the clinical outcomes? Lastly, we report how different treatments for hyperthyroidism benefit thyroid hormone-induced osteoporosis.

1. Introduction

The World Health Organization (WHO) defines osteoporosis as bone mineral density (BMD) 2.5 or more standard deviations below that of a young adult (T score) at any site. Characteristics of osteoporosis include increased risk of fragility fractures, a deterioration of bone architecture, and in addition to low bone mass. The distinction of secondary osteoporosis is applied to low BMD as a result of factors beyond postmenopausal and senile osteoporosis. The myriad of secondary causes includes glucocorticoid therapy, connective tissue disorders, malabsorption syndromes, diabetes mellitus, and hyperthyroidism [1]. This review will focus on the molecular and clinical consequences of hyperthyroidism on bone metabolism through a series of focused questions.

Key Points regarding the Impact of Hyperthyroidism on Bone Health

(1) In hyperthyroidism, bone turnover is accelerated twofold, with a net loss of bone.

(2) Receptors for thyroid hormones and TSH are present in bone. These hormones may act directly on both osteoclasts and osteoblasts.

(3) The severity of hyperthyroidism correlates with the decrease in BMD and the increase in fracture risk.

(4) Hyperthyroidism affects cortical bone to a greater extent than trabecular bone and is best measured by BMD at the distal forearm.

(5) Women over 65 years old with a TSH <0.1 have the greatest fracture risk.

(6) Normalizing thyroid function alone is able to effect some reversal of bone loss.

(7) Antithyroid therapy combined with Vitamin D and bisphosphonates is most effective at normalizing bone density. There is no role for calcitonin.

(8) Subclinical hyperthyroidism is surprisingly prevalent, estimated to be present in up to 24% of those over age 60 who receive thyroxine replacement.

(9) Subclinical hyperthyroidism contributes to an estimated additional 1% bone loss per year in these individuals.

2. Normal and Pathological Mechanism of Thyroid Hormone Action on Bone Metabolism

Question 1. How do the elevated thyroid hormone levels in thyrotoxicosis result in accelerated bone loss?

2.1. Normal Molecular Action of Thyroid Hormones on Bone Metabolism. As a result of pituitary TSH stimulation, the thyroid gland secretes two thyroid hormones, T4 (3,5,3′,5′-L-tetraiodothyronine) along with a small amount of T3 (3,5,3′-L-triiodothyronine), which is significantly more biologically active than T4. Additionally, T3 is peripherally created by the enzymatic conversion of T4 into T3 by the deiodinase enzymes D1 and D2. A third deiodinase enzyme, D3, converts T3 and T4 into inactive hormone metabolites reverse T4 (rT2) and rT3 [2]. Intracellular levels of T3 are determined by the relative action of the three deiodinases [3].

T3 action is mediated by its nuclear T3 hormone receptors (TRs). Two receptor isoforms, TRα and TRβ, exist [4, 5] and their subtypes TRα1, TRα2, and TRβ1 mediate T3 action though their wide distribution in tissues. There is extensive expression of thyroid hormone receptors within bone, indicating that thyroid hormones can influence this tissue type. Expression of TRα and TRβ has been identified in chondrocytes, osteoblasts, and osteoblastoma and osteoclastoma cell lines [4, 6]. TRα1 expression in bone is about 10-fold higher than TRβ1 [7], and studies of genetically modified mice have implicated TRα1 as the primary mediator of T3 action on bone [7–10]. The engineered absence of all TRα1 receptors in a mutant mouse model (TRα (0/0), display developmentally delayed ossification, growth retardation, and impaired bone mineralization despite being euthyroid, highlighting the requirement for thyroid hormone in normal bone metabolism. In contrast, TRβ mutant mice TRβ (−/−) exhibit normal skeletal development in young mice. There was a persistent elevation of T3 and T4 levels in these TRβ (−/−) mice related to disruption of normal negative hormonal feedback at the hypothalamus and pituitary through TRβ. The excessive stimulation of TRα1 in these adult mice from the elevated T3 and T4 levels resulted in accelerated bone remodeling leading to adult osteoporosis [7–11]. Clinically, this is similar to the observations in humans that prolonged hyperthyroidism results in accelerated bone loss.

Classically, the deleterious effects on BMD in hyperthyroidism have been attributed to high levels of the circulating thyroid hormones T4 and T3. However, more recent publications have also implicated low TSH levels alone in the development of reduced skeletal integrity. This is clinically relevant, as subclinical hyperthyroidism is defined by normal T3 and T4 levels with an isolated suppression of TSH and has been correlated with changes to human bone density over time [12–14]. The TSH receptor (TSHR) is predominantly expressed in cells of thyroid follicles; however, TSHR expression has also been demonstrated in a variety of tissue including osteoblasts and osteoclasts [15, 16], as well as kidney, brain, heart, testis, lymphocytes, and adipose tissue [17]. The presence of TSHR in osteoclasts and osteoblasts has led investigators to determine what direct effects TSH may exert on bone metabolism. Abe et al. [15] carried out experiments with mice carrying deletions for both (homozygous) or one (heterozygous) alleles of the TSHR. Homozygous TSHR deletion mice had low levels of T3 and T4 with high TSH, low BMD, and required exogenous thyroid administration for normal growth. Interestingly, the loss of BMD was not reversed with thyroid hormone supplementation, indicating that TSH acting through its hormone receptor is necessary for alterations in bone metabolism. Heterozygous mice had normal levels of T3, T4, and TSH; however, they also exhibited a significant reduction of BMD. Both groups of mice had osteoporosis due to high bone turnover, leading them to postulate that TSH action via its receptor acts as a negative regulator of bone turnover [15, 18]. Other studies have suggested that the action of TSH may also indirectly affect bone integrity through increasing deiodinase D2 activity in osteoblasts which results in local T3 elevations (by conversion of T4 to T3) as a consequence [19]. It remains unclear whether it is the action of excessive thyroid hormones, the lack of TSH, or a combination of both that is responsible for bone loss in the hyperthyroid state.

2.2. The Significance of Thyroid Hormone Excess on Rates of Bone Remodeling. The continuous process of bone remodeling through balanced resorption and deposition is essential to maintain the integrity and strength of the human skeleton [20]. During remodeling, osteoclasts degrade and resorb the old bony matrix, while new bone is deposited by osteoblasts. The normal bone remodeling cycle lasts 150–200 days (approximately <7 months), and any disturbances in this equilibrium lead to excessive/disordered bone formation or uncompensated bone loss. In the hyperthyroid state, the cycle occurs in roughly half of the normal time (3-4 months), and this accelerated rate of bone turnover creates an increased number of osteoclast resorption sites and increases the ratio of resorption to bone formation ultimately causing osteoporosis from cumulative new bone loss [21–23]. Using iliac crest biopsy specimens from hyperthyroid patients (n = 15) and age and sex-matched controls to reconstruct bone remodeling curves, it was determined that there was a 9.6% loss of mineralized bone with each cycle in the hyperthyroid group [24]. Conversely, hypothyroidism prolongs the remodeling process, resulting in reduced bone turnover and a 17%

increase in mineralized bone as a consequence of a 700-day cycle [25].

2.3. The Significance of Thyroid Hormone Excess on Mineral Metabolism. The accelerated bone remodeling cycle in hyperthyroid states results in increased bone resorption and a subsequent increased release of calcium into systemic circulation [26, 27]. High levels of calcium, specifically in the ionized form, are present in up to 8% of patients with hyperthyroidism [26, 28]. High levels of serum calcium inhibit parathyroid hormone (PTH) secretion and create a negative calcium balance through prolonged urinary and fecal losses. Decreased PTH secretion causes hypercalciuria as a protective mechanism against hypercalcemia [28]. With low PTH, Vitamin D is not converted into its active form, leading to low gastrointestinal calcium and phosphorous absorption and resultant fecal calcium losses [29–31].

In addition to the release of calcium from bone into serum, levels of bone turnover markers are also elevated. Serum concentrations of alkaline phosphate, osteocalcin, osteoprotegerin, and FGF-23 correlate directly with the severity of biochemical hyperthyroidism [32–37]. Interestingly, bone turnover markers remain high for months after treatment due to the persistent increase in osteoblast activity, despite normalization of thyroid hormone levels in the serum [32–37].

In contrast, the urinary bone turnover marker, collagen-derived pyridinium cross-links, rapidly normalizes with correction of thyroid hormone serum levels. In the presence of elevated free T3 (fT3), collagen-derived pyridinium cross-links are elevated in urine; however, they rapidly return to normal shortly after initiation of therapy [38, 39].

3. Clinical Consequences of Hyperthyroidism on Bone Mineral Density and Fracture Risk

Question 2. Does the severity and duration of thyrotoxicosis correlate with the rate, extent and location of bone loss?

3.1. The Impact of Thyrotoxicosis on Bone Density (BMD). Osteoporosis is a uniform feature of untreated and sustained thyrotoxicosis. Numerous reports have described a consistent decrease in BMD and increase in fracture risk in untreated overt hyperthyroidism [40]. This effect on BMD is evident in both pre- and postmenopausal women [41]. Hyperthyroidism differentially influences trabecular and cortical bone metabolism, with the latter more predominantly affected [42]. An early study demonstrated up to 40% increase in osteoclast resorption in cortical bone, compared to 2.7% reduction in trabecular bone volume [43]. Recently, a cross-sectional, population-based study of euthyroid and hyperthyroid women over 40 years of age evaluated the correlation between serum TSH levels and BMD of the distal and ultradistal forearm [44]. This is the site most profoundly affected by parathyroid hormone dysregulation [45]. The study, which reported a statistically higher risk of osteoporosis at the radius in hyperthyroidism, found a stronger association between low TSH and low BMD in distal (cortical) bone in comparison to the ultradistal (trabecular) bone of the radius, complementing previous publications recommending distal forearm BMD measurements as appropriate for studying thyroid effects on bone [42, 44]. Other areas of the skeleton that have demonstrated significant BMD decreases associated with hyperthyroidism include the lumbar spine, total hip and femoral neck. A 2010 cross-sectional study evaluating BMD in premenopausal women, as analyzed by DXA, revealed significantly lower BMD in hyperthyroid women compared to controls at the lumbar spine (0.928 versus 0.991), the total hip (0.838 versus 0.917), and the femoral neck (0.774 versus 0.832) [46]. In contrast to the distal radius, the decrease of BMD in the lumbar spine, total hip, or femoral neck does not appear to be associated with the duration of overt hyperthyroidism [46, 47].

The severity of hyperthyroidism appears to influence the degree of bone loss and increase the probability of osteoporosis when looking at the relative degree of TSH suppression, rather than looking at free T4 and free T3 levels. The aforementioned cross-sectional study by Svare et al. [44] reported a statistically significant decrease in BMD in hyperthyroid women with TSH <0.50 mU/L (reference range: 0.50–1.49 mU/L), with the highest association seen with TSH <0.10 mU/L. No statistically significant difference was observed in the BMD of hyperthyroid patients versus controls with TSH above 0.50 mU/L [44]. A 2007 analysis using data from the U.S. National Health and Nutrition Examination Survey (NHANES) evaluated the BMD in postmenopausal women and reported an odds ratio (OR) of 3.4 relating osteoporosis to TSH levels <1.8 mU/L, compared to an OR of 2.2 for TSH levels ≥1.8–4.5 mU/L [48]. The report also found that as TSH levels increased over the reference range (TSH 0.39–4.6 mU/L), a statistically significant increase was observed in BMD [48].

3.2. The Impact of Thyrotoxicosis on Fracture Risk. Thyrotoxicosis is an established risk factor for fracture later in life [49–51]. A prospective cohort study followed women over 65 years old for 4 years to evaluate fracture incidence and risk ($n = 686$) [52]. A TSH level of <0.1 mU/L resulted in a 4.5-fold risk of vertebral fracture and a 3.6-fold increase of hip fracture [52]. The fracture risk was much less pronounced with relatively higher TSH levels of 0.1–0.5 mU/L (normal range: 0.5–5.5 mU/L). The same report also found that a history of hyperthyroidism (regardless of duration or degree), after adjustment for TSH concentration and BMD, remains an independent risk factor for hip fracture [52]. Support for these finding was reported in a more recent cross-sectional study ($n = 6722$), which estimated a 31% higher fracture risk (forearm, hip, and vertebral) in patients with TSH <0.10 mU/L compared to TSH 1.0–1.49 mU/L [44].

4. Reversibility of Hyperthyroidism-Induced Bone Loss with Therapy

4.1. Targeted Antithyroid Therapy and Clinical Fracture Risk Outcomes. The choice of initial antithyroid therapy used to correct the hyperthyroidism may be clinically important,

as highlighted by studies looking at subsequent fracture risk. In a historical follow-up study of 617 patients with toxic goiter, patients treated with radioactive iodine alone, rather than being cotreated with antithyroid medications, had an increased fracture risk at the spine and forearm in comparison to age- and gender-matched control [53]. This increase in fracture risk from controls was not observed in patients who were also cotreated with methimazole [53]. It was hypothesized by these authors that patients treated with combination therapy may have had more severe initial presentation of hyperthyroidism which resulted in earlier diagnosis and management, with subsequently less time for bone loss to occur [53]. It will be of great importance for future clinical management decisions to repeat this study in a larger controlled prospective manner to determine if duel initial therapy does in fact provide improved long-term protection from fractures.

Question 3. Is the bone loss or osteoporosis due to hyperthyroidism reversible by normalizing thyroid function?

The initiation of antithyroid therapy and successful achievement of an euthyroid state can reverse the extent of osteoporosis induced by overt hyperthyroidism [40, 54–58]. Numerous studies have evaluated BMD after the successful treatment of hyperthyroidism and reported a significant, though incomplete, recovery of bone density with effective antithyroid treatment within the first years after initiation of therapy [54, 56, 59–66]. Despite significant increases in BMD at the lumbar spine, femoral neck, and distal radius after 9–12 months of antithyroid therapy, bone density remained 5–16% lower than controls at 18–24 months of followup [50, 67].

At least three reports suggest after several years of euthyroidism, thyrotoxicosis-induced osteoporosis can be restored to normal [55, 56, 68]. BMD has been observed to return to a normal level, or that compared to healthy controls, after 3 to 6 years of euthyroid state after successful antithyroid therapy [40, 55, 65, 69].

4.2. Medical Treatment Options for Secondary Osteoporosis due to Hyperthyroidism

4.2.1. Combination of Bisphosphonate and Antithyroid Therapy. Studies carried out with rodents have established that bisphosphonate therapy ameliorates the excessive bone loss caused by hyperthyroidism due to excessive T4 hormone supplementation [70]. Research in humans has evaluated the BMD and fracture risk outcomes using antithyroid drug monotherapy compared to the combination of bisphosphonates and antithyroid treatments. Lupoli et al. [71] examined the addition of alendronate to methimazole therapy in 40 pre- and postmenopausal hyperthyroid women and age-matched controls with no history of thyroid disease. BMD and osteocalcin levels were evaluated at baseline and 6 and 12 months after initiation of either methimazole (MMI) or MMI and alendronate combination therapy. At its conclusion, the study revealed a statistically significant clinical and molecular benefit in the combination therapy arm: a significant increase in BMD and decrease in the bone turnover marker osteocalcin. In addition this was independent of estrogen levels as both pre- and postmenopausal women exhibited significant increases in BMD and decreases in osteocalcin levels from basal values after 12 months of treatment [71]. Fittipaldi et al. [72] examined the effect of combination therapy in elderly male subjects with hyperthyroidism and osteoporosis. The results were similar to the effect seen in women; a detectable mean increase in BMD at the lumbar spine and femoral neck was significantly higher in patients treated with the combination of MMI and alendronate (6.2% and 2.1%, resp.) than in those treated with MMI alone (2.0% and 1.4%, resp.) [72]. Recently, another bisphosphonate, risedronate, were evaluated for its combination therapy effects on BMD and bone resorption markers in males with Graves' disease (n = 27, mean age = 43.7). Comparable with previous investigations, the increase in BMD at the lumbar spine, femoral neck, and distal radius was significantly higher after 12 months of combination treatment [73].

4.2.2. Vitamin D. Therapy of hyperthyroidism-induced osteoporosis includes adequate Vitamin D supplementation and bisphosphonate therapy, in addition to antithyroid treatment. The continuous bone resorption and high serum calcium levels seen in sustained hyperthyroidism result in decreased levels of active Vitamin D, suggesting a therapeutic role for oral Vitamin D supplementation. A 2010 study examined the relationship between Vitamin D levels and BMD in patients with newly diagnosed hyperthyroidism [74]. The report revealed significantly lower mean Z-scores (age-matched controls) and overall BMD values in vitamin D deficient patients (<25 nmol/L) compared to hyperthyroid patients with sufficient vitamin D levels (>25 nmol/L) [74], supporting the inclusion of Vitamin D supplementation where deficiencies are detected.

4.2.3. Calcitonin. As a result of its potent inhibition of osteoclast activity, calcitonin administration has also been investigated as a potential adjunct to therapy of hyperthyroidism-induced osteoporosis when used in combination with antithyroid medications. No additional benefit was seen in BMD or bone resorption markers at any dose of intranasal calcitonin although the BMD was significantly increased in all patients treated with antithyroid therapy and intranasal calcitonin at varying doses for 9 months [54]. The conclusion was that no additional benefit beyond reaching the euthyroid state was conferred by the addition of calcitonin [54].

5. Clinical Bone Disease in Subclinical Hyperthyroidism

Question 4. What impact does menopause or gender have on clinical outcomes of bone turnover in subclinical hyperthyroidism?

Subclinical hyperthyroidism is a biochemical definition, with suppressed or undetectable TSH levels and normal concentrations of T3 and T4. Subclinical hyperthyroidism

incidence increases with age, especially in women, and is present in an estimated 1.5% of women over the age of 60 [75]. Serum TSH levels may spontaneously return to normal, as can be seen in patients with subclinical hyperthyroidism caused by Graves' disease or may progress to overt hyperthyroidism, more commonly in patients with autonomous thyroid nodules or multinodular goiters [75]. Exogenous hyperthyroidism is considered to exist due to iatrogenic overreplacement with thyroid hormone supplementation, such as during long-term management of differentiated thyroid carcinoma patients.

5.1. The Significance of Endogenous Subclinical Hyperthyroidism on BMD. Evaluation of the association between the presence of subclinical hyperthyroidism and the risk of development of osteoporosis has produced conflicting results from many small studies; therefore, BMD and fracture risk in this population are better examined by a subdivision dependent on estrogen levels found in pre- and postmenopausal women.

5.1.1. Postmenopausal Women, BMD, and Subclinical Hyperthyroidism. Subclinical hyperthyroidism has consistently been correlated with an increased risk of reduced BMD [12, 13, 50, 76–78]. Foldes et al. [14] conducted a cross-sectional study ($n = 37$) measuring BMD in pre- and postmenopausal women with endogenous subclinical hyperthyroidism. It was observed that the BMD was not significantly affected in premenopausal patients, in direct contrast to postmenopausal women who had significantly decreased BMD at the femoral neck and radius, sites of predominantly cortical bone.

5.1.2. Premenopausal Women, BMD, and Subclinical Hyperthyroidism. Multiple studies of premenopausal women with subclinical hyperthyroidism, either in comparison to normal controls or postmenopausal subjects, have consistently found normal or near-normal BMD levels. It is unclear if such studies have been able to remove the bias of age, as postmenopausal women may just have had longer durations of low TSH [13, 14, 76, 78–84]. Two small studies have reported statistically significant reduction in BMD in the femoral neck, but not in the lumbar spine, of premenopausal women, in comparison to significant reduction at both sites in postmenopausal patients [84, 85]. Similarly, the majority of studies have revealed normal serum markers of bone turnover, with the exception of the aforementioned studies, which noted significant increase in bone resorption markers in both pre- and postmenopausal women [14, 80, 82, 86, 87].

5.1.3. Males, BMD, and Subclinical Hyperthyroidism. Nearly half of the cases of osteoporosis in men are found to have an underlying secondary cause. The effects on BMD in male patients with endogenous subclinical hyperthyroidism have not been widely studied or reported. A recent retrospective analysis of hip fracture risk in patients with subclinical thyroid dysfunction revealed a hazard ratio (HR) of 4.91 in men with endogenous subclinical hyperthyroidism in comparison to a HR of 2.42 in postmenopausal females of the same category [88]. The reason for the increased fracture risk in males is unexpected, and further statistical analysis of androgens, TSH, T4, and T3 levels may help clarify the risks involved.

5.2. The Importance of Endogenous Subclinical Hyperthyroidism on Increased Fracture Risk. Subclinical hyperthyroidism has also been implicated with an increased incidence of fracture at any site. A retrospective study ($n = 2004$) examined fracture risk patients with subclinical hyperthyroidism, defined as a TSH below the reference range (≤ 0.4 mU/L) and a normal total T4 and T3, to age- and gender-matched controls [89]. The results revealed a hazard ratio of 1.25 for osteoporotic fracture. This association was lost, however, when patients were included who converted into either overt hyperthyroidism or the euthyroid state during the follow-up period (median: 5.6 years) [89].

6. Reversibility of Subclinical Hyperthyroidism-Induced Bone Loss with Therapy

Question 5. How well does low bone density recover through correction of subclinical hyperthyroidism alone?

There is an established link between subclinical hypothyroidism and BMD decreases in postmenopausal women, leading investigators to test the efficacy of antithyroid therapy on bone density recovery in these patients. Studies in this subset of patients with subclinical hyperthyroidism have revealed a strong association with improvement in BMD within as little as 6 months of reaching euthyroidism [64, 90–92]. A prospective study by Mudde et al. [76] followed postmenopausal women with subclinical hyperthyroidism for a 2-year period following treatment with MMI and compared them to untreated controls. Although significant changes in bone turnover serum markers were not seen in either group, the mean BMD in women treated with MMI was significantly higher than in those in the untreated group [76]. Faber et al. [93] conducted a prospective study examining the effects of radioiodine therapy on the BMD of postmenopausal women with subclinical hyperthyroidism secondary to nodular goiter. The spine BMD in the treated patients increased by 1.9% at 1-year followup and remained increased by 1.5% after 2 years. BMD at the hip was also increased by 2.3% after 1 year of treatment and remained 1.7% increased after 2 years. These results were significant when compared to the untreated controls, in whom BMD decreased by about 2% per year at the hip and the spine [93]. A recent prospective study of seventeen women over the age of 65 with subclinical hyperthyroidism due to nodular goiter and treated with radioiodine showed a similar effect on BMD after achievement of euthyroidism [94]. After 1 year of treatment with radioiodine, average BMD had increased by 1.9% at the femoral neck and by 1.6% at the lumbar spine in the twelve patients who achieved euthyroidism. Conversely, in the four patients who continued to have subclinical hyperthyroidism despite one year of therapy, the average BMD had decreased by 2% at the femoral neck and by 1.8% at the lumber spine at followup [94].

As mentioned previously, subclinical hyperthyroidism in premenopausal women is typically associated with a normal BMD and no alteration in serum bone turnover markers. Therefore, the usefulness of treatment of this group has been put into question. Nonetheless, a small prospective randomized trial evaluated BMD in premenopausal women after 6 months of antithyroid therapy and concluded that no difference in BMD between treated patients and controls was found [95]. This is an important finding for clinical management, as no measureable clinical benefit to bone health through active treatment was detected.

7. Iatrogenic Subclinical Hyperthyroidism and Negative Outcomes to Bone Density

Question 6. How common is subclinical hyperthyroidism in the general population and what clinical consequences does this have on their bone health?

7.1. Prevalence of Iatrogenic Subclinical Hyperthyroidism. The prevalence of exogenous subclinical hyperthyroidism is much more common than the endogenous form, due to the widespread use of thyroid hormone supplementation and its inclusion in many herbal and weight loss formulations [75]. An estimated 3% of women over the age of 60 are taking exogenous T4 (thyroxine) for medically indicated purposes, either for TSH suppression or as replacement of iatrogenic or endogenous hypothyroidism [96]. Full replacement doses of levothyroxine are crudely approximated at <1.6 μg/kg, and suppressive doses result in a decreased TSH serum level [97]. In a community-based study of 1210 adults over 60 years old, Parle et al. [96] evaluated the prevalence of thyroid dysfunction. It was found that 24% of these patients were overreplaced with thyroid hormone, having measurably low TSH levels. Congruent with this report, a recent review of subclinical thyroid disease by Cooper and Biondi [75] estimated low TSH levels in 20–40% of patients treated with thyroid hormone. These studies highlight the surprising prevalence of community subclinical hyperthyroidism.

7.2. The Impact of Exogenous Subclinical Hyperthyroidism on Bone Mineral Density. Euthyroid patients who are treated with oral T4 and sustain serum TSH levels within the normal reference range have not been found to have changes in their BMD regardless of menstrual status [6, 98]. Because of wide variations in outcomes between pre- and postmenopausal women, the extent of bone loss associated with exogenous thyroid therapy is best examined individually within these groups of women.

7.2.1. Postmenopausal Women. As with endogenous subclinical hyperthyroidism, postmenopausal women taking excess exogenous thyroid hormone have been found to be at risk for hyperthyroid-induced skeletal effects [99]. Significant reductions of BMD in postmenopausal women treated with thyroxine are a universal finding [34, 61, 83, 100–104]. A meta-analysis by Faber and Galloe [90] reported a significant reduction of BMD by 9% after 10 years of thyroxine treatment in postmenopausal women compared to controls. This value implies that subclinical hyperthyroidism induced by thyroxine administration confers an additional annual bone loss of nearly 1% in postmenopausal women who already have an estimated 1–2.5% annual bone loss. Uzzan et al. [105] demonstrated similar loss values in their meta-analysis, with BMD losses of 7% of the spine, 5% of the femoral neck, 9% of the trochanter and Ward's triangle, and 7% of the distal radius. These values may correspond to a 12–44% lifetime risk of hip fracture conferred from bone loss of 6–10% over a 10-year period [106]. Clearly, these studies highlight the need for clinical vigilance in optimizing replacement levels to reduce adverse effects on bone metabolism.

7.2.2. Premenopausal Women. The main reason for minimal bone loss in premenopausal women is thought to be due to preserved estrogen production; therefore, the impact of thyroxine overreplacement on bone mass should exhibit a lesser impact than postmenopausal women [97]. Consistent with this notion, suppressive doses of thyroxine have not been implicated in significant reductions of BMD in premenopausal women in large population analyses and review [6, 90, 99, 105, 107, 108]. A meta-analysis revealed that BMD in premenopausal women treated with thyroxine for 8.5 years (90% with TSH below the reference range) had a mean reduction in BMD of 2.7%, a value corresponding to 0.3% annual bone loss throughout the duration of treatment, a number found to be nonsignificant by the investigators in comparison to controls [90].

7.2.3. Males. Few studies have included male patients evaluating the effects of exogenous thyroid hormones on BMD. Meta analyses and literature reviews have concluded that no significant effect on BMD has been observed in men receiving suppressive thyroxine therapy [6, 99, 107].

7.3. Fracture Risk in Subclinical Hyperthyroidism due to Exogenous Overreplacement. There is an increased risk of bone fracture in individuals who are overreplaced with exogenous thyroid hormone. Fracture risk is related to the degree of TSH suppression (TSH < 0.1 mU/L versus TSH 0.1–0.5 mU/L) and patient factors, such as age [52]. For women with TSH values in the normal range, T4 replacement does not confer a risk for fracture [52]. A recent observational cohort study examined fracture risk in patients >18 years old (mean age: 60.3 females, 61.8 males) on long-term T4 therapy with a median follow-up of 4.5 years (n = 17,684) [109]. No increase in fracture risk was found in patients with low TSH concentrations (0.04–0.4 mU/L) compared to normal range TSH levels (0.4–4.0 mU/L); however, they reported a two-fold increase in fracture risk in patients who had undetectable TSH levels (≤0.03 mU/L) [109]. Fracture risk is further increased in postmenopausal women with suppressed or undetectable TSH levels (<0.1 mU/L) due to T4 replacement with as much as a four-fold increase in vertebral and hip fractures after 4 years of followup compared to controls with normal TSH values (>0.5 mU/L) [52]. Therefore, the greatest risk lies with those with the lowest TSH values and those who are postmenopausal.

Question 7. Can bone loss due to exogenous thyroid hormone overreplacement be reversed?

7.3.1. Efficacy of Correction of Thyroid Replacement Dosing on Bone Recovery. Appropriate thyroxine supplementation in euthyroid individuals who maintain TSH levels within the reference range has not been implicated in BMD reduction [6]. It has been suggested that titration of suppressive thyroxine therapy is imperative in protecting the negative skeletal effects of subclinical hyperthyroidism [97]. In a small clinical trial, Appetecchia [110] examined the effects on BMD and thyroxine suppression therapy in 200 pre- and postmenopausal women with nodular goiter. The result revealed that thyroxine therapy titrated to doses that normalized TSH levels (0.27–4.20 mU/mL) did not have any deleterious effects on BMD regardless of menopause status [110]. First-line management clearly indicates that thyroxine dosing should be monitored and adjusted to prevent ongoing bone loss.

7.3.2. Calcium Supplementation. A prospective study by Kung and Yeung [83] evaluated the role of calcium supplementation in 46 postmenopausal women treated with suppressive doses of thyroxine. The women were divided into groups of either 1000 mg calcium daily or placebo and followed for 2 years. At 6-month intervals and at the conclusion of the study, BMD was determined for the study participants. The results revealed that the patients supplemented with calcium had stable BMD, whereas patients in the placebo cohort had significant bone loss of 5% at the lumbar spine, 6.7% at the hip, 4.7% at the trochanter, and 8.8% at Ward's triangle, and BMD was significantly lower than the supplemented group [83]. The researchers concluded that suppressive thyroxine therapy should be supplemented with calcium to prevent BMD loss in treated patients [83].

7.3.3. Adjunct Estrogen Replacement Therapy in Postmenopausal Women. Effects of exogenous subclinical hyperthyroidism on bone are minimal in premenopausal women presumably due to the positive effects of estrogen on skeletal health. A cross-sectional study by Schneider et al. [97] evaluated the impact of concomitant estrogen therapy in 991 postmenopausal women taking levothyroxine in replacement or suppressive doses. Women treated with estrogen and thyroid hormone has significantly higher BMD than those taking thyroid hormones alone. BMD in the estrogen replacement group was 12.9% higher at the midshaft radius, 8.1% higher at the hip, 7.8% higher at the lumbar spine, and 17.7% higher at the ultradistal radius [97]. With adjustments for age, BMI, smoking, and concurrent use of corticosteroids and thiazide, the BMD in women taking both levothyroxine and estrogen was comparable to that of women solely taking estrogen, without use of thyroid hormone [97]. The positive effects of estrogen are presumed to oppose the negative calcium balance seen in the hyperthyroid state, by increased absorption and decreased excretion of calcium [111]. However, the direct effect of estrogen on osteoblasts and the increase in calcitonin following estrogen administration are also thought to play a role. Although estrogen is clearly effective in modulating bone metabolism and was routinely prescribed prior to the Women's Health Initiative, it is a controversial supplement. Given the efficacy of alternative, nonhormonal therapies (below) estrogen is not recommended solely for the prevention or treatment of osteoporosis [112].

7.3.4. Bisphosphonates. Bisphosphonates are indicated for the treatment of osteoporosis induced by overt hyperthyroidism. In a randomized trial, Rosen et al. [81] evaluated the efficacy of bisphosphonates in patients taking suppressive doses of thyroxine who fit the biochemical criteria for subclinical hyperthyroidism (normal freeT4 in 97% of cases). Men and postmenopausal women were randomized to either taking thyroxine and placebo, or thyroxine with addition of 30 mg of intravenous pamidronate every 3 months and were followed for 2 years. The bisphosphonate-treated group showed significant increases in BMD at the spine (4.3%), total hip (1.4%), and trochanter (3.0%) compared to patients solely receiving thyroid hormone [81].

7.3.5. Calcitonin. Similar to the conclusions discussed previously in overt hyperthyroidism, the use of intranasal calcitonin has not been found to benefit BMD in patients treated with exogenous subclinical hyperthyroidism. Kung and Yeung [83] found no additional benefit of intranasal calcitonin on BMD in postmenopausal women supplemented with 1000 mg of calcium daily.

8. Conclusions and Key Points

Regulation of skeletal development and maintenance of skeletal integrity is regulated in part by a normal balance of thyroid hormones. Rodent studies have implicated both thyroid hormones and pituitary TSH in regulation of bone remodeling, although it remains unclear if T3, TSH, or the combination of both are responsible for the increased turnover and subsequent osteoporosis observed in the hyperthyroid state. Overt hyperthyroidism leads to osteoporosis and increased fracture risk in patients regardless of their age or gender. Prevention of fractures attributed to low BMD in hyperthyroid patients begins with antithyroid therapy, as reversibility of hyperthyroid-induced osteoporosis is evident a few years after achievement of an euthyroid state. Supplementation with Vitamin D and bisphosphonates may further increase BMD in these patients. The fine control of bone integrity by thyroid hormones is exhibited by the detrimental consequences on BMD and increased fracture risk by subclinical hyperthyroidism in postmenopausal women and men, and in postmenopausal women treated with suppressive doses of thyroid hormone. Postmenopausal women and men with endogenous subclinical hyperthyroidism should be considered for bone density measurement and subsequent antithyroid therapy. Postmenopausal women on thyroid supplementation in suppressive doses should have bone density testing, with appropriate titration of suppressive therapy, and supplementation with calcium and bisphosphonates.

References

[1] C. Gennari, G. Martini, and R. Nuti, "Secondary osteoporosis," *Aging*, vol. 10, no. 3, pp. 214–224, 1998.

[2] J. H. D. Bassett and G. R. Williams, "The molecular actions of thyroid hormone in bone," *Trends in Endocrinology and Metabolism*, vol. 14, no. 8, pp. 356–364, 2003.

[3] A. C. Bianco, D. Salvatore, B. Gereben, M. J. Berry, and P. R. Larsen, "Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases," *Endocrine Reviews*, vol. 23, no. 1, pp. 38–89, 2002.

[4] J. A. Wexler and J. Sharretts, "Thyroid and bone," *Endocrinology and Metabolism Clinics of North America*, vol. 36, no. 3, pp. 673–705, 2007.

[5] C. B. Harvey, J. H. D. Bassett, P. Maruvada, P. M. Yen, and G. R. Williams, "The rat thyroid hormone receptor (TR) Deltabeta3 displays cell-, TR isoform-, and thyroid hormone response element-specific actions," *Endocrinology*, vol. 148, no. 4, pp. 1764–1773, 2007.

[6] E. Murphy and G. R. Williams, "The thyroid and the skeleton," *Clinical Endocrinology*, vol. 61, no. 3, pp. 285–298, 2004.

[7] P. J. O'Shea, C. B. Harvey, H. Suzuki et al., "A thyrotoxic skeletal phenotype of advanced bone formation in mice with resistance to thyroid hormone," *Molecular Endocrinology*, vol. 17, no. 7, pp. 1410–1424, 2003.

[8] P. J. O'Shea, J. H. D. Bassett, S. Sriskantharajah, H. Ying, S. Cheng, and G. R. Williams, "Contrasting skeletal phenotypes in mice with an identical mutation targeted to thyroid hormone receptor α1 or β," *Molecular Endocrinology*, vol. 19, no. 12, pp. 3045–3059, 2005.

[9] J. H. D. Bassett and G. R. Williams, "The skeletal phenotypes of TRα and TBβ mutant mice," *Journal of Molecular Endocrinology*, vol. 42, no. 4, pp. 269–282, 2009.

[10] K. Gauthier, M. Plateroti, C. B. Harvey et al., "Genetic analysis reveals different functions for the products of the thyroid hormone receptor α locus," *Molecular and Cellular Biology*, vol. 21, no. 14, pp. 4748–4760, 2001.

[11] J. H. D. Bassett, K. Nordström, A. Boyde et al., "Thyroid status during skeletal development determines adult bone structure and mineralization," *Molecular Endocrinology*, vol. 21, no. 8, pp. 1893–1904, 2007.

[12] G. Grimnes, N. Emaus, R. M. Joakimsen, Y. Figenschau, and R. Jorde, "The relationship between serum TSH and bone mineral density in men and postmenopausal women: the Tromsø study," *Thyroid*, vol. 18, no. 11, pp. 1147–1155, 2008.

[13] W. Y. Lee, K. W. Oh, E. J. Rhee et al., "Relationship between subclinical thyroid dysfunction and femoral neck bone mineral density in women," *Archives of Medical Research*, vol. 37, no. 4, pp. 511–516, 2006.

[14] J. Foldes, G. Tarjan, M. Szathmari, F. Varga, I. Krasznai, and C. Horvath, "Bone mineral density in patients with endogenous subclinical hyperthyroidism: is this thyroid status a risk factor for osteoporosis?" *Clinical Endocrinology*, vol. 39, no. 5, pp. 521–527, 1993.

[15] E. Abe, R. C. Marians, W. Yu et al., "TSH is a negative regulator of skeletal remodeling," *Cell*, vol. 115, no. 2, pp. 151–162, 2003.

[16] J. A. Tsai, A. Janson, E. Bucht et al., "Weak evidence of thyrotropin receptors in primary cultures of human osteoblast-like cells," *Calcified Tissue International*, vol. 74, no. 5, pp. 486–491, 2004.

[17] T. Davies, R. Marians, and R. Latif, "The TSH receptor reveals itself," *Journal of Clinical Investigation*, vol. 110, no. 2, pp. 161–164, 2002.

[18] L. Sun, T. F. Davies, H. C. Blair, E. Abe, and M. Zaidi, "TSH and bone loss," *Annals of the New York Academy of Sciences*, vol. 1068, no. 1, pp. 309–318, 2006.

[19] T. Morimura, K. Tsunekawa, T. Kasahara et al., "Expression of type 2 iodothyronine deiodinase in human osteoblast is stimulated by thyrotropin," *Endocrinology*, vol. 146, no. 4, pp. 2077–2084, 2005.

[20] L. J. Raggatt and N. C. Partridge, "Cellular and molecular mechanisms of bone remodeling," *Journal of Biological Chemistry*, vol. 285, no. 33, pp. 25103–25108, 2010.

[21] M. S. Lee, S. Y. Kim, M. C. Lee et al., "Negative correlation between the change in bone mineral density and serum osteocalcin in patients with hyperthyroidism," *Journal of Clinical Endocrinology and Metabolism*, vol. 70, no. 3, pp. 766–770, 1990.

[22] M. D. Fallon, H. M. Perry III, and M. Bergfeld, "Exogenous hyperthyroidism with osteoporosis," *Archives of Internal Medicine*, vol. 143, no. 3, pp. 442–444, 1983.

[23] S. A. Fraser, J. B. Anderson, D. A. Smith, and G. M. Wilson, "Osteoporosis and fractures following thyrotoxicosis," *The Lancet*, vol. 1, no. 7707, pp. 981–983, 1971.

[24] E. F. Eriksen, L. Mosekilde, and F. Melsen, "Trabecular bone remodeling and bone balance in hyperthyroidism," *Bone*, vol. 6, no. 6, pp. 421–428, 1985.

[25] F. Melsen and L. Mosekilde, "Trabecular bone mineralization lag time determined by tetracycline double-labeling in normal and certain pathological conditions," *Acta Pathologica et Microbiologica Scandinavica A*, vol. 88, no. 2, pp. 83–88, 1980.

[26] L. Mosekilde, E. F. Eriksen, and P. Charles, "Effects of thyroid hormones on bone and mineral metabolism," *Endocrinology and Metabolism Clinics of North America*, vol. 19, no. 1, pp. 35–63, 1990.

[27] C. B. Harvey, P. J. O'Shea, A. J. Scott et al., "Molecular mechanisms of thyroid hormone effects on bone growth and function," *Molecular Genetics and Metabolism*, vol. 75, no. 1, pp. 17–30, 2002.

[28] D. Frizel, A. Malleson, and V. Marks, "Plasma levels of ionised calcium and magnesium in thyroid disease," *The Lancet*, vol. 1, no. 7504, pp. 1360–1361, 1967.

[29] B. Jastrup, L. Mosekilde, and F. Melsen, "Serum levels of vitamin D metabolites and bone remodelling in hyperthyroidism," *Metabolism*, vol. 31, no. 2, pp. 126–132, 1982.

[30] G. Karsenty, P. Bouchard, A. Ulmann, and G. Schaison, "Elevated metabolic clearance rate of 1α,25-dihydroxyvitamin D3 in hyperthyroidism," *Acta Endocrinologica*, vol. 110, no. 1, pp. 70–74, 1985.

[31] F. B. Thomas, J. H. Caldwell, and N. J. Greenberger, "Steatorrhea in thyrotoxicosis. Relation to hypermotility and excessive dietary fat," *Annals of Internal Medicine*, vol. 78, no. 5, pp. 669–675, 1973.

[32] S. E. Park, M. A. Cho, S. H. Kim et al., "The adaptation and relationship of FGF-23 to changes in mineral metabolism in Graves' disease," *Clinical Endocrinology*, vol. 66, no. 6, pp. 854–858, 2007.

[33] D. R. Garrel, P. D. Delmas, L. Malaval, and J. Tourniaire, "Serum bone Gla protein: a marker of bone turnover in hyperthyroidism," *Journal of Clinical Endocrinology and Metabolism*, vol. 62, no. 5, pp. 1052–1055, 1986.

[34] C. Guo, A. P. Weetman, and R. Eastell, "Longitudinal changes of bone mineral density and bone turnover in postmenopausal women on thyroxine," *Clinical Endocrinology*, vol. 46, no. 3, pp. 301–307, 1997.

[35] J. Toivonen, R. Tähtelä, K. Laitinen, J. Risteli, and M. J. Välimäki, "Markers of bone turnover in patients with differentiated thyroid cancer with and following withdrawal of thyroxine

suppressive therapy," *European Journal of Endocrinology*, vol. 138, no. 6, pp. 667–673, 1998.

[36] D. S. Cooper, M. M. Kaplan, and E. C. Ridgway, "Alkaline phosphatase isoenzyme patterns in hyperthyroidism," *Annals of Internal Medicine*, vol. 90, no. 2, pp. 164–168, 1979.

[37] G. Amato, G. Mazziotti, F. Sorvillo et al., "High serum osteoprotegerin levels in patients with hyperthyroidism: effect of medical treatment," *Bone*, vol. 35, no. 3, pp. 785–791, 2004.

[38] R. D. Harvey, K. C. McHardy, I. W. Reid et al., "Measurement of bone collagen degradation in hyperthyroidism and during thyroxine replacement therapy using pyridinium cross-links as specific urinary markers," *Journal of Clinical Endocrinology and Metabolism*, vol. 72, no. 6, pp. 1189–1194, 1991.

[39] J. M. MacLeod, K. C. McHardy, R. D. Harvey et al., "The early effects of radioiodine therapy for hyperthyroidism on biochemical indices of bone turnover," *Clinical Endocrinology*, vol. 38, no. 1, pp. 49–53, 1993.

[40] P. Vestergaard and L. Mosekilde, "Hyperthyroidism, bone mineral, fracture risk-a meta-analysis," *Thyroid*, vol. 13, no. 6, pp. 585–593, 2003.

[41] C.-H. Chen, J.-F. Chen, B.-Y. Yang et al., "Bone mineral density in women receiving thyroxine suppressive therapy for differentiated thyroid carcinoma," *Journal of the Formosan Medical Association*, vol. 103, no. 6, pp. 442–447, 2004.

[42] S. L. Greenspan and F. S. Greenspan, "The effect of thyroid hormone on skeletal integrity," *Annals of Internal Medicine*, vol. 130, no. 9, pp. 750–758, 1999.

[43] G. S. Bianchi, P. Meunier, P. Courpron, C. Edouard, J. Bernard, and G. Vignon, "Osseous effects of hyperthyroidism," *Revue du Rhumatisme et des Maladies Osteo-Articulaires*, vol. 39, no. 1, pp. 19–32, 1972.

[44] A. Svare, T. I. L. Nilsen, T. Bjøro, S. Forsmo, B. Schei, and A. Langhammer, "Hyperthyroid levels of TSH correlate with low bone mineral density: the HUNT 2 study," *European Journal of Endocrinology*, vol. 161, no. 5, pp. 779–786, 2009.

[45] J. Kosowicz, D. Baszko-Błaszyh, W. Horst-Sikorska, and A. Baumann-Antczak, "Bone mineral density in primary hyperparathyroidism," *Polskie Archiwum Medycyny Wewnetrznej*, vol. 101, no. 2, pp. 133–138, 1999.

[46] T. Boonya-Ussadorn, B. Punkaew, and N. Sriassawaamorn, "A comparative study of bone mineral density between premenopausal women with hyperthyroidism and healthy premenopausal women," *Journal of the Medical Association of Thailand*, vol. 93, supplement 6, pp. S1–S5, 2010.

[47] J. Linde and T. Friis, "Osteoporosis in hyperthyroidism estimated by photon absorptiometry," *Acta Endocrinologica*, vol. 91, no. 3, pp. 437–448, 1979.

[48] M. S. Morris, "The association between serum thyroid-stimulating hormone in its reference range and bone status in postmenopausal American women," *Bone*, vol. 40, no. 4, pp. 1128–1134, 2007.

[49] B. Wejda, G. Hintze, B. Katschinski, T. Olbricht, and G. Benker, "Hip fractures and the thyroid: a case-control study," *Journal of Internal Medicine*, vol. 237, no. 3, pp. 241–247, 1995.

[50] S. R. Cummings, M. C. Nevitt, W. S. Browner et al., "Risk factors for hip fracture in white women," *New England Journal of Medicine*, vol. 332, no. 12, pp. 767–773, 1995.

[51] T. J. Allain and A. M. McGregor, "Thyroid hormones and bone," *Journal of Endocrinology*, vol. 139, no. 1, pp. 9–18, 1993.

[52] D. C. Bauer, B. Ettinger, M. C. Nevitt, and K. L. Stone, "Risk for fracture in women with low serum levels of thyroid-stimulating hormone," *Annals of Internal Medicine*, vol. 134, no. 7, pp. 561–568, 2001.

[53] P. Vestergaard, L. Rejnmark, J. Weeke, and L. Mosekilde, "Fracture risk in patients treated for hyperthyroidism," *Thyroid*, vol. 10, no. 4, pp. 341–348, 2000.

[54] E. Jódar, M. Muñoz-Torres, F. Escobar-Jiménez, M. Quesada, J. D. Luna, and N. Olea, "Antiresorptive therapy in hyperthyroid patients: longitudinal changes in bone and mineral metabolism," *Journal of Clinical Endocrinology and Metabolism*, vol. 82, no. 6, pp. 1989–1994, 1997.

[55] B. L. Langdahl, A. G. R. Loft, E. F. Eriksen, L. Mosekilde, and P. Charles, "Bone mass, bone turnover and body composition in former hypothyroid patients receiving replacement therapy," *European Journal of Endocrinology*, vol. 134, no. 6, pp. 702–709, 1996.

[56] H. Karga, P. D. Papapetrou, A. Korakovouni, F. Papandroulaki, A. Polymeris, and G. Pampouras, "Bone mineral density in hyperthyroidism," *Clinical Endocrinology*, vol. 61, no. 4, pp. 466–472, 2004.

[57] P. Lakatos, "Thyroid hormones: beneficial or deleterious for bone?" *Calcified Tissue International*, vol. 73, no. 3, pp. 205–209, 2003.

[58] A. Siddiqi, J. M. Burrin, K. Noonan et al., "A longitudinal study of markers of bone turnover in Graves' disease and their value in predicting bone mineral density," *Journal of Clinical Endocrinology and Metabolism*, vol. 82, no. 3, pp. 753–759, 1997.

[59] A. Serraclara, E. Jódar, F. Sarabia, and F. Hawkins, "Bone mass after long-term euthyroidism in former hyperthyroid women treated with 131I: influence of menopausal status," *Journal of Clinical Densitometry*, vol. 4, no. 3, pp. 249–255, 2001.

[60] S. H. Toh, B. C. Claunch, and P. H. Brown, "Effect of hyperthyroidism and its treatment on bone mineral content," *Archives of Internal Medicine*, vol. 145, no. 5, pp. 883–886, 1985.

[61] T. Diamond, J. Vine, R. Smart, and P. Butler, "Thyrotoxic bone disease in women: a potentially reversible disorder," *Annals of Internal Medicine*, vol. 120, no. 1, pp. 8–11, 1994.

[62] J. Franklyn, J. Betteridge, R. Holder, J. Daykin, J. Lilley, and M. Sheppard, "Bone mineral density in thyroxine treated females with or without a previous history of thyrotoxicosis," *Clinical Endocrinology*, vol. 41, no. 4, pp. 425–432, 1994.

[63] D. J. Grant, M. E. T. McMurdo, P. A. Mole, and C. R. Paterson, "Is previous hyperthyroidism still a risk factor for osteoporosis in post-menopausal women?" *Clinical Endocrinology*, vol. 43, no. 3, pp. 339–345, 1995.

[64] L. J. S. Greenlund, K. S. Nair, and M. D. Brennan, "Changes in body composition in women following treatment of overt and subclinical hyperthyroidism," *Endocrine Practice*, vol. 14, no. 8, pp. 973–978, 2008.

[65] N. Udayakumar, M. Chandrasekaran, M. H. Rasheed, R. V. Suresh, and S. Sivaprakash, "Evaluation of bone mineral density in thyrotoxicosis," *Singapore Medical Journal*, vol. 47, no. 11, pp. 947–950, 2006.

[66] S. T. Chen, M. J. Huang, J. H. Juang et al., "Bone changes in hyperthyroidism—a preliminary study," *Changgeng Yi Xue Za Zhi*, vol. 13, no. 4, pp. 274–281, 1990.

[67] E. Jódar, M. Muñoz-Torres, F. Escobar-Jiménez, M. Quesada-Charneco, and J. D. Luna Del Castillo, "Bone loss in hyperthyroid patients and in former hyperthyroid patients controlled on medical therapy: influence of aetiology and menopause," *Clinical Endocrinology*, vol. 47, no. 3, pp. 279–285, 1997.

[68] J. Rosen, B. Density, and A. Adler, "Changes in lumbar of euthyroidism," 1992.

[69] E. Murphy, J. H. D. Bassett, and G. R. Williams, "Disorders of calcium," *Practitioner*, vol. 250, no. 1686, pp. 4–8, 2006.

[70] S. Zeni, C. Gomez-Acotto, and C. Mautalen, "The effect of olpadronate in ovariectomized thyroxine-treated rats," *Bone*, vol. 21, no. 4, pp. 329–333, 1997.

[71] G. Lupoli, V. Nuzzo, C. Di Carlo et al., "Effects of alendronate on bone loss in pre- and postmenopausal hyperthyroid women treated with methimazole," *Gynecological Endocrinology*, vol. 10, no. 5, pp. 343–348, 1996.

[72] M. R. Fittipaldi, F. Fonderico, G. Vitale et al., "Osteoporosis treatment in elderly hyperthyroid male patients," *Journal of Endocrinological Investigation*, vol. 25, no. 10, pp. 98–100, 2002.

[73] T. Majima, Y. Komatsu, K. Doi et al., "Clinical significance of risedronate for osteoporosis in the initial treatment of male patients with Graves' disease," *Journal of Bone and Mineral Metabolism*, vol. 24, no. 2, pp. 105–113, 2006.

[74] D. K. Dhanwal and N. Gupta, "Bone mineral density trends in Indian patients with hyperthyroidism—effect of antithyroid therapy," *Journal of Association of Physicians of India*, vol. 59, no. 9, pp. 561–567, 2011.

[75] D. S. Cooper and B. Biondi, "Subclinical thyroid disease," *The Lancet*, vol. 379, no. 9821, pp. 1142–1154, 2012.

[76] A. H. Mudde, A. J. H. M. Houben, and A. C. Nieuwenhuijzen Kruseman, "Bone metabolism during anti-thyroid drug treatment of endogenous subclinical hyperthyroidism," *Clinical Endocrinology*, vol. 41, no. 4, pp. 421–424, 1994.

[77] D. S. Cooper, "Approach to the patient: approach to the patient with subclinical hyperthyroidism," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 1, pp. 3–9, 2007.

[78] B. Ugur-Altun, A. Altun, E. Arikan, S. Guldiken, and A. Tugrul, "Relationships existing between the serum cytokine levels and bone mineral density in women in the premenopausal period affected by Graves' disease with subclinical hyperthyroidism," *Endocrine Research*, vol. 29, no. 4, pp. 389–398, 2003.

[79] B. Biondi, A. Palmieri, M. Klain, M. Schlumberger, S. Filetti, and G. Lombardi, "Subclinical hyperthyroidism: clinical features and treatment options," *European Journal of Endocrinology*, vol. 152, no. 1, pp. 1–9, 2005.

[80] A. Gürlek and O. Gedik, "Effect of endogenous subclinical hyperthyroidism on bone metabolism and bone mineral density in premenopausal women," *Thyroid*, vol. 9, no. 6, pp. 539–543, 1999.

[81] H. N. Rosen, A. C. Moses, J. Garber et al., "Randomized trial of pamidronate in patients with thyroid cancer: bone density is not reduced by suppressive doses of thyroxine, but is increased by cyclic intravenous pamidronate," *Journal of Clinical Endocrinology and Metabolism*, vol. 83, no. 7, pp. 2324–2330, 1998.

[82] H. N. Rosen, A. C. Moses, C. Gundberg et al., "Therapy with parenteral pamidronate prevents thyroid hormone-induced bone turnover in humans," *Journal of Clinical Endocrinology and Metabolism*, vol. 77, no. 3, pp. 664–669, 1993.

[83] A. W. C. Kung and S. S. Yeung, "Prevention of bone loss induced by thyroxine suppressive therapy in postmenopausal women: the effect of calcium and calcitonin," *Journal of Clinical Endocrinology and Metabolism*, vol. 81, no. 3, pp. 1232–1236, 1996.

[84] L. Tauchmanovà, V. Nuzzo, A. Del Puente et al., "Reduced bone mass detected by bone quantitative ultrasonometry and DEXA in pre- and postmenopausal women with endogenous subclinical hyperthyroidism," *Maturitas*, vol. 48, no. 3, pp. 299–306, 2004.

[85] P. W. Rosario, "Bone and heart abnormalities of subclinical hyperthyroidism in women below the age of 65 years," *Arquivos Brasileiros de Endocrinologia e Metabologia*, vol. 52, no. 9, pp. 1448–1451, 2008.

[86] E. De Menis, G. Da Rin, I. Roiter, P. Legovini, G. Foscolo, and N. Conte, "Bone turnover in overt and subclinical hyperthyroidism due to autonomous thyroid adenoma," *Hormone Research*, vol. 37, no. 6, pp. 217–220, 1992.

[87] M. Garton, I. Reid, N. Loveridge et al., "Bone mineral density and metabolism in premenopausal women taking L-thyroxine replacement therapy," *Clinical Endocrinology*, vol. 41, no. 6, pp. 747–755, 1994.

[88] J. S. Lee, P. Bůžková, H. A. Fink et al., "Subclinical thyroid dysfunction and incident hip fracture in older adults," *Archives of Internal Medicine*, vol. 170, no. 21, pp. 1876–1883, 2010.

[89] T. Vadiveloo, P. T. Donnan, L. Cochrane, and G. P. Leese, "The thyroid epidemiology, audit, and research study (TEARS): morbidity in patients with endogenous subclinical hyperthyroidism," *Journal of Clinical Endocrinology and Metabolism*, vol. 96, no. 5, pp. 1344–1351, 2011.

[90] J. Faber and A. M. Galloe, "Changes in bone mass during prolonged subclinical hyperthyroidism due to L-thyroxine treatment: a meta-analysis," *European Journal of Endocrinology*, vol. 130, no. 4, pp. 350–356, 1994.

[91] P. Vestergaard, L. Mosekilde, and K. Brixen, "Treatment of osteoporosis with antiresorptive drugs (vitamin D, calcium, estrogen and gestagen, raloxifene, bisphosphonates and calcitonin)," *Ugeskrift for Laeger*, vol. 167, no. 8, pp. 883–887, 2005.

[92] S. Buscemi, S. Verga, S. Cottone et al., "Favorable clinical heart and bone effects of anti-thyroid drug therapy in endogenous subclinical hyperthyroidism," *Journal of Endocrinological Investigation*, vol. 30, no. 3, pp. 230–235, 2007.

[93] J. Faber, I. W. Jensen, L. Petersen, B. Nygaard, L. Hegedüs, and K. Siersbæk-Nielsen, "Normalization of serum thyrotrophin by means of radioiodine treatment in subclinical hyperthyroidism: effect on bone loss in postmenopasual women," *Clinical Endocrinology*, vol. 48, no. 3, pp. 285–290, 1998.

[94] P. W. Rosario, "Radioiodine therapy in elderly patients with subclinical hyperthyroidism due to non-voluminous goiter and its effects on bone metabolism," *Arquivos Brasileiros de Endocrinologia & Metabologia*, vol. 57, no. 2, pp. 144–147, 2013.

[95] O. Yönem, H. S. Dokmetas, S. M. Aslan, and T. Erselcan, "Is antithyroid treatment really relevant for young patients with subclinical hyperthyroidism?" *Endocrine Journal*, vol. 49, no. 3, pp. 307–314, 2002.

[96] J. V. Parle, J. A. Franklyn, K. W. Cross, S. C. Jones, and M. C. Sheppard, "Prevalence and follow-up of abnormal thyrotrophin (TSH) concentrations in the elderly in the United Kingdom," *Clinical Endocrinology*, vol. 34, no. 1, pp. 77–83, 1991.

[97] D. L. Schneider, E. L. Barrett-Connor, and D. J. Morton, "Thyroid hormone use and bone mineral density in elderly women. Effects of estrogen," *Journal of the American Medical Association*, vol. 271, no. 16, pp. 1245–1249, 1994.

[98] F. W. Hanna, R. J. Pettit, F. Ammari, W. D. Evans, D. Sandeman, and J. H. Lazarus, "Effect of replacement doses of thyroxine on bone mineral density," *Clinical Endocrinology*, vol. 48, no. 2, pp. 229–234, 1998.

[99] K. A. Heemstra, N. A. T. Hamdy, J. A. Romijn, and J. W. A. Smit, "The effects of thyrotropin-suppressive therapy on bone metabolism in patients with well-differentiated thyroid carcinoma," *Thyroid*, vol. 16, no. 6, pp. 583–591, 2006.

[100] C. Cipriani, E. Romagnoli, A. Scarpiello, M. Angelozzi, T. Montesano, and S. Minisola, "Phalangeal quantitative ultrasound and bone mineral density in evaluating cortical bone loss: a study in postmenopausal women with primary hyperparathyroidism and subclinical Iatrogenic hyperthyroidism," *Journal of Clinical Densitometry*, vol. 12, no. 4, pp. 456–460, 2009.

[101] D. S. Ross, R. M. Neer, E. C. Ridgway, and G. H. Daniels, "Subclinical hyperthyroidism and reduced bone density as a possible result of prolonged suppression of the pituitary-thyroid axis with L-thyroxine," *American Journal of Medicine*, vol. 82, no. 6, pp. 1167–1170, 1987.

[102] T. L. Paul, J. Kerrigan, A. M. Kelly, L. E. Braverman, and D. T. Baran, "Long-term L-thyroxine therapy is associated with decreased hip bone density in premenopausal women," *Journal of the American Medical Association*, vol. 259, no. 21, pp. 3137–3141, 1988.

[103] G. De Rosa, A. Testa, D. Giacomini, C. Carrozza, P. Astazi, and P. Caradonna, "Prospective study of bone loss in pre- and post-menopausal women on L- thyroxine therapy for non-toxic goitre," *Clinical Endocrinology*, vol. 47, no. 5, pp. 529–535, 1997.

[104] J. M. Stock, M. I. Surks, and J. H. Oppenheimer, "Replacement dosage of L-thyroxine in hypothyroidism. A re-evaluation," *New England Journal of Medicine*, vol. 290, no. 10, pp. 529–533, 1974.

[105] B. Uzzan, J. Campos, M. Cucherat, P. Nony, J. P. Boissel, and G. Y. Perret, "Effects on bone mass of long term treatment with thyroid hormones: a meta-analysis," *Journal of Clinical Endocrinology and Metabolism*, vol. 81, no. 12, pp. 4278–4289, 1996.

[106] L. J. Melton III, E. Ardila, C. S. Crowson, W. M. O'Fallon, and S. Khosla, "Fractures following thyroidectomy in women: a population-based cohort study," *Bone*, vol. 27, no. 5, pp. 695–700, 2000.

[107] J. J. Nicholls, M. J. Brassill, G. R. Williams, and J. H. D. Bassett, "The skeletal consequences of thyrotoxicosis," *The Journal of Endocrinology*, vol. 213, pp. 209–221, 2012.

[108] M. L. Quan, J. L. Pasieka, and O. Rorstad, "Bone mineral density in well-differentiated thyroid cancer patients treated with suppressive thyroxine: a systematic overview of the literature," *Journal of Surgical Oncology*, vol. 79, no. 1, pp. 62–69, 2002.

[109] R. W. Flynn, S. R. Bonellie, R. T. Jung, T. M. MacDonald, A. D. Morris, and G. P. Leese, "Serum thyroid-stimulating hormone concentration and morbidity from cardiovascular disease and fractures in patients on long-term thyroxine therapy," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 1, pp. 186–193, 2010.

[110] M. Appetecchia, "Effects on bone mineral density by treatment of benign nodular goiter with mildly suppressive doses of L-thyroxine in a cohort women study," *Hormone Research*, vol. 64, no. 6, pp. 293–298, 2005.

[111] C. Christiansen and R. Lindsay, "Estrogens, bone loss and preservation," *Osteoporosis International*, vol. 1, no. 1, pp. 7–13, 1990.

[112] G. L. Anderson and M. Limacher, "Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the women's health initiative randomized controlled trial," *Journal of the American Medical Association*, vol. 291, no. 14, pp. 1701–1712, 2004.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 351670, 6 pages
http://dx.doi.org/10.1155/2013/351670

Clinical Study

A Study on Bone Mass in Elderly Chinese Foot-Binding Women

Yi Pan,[1] Ling Qin,[2] Mian Xu,[1] Yin He,[1] Juan Bao,[1] Xian Guo,[1] and Jun Shu[1]

[1] *Department of Endocrinology, The Second Affiliated Hospital of Kunming Medical University, No. 374 The Dianmian Avenue, Wuhua, Kunming, Yunnan Province 650101, China*
[2] *Department of Orthopedics, Medical College of the Chinese University of Hong Kong, Shatin, New Territories, Hong Kong*

Correspondence should be addressed to Jun Shu; shujun37@yahoo.com.cn

Received 9 January 2013; Accepted 11 June 2013

Academic Editor: Ling-Qing Yuan

The aim of this study is to understand the influences of the social custom of foot binding on female osteoporosis by means of comparing and analyzing the lumbar vertebrae and hip bone mass differences between the foot-binding aged women and unbound women of the same age at Qujing District of Yunnan Province. Of the examined people, 81.37% suffer from osteoporosis on the basis of lumbar vertebra (L1–L4) and femoral neck BMD, of which 82.14% for the foot-binding group and 80.44% for the unbound group. There is no statistical difference for the osteoporosis morbidity of the two groups. Compare the BMD value for various vertebrae, femoral neck, and rehabilitation of the two groups and find the BMD value for the other parts have no statistical difference except the BMD value of L1 centrum, which shows that foot binding does not significantly influence the overall bone mineral density of foot-binding women.

1. Introduction

Foot binding is a special custom from the ancient times to modern times; mainly for women of Han nationality, they use cotton and silk to swathe the feet of girls to make the front end sharp and restrict the free growth of both feet. With the gradual growth and development of feet, the arches of foot are extruded high and eventually form the special foot shape of "three-inch bound feet."

Foot binding violates the normal development of limbs but is also an important education, morality, behavior, beautifying, and life standard for Han women for thousands of years. With the changes and progress of times, modern civilization has thoroughly abandoned the deformity custom which binds women, the remaining foot-binding women in our society are gradually disappear with time going by, and the foot binding is going to meet its death, which has spread throughout China for thousands of years. To avoid the unique Chinese traditional custom disappearing with time passing by and to reserve more relevant social data of foot-binding behavior, we made a survey on the foot-binding women of Yunnan Region from the medical view.

2. Data and Method

2.1. Exclusion Standard. Take inquiry for the selected foot-binding aged women and unbound women at Sanchahe Town, Luliang County, Qujing City, and Yunnan Province, to exclude diseases that may influence bone metabolism, such as liver and kidney disease, diabetes mellitus, thyroid and parathyroid disease, metabolic bone disease, ovariectomization, and subtotal gastrectomy; to exclude these with the medical history that they have been proved to have vertebral fracture and femoral fracture by X-ray examination in recent 3 months; to exclude these taking the drugs that may affect bone metabolism in recent 3 months, such as selective estrogen regulator, calcitonin, compound steroid hormones, bisphosphonate, thyroxine, parathyrin, or other antiosteoporosis drugs.

2.2. General Data. Totally 308 qualified testers are selected, who are all local countryside people of Han nationality. Of them, there are 204 foot-binding women, aged between 65 and 88, and averagely 76 and 104 unbound women, aged between 64 and 87, averagely 73. Select 102 healthy testers

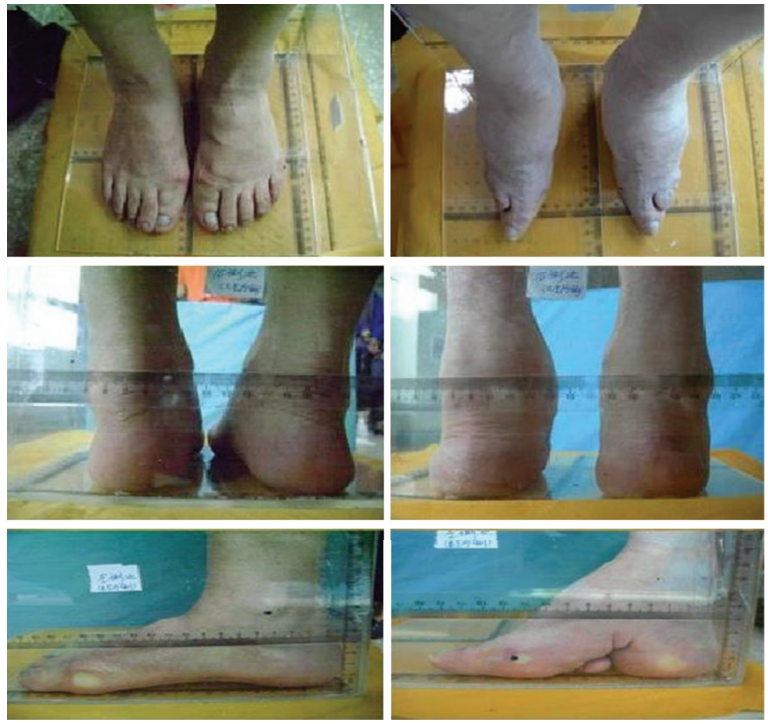


Figure 1: Comparison between the foot-binding deformity and normal foot.

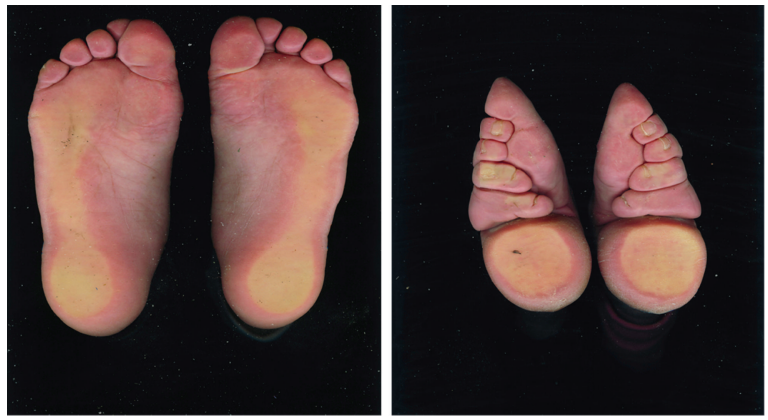


Figure 2: Comparison of pelma print scanning picture between the foot-binding deformity and normal foot.

from the above 308 testers for bone mass and bone density measurement, of which 56 are from the foot-binding group, aged between 67–85, and averagely 74.8; 46 are from the unbound group, aged between 66 and 85, and averagely 72.5.

2.3. Test Method. Measure the height, weight, waistline, and hipline of all testers and then, use body mass index and waist-hip ratio (WHR) for evaluation and comparative analysis. Bone mass and bone density measurement adopts GE-Lunar-Prodigy DXA for the lumber vertebra (L1–L4) and hip examination.

3. Result

3.1. Foot-Binding Shape Comparison. By appearance-shape comparison between the foot-binding deformity and normal foot, the photo comparison of both feet of the testers is shown in Figure 1, and pelma print scanning picture comparison is shown in Figure 2.

From Figures 1 and 2, we can see that the appearance-shape characteristics of the foot-binding deformity are as follows: (1) foot is of triangle shape, with small front part which is similar to the top end of an awl, and large heel which is similar to a circular; (2) the 2nd–5th toes bend inwards at the pelma, the forefoot extrudes and draws close to the heel,

Table 1: General health indicators of normal foot group ($n = 104$) and foot-binding group ($n = 204$).

	Normal foot group ($\overline{x} \pm s$)	Foot-binding group ($\overline{x} \pm s$)	t	P
Age (Years)	72.95 ± 4.50	75.99 ± 4.58	−5.482	0.001
Height (cm)	149.32 ± 6.12	148.36 ± 6.03	1.302	0.194
Weight (kg)	49.03 ± 8.55	45.73 ± 7.33	3.485	0.001
BMI	21.95 ± 3.36	20.79 ± 3.19	2.928	0.004
Waistline (cm)	72.81 ± 7.92	72.37 ± 7.68	0.465	0.642
Hipline (cm)	85.55 ± 6.46	84.46 ± 5.60	1.512	0.131
Left vision	0.75 ± 1.14	0.55 ± 0.94	1.605	0.110
Right vision	0.74 ± 1.16	0.55 ± 0.96	1.465	0.144
Age of menopause (years)	47.49 ± 4.52	48.05 ± 3.72	1.160	0.247
Gravidity	6.77 ± 2.22	7.64 ± 2.86	2.689	0.008
Parity	6.21 ± 2.13	7.02 ± 2.63	2.724	0.007
Lactation month time	127.80 ± 58.35	125.57 ± 61.07	−0.304	0.762
Footbinding age (years)		8.39 ± 3.16		

Table 2: Bodily form contrast of normal foot group ($n = 104$) and foot-binding group ($n = 204$).

	BMI				WHR	
	Slants thin	Normal	Overweight	Fat	Noncentral obesity	Central obesity
Normal foot group (%)	15 (14.7)	61 (58.7)	25 (24.5)	3 (2.9)	54 (51.9)	50 (48.1)
Foot-binding group (%)	56 (27.5)	112 (54.9)	30 (14.7)	6 (2.9)	96 (47.1)	108 (52.9)
χ^2	8.605				0.472	
P	0.035				0.492	

there is a horizontal hollowing at the middle of the pelma, and the 5th toe is usually pressed in the hollowing; (3) arch of foot is extruded to rise, acrotarsium bulges upwards, and the forefoot has obvious plantar flexion; (4) after binding, the whole foot is obviously smaller than normal foot.

3.2. General Health Condition Analysis. Divide the testers into the foot-binding group and unbound group and adopt the statistical method of group T examination to compare the differences in health index of the groups, such as height, weight, weight index, waistline, hipline, binocular vision, age of menopause, pregnancy and parity times, and breast-feed time. For results, please refer to Table 1.

From Table 1, we can see that there is statistical difference in the weight, weight index, and pregnancy and parity times between the two groups ($P < 0.05$). The weight and weight index of the foot-binding group are lower than the unbound group, and the average pregnancy and parity times are higher than the unbound group.

Process the obtained height, weight, waistline, and hipline data from measurement and adopt weight index (BMI) and waist-hip ratio (WHR) for comparative analysis. BMI = weight (kg)/height $(m)^2$; WHR = waistline (cm)/hipline (cm). Divide the testers into 4 groups according to BMI value as follows: thin: BMI < 18.5; normal: 18.5 ≤ BMI < 24; overweight: 24 ≤ BMI < 28; obesity: BMI ≥ 28; take WHR = 0.8 as the boundary to divide the testers into two groups, that is, noncentral obesity and central obesity, and adopt Chi-square test to compare the body shape differences between the foot-binding group and unbound group, and the result is shown in Table 2. Adopt the statistical method of group T examination to compare the BMI and WHR differences of the foot-binding group and unbound group, and the result is shown in Table 3. Adopt the grouping method according to ages to compare BMI and WHR differences of each age group. The age groups include the following: group below 70, group of 71–75, group of 76–80, and group above 81. After grouping according to ages, the comparison result of BMI and WHR of the foot-binding group and unbound group is shown in Table 4.

Table 3: Comparison of BMI and WHR of normal foot group ($n = 104$) and foot-binding group ($n = 204$).

	Foot-binding group	Normal foot group	T	P
BMI	20.79 ± 3.19	21.95 ± 3.36	−2.928	0.004
WHR	0.856 ± 0.055	0.850 ± 0.052	0.859	0.391

For the above data, we can see, in general, the foot-binding group is thinner than the unbound group, weight index is more ideal, but WHR has no statistical difference (Tables 2 and 3). After grouping according to ages, the comparison result of BMI and WHR of the foot-binding group and unbound group has no statistical difference, which shows that, foot binding does not influence the height, weight, waistline, or hipline of foot-binding women (Table 4).

3.3. Osteoporosis Morbidity Comparison. Take the lumber vertebra (L1–L4) or femoral neck BMD-T value as standard (take whichever is the lower of the both) for DXA examination testers, if T value is ≥−1.0, the bone mass is normal; if

Table 4: Body comparison according to age groups between normal foot group (n = 104) and foot-binding group (n = 204).

Grouping	BMI		T	P	WHR		T	P
	Foot-binding group	Normal foot group			Foot-binding group	Normal foot group		
Group below 70	21.64 ± 3.71	22.06 ± 2.92	−0.465	0.644	0.842 ± 0.065	0.847 ± 0.052	−0.325	0.747
Group of 71–75	21.75 ± 2.95	22.40 ± 3.62	−1.691	0.092	0.844 ± 0.052	0.857 ± 0.052	−1.335	0.184
Group of 76–80	20.47 ± 3.14	20.81 ± 2.97	−0.454	0.651	0.843 ± 0.050	0.835 ± 0.050	1.328	0.185
Group above 81	21.16 ± 3.48	22.66 ± 5.01	−0.858	0.396	0.878 ± 0.055	0.879 ± 0.060	−0.009	0.993

Table 5: Osteoporosis diagnosis rate between normal foot group (n = 46) and foot-binding group (n = 56).

	Normal	Osteopenia	Osteoporosis
Normal foot group	2.17%	17.39%	80.44%
Foot-binding group	3.57%	14.29%	82.14%
Whole	2.94%	15.69%	81.37%
χ^2			0.049
P			0.826

T value is between −1 ~ −2.5, it is osteopenia; if T value is ≤−2.5 or more, it is osteoporosis. The statistical result for the osteoporosis morbidity between the foot-binding group and the unbound group is shown in Table 7. The overall osteoporosis morbidity for all testers is 81.37%, of which 82.14% for the foot-binding group and 80.44% for the unbound group. Adopt Chi-square test to compare the osteoporosis morbidity between the two groups, and there is no statistical difference in the result (Table 5), which shows, the foot binding does not influence the osteoporosis morbidity for local aged women, but the aged women, especially rural women, have a high osteoporosis morbidity (Table 5).

3.4. DXA Result Analysis. Carry out DXA examination for L1, L2, L3, L4, L1–L4, femoral neck, and rehabilitation of 102 health testers and adopt the statistical method of group T examination to compare the difference between the two groups on BMC of each lumber interbody, BA, and BMD value of each lumber interbody, femoral neck, and rehabilitation (Table 6).

We can see that (1) for lumbar vertebra, the two groups have no statistical difference for L2 and L3 bone size but have statistical differences on BMD value of L1 vertebral body, and the other values have no statistical difference; (2) the whole lumbar vertebra (L1–L4) and femoral neck, rehabilitation BMD value, and T value have no statistical difference, which shows that the foot binding does not influence the overall BMD of the lumbar vertebra and hip of the foot-binding people, and the two groups have equivalent risks of lumbar vertebra and hip fracture.

Meanwhile, no matter for the foot-binding group or the unbound group, their bone mineral density is significantly lower than femoral neck, which shows that, for aged women, the risk of lumber vertebra fracture is higher than that of femoral neck (Table 7).

As the morbidity of osteoporosis has clear relevance with age [1, 2], so age grouping is adopted for further comparison. The age groups include the following: group below 70, group of 71–75, group of 76–80, and group above 81, with statistical method of independent sample t examination. The result is shown in Table 8.

From the results in Table 8, we can see, after grouping according to ages, the two groups have no statistical difference for lumbar vertebra T value and hip T value comparison, which shows that there is little difference in the BMD of the lumber vertebra and hip of testers of both groups, foot binding does not obviously influence the BMD of the lumber vertebra and hip of foot-binding people, and the two groups have equivalent risks of lumbar vertebra and hip fracture.

4. Discussion

4.1. Appearance-Shape Observation for Foot-Binding Deformity. By visual observation for photo of both feet and pelma print canning of the testers to compare and analyze the appearance shape of the foot-binding deformity and normal foot, we can find the appearance-shape characteristics of foot-binding deformity as follows: (1) foot is of triangle shape, with small front part which is similar to the top end of an awl, and large heel which is similar to a circular; (2) the 2nd–5th toes bend inwards at the pelma, the forefoot extrudes and draws close to the heel, there is a horizontal hollowing at the middle of the pelma, and the 5th toe is usually pressed in the hollowing; (3) arch of foot is extruded to rise, acrotarsium bulges upwards, and the forefoot has obvious plantar flexion; (4) after binding, the whole foot is obviously smaller than normal foot.

4.2. DXA Examination Result for Lumber Vertebra and Hip. It is theoretically predicted that, because of foot binding, with action difficulty and the limited scope of activities, they rarely take part in social activities and housework; thus, the bone mass of the lumbar vertebra and hip of foot-binding women should be obviously lower than that of unbound women.

According to the actual examination result, after grouping according to ages, there is no statistical difference for lumbar vertebra T value and hip T value of both groups, which shows that there is little difference in the BMD of the lumber vertebra and hip of testers of both groups, foot binding does not obviously influence the BMD of the lumber vertebra and hip of foot-binding people, and the two groups have equivalent risks of lumbar vertebra and hip fracture. It may be because the testers are all from rural areas; after foot binding, no matter what physiological differences they have, there is no obvious difference between labor opportunity and labor intensity of them. Especially for testers, at the youth age when

Table 6: Lumbar spine and hip DXA data analysis between normal foot group ($n = 46$) and foot-binding group ($n = 56$).

	Normal foot group	Foot-binding group	T	P
BMC g				
L1	6.870 ± 1.492	6.463 ± 1.576	−1.320	0.19
L2	7.717 ± 1.875	7.685 ± 1.763	−0.088	0.93
L3	9.247 ± 2.251	9.567 ± 2.255	0.708	0.48
L4	10.833 ± 2.469	10.951 ± 2.426	0.24	0.811
L1–L4	34.668 ± 7.307	34.401 ± 7.306	−0.182	0.856
BA cm^2				
L1	9.768 ± 1.211	10.170 ± 1.217	1.653	0.101
L2	10.757 ± 1.446	11.329 ± 1.588	1.87	0.064
L3	12.049 ± 1.616	12.712 ± 1.532	2.109	0.037
L4	13.521 ± 1.999	13.809 ± 1.894	0.742	0.46
L1–L4	46.095 ± 4.940	47.735 ± 5.472	1.563	0.121
BMD g/cm^2				
L1	0.702 ± 0.119	0.631 ± 0.113	−0.306	0.003
L2	0.713 ± 0.126	0.676 ± 0.114	−1.548	0.125
L3	0.764 ± 0.134	0.749 ± 0.135	−0.546	0.586
L4	0.805 ± 0.156	0.792 ± 0.137	−0.445	0.658
L1–L4	0.750 ± 0.126	0.718 ± 0.117	−1.317	0.191
Femoral neck	0.664 ± 0.092	0.647 ± 0.085	−0.939	0.35
Rehabilitation	0.737 ± 0.118	0.709 ± 0.095	−1.348	0.181
T value (L1–L4)	−3.02 ± 1.01	−3.257 ± 0.96	−1.206	0.231
T value (femoral neck)	−1.615 ± 0.96	−1.870 ± 0.79	−1.472	0.144

Table 7: The lumbar spine and femoral neck bone mineral density (normal foot group $n = 46$; foot-binding group $n = 56$).

	T value (L1–L4)	T value (femoral neck)	T	P
Normal foot group	−3.02 ± 1.01	−1.615 ± 0.96	6.839	0.000
Foot-binding group	−3.257 ± 0.96	−1.870 ± 0.79	8.349	0.000

the bone mass accumulates, they all need to take part in labor of the same intensity to make life, thus, making the influence of foot binding on body bone mass to the minimum. The result demonstrates again from another aspect that exercise and sports play an important role in accumulating peak bone mass and delaying bone mass declination [3–5].

Besides, L1 centrum BMD value of the foot-binding group is significantly lower than the normal people; maybe we can deduct that foot binding has little influence on the lower part of axial skeleton of human body because foot binding changes the way women walk. Foot-binding people walk with both heels down to the ground; the driving force is all on the muscle of the thigh and depends on the activity of knee joint. In this case, the muscle of hip and thigh is more developed, therefore, the lower part of lumbar vertebra always drives exercise, and BMD declination is not obvious. Whether foot binding influences BMD of the medium and upper part bone of the axial skeleton can be further confirmed by thoracic vertebra DXA examination.

4.3. Measuring Result of Weight Index and WHR. Current researches have proved that height and weight are important factors to influence BMD of human body [6]. WHR is the ratio between waistline and hipline, and also the index to predict obesity at early stage [7]. Theoretically, after foot binding, people shall always suffer from continuous pain, and their diet and sleep are both disturbed, causing great influence on their body growth and development. Therefore, the weight index of the foot-binding group is lower than the unbound group, and the testers of the foot-binding group are generally light weight group (BMI < 18.5).

Secondly, the 2nd–5th toes of the pelma of foot-binding people twine at the pelma, and the forefoot only has the halluces to carry the load, which changes the structure of 3 points of normal foot which carry the load. Besides, the foot arch on the pelma to buffer the force disappears, while walking, their heel falls down to the ground, and the strength of the thigh is used to take a step; eventually, thigh muscle is developed, calf muscle shrinks, and the stress is applied to the knee joint and hip joint until hip joint changes. As a result, after many years' foot-binding life, the hip line of the foot-binding people should be larger than that of the unbound people, and the WHR of the foot-binding people should be smaller than that of the unbound people. But the details for how the skeleton of hip joint is changed still need further comparison and analysis after double hip plain film examination.

From the measurement result, we can see that the testers from the foot-binding group are generally thinner than the testers from the unbound group, and their weight index is more ideal. But after grouping according to ages, there is no statistical difference in weight index and WHR of both groups, which shows that foot binding does not obviously

Table 8: Comparison of T values of the lumbar spine and hip T values in the different age groups between normal foot group and foot-binding group.

Grouping	Lumbar T value		T	P	Hip T value		T	P
	Foot-binding group	Normal foot group			Foot-binding group	Normal foot group		
Group below 70	−2.94 ± 0.57	−2.96 ± 1.16	0.029	0.977	−1.73 ± 0.69	−1.40 ± 1.21	−0.669	0.511
Group of 71–75	−3.29 ± 1.07	−3.21 ± 1.00	−0.291	0.773	−1.85 ± 0.93	−1.84 ± 0.84	−0.020	0.984
Group of 76–80	−3.35 ± 1.01	−2.69 ± 0.84	−1.540	0.137	−1.93 ± 0.65	−1.44 ± 0.68	−1.672	0.108
Group above 81	−3.15 ± 0.61	−2.75 ± 0.21	−0.852	0.442	−1.98 ± 0.69	−1.55 ± 0.78	−0.692	0.527

influence the height, weight, waistline, and hipline of the foot-binding people. No matter they are from the foot-binding group or the unbound group, their weight index and WHR are basically within the ideal scope.

5. Conclusion

Although foot binding influences the walking posture and even life of the foot-binding people by means of changing foot shape, it has little influence on the bone mass of the whole body of the foot-binding people, except partial bones. The aged women of the rural area of Qujing District of Yunnan Province suffer from a high osteoporosis morbidity, and their risk for lumbar vertebra fracture is higher than the femoral neck.

References

[1] W. Yuan, "Affect of body fat on DXA bone density measurement results and diagnosis of osteoporosis," *International Journal of Pathology and Clinical Medicine*, vol. 29, no. 3, pp. 272–276, 2009.

[2] X. Yueyong, S. Gang, and H. Boxun, "Quantitative CT measurement of the value of the normal human vertebrae aging changes of bone mineral density predict senile osteoporotic fractures," *Chinese Journal of Gerontology*, vol. 15, no. 4, pp. 201–204, 1995.

[3] K. Kerschan, Y. Alacamlioglu, and J. Kollmitzer, "Functional impact of unvarying exercise program in women after menopause," *American Journal of Physical Medicine & Rehabilitation*, vol. 77, no. 4, pp. 326–332, 1998.

[4] L. Welsh and O. M. Rutherford, "Hip bone mineral density is improved by high-impact aerobic exercise in postmenopausal women and men over 50 years," *European Journal of Applied Physiology and Occupational Physiology*, vol. 74, no. 6, pp. 511–517, 1996.

[5] B. A. Michel, D. A. Bloch, and J. F. Fries, "Weight-bearing exercise, overexercise, and lumbar bone density over age 50 years," *Archives of Internal Medicine*, vol. 149, no. 10, pp. 2325–2329, 1989.

[6] Z. Gang, "Influencing factors of the human body bone mineral density," *Foreign Medical Sciences*, vol. 31, no. 3, pp. 184–187, 2004.

[7] S. Yusuf, S. Hawken, S. Ôunpuu et al., "Obesity and the risk of myocardial infarction in 27 000 participants from 52 countries: a case-control study," *The Lancet*, vol. 366, no. 9497, pp. 1640–1649, 2005.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 786574, 6 pages
http://dx.doi.org/10.1155/2013/786574

Research Article

Puerarin Suppress Apoptosis of Human Osteoblasts via ERK Signaling Pathway

Ling-juan Liu,[1] Li-qun Liu,[1] Tao Bo,[1] Shi-jun Li,[1] Zhen Zhu,[1] Rong-rong Cui,[2] and Ding-an Mao[1]

[1] *Department of Pediatrics, The Second Xiang-Ya Hospital, Central South University, 139 Middle Renmin Road, Changsha, Hunan 410011, China*
[2] *Institute of Metabolism and Endocrinology, The Second Xiang-Ya Hospital, Central South University, 139 Middle Renmin Road, Changsha, Hunan 410011, China*

Correspondence should be addressed to Rong-rong Cui; rongrongcui530@hotmail.com and Ding-an Mao; mda5611@sina.com

Received 3 January 2013; Revised 31 March 2013; Accepted 10 April 2013

Academic Editor: Guang-Da Xiang

Puerarin, the main isoflavone glycoside extracted from Radix Puerariae, is an isoflavone traditional Chinese herb. Previous studies have demonstrated that puerarin could regulate osteoblast proliferation and differentiation to promote bone formation. However, the effect of puerarin on the process of human osteoblasts (hOBs) apoptosis is still unclear. In this study, we detected the function of puerarin on serum-free-induced cell apoptosis using ELISA and TUNEL arrays and then found that the mortality of hOBs was significantly decreased after exposure to 10^{-10}–10^{-6} M puerarin and reached the maximal antiapoptotic effect at the concentration of 10^{-8} M. In addition, compared with the control group, puerarin notably increased the Bcl-2 protein levels while it decreased the Bax protein levels in the hOBs in a dose-dependent way. 10^{-7} M puerarin decreased the Bax/Bcl-2 ratio with a maximal decrease to 0.08. Moreover, puerarin activated ERK signaling pathways in hOBs, and the antiapoptotic effect induced by puerarin was abolished by incubation of ERK inhibitor PD98059. Similarly, the estrogen receptor antagonist ICI182780 also suppressed the inhibitory effect of puerarin on hOBs apoptosis. In conclusion, puerarin could prevent hOBs apoptosis via ERK signaling pathway, which might be effective in providing protection against bone loss and bone remolding associated with osteoporosis.

1. Introduction

Osteoblasts, mononuclear specialized cells derived from mesenchymal precursor cells, are responsible for bone formation, deposition, and mineralization [1, 2], playing an essential role in the maintenance of the stability of bone microarchitecture. Osteoblast apoptosis, induced by various pathological and physiological factors (e.g., estrogen loss, glucocorticoids, weightlessness, and aging), breeds a series of bone disorders. Osteoporosis is the most prevalent bone disorder affecting the elders, which is characterized by an imbalance between bone formation and bone resorption [3–5]. Several studies have showed that apoptosis might be the third most common cause of osteoporosis, and 60–80% of osteoblasts were estimated to originally assembled at the resorption pit die by apoptosis [6]. Therefore, apoptosis is generally served as the pivotal target for prevention and/or ameliorate osteoporosis.

Puerarin, 7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-β-D-glucopyranoside ($C_{12}H_{20}C_9$), is one of the major isoflavonoid compounds extracted from the root of a wild leguminous creeper [7, 8]. It possesses estrogen-like structure and moderates estrogenic activity. As a famous phytoestrogen, current studies have established that puerarin provides a strong protection against osteoporosis through facilitating osteoblast proliferation and differentiation [9, 10]. Although it acts as an important regulatory factor for cell death, the role of puerarin on osteoblast apoptosis and its underlying mechanism of action are still unclear. As we know osteoblasts apoptosis is an extremely complicated event; a series of proteins and signaling pathways (e.g., Bcl-2 family proteins, ERK, MAPK, APJ/PI3-K/Akt, JAK2, and Fas) are reported to be involved in this process in vitro and in vivo [11–15]. In present study, we aim to detect the impact of puerarin on the serum-deprivation-induced hOBs apoptosis

along with observing the expression of Bcl-2 and Bax protein using western blotting and the cell signal pathway involved.

2. Materials and Methods

2.1. Reagents. Puerarin and 17β-estradiol were purchased from Sigma, Inc. (USA). β-actin, anti-Bcl-2, Bax, ERK, p-ERK antibodies, anti-mouse, and -rabbit IgG peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology Inc. (Waltham, MA, USA). ICI 182780 and PD98059 were purchased from Calbiochem Corp. (San Diego, CA, USA).

2.2. Cell Cultures. hOBs were obtained from traffic accident victims suffering surgery, and this procedure was approved by the local research ethics committee as previously mentioned [16]. Cells isolated from femur were cultured in a-MEM medium (Gibico-BRL. Corp., NY, USA) containing 15% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were identified as osteoblasts by the expression of ALP, collagen type I and osteocalcin, and formation of mineralized nodules as lately described [12].

To determine the effect of puerarin on apoptosis of hOBs, the cells were seeded for 24 h followed by culturing for 24 h in serum-free medium, then treated with 0, 10^{-9} M, 10^{-8} M, 10^{-7} M puerarin, or 17 β-estradiol (E2, 10 μM) for 48 h. To analyze the effect of ERK or estrogen receptor (ER) inhibition on hOB apoptosis, hOBs were pretreated with 10 μM ERK inhibitor PD98059 or ER antagonist ICI 182780 for 3 h anterior to incubation with 10^{-8} M puerarin for 48 h.

2.3. Western Blot Analysis. Immunoblotting was carried out as before [17]. Total protein was extracted with RIPA lysis buffer (Beyotime, China), then protein concentration determined by a Bradford assay, and equal amounts of protein were loaded onto SDS-PAGE and then transferred to PVDF membranes (Invitrogen, Carlsbad, CA, USA). Thereafter, the membranes were blocked with 5% nonfat milk in PBS for 1 h at room temperature and then incubated with anti-Bcl-2, anti-Bax (1 : 500), or anti-β-actin (1 : 1000) antibodies overnight at 4°C. Resultant protein bands after incubation with a proper secondary antibody were visualized by chemiluminescence. The absorbance values of target proteins were analyzed through Gel-Pro 4.0 gel image analysis software. The absorbance ratio of each protein to internal reference was represented as the relative amount of target proteins [18].

2.4. Measurement of ERK Activation. hOBs were treated with 10^{-8} M puerarin for 0–45 min. The cell layers were washed twice with cold PBS and then lysed with a buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 mM NaH_2PO_4, 10% glycerol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM ABSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Western blot analysis was carried out as previously described [19, 20]. Equal amount of proteins were transferred onto PVDF membranes, then incubated with anti-ERK or anti-p-ERK monoclonal antibodies (1 : 500). The ECL detection kit was used for detection.

2.5. Measurement of Cell Apoptosis

2.5.1. Cell Death ELISA Detection. Cell death ELISA detection was performed for detecting the apoptosis levels as previously described [21]. Cell death detection ELISA kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany) was used for detection, according to the kit protocol. Briefly, cells were plated at a density of 10,000 cells/well in 24-well plates for 1 day followed by culture in serum-free medium for 48 h in the absence or presence of 0–10^{-6} M puerarin or 10 μM E2. Cells were rinsed with PBS and incubated with 0.5 mL of lysis buffer at 4°C for 30 min, then centrifuged for 10 min at 15,000 rpm. Aliquots of the supernatant were tested for the rate of apoptosis through the cell death detection kit.

2.5.2. TUNEL Assay. Terminal deoxynucleotidyl transferase-mediated deoxyribonucleotide triphosphate nick end-labeling (TUNEL) was generally used to assess cell death. hOBs were washed with PBS after cultured in serum-deprivation medium for 48 h in the absence or presence of 10^{-8} M puerarin and then fixed with 4% paraformaldehyde for 30 min at room temperature. Thereafter, the cells were incubated with the TUNEL reaction mixture(Roche Molecular Biochemicals, Indianapolis, IN) for 60 min at 37°C followed by labeling with fluorescein isothio cyanate (FITC)-conjugated anti-fluorescein anti-goat antibody (Fab fragment) for an additional 30 min. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Finally, TUNEL-positive cells were photographed on an Olympus microscope.

2.6. Statistical Analysis. The data are presented as mean ± standard deviation (SD). Statistical analyses of the data were performed through one-way analysis of variance (ANOVA) and the LSD post hoc test for multiple comparisons. $P < 0.05$ was considered the statistical significant difference. All experiments were repeated at least three times.

3. Results

3.1. Puerarin Protects the hOBs from Serum-Free-Induced Apoptosis. Our study used ELISA assays to assess hOBs apoptosis cultured in serum-depravation medium for 48 h in the presence of 0–10^{-6} M puerarin or 10 μM E2. Results showed that after exposure to puerarin the apoptotic cells at 10^{-10} M (2.23 ± 0.14 ELISA absorbance units), 10^{-9} M (1.89 ± 0.16 ELISA absorbance units), 10^{-8} M (1.54 ± 0.13 ELISA absorbance units), 10^{-7} M (1.62 ± 0.15 ELISA absorbance units), and 10^{-6} M (1.58 ± 0.12 ELISA absorbance units) puerarin were less than those of the negative control group (2.51 ± 0.11 ELISA absorbance units, all $P < 0.05$), while they were higher than the positive group exposed to 10 μM E2 (1.30 ± 0.12 ELISA absorbance units, $P < 0.05$). Puerarin

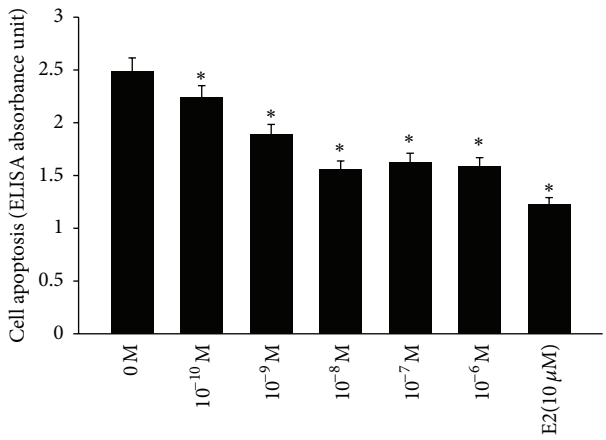


Figure 1: Puerarin protected the hOBs from serum-free-induced apoptosis. Cells were treated with 0–10^{-6} M puerarin, and cells exposed to 10 μM E2 served as the positive control. Apoptosis were measured by ELISA according to the kit specification. The bars represent the mean ± SD ($n = 6$), $^{*}P < 0.05$, as compared with the control cells.

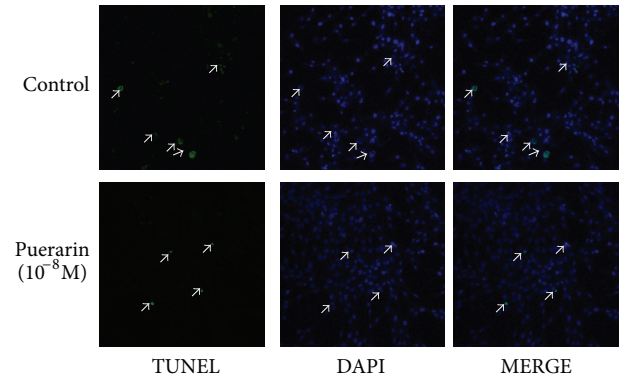


Figure 2: Effect of puerarin on apoptosis of hOBs measured by TUNEL assay. Cells were exposed to 10^{-8} M puerarin for 48 h, while the control group was incubated with serum-free medium for 48 h. Apoptotic nuclei were detected by TdT-mediated dUTP nick end-labeling (TUNEL). Arrows show apoptotic cells. Original magnification ×100.

reached the maximal antiapoptotic effect at the concentration of 10^{-8} M (Figure 1). The results of TUNEL assay also indicated that 10^{-8} M puerarin significantly decreases hOBs apoptosis caused by serum deprivation compared to the control ($P < 0.05$, Figure 2).

3.2. Effects of Puerarin on the Expression of Bcl-2 and Bax in hOBs. Bcl-2 and Bax are the essential members of Bcl-2 family which involved in the process of apoptosis. Western blot analysis was used to detect the expression of Bcl-2 and Bax in hOBs incubated with 0, 10^{-9} M, 10^{-8} M, and 10^{-7} M puerarin. As a result, we found that puerarin increased the levels of Bcl-2 protein, while it decreased the expression of Bax in hOBs in a dose-dependent manner (all $P < 0.05$, Figure 3). The Bax/Bcl-2 ratio was set to 1 in the control group, and 10^{-7} M puerarin could downregulate the Bax/Bcl-2 ratio with a maximal decrease to 0.08 (all $P < 0.05$, Figure 3).

3.3. Puerarin Activated ERK Signaling Pathway in hOBs. ERK signaling cascades is a classic pathway involved in the regulation of cell death. According to our results, 10^{-8} M was the optimum concentration for puerarin to produce the best antiapoptotic protection. Therefore, we used western blotting to confirm the effects of 10^{-8} M puerarin on ERK phosphorylation and found that the levels of phosphorylated ERK was upregulated after 5 min incubation with puerarin compared with the control group ($P < 0.05$). Additionally, this effects was time-dependent with the peak activation of ERK at 45 min of incubation ($P < 0.05$, Figure 4).

3.4. ERK Signaling Pathway and Estrogen Receptors Mediated the Antiapoptotic Effects of Puerarin in hOBs. After detecting that puerarin could activate ERK signaling pathway in hOBs, we further verified whether the puerarin-mediated activation of ERK participates in apoptosis. We examined apoptosis after incubation with 10^{-8} M puerarin and/or PD98059 by ELISA, then detected that the protection effect of puerarin on hOBs was blocked by PD98059, which had little effects on apoptosis alone ($P < 0.05$, Figure 5). Moreover, puerarin was proved to have estrogen-like structure and moderate estrogenic activity, and we found here that ICI 182780 did not affect the process of apoptosis but significantly suppressed the antiapoptotic effect of puerarin on hOBs ($P < 0.05$, Figure 5).

4. Discussion

Osteoblast apoptosis is generally regarded as a key component of bone turnover, repair, and regeneration [22]. It is reported that approximately 50–70% of osteoblasts undergo apoptosis during bone regeneration [23]. Puerarin, an isoflavone traditional Chinese herb, is reported to significantly facilitate the survival rate of osteoblasts, and the puerarin-treated rats also displayed a higher rate of bone formation in the osteoblast implants than the control, suggesting that puerarin might regulate osteoblast proliferation and differentiation to promote bone formation in osteoblast implants [24, 25]. In present research, we demonstrated that puerarin alleviates apoptosis of hOBs induced by serum deprivation through activating ERK signaling pathway.

Cell apoptosis is an essential process in maintaining the homeostasis under normal conditions [26]. Members of the Bcl-2 family, including Bcl-2 and Bax, are the main regulators of apoptosis which promote (Bax) or inhibit (Bcl-2) apoptosis [27]. Each of them regulates apoptosis independently [28]. Furthermore, Bcl-2 protein forms heterodimer complexes with Bax proteins, leading to the release of cytochrome C from the mitochondria and induction of cell apoptosis [29]. Previous reporters have proved that puerarin acts on a variety of apoptosis through regulating the expression of Bcl-2 family proteins in vitro, including vascular endothelial cells, vascular smooth muscle cells, and human neurons [30–32]. Here we

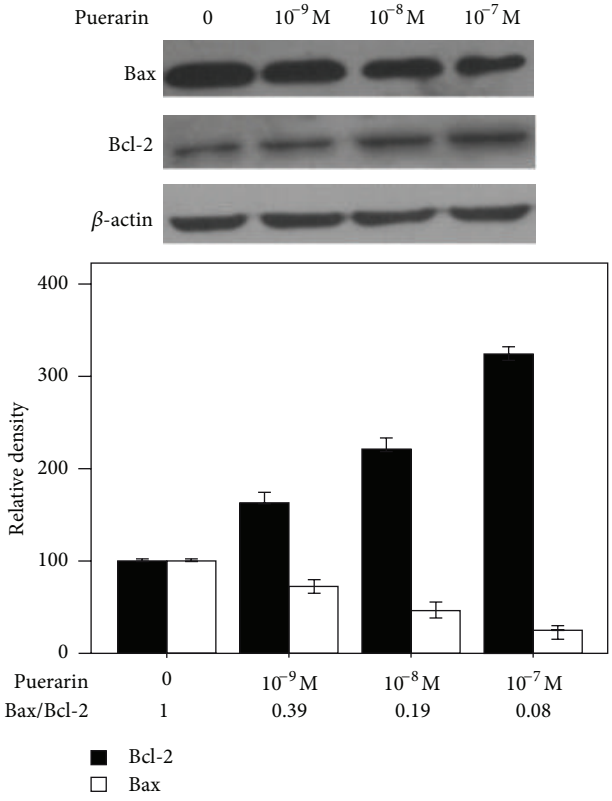


Figure 3: Effects of puerarin on the expression of Bcl-2 and Bax in hOBs. Cells were treated with 0, 10^{-9} M, 10^{-8} M, and 10^{-7} M puerarin for 48 h before collecting proteins. Cell lysates were subjected to western blot analysis and incubated with anti-Bax, anti-Bcl-2, or anti-β-actin monoclonal antibodies. Gel-Pro 4.0 gel image analysis software was used to analyze the absorbance values of Bcl-2 and Bax.

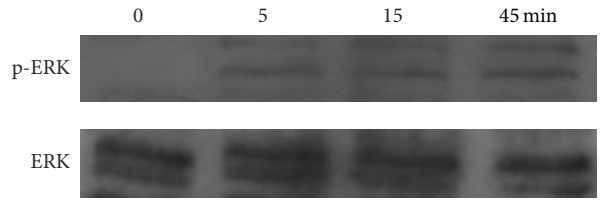


Figure 4: Effects of puerarin on ERK signaling pathways in hOBs. Anti-ERK and anti-p-ERK monoclonal antibodies were used to perform in western blotting. Cells were exposed to 10^{-8} M puerarin for 0–45 min. The levels of ERK and p-ERK were measured by densitometry of autoradiographs.

discovered that puerarin protects hOBs from serum-free-induced apoptosis by upregulating the expression of Bcl-2 and downregulating the expression of Bax and then significantly decreasing the Bax/Bcl-2 ratio in a dose-dependent manner, suggesting that the Bcl-2 family participates in the regulation of the prevention hOBs from apoptosis by puerarin.

To gain further insight into the mechanisms by which puerarin suppresses hOBs apoptosis, we examined ERK signaling pathways. Extracellular signal-regulated kinase

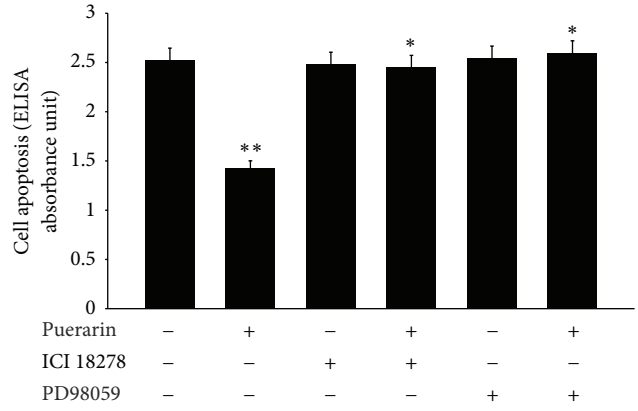


Figure 5: ERK signaling pathway and estrogen receptor mediated the antiapoptotic effects of puerarin in hOBs. Cells were incubated with PD98059 (10 μM) and/or ICI 182780 (10 μM) for 3 h prior to treatment with 10^{-8} M puerarin for 48 h. The bars represented the mean ± SD ($n = 6$). $^{*}P < 0.05$, as compared with the puerarin-treated cells; $^{**}P < 0.05$, as compared with the serum-free treated cells.

(ERK1/2), located at both the cytoplasm and the nucleus of cells, is a multifunctional serine/threonine kinases which induce various of substrates phosphorylation localized in all cellular compartments [33, 34]. It is believed that ERK is involved mainly in the activation of nuclear transcription factors that control cell proliferation, differentiation and apoptosis [35]. Currently, osteoblasts apoptosis induced by serum deprivation was proved to be suppressed significantly through activating the ERK signaling pathway [12, 36]. Present study we investigated the effect of puerarin on ERK and found that puerarin activated ERK phosphorylation and inhibited hOBs apoptosis. This protection was eliminated through pretreatment with PD98059, indicating that ERK signaling pathway was the key link in the antiapoptotic effects of puerarin on hOBs.

Additionally, it is well known that ERs are the major targets for estrogen acting on osteoblasts apoptosis [37]. As a phytoestrogen, puerarin was reported to be closely connected with ERs. In the study of Tiyasatkulkovit et al. [38], ER antagonist ICI182780 substantially inhibited the facilitation of puerarin on osteoblast differentiation. These present findings corroborated that ICI182780 blocked the effects of puerarin on hOBs apoptosis just as was expected, supporting the conclusion that puerarin suppressed hOBs apoptosis in an ER-dependent manner.

5. Conclusion

The present study has confirmed the potential benefit of puerarin on hOBs apoptosis in vitro, which is mediated by ERK signaling pathway. It is well known that hOBs apoptosis plays an essential role in the process of osteoporosis. Consequently, we conclude here that puerarin might ameliorate bone loss and promote bone remolding after subjecting to osteoporosis. Further studies are needed to affirm the effect of puerarin on osteoporosis in vivo.

Acknowledgments

This work was supported by Grants 81000313, 30900622, and 81070246 from the China National Natural Scientific Foundation; Grant 078202042 from Scholarship Award for Excellent Doctoral Student granted by Ministry of Education; Central South University "Excellent Doctoral Dissertation of training support program."

References

[1] H. Taipaleenmäki, L. Bjerre Hokland, L. Chen, S. Kauppinen, and M. Kassem, "Mechanisms in endocrinology: microRNAs: targets for enhancing osteoblast differentiation and bone formation," *European Journal of Endocrinology*, vol. 166, no. 3, pp. 359–371, 2012.

[2] C. V. Gurban and O. Mederle, "The OPG/RANKL system and zinc ions are promoters of bone remodeling by osteoblast proliferation in postmenopausal osteoporosis," *Romanian Journal of Morphology and Embryology*, vol. 52, supplement 3, pp. 1113–1119, 2011.

[3] N. Maruotti, A. Corrado, A. Neve, and F. P. Cantatore, "Bisphosphonates: effects on osteoblast," *European Journal of Clinical Pharmacology*, vol. 68, no. 7, pp. 1013–1018, 2012.

[4] A. Gohel, M. B. McCarthy, and G. Gronowicz, "Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts in vivo and in vitro," *Endocrinology*, vol. 140, no. 11, pp. 5339–5347, 1999.

[5] H. Li, W. Qian, X. Weng et al., "Glucocorticoid receptor and sequential P53 activation by dexamethasone mediates apoptosis and cell cycle arrest of osteoblastic MC3T3-E1 cells," *PLoS One*, vol. 7, no. 6, Article ID e37030, 2012.

[6] S. C. Manolagas, "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis," *Endocrine Reviews*, vol. 21, no. 2, pp. 115–137, 2000.

[7] F. S. Chueh, C. P. Chang, C. C. Chio, and M. T. Lin, "Puerarin acts through brain serotonergic mechanisms to induce thermal effects," *Journal of Pharmacological Sciences*, vol. 96, no. 4, pp. 420–427, 2004.

[8] R. M. Han, Y. X. Tian, E. M. Becker, M. L. Andersen, J. P. Zhang, and L. H. Skibsted, "Puerarin and conjugate bases as radical scavengers and antioxidants: molecular mechanism and synergism with β-carotene," *Journal of Agricultural and Food Chemistry*, vol. 55, no. 6, pp. 2384–2391, 2007.

[9] P. P. Wang, X. F. Zhu, L. Yang et al., "Puerarin stimulates osteoblasts differentiation and bone formation through estrogen receptor, p38 MAPK, and Wnt/β-catenin pathways," *Journal of Asian Natural Products Research*, vol. 14, no. 9, pp. 897–905, 2012.

[10] M. Y. Zhang, H. Qiang, H. Q. Yang, X. Q. Dang, and K. Z. Wang, "In vitro and in vivo effects of puerarin on promotion of osteoblast bone formation," *Chinese Journal of Integrative Medicine*, vol. 18, no. 4, pp. 276–282, 2012.

[11] K. M. Wiren, A. R. Toombs, A. A. Semirale, and X. Zhang, "Osteoblast and osteocyte apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio," *Bone*, vol. 38, no. 5, pp. 637–651, 2006.

[12] X. Zhu, Y. Jiang, P. F. Shan et al., "Vaspin attenuates the apoptosis of human osteoblasts through ERK signaling pathway," *Amino Acids*, vol. 44, no. 3, pp. 961–968, 2013.

[13] H. Xie, L. Q. Yuan, X. H. Luo et al., "Apelin suppresses apoptosis of human osteoblasts," *Apoptosis*, vol. 12, no. 1, pp. 247–254, 2007.

[14] O. Morales, M. K. R. Samuelsson, U. Lindgren, and L. A. Haldosén, "Effects of 1α,25-dihydroxyvitamin D3 and growth hormone on apoptosis and proliferation in UMR 106 osteoblast-like cells," *Endocrinology*, vol. 145, no. 1, pp. 87–94, 2004.

[15] G. Kogianni, V. Mann, F. Ebetino et al., "Fas/CD95 is associated with glucocorticoid-induced osteocyte apoptosis," *Life Sciences*, vol. 75, no. 24, pp. 2879–2895, 2004.

[16] Y. S. Liu, Y. Lu, W. Liu et al., "Connective tissue growth factor is a downstream mediator for preptin-induced proliferation and differentiation in human osteoblasts," *Amino Acids*, vol. 38, no. 3, pp. 763–769, 2010.

[17] L. Q. Yuan, W. Liu, R. R. Cui et al., "Taurine inhibits osteoclastogenesis through the taurine transporter," *Amino Acids*, vol. 39, no. 1, pp. 89–99, 2010.

[18] E. Bona, H. Hagberg, E. M. Løberg, R. Bågenholm, and M. Thoresen, "Protective effects of moderate hypothermia after neonatal hypoxia- ischemia: short- and long-term outcome," *Pediatric Research*, vol. 43, no. 6, pp. 738–745, 1998.

[19] P. F. Shan, Y. Lu, R. R. Cui, Y. Jiang, L. Q. Yuan, and E. Y. Liao, "Apelin attenuates the osteoblastic differentiation of vascular smooth muscle cells," *PLoS One*, vol. 6, no. 3, Article ID e17938, 2011.

[20] L. Q. Yuan, J. H. Zhu, H. W. Wang et al., "RANKL is a downstream mediator for insulin-induced osteoblastic differentiation of vascular smooth muscle cells," *PLoS One*, vol. 6, no. 12, Article ID e29037, 2011.

[21] R. R. Cui, D. A. Mao, L. Yi et al., "Apelin suppresses apoptosis of human vascular smooth muscle cells via APJ/PI3-K/Akt signaling pathways," *Amino Acids*, vol. 39, no. 5, pp. 1193–1200, 2010.

[22] J. M. Hock, V. Krishnan, J. E. Onyia et al., "Osteoblast apoptosis and bone turnover," *Journal of Bone and Mineral Research*, vol. 16, no. 6, pp. 975–984, 2001.

[23] G. M. Bran, J. Stern-Straeter, K. Hörmann, F. Riedel, and U. R. Goessler, "Apoptosis in bone for tissue engineering," *Archives of Medical Research*, vol. 39, no. 5, pp. 467–482, 2008.

[24] Y. P. Hwang and H. G. Jeong, "Mechanism of phytoestrogen puerarin-mediated cytoprotection following oxidative injury: estrogen receptor-dependent up-regulation of PI3K/Akt and HO-1," *Toxicology and Applied Pharmacology*, vol. 233, no. 3, pp. 371–381, 2008.

[25] M. Y. Zhang, H. Qiang, H. Q. Yang, X. Q. Dang, and K. Z. Wang, "In vitro and in vivo effects of puerarin on promotion of osteoblast bone formation," *Chinese Journal of Integrative Medicine*, vol. 18, no. 4, pp. 276–282, 2012.

[26] H. E. Abud, "Shaping developing tissues by apoptosis," *Cell Death and Differentiation*, vol. 11, no. 8, pp. 797–799, 2004.

[27] M. Bras, B. Queenan, and S. A. Susin, "Programmed cell death via mitochondria: different modes of dying," *Biochemistry*, vol. 70, no. 2, pp. 231–239, 2005.

[28] C. M. Knudson and S. J. Korsmeyer, "Bcl-2 and bax function independently to regulate cell death," *Nature Genetics*, vol. 16, no. 4, pp. 358–363, 1997.

[29] H. Zhang, Y. Liu, M. Lao, Z. Ma, and X. Yi, "Puerarin protects Alzheimer's disease neuronal cybrids from oxidant-stress induced apoptosis by inhibiting pro-death signaling pathways," *Experimental Gerontology*, vol. 46, no. 1, pp. 30–37, 2011.

[30] R. L. Shi and J. J. Zhang, "Protective effect of puerarin on vascular endothelial cell apoptosis induced by chemical hypoxia in vitro," *Yaoxue Xuebao*, vol. 38, no. 2, pp. 103–107, 2003.

[31] L. H. Zhu, L. Wang, D. Wang et al., "Puerarin attenuates high-glucose-and diabetes-induced vascular smooth muscle cell proliferation by blocking PKCbeta2/Rac1-dependent signaling," *Free Radical Biology and Medicine*, vol. 48, no. 4, pp. 471–482, 2010.

[32] J. Q. Han, K. Y. Yu, and M. He, "Effects of puerarin on the neurocyte apoptosis and p-Akt (Ser473) expressions in rats with cerebral ischemia/reperfusion injury," *Zhongguo Zhong Xi Yi Jie He Za Zhi*, vol. 32, no. 8, pp. 1069–1072, 2012.

[33] C. J. Tai, C. H. Lee, H. C. Chen et al., "High nuclear expression of phosphorylated extracellular signal-regulated kinase in tumor cells in colorectal glands is associated with poor outcome in colorectal cancer," *Annals of Diagnostic Pathology*, vol. 17, no. 2, pp. 165–171, 2013.

[34] M. Tasyriq, I. A. Najmuldeen, L. L. In et al., "7α-hydrox-β-sitosterol from Chisocheton tomentosus induces apoptosis via dysregulation of cellular Bax/Bcl-2 ratio and cell cycle arrest by downregulating ERK1/2 activation," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 765316, 12 pages, 2012.

[35] Q. H. Liang, Y. Jiang, X. Zhu et al., "Ghrelin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the ERK pathway," *PLoS One*, vol. 7, no. 4, Article ID e33126, 2012.

[36] L. Y. Zhang, Y. Y. Zhou, F. Chen et al., "Taurine inhibits serum deprivation-induced osteoblast apoptosis via the taurine transporter/ERK signaling pathway," *Brazilian Journal of Medical and Biological Research*, vol. 44, no. 7, pp. 618–623, 2011.

[37] R. S. Weinstein, C. A. O'Brien, R. L. Jilka et al., "Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERα," *Journal of Bone and Mineral Research*, vol. 25, no. 4, pp. 769–781, 2010.

[38] W. Tiyasatkulkovit, N. Charoenphandhu, K. Wongdee et al., "Upregulation of osteoblastic differentiation marker mRNA expression in osteoblast-like UMR106 cells by puerarin and phytoestrogens from Pueraria mirifica," *Phytomedicine*, vol. 19, no. 13, pp. 1147–1155, 2012.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 378632, 5 pages
http://dx.doi.org/10.1155/2013/378632

Research Article

Relation of Visceral and Subcutaneous Adipose Tissue to Bone Mineral Density in Chinese Women

Ling Wang,[1] Wei Wang,[2] Li Xu,[1] Xiaoguang Cheng,[1] Yimin Ma,[1] Dan Liu,[1] Zhe Guo,[1] Yongbin Su,[1] and Qianqian Wang[3]

[1] *Department of Radiology, Beijing Jishuitan Hospital, Beijing 100035, China*
[2] *Department of Radiology, Hong Kong University Shenzhen Hospital, Shenzhen 518053, China*
[3] *Department of Epidemiology, Beijing Jishuitan Hospital, Beijing 100035, China*

Correspondence should be addressed to Xiaoguang Cheng; xiao65@263.net

Received 3 January 2013; Revised 7 May 2013; Accepted 10 May 2013

Academic Editor: Ling-Qing Yuan

The relationship between adipose and bone tissues is still being debated. The purpose of our study was to evaluate whether the distribution and volume of abdomen adipose tissue are correlated to trabecular bone mineral density in the lumbar spine. In this cross-sectional study, 320 Chinese women, being divided into two groups according to age ≥55 years and <55 years, were evaluated with quantitative computed tomography (QCT) of the spine to simultaneously evaluate the average trabecular BMD of L2–L4, VAT, and SAT. Possible covariates of height, weight, age, and comorbidities were considered. In the <55-year-old sample, multiple linear regression analyses indicated that VAT volume was negatively correlated to trabecular BMD (P value = 0.0003) and SAT volume had no correlation to trabecular BMD. In contrast, there was no significant correlation between VAT or SAT and BMD in the ≥55-year-old sample. Our results indicate that high VAT volume is associated with low BMD in Chinese women aged <55 years and SAT has no relation with BMD.

1. Introduction

Low bone mineral density (BMD) has long been established as an important risk factor for hip fracture or lumbar fracture [1]. It follows that knowledge of the other factors, such as obesity, smoking, alcohol intake, drugs intake, and long-term bed rest, influencing BMD is crucial for preventing and treating osteoporotic disease. Among these factors, obesity was previously thought to have a positive influence on the maintenance of BMD [2–5]. However, recent studies have documented an obesity paradox of lower bone density in obese than normal weight subjects with particular conditions [6, 7]. A number of studies reported the potential physiological mechanisms that may lead to obesity paradoxes [8–10], but the topic is far from being definitively settled.

Little is known regarding the distribution of adipose tissue in terms of visceral (VAT) and subcutaneous (SAT) compartments on bone fragility. Although there is evidence that amount of visceral adipose tissue (VAT) plays a deleterious role in many other diseases [11], there is little information on how VAT and SAT affect bone mineral density [12–14]. Therefore, further studies are needed to explore the possible effects of VAT and SAT on BMD.

To our knowledge, there have been no previous studies on the possible independent effects of VAT and SAT on BMD in Chinese women. The purpose of our study was to investigate whether the distribution of abdomen adipose tissues influences trabecular BMD of the lumbar spine. We chose to use cross-sectional images using quantitative computed tomography to simultaneously estimate the volumes of VAT and SAT as well as vertebral trabecular bone density.

2. Materials and Methods

2.1. Subjects. Subjects included 320 Chinese women aged 19–86 years having QCT examinations from February 2010 through October 2012. Some subjects had their first QCT examination at our hospital for an assessment of their bone mineral density. The others were recruited from a pool of

outpatients for abdomen or Lumbar spine CT scan. The QCT dataset could be achieved with calibration phantom scanned beneath the body simultaneously, without additional radiation and relocation of the patients. These patients completed written informed consent forms before any measurement. The menopausal status could not be confirmed in all subjects, as some of them could not be able to accurately determine when they begin to be menopausal or did not remember accurate age of menopause. An epidemiological survey including 15083 subjects indicates that the mean age at spontaneous menopause was 50.6 ± 3.7 years old in Chinese women [15]. Prior pointed out that the term "perimenopause" could be used to characterize women between the ages 45 and 55, given the lack of clarity about the onset of the perimenopause [16]. So the subjects were divided into two groups according to ages ≥55 and <55. Most of the subjects aged ≥55 may be postmenopausal to further overcome perimenopausal effect. Participants who had used or were using drugs that have an influence on bone metabolism were excluded. The exclusion criteria also included diabetes, thyroid and parathyroid disease, and liver or renal disease. The study was approved by the Ethics Committee of the Beijing Jishuitan Hospital, 4th Clinical College of Peking University.

2.2. Anthropometry. Height and weight were measured to the nearest 0.1 cm and 0.1 kg respectively when the subjects wore the underwear. All values were recorded as the mean of two repeated measures. BMI was calculated as the weight (kg) divided by the square of the height (meters).

2.3. QCT Measurements of BMD. All subjects underwent cross-sectional CT scan of the abdomen from the level of the second to the fourth lumbar vertebral body (L2–L4) with the same CT scanner (Aquilion, 16 Toshiba, Tokyo, Japan). Scan parameters were 120 kV, 100 mAs, 1 mm slice thickness, and 40 cm field of view (FOV). Trabecular bone mineral density (BMD) measurement of L2–L4 was performed using a software package: QCT PRO 4.2.3 (Mindways, Austin, TX, USA). Subjects were positioned supine on the CT table with the same Mindways CT calibration phantom placed under the subjects to cover levels L2 to L4. Images were transferred to the QCT PRO PC (2007 Mindways Software, Version 4.2.3; Mindways, Austin, TX) by the image transfer utility set up on the CT scanner. A region of interest within trabecular bone of each of three vertebral bodies was placed semiautomatically for the BMD measurement, so as to avoid cortical bone and posterior veins. The average trabecular BMD of L2–L4 was calculated. The precision for this technique is less than 1.5% [17, 18].

2.4. QCT Measurements of Adipose Tissue. We measured adipose tissue in L4 level slice. This slice typically intersects the umbilicus and is consistent with other CT protocols for VAT measures. The umbilicus cross-section was chosen because it has the maximum ratio of fat to total tissue area and the visceral fat area at the umbilical region has been found to be strongly correlated with visceral fat volume ($r = 0.921$ in males and 0.931 in females) [19, 20]. On the same CT images, measurements of total adipose area (TAA) and visceral adipose area (VAA) were semi-automatically completed by the commercial software package: "Tissue Composition Module" Beta 1.0 (Mindways, Austin, TX, USA). For the purposes of this study, SAT was defined as the area of adipose tissues between the skin and the rectus muscles of the abdomen, the external oblique muscles, the broadest muscle of the back, and the erector muscles of the spine at the level of L4. VAT was defined as all intra-abdominal adipose tissue area within the abdominal cavity of rectus, external oblique, lumbar quadrate, and psoas muscles. All the measurements were carried out by a single trained in the QCT techniques.

2.5. Statistical Analysis. Statview 9.0.1 (SAS Institute Inc., Cary, NC) was used for the statistical analysis. Results are presented as mean ± SD. All variables were checked for outliers and normality using Shapiro-wilk tests. Because all continuous variables were nonnormally distributed, associations among the independent variables were explored using nonparametric Spearman rank correlation coefficients. Multiple linear regression was used to assess the relationships between abdominal adiposity and trabecular BMD. Statistical significance was accepted at $P < 0.05$.

3. Results

The descriptive statistics for the samples are shown in Table 1. The age of young and elder sample ranged from 19 to 54 years and from 55 to 86 years, with a mean and SD of 41.51 ± 10.60 years and 66.1 ± 7.34 years, respectively. In the group aged <55 years, nonparametric Spearman rank correlation coefficient analysis showed average trabecular BMD to be negatively correlated with age and BMI, whereas there was no association between average trabecular BMD and weight ($P = 0.26$) (Table 2). There was an inverse correlation between average trabecular BMD and VAT ($r = -0.52$, $P < 0.0001$) (Figure 1), which remained significant after adjustment for age and BMI using multiple liner regression analysis ($P = 0.01$) (Table 4). TAT was found to be not correlated with trabecular BMD using regression analysis (Tables 2 and 4). Whereas spearman correlation between SAT and average trabecular BMD was negative (Figure 2), multiple regression analysis showed that SAT, after accounting for age and BMI, had no correlation with BMD ($P = 0.88$). In contrast to the significant correlation found in the group aged <55 years, there was no significant association between any adiposity and average trabecular BMD in the group aged ≥55 years (all $P > 0.05$) (Tables 3 and 5).

4. Discussion

Our results indicate that VAT may be deleterious to BMD but that SAT appears not to be correlated with BMD in the young Chinese women. There also appears to be no correlation between abdominal adipose tissue and trabecular BMD in the elder Chinese women. More fat accumulation is a known risk factor for cardiovascular disease, hypertension,

Table 1: Descriptive characteristics of the subjects.

	Mean ± SD (range)	
	Age < 55 (n = 89)	Age ≥ 55 (n = 231)
Age (years)	41.51 ± 10.60 (19.00–54.00)	66.10 ± 7.45 (55.00–86.00)
Height (cm)	162.01 ± 6.83 (150.00–180.00)	160.88 ± 7.30 (140.00–190.00)
Weight (kg)	61.60 ± 11.48 (40.00–100.00)	66.26 ± 12.46 (40.00–110.00)
Average BMD (mg/cm^3)	130.86 ± 35.56 (45.19–211.95)	66.92 ± 31.63 (4.95–218.34)
BMI (kg/m^2)	23.45 ± 4.00 (16.60–33.20)	25.54 ± 4.18 (16.02–40.90)
TAT (cm^2)	280.81 ± 115.10 (79.85–641.84)	357.88 ± 107.05 (67.85–623.44)
VAT (cm^2)	106.33 ± 51.63 (22.61–289.31)	159.24 ± 54.79 (37.69–326.27)
SAT (cm^2)	174.48 ± 74.83 (51.70–467.14)	198.64 ± 70.31 (23.60–384.82)

BMD: bone mineral density, BMI: body mass, TAT: total adipose tissue, VAT: visceral adipose tissue, and SAT: subcutaneous adipose tissue.

Table 2: Correlations (r) between adiposity, age, BMI, and BMD in group aged <55 years.

	Age	Average BMD	BMI	TAT	VAT	SAT
Average BMD	−0.68					
BMI	0.38	−0.22				
TAT	0.53	−0.39	0.67			
VAT	0.69	−0.52	0.59	0.89		
SAT	0.35	−0.23	0.59	0.93	0.67	

All other correlations are significant; $P < 0.05$.

Table 3: Correlations (r) between adiposity, age, BMI, and BMD in group aged ≥55 years.

	Age	Average BMD	BMI	TAT	VAT	SAT
Average BMD	−0.55					
BMI	−0.12[a]	0.13[d]				
TAT	0.05[b]	0.02[e]	0.56			
VAT	0.18	−0.02[f]	0.43	0.82		
SAT	−0.07[c]	0.05[g]	0.51	0.87	0.46	

All other correlations are significant; $P < 0.05$.
[a] $P = 0.06$.
[b] $P = 0.49$.
[c] $P = 0.27$.
[d] $P = 0.06$.
[e] $P = 0.76$.
[f] $P = 0.74$.
[g] $P = 0.43$.

and diabetes; however, the role of abdominal adiposity on BMD is still being debated. Recent studies suggest the adipose tissue is detrimental to the maintenance of BMD [21–23] and a number of physiological mechanism studies have confirmed this hypothesis [8–10, 24, 25]. Though abdominal

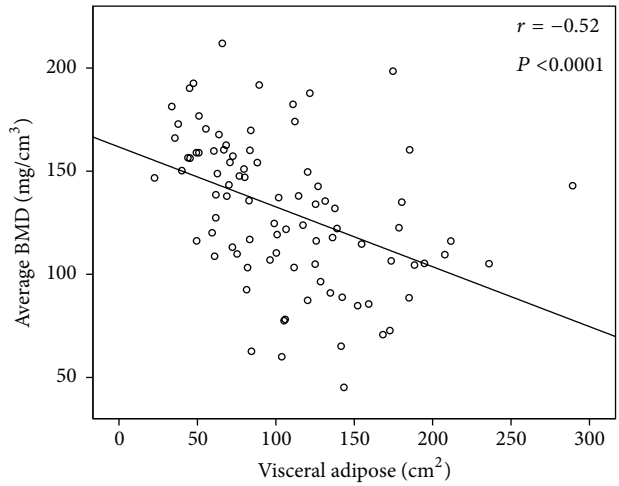


Figure 1: Correlation between average bone mineral density (mg/cm^3) and visceral adipose tissue in group aged <55. "r" is the Spearman correlation coefficient.

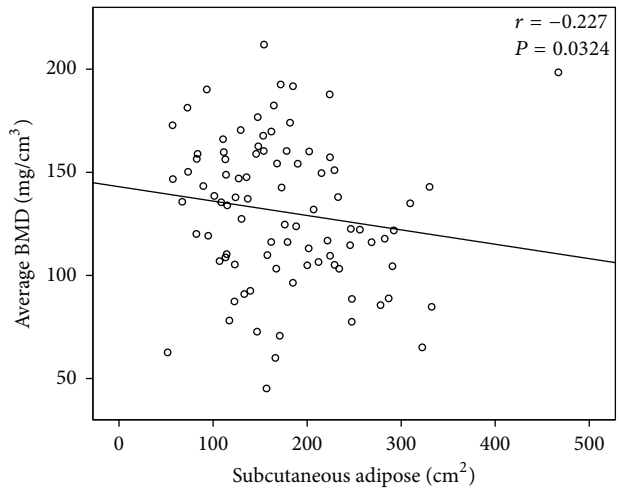


Figure 2: Correlation between average bone mineral density (mg/cm^3) and subcutaneous adipose tissue in group aged <55. "r" is the Spearman correlation coefficient.

Table 4: Multiple linear regression including TAT, VAT, and SAT as independent predictors of BMD in group aged <55, adjusting for BMI and age.

	TAT		VAT		SAT	
	β	P	β	P	β	P
Average BMD	−0.04	0.32	−0.22	0.01	0.0079	0.87

Table 5: Multiple linear regression including TAT, VAT, and SAT as independent predictors of BMD in group aged ≥55, adjusting for BMI and age.

	TAT		VAT		SAT	
	β	P	β	P	β	P
Average BMD	0.02	0.35	0.05	0.16	0.008	0.79

adiposity may have a direct effect on skeletal loading and may have a positive effect on BMD [26], the relation between adipose tissue and bone is complicated. Both osteoblasts and adipocytes originate from a common progenitor and bone marrow skeletal stem cells MSC [8], and their differentiation is regulated through the PPAR-γ (peroxisome proliferators activated receptor-gamma) pathway. Activation of PPAR-γ drives the differentiation of MSC towards adipocytes over osteoblasts [9]. Furthermore, the neuropeptide Y (NPY) system acts to regulate both bone and fat tissue in a coordinated manner, and this remains a strong candidate for mediating interactions between these two tissues [10]. Secretion from adipocytes may have both negative and positive effects on bone [9].

Visceral and subcutaneous adipose tissues express different adipokines. Visceral fat induces an increased risk of cardiovascular and metabolic complications, whereas subcutaneous fat exerts some still undefined protective actions [27]. Our data confirms the hypothesis that the different distributions of abdominal adipose tissue may have different influences on trabecular BMD. A recent study suggests that visceral fat is detrimental to femur structure and strength, whereas subcutaneous fat is beneficial to bone [28]. This suggests the same relationship between VAT and bone that we found in our study. However, it would appear that SAT may have a different relationship with bone compared to our study, although our study involved measurement of the trabecular BMD of lumbar spine and not the femur. It may be that the subcutaneous adipose tissue exerts mechanical stress on bone and therefore acts positively on femur structure and strength.

Other studies confirmed the negative relationship between VAT and bone [21–23]. Nevertheless, the results of SAT and bone in observational studies are somewhat controversial. Both negative [23, 29] and positive [28, 30] associations between SAT and bone mass have been reported. In our study, the average trabecular BMD is negatively associated with SAT in the group aged <55, whereas no relation was found in regression analysis adjusting for BMI and age ($P = 0.88$). This suggests that the relation between adiposity and BMD may be confounded by BMI and age.

We used QCT to assess the BMD of vertebral body. Most previous studies have assessed bone parameters by DXA. Area bone mineral density is dependent on skeletal size, so it will correlate with any other variable (such as lean mass) which is also dependent on skeletal size. DXA measures area bone density (g/cm^2) so is also influenced by bone size, as well as the mineral density of the bone being assessed [31]. This limitation leads inevitably to a relationship between body mass and bone mass or areal density. It is therefore important to produce a measurement of bone mineral density that takes account of this problem. This can be done by using QCT, which directly assesses volumetric bone density [26]. Furthermore, fat layering introduces error and decreases the reproducibility of DXA spine and hip BMD measurements in human volunteers. Although overlying fat also affects QCT BMD measurements, the error is smaller and more uniform than with DXA BMD [32]. QCT measures trabecular BMD and true volumetric bone density, irrespective of bone size. So the measurements of QCT may demonstrate a more accurate relationship between adiposity and BMD.

Although the subject populations where divided on an age-related basis, our results indicate that the relationship between adiposity and bone may be different when separating pre- and postmenopausal women. The menopausal transition is associated with substantial bone loss but a gain in fat mass. Numerous cross-sectional studies show an onset of bone loss at the average age of menopause and lack of consistent; normal ovulation is associated with accelerated bone loss [16]. The negative relation between VAT and BMD in the group aged <55 is consistent with previous studies. Katzmarzyk et al. have recently suggested that VAT was negatively associated with BMD in the younger age group ($\beta = -0.054$; $P = 0.0001$) but not in the older age group ($\beta = -0.002$; $P = 0.86$) [23]. The reason for this discrepancy between the two aged groups is not clear. Further studies are needed to investigate possible underlying mechanisms.

Our study has several limitations. Firstly, the study is cross-sectional. Secondly, it is not population based and some participants visited the hospital for BMD measurements. Because it is likely that they had lower BMD, associations might have been underestimated. Finally, data on alcohol intake, lifestyle habits, and menopausal age were not available.

5. Conclusion

Our results indicate that high VAT volume is associated with low BMD in Chinese women of age <55 and SAT has no relation with BMD, and there also appears to be no correlation between abdominal adipose tissue and trabecular BMD in the elder Chinese women. For the young Chinese women, obesity, especially visceral adipose accumulation, may not only be a risk factor for many diseases but also be detrimental to bone mineral density.

Acknowledgment

This study was supported by a Grant from Beijing high levels of health technical talent team of construction project (no. 2009-02-03).

References

[1] J. A. Kanis, H. Johansson, A. Oden, and E. V. McCloskey, "Assessment of fracture risk," *European Journal of Radiology*, vol. 71, no. 3, pp. 392–397, 2009.

[2] S. Khosla, E. J. Atkinson, B. L. Riggs, and L. J. Melton III, "Relationship between body composition and bone mass in women," *Journal of Bone and Mineral Research*, vol. 11, no. 6, pp. 857–863, 1996.

[3] E. M. Clark, A. R. Ness, and J. H. Tobias, "Adipose tissue stimulates bone growth in prepubertal children," *Journal of Clinical Endocrinology and Metabolism*, vol. 91, no. 7, pp. 2534–2541, 2006.

[4] I. R. Reid, L. D. Plank, and M. C. Evans, "Fat mass is an important determinant of whole body bone density in premenopausal

women but not in men," *Journal of Clinical Endocrinology and Metabolism*, vol. 75, no. 3, pp. 779–782, 1992.

[5] D. R. Taaffe, J. A. Cauley, M. Danielson et al., "Race and sex effects on the association between muscle strength, soft tissue, and bone mineral density in healthy elders: the health, aging, and body composition study," *Journal of Bone and Mineral Research*, vol. 16, no. 7, pp. 1343–1352, 2001.

[6] C. S. Chang, Y. F. Chang, M. W. Wang et al., "Inverse relationship between central obesity and osteoporosis in osteoporotic drug naive elderly females: the tianliao old people (TOP) study," *Journal of Clinical Densitometry*, vol. 16, no. 2, pp. 204–211, 2012.

[7] J. A. Shepherd, "Is osteoporosis an obesity paradox?" *Journal of Clinical Densitometry*, vol. 16, no. 2, pp. 131–132, 2012.

[8] M. Dominici, K. Le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.

[9] L. Zhao, Y. Liu, P. Liu, J. Hamilton, R. R. Recker, and H. Deng, "Relationship of obesity with osteoporosis," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 5, pp. 1640–1646, 2007.

[10] Y. Shi and P. A. Baldock, "Central and peripheral mechanisms of the NPY system in the regulation of bone and adipose tissue," *Bone*, vol. 50, no. 2, pp. 430–436, 2012.

[11] G. A. Rosito, J. M. Massaro, U. Hoffmann et al., "Pericardial fat, visceral abdominal fat, cardiovascular disease risk factors, and vascular calcification in a community-based sample the framingham heart study," *Circulation*, vol. 117, no. 5, pp. 605–613, 2008.

[12] M. M. Ibrahim, "Subcutaneous and visceral adipose tissue: structural and functional differences," *Obesity Reviews*, vol. 11, no. 1, pp. 11–18, 2010.

[13] M. Alvehus, J. Burén, M. Sjöström, J. Goedecke, and T. Olsson, "The human visceral fat depot has a unique inflammatory profile," *Obesity*, vol. 18, no. 5, pp. 879–883, 2010.

[14] R. Rosmond, Y. C. Chagnon, G. Holm et al., "A glucocorticoid receptor gene marker is associated with abdominal obesity, leptin, and dysregulation of the hypothalamic-pituitary-adrenal axis," *Obesity Research*, vol. 8, no. 3, pp. 211–218, 2000.

[15] H. Chen, Y. Feng, H. Shu et al., "Survey on menopausal age and menstruation span in women in Pudong district of Shanghai," *Zhonghua fu chan ke za zhi*, vol. 45, no. 6, pp. 415–419, 2010.

[16] J. C. Prior, "Perimenopause: the complex endocrinology of the menopausal transition," *Endocrine Reviews*, vol. 19, no. 4, pp. 397–428, 1998.

[17] M. Bligh, L. Bidaut, R. A. White, W. A. Murphy Jr., D. M. Stevens, and D. D. Cody, "Helical multidetector row quantitative computed tomography (QCT) precision," *Academic Radiology*, vol. 16, no. 2, pp. 150–159, 2009.

[18] T. F. Lang, J. Li, S. T. Harris, and H. K. Genant, "Assessment of vertebral bone mineral density using volumetric quantitative CT," *Journal of Computer Assisted Tomography*, vol. 23, no. 1, pp. 130–137, 1999.

[19] G. A. Borkan, S. G. Gerzof, and A. H. Robbins, "Assessment of abdominal fat content by computed tomography," *American Journal of Clinical Nutrition*, vol. 36, no. 1, pp. 172–177, 1982.

[20] K. Furukawa, T. Katabami, Y. Nakajima et al., "Evaluation of whole-abdominal fat volume by 700-slice CT scanning and comparison with the umbilical fat area anthropometric indices," *Obesity Research and Clinical Practice*, vol. 4, no. 2, pp. e111–e117, 2010.

[21] M. A. Bredella, M. Torriani, R. H. Ghomi et al., "Determinants of bone mineral density in obese premenopausal women," *Bone*, vol. 48, no. 4, pp. 748–754, 2011.

[22] M. Russell, N. Mendes, K. K. Miller et al., "Visceral fat is a negative predictor of bone density measures in obese adolescent girls," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 3, pp. 1247–1255, 2010.

[23] P. T. Katzmarzyk, T. V. Barreira, D. M. Harrington, A. E. Staiano, S. B. Heymsfield, and J. M. Gimble, "Relationship between abdominal fat and bone mineral density in white and African American adults," *Bone*, vol. 50, no. 2, pp. 576–579, 2012.

[24] A. Krings, S. Rahman, S. Huang, Y. Lu, P. J. Czernik, and B. Lecka-Czernik, "Bone marrow fat has brown adipose tissue characteristics, which are attenuated with aging and diabetes," *Bone*, vol. 50, no. 2, pp. 546–552, 2012.

[25] B. M. Abdallah and M. Kassem, "New factors controlling the balance between osteoblastogenesis and adipogenesis," *Bone*, vol. 50, no. 2, pp. 540–545, 2012.

[26] I. R. Reid, "Relationships among body mass, its components, and bone," *Bone*, vol. 31, no. 5, pp. 547–555, 2002.

[27] C. Mammi, M. Calanchini, A. Antelmi et al., "Androgens and adipose tissue in males: a complex and reciprocal interplay," *International Journal of Endocrinology*, vol. 2012, Article ID 789653, 8 pages, 2012.

[28] V. Gilsanz, J. Chalfant, A. O. Mo, D. C. Lee, F. J. Dorey, and S. D. Mittelman, "Reciprocal relations of subcutaneous and visceral fat to bone structure and strength," *Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 9, pp. 3387–3393, 2009.

[29] T. Yamaguchi, I. Kanazawa, M. Yamamoto et al., "Associations between components of the metabolic syndrome versus bone mineral density and vertebral fractures in patients with type 2 diabetes," *Bone*, vol. 45, no. 2, pp. 174–179, 2009.

[30] L. M. Yerges-Armstrong, I. Miljkovic, J. A. Cauley et al., "Adipose tissue and volumetric bone mineral density of older Afro-Caribbean men," *Journal of Bone and Mineral Research*, vol. 25, no. 10, pp. 2221–2228, 2010.

[31] I. R. Reid, "Fat and bone," *Archives of Biochemistry and Biophysics*, vol. 503, no. 1, pp. 20–27, 2010.

[32] E. W. Yu, B. J. Thomas, J. K. Brown, and J. S. Finkelstein, "Simulated increases in body fat and errors in bone mineral density measurements by DXA and QCT," *Journal of Bone and Mineral Research*, vol. 27, no. 1, pp. 119–124, 2012.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 316783, 6 pages
http://dx.doi.org/10.1155/2013/316783

Review Article

Silicon: A Review of Its Potential Role in the Prevention and Treatment of Postmenopausal Osteoporosis

Charles T. Price, Kenneth J. Koval, and Joshua R. Langford

Orlando Health Department of Orthopedic Surgery, 1222 Orange Avenue, Orlando, FL 32806, USA

Correspondence should be addressed to Charles T. Price; charles.price@orlandohealth.com

Received 23 December 2012; Accepted 23 April 2013

Academic Editor: Cory Xian

Physicians are aware of the benefits of calcium and vitamin D supplementation. However, additional nutritional components may also be important for bone health. There is a growing body of the scientific literature which recognizes that silicon plays an essential role in bone formation and maintenance. Silicon improves bone matrix quality and facilitates bone mineralization. Increased intake of bioavailable silicon has been associated with increased bone mineral density. Silicon supplementation in animals and humans has been shown to increase bone mineral density and improve bone strength. Dietary sources of bioavailable silicon include whole grains, cereals, beer, and some vegetables such as green beans. Silicon in the form of silica, or silicon dioxide (SiO_2), is a common food additive but has limited intestinal absorption. More attention to this important mineral by the academic community may lead to improved nutrition, dietary supplements, and better understanding of the role of silicon in the management of postmenopausal osteoporosis.

1. Introduction

Nutrition, exercise, and lifestyle are recognized as important factors in the management of osteoporosis [1, 2]. Dietary supplementation with calcium and vitamin D decreases the risk of fractures and improves the effectiveness of pharmacological management [1, 3–5]. In addition to calcium and vitamin D, a wide range of nutritional supplements have been recommended to improve low bone density, but the evidence of benefit is limited [6, 7]. The absence of evidence may mean that more study is required, or it may mean that supplementation is unnecessary.

Many essential nutrients behave synergistically, for example, vitamin D and vitamin K in the production and activation of osteocalcin. Vitamin D stimulates the production of osteocalcin, while vitamin K carboxylates osteocalcin for improved bone toughness [8, 9]. Thus, there may be several micronutrients that should be supplemented in addition to calcium and vitamin D as part of the management of osteoporosis. The US National Institutes of Health have documented that more than half of the adults in the USA are insufficient in dietary intake of magnesium, vitamin K, vitamin C, and other nutrients that are essential for bone health [10–12]. One mineral that warrants attention is silicon because of the growing body of the scientific literature that recognizes silicon's importance for bone health [13, 14].

Silicon is an essential mineral for bone formation [15, 16]. In 1970, Edith M. Carlisle, Ph.D., published a brief paper in *Science* titled "*Silicon: a possible factor in bone calcification*" [17]. She performed quantitative electron probe analysis of silicon content in young mice and rats. Carlisle concluded that silicon is important as an initiator of mineralization because silicon is highly concentrated in immature osteoid but declines as calcium content rises in mature bone. Another study by Carlisle reported that silicon supplementation accelerates the rate of bone mineralization [18]. She continued her research with several additional studies including a publication in 1981 titled "*Silicon: a requirement in bone formation independent of vitamin* D_1" [15]. In this study, bones of silicon-deficient chicks contained less collagen than the bones of silicon-supplemented chicks regardless of vitamin D levels. Carlisle concluded that silicon had an effect on collagen to make the bone matrix more calcifiable. The essential nature of silicon for skeletal development was also confirmed by Schwarz and Milne in 1972 and by Nielsen and Sandstead in 1974 [16, 19]. No other researchers reported the role of silicon

in bone health until 1993, when Hott et.al. published a study of the effects of silicon supplementation on bone density in ovariectomized rats [20]. They reported that silicon reduced bone resorption and increased bone formation in the animal model of postmenopausal osteoporosis.

Since 2002, there has been increased research regarding the role of silicon in a variety of tissues including bone [13, 14, 21, 22]. The purpose of this report is to review the role of silicon as an essential element for bone formation and maintenance. A secondary purpose is to call attention to this nutritional component so that more research may be directed towards the study of silicon for the management of osteoporosis. It is possible that silicon supplementation should be considered in addition to supplementation with other vitamins and minerals for the management of patients with low bone density.

2. Silicon Chemistry

Silicon is a *chemical element*, which has the symbol **Si** and an atomic weight of 28. It is classified as a semiconductor with electrical properties that are intermediate between metal and nonmetal elements. Crystalline silicon has piezoresistive properties that are utilized in micropressure transducers and computer electronics. Silicon rarely occurs as a pure free element in nature. It forms strong bonds with oxygen and generally exists as silica or silicate compounds. Silica is the general term for inorganic compounds containing silicon and oxygen. The silicon dioxide (SiO_2) form is a major component of sand, granite, quartz, and other types of rocks, clays, and gems in the Earth's crust [22]. Thus, silicon is the second most abundant element in the Earth's crust. Silicon dioxide is poorly soluble in water and has many industrial applications including abrasives, electronics, and construction. Industrial food preparation uses silica powder to decrease foaming, reduce caking of powders, or clarify liquids. Another compound form of silicon is Silicone. Silicones are polymeric compounds with a silicon-oxygen-silicon (Si-O-Si) backbone. These polymers can be linked together to form rubber-like materials that are used for many purposes including plumbing, dental applications, medical implants, tubing, lubrication, and insulation. Neither silicon dioxide nor silicone rubber compounds are useful dietary sources because they have poor water solubility and poor biological availability [22, 23]. In contrast, water-soluble forms of silicon are more biologically available. Silicon in geological formations, especially in volcanic areas, may gradually dissolve to produce soluble forms of silicon in artesian waters [22, 24, 25]. Monomethylsilanetriol (MMST), or CH_3-Si-$(OH)_3$, is a commercially available liquid form of silicon that has biological availability and is used as a liquid nutritional supplement [26].

Water-soluble forms of silicon are absorbed in the intestinal tract, with excess amounts eliminated by the kidneys within 4–8 hours following ingestion [14]. Thus, it is unlikely for silicon to accumulate in excessive amounts in healthy individuals. Oral toxicity from elemental or organic silicon has not been identified in animals or humans even when rats and mice have been fed up to 1000 times the normal dietary intake [26]. Patients on dialysis may accumulate silicon because renal failure prevents the excretion of silicon. Serum silicon levels up to ten times normal have been reported in patients with renal failure, but no adverse effects have been associated with these levels [27, 28]. There have been rare cases of silica renal stones in patients who were also consuming large quantities of magnesium in the form of magnesium trisilicate antacids [22, 29]. Thus, adverse effects from oral silicon have not been observed in healthy individuals.

3. Silicon's Role in Bone Formation

Silicon is bound to glycosaminoglycans and has an important role in the formation of cross-links between collagen and proteoglycans [15, 30, 31]. Silicon is present in all body tissues, but the tissues with the highest concentrations of silicon are bone and other connective tissue including skin, hair, arteries, and nails [14]. In vitro studies have demonstrated that silicon stimulates type 1 collagen synthesis and osteoblast differentiation [32]. Studies in rats have demonstrated that silicon at physiological levels improves calcium incorporation in bone when compared to rats that are deficient in silicon [20, 33, 34]. Thus, silicon is an essential element for bone formation [15, 16].

The exact sequence of mineralization is unknown, but Carlisle concluded that silicon probably acts by making the bone matrix more calcifiable [15]. Silicon concentrations in osteoid are 25 times greater than in surrounding areas and the silicon content gradually declines as calcification occurs [17]. Silicon is a known semiconductor of electrical charges. Silicon crystals are used in microscopic pressure transducers because they have a piezoresistive effect when subjected to stress [35]. It is also known that the collagen matrix of immature bone has piezoelectric properties that generate electrical potentials when subjected to strain. Bone mineralization occurs in the electronegative areas that are generated by compression [36]. It is possible that silicon plays a role in the electrochemical process of mineralization, but the precise biological role of silicon remains unknown.

Studies of dietary silicon supplementation in growing animals have reported improved bone quality by direct measurements of bone strength and density for quail, broiler chickens, and rainbow trout [37–39]. A randomized blind study of racing quarter horses compared a control group to three different levels of dietary silicon supplementation [40]. The supplemented horses began their diets at six months of age and continued for 18 months including a six-month training and racing period. Race times, lameness, fractures, and serum silicon levels were recorded during the period of study. At the completion of the study, it was determined that the horses with medium and high levels of silicon supplementation had significantly faster race times and greater training distances before the first breakdown. The horses with the highest level of silicon supplementation also had increased bone mineral density in the third metacarpal [40].

Direct measurements of bone mass and strength in numerous animal models have demonstrated the beneficial effects of silicon supplementation to increase bone mineral density and to reduce bone fragility [20, 33, 37–43].

The ovariectomized rat is a standard model for postmenopausal bone loss [44]. Five reports have been published using this model to study the effects of dietary silicon on bone metabolism [20, 41–43, 45]. Hott et al. compared physiological levels to low levels of dietary silicon in ovariectomized rats [20]. The mineral apposition and bone formation rate was 30% greater in the group with physiological silicon intake. The silicon-supplemented group also had less bone resorption. An experimental study by Calomme et al. demonstrated increased femoral bone density when physiological levels of silicon supplementation were added to the standard diet [45]. Additional research in postmenopausal animal models used high levels of supplemental dietary silicon (20 mg/kg/day) [41–43]. These high levels of silicon stimulated bone formation, increased bone mineral density, and decreased calcium excretion in the urine. This effect of increased bone formation has even been noted in calcium-deficient rat models although calcium supplementation combined with silicon supplementation produced greater bone mineral density [43].

Although silicon supplementation is associated with increased bone mineral density, the exact mechanism for this action has not been identified. Serum measurements of bone turnover have been inconsistent, while markers of bone matrix formation are consistently increased. This may indicate that silicon improves mineralization without affecting the rate of bone formation or bone loss. There may also be an effect on collagen that improves bone strength independent of mineral density [46]. In contrast to studies reporting improved bone strength, two experimental studies in rats have reported small reductions in bone strength when excessively high and prolonged levels of dietary silicon were added to the diet [43, 47]. This may represent an antagonistic effect of excessive silicon that decreases intestinal absorption of calcium and magnesium when very high amounts of silicon are provided in the diet [47].

Silicon also has biological activity for bone formation when incorporated into calcium phosphate bioceramics [48–50]. These bioceramic materials are used as bone graft substitutes to augment or replace autogenous bone grafts for orthopedic surgical procedures. Calcium phosphate ceramics without silicon substitution are considered osteoconductive because they provide a scaffold for resorption and replacement by bone through osteoclastic resorption and osteoblastic deposition of new bone [36]. Substitution of less than 1% of the phosphate groups (PO_4) with silicate ions (SiO_4) enhances the biological activity of the material [48, 50] and creates osteoinductive properties. Coathup et al. compared implantation of calcium phosphate to implantation of silicate-substituted calcium phosphate into the paraspinal muscles of sheep [48]. The silicate-substituted calcium phosphate demonstrated osteoinductive properties and significantly increased the amount of bone that formed compared to the calcium phosphate implants. The osteoinductive properties of silicate-substituted calcium phosphate ceramics have been reported by other researchers [50]. The exact mechanism of this enhanced bone formation is uncertain [49, 51]. One explanation is that silicon in the ceramic generates a more electronegative surface that promotes bone formation. Another explanation is that elemental silicon is released during resorption of the ceramic material and directly stimulates the differentiation and proliferation of osteoblasts. Regardless of the mechanism of action, there is an increasing confirmation that silicon plays a role in bone formation.

4. Osteoporosis and Silicon Intake

Average daily dietary intake of silicon is 20–50 mg for European and North American populations [14]. Daily intake of silicon is higher in China and India (140–200 mg/day) where grains, fruits, and vegetables form a larger part of the diet [52, 53]. China and India also have the lowest prevalence of hip fractures compared to all other regions of the world [54].

Diets containing more than 40 mg/day of silicon have been positively associated with increased femoral bone mineral density compared to dietary intake of less than 14 mg/day [55]. A study of postmenopausal Scottish women determined that average daily intake of silicon was 18.6 mg/day which was lower than a standard British diet that contains approximately 30 mg/day [56, 57]. Dietary intake of silicon declines with age by approximately 0.1 mg/year [58]. In a North American epidemiological study, none of the postmenopausal women achieved 40 mg/day of dietary silicon intake [55].

Two epidemiological studies have reported the relationship between dietary silicon intake and osteoporosis [55, 59]. Increased silicon intake correlated with increased bone mineral density for men, premenopausal women, and postmenopausal women on hormone replacement therapy (HRT). Silicon intake and bone mineral density did not correlate for post-menopausal women who were not on HRT [55]. Macdonald et al. noted that estrogen status may be important for silicon metabolism and suggested that silicon and estrogen may interact synergistically [21]. However, this does not explain the increased bone mineral density in men with increased silicon intake, and the amount of dietary silicon intake by postmenopausal women was generally low. None of the postmenopausal groups achieved more than 40 mg/day of dietary silicon intake which is the amount associated with increased bone mineral density in men and premenopausal women. It is known that estrogen increases the intestinal absorption of calcium [60] so, it is possible that estrogen also influences the intestinal absorption of silicon. Thus, there may be a role for silicon supplementation to increase silicon absorption in post-menopausal women who are not on HRT, but more research is needed to determine the link between estrogen and silicon.

Silicon supplementation has had limited study as a method to increase bone mineral density in women with postmenopausal osteoporosis [61, 62]. Intramuscular injections of silicon as monomethyl trisilanol at a dose of 50 mg twice a week for four months were administered to postmenopausal women with osteoporosis. This treatment was compared to etidronate, fluoride, magnesium, and controls [61]. Patients in all groups received 1000 mg of calcium and 500 IU of Vitamin D daily. A significant improvement in femoral bone density was noted in the silicon group compared to the other groups. Vertebral bone density improved more with administration of magnesium and etidronate than with silicon.

This is consistent with a study of silicon supplementation in ovariectomized rats that demonstrated a significant increase in femoral bone mineral density and only marginal increases in lumbar bone density [45].

Another study of silicon supplementation in women with osteoporosis evaluated change in trabecular bone volume measured by iliac crest biopsy following a period of treatment [62]. Three groups consisted of controls, parenteral administration of 16.5 mg/wk of silicon for four months, or oral supplementation with 27.5 mg/wk for three months. Participants consumed their normal diets, but supplemental calcium or vitamin D was not added. The two groups with supplemental silicon had significant increases in trabecular bone volume compared to the control group [62].

A more recent study was conducted in osteopenic women using 3, 6, or 12 mg of silicon supplementation compared to controls [46]. All four groups received calcium and vitamin D supplementation but no other forms of treatment. After one year, the control group had a decrease in femoral bone density, while the groups with silicon supplementation maintained bone density. However, the difference was not statistically significant. It should be noted that average dietary silicon intake ranges from 20–50 mg per day in the United States; so, the levels of silicon supplementation in this study was low, even for the 12 mg/day group.

Serum markers of bone turnover instead of direct measurements of bone mineral density have also been studied following silicon supplementation [46, 63]. These studies have been inconclusive. One was a short-term study of 12 weeks that did not show any measurable changes [63]. The other study reported a significant positive change in the markers for type I collagen formation (PINP) but no change in other markers of bone turnover [46].

Based on these reports in postmenopausal women and in experimental models of postmenopausal osteoporosis, there is evidence that moderate silicon supplementation has a beneficial effect on bone mineralization and bone density, that is, independent of other factors.

5. Dietary Sources of Silicon

Principle sources of dietary silicon are whole grains, fruits, beverages, and vegetables in that order [14, 22, 56, 64] (Table 1). Unrefined cereals and grains have high silicon content, especially oats and oat bran. Rice hulls and husks are rich sources of silicon. Beer has high silicon content due to the processing of barley and hops. Meats, dairy products, and refined flours have little silicon content. Drinking water can be a source of silicon depending on the source and method of processing [14, 24]. Hard water typically has higher silicon levels than soft water. Initial purification of drinking water by flocculation decreases silicon content in tap water [22, 64].

The relationship between bone density and consumption of beer, wine, and liquor was evaluated by Tucker et al. [65]. They found that moderate consumption of alcohol was associated with increased bone mineral density in men and postmenopausal women when the source of alcohol was beer or wine but not when the source was liquor. This suggests that components other than alcohol may influence bone density.

Table 1: Common dietary sources of silicon [58, 64].

Dietary source	Portion size	mg/portion
Beer	12 oz	8.25 mg
Red wine	4 oz	1.70 mg
Raisins	100 gm	8.25 mg
Green beans	250 gm	6.10 mg
High-bran cereal	100 gm	10.17 mg
Whole grain bread	200 gm	4.50 mg
Mineral water	0.5 L	0–40 mg depending on brand
Brown rice with husks	100 gm	2.07 mg

Another study demonstrated that nonalcoholic beer acutely reduced markers of bone resorption [66]. However, the same study demonstrated that moderate intake of ethanol alone also decreased markers of bone resorption. Although Tucker and Sripanyakorn et al. suggested that the silicon in beer had a moderate effect on bone formation independent of ethanol, the short-term effects of silicon ingestion on markers of bone resorption could not be demonstrated [65, 66].

The bioavailability of silicon for intestinal absorption depends on the solubility of the silicon compound [22, 58]. Silicon levels are high in bananas but the silicon is highly polymerized and poorly absorbed [24]. Absorption of silicon is best from whole grains and grain products (breakfast cereals, breads, rice, and pasta). The silicon uptake from green beans and dried fruits is intermediate [58]. Orthosilicic acid is soluble and absorbable form of silica, that is, present in beer, some beverages, and some drinking water. High levels of orthosilicic acid are found in natural sources of water from volcanic areas [58].

Silicon is also available in some nutritional supplements with varying amounts of bioavailability [24]. Poor absorption is noted for antacids containing silicon such as magnesium trisilicate. Supplemental monomethyl silanetriol (MMST) is an absorbable form of silicon while choline-stabilized orthosilicic acid is intermediate. In general, the smaller molecules, or monomeric forms, are better absorbed than the larger, highly polymerized, or oligomeric forms [24].

In the absence of evidence of oral toxicity in animals or humans, safe upper levels for humans have been recommended with a maximum range of 700–1,750 mg/day [22, 26]. Thus, it is unlikely that modest nutritional supplementation would cause adverse effects in humans with normal renal function.

6. Summary

Optimum therapy for postmenopausal osteoporosis includes a balanced approach of prevention, exercise, nutrition, early diagnosis, and appropriate treatment. While there are numerous factors that contribute to bone health and to therapy for postmenopausal osteoporosis, silicon is also a mineral that is increasingly recognized as an essential nutrient for bone formation and maintenance. More attention to this important nutrient by the medical community may lead to improved dietary supplements and better understanding of the role of silicon in management of postmenopausal osteoporosis.

References

[1] United States Department of Health and Human Services, *Bone Health and Osteoporosis: A Report of the Surgeon General*, Department of Health and Human Services, Office of the Surgeon General, Rockville, Md, USA, 2004.

[2] J.-J. Body, P. Bergmann, S. Boonen et al., "Non-pharmacological management of osteoporosis: a consensus of the Belgian Bone Club," *Osteoporosis International*, vol. 22, no. 11, pp. 2769–2788, 2011.

[3] A. Carmel, A. Shieh, H. Bang, and R. S. Bockman, "The 25(OH)D level needed to maintain a favorable bisphosphonate response is ≥ 33 ng/ml," *Osteoporosis International*, vol. 23, no. 10, pp. 2479–2487, 2012.

[4] H. A. Bischoff-Ferrari, B. Dawson-Hughes, J. A. Baron et al., "Calcium intake and hip fracture risk in men and women: a meta-analysis of prospective cohort studies and randomized controlled trials," *American Journal of Clinical Nutrition*, vol. 86, no. 6, pp. 1780–1790, 2007.

[5] H. A. Bischoff-Ferrari, W. C. Willett, E. J. Orav et al., "A pooled analysis of vitamin D dose requirements for fracture prevention," *The New England Journal of Medicine*, vol. 367, no. 1, pp. 40–49, 2012.

[6] J. W. Nieves, "Skeletal effects of nutrients and neutraceuticals, beyond calcium and vitamin D," *Osteoporosis Inernational*, vol. 24, no. 3, pp. 771–786, 2013.

[7] S. Levis and V. S. Lagari, "The role of diet in osteoporosis prevention and management," *Current Osteoporosis Reports*, vol. 10, no. 4, pp. 296–302, 2012.

[8] T. Ushiroyama, A. Ikeda, and M. Ueki, "Effect of continuous combined therapy with vitamin K_2 and vitamin D_3 on bone mineral density and coagulofibrinolysis function in postmenopausal women," *Maturitas*, vol. 41, no. 3, pp. 211–221, 2002.

[9] A. A. Poundarik, T. Diab, G. E. Sroga et al., "Dilational band formation in bone," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 47, pp. 19178–19183, 2012.

[10] R. B. Ervin, C. Y. Wang, J. D. Wright, and J. Kennedy-Stephens, *Dietary Intake of Selected Minerals for the United States Population: 1999-2000*, Advance Data from Vital Health Statistics, no. 341, National Center for Health Statistics, Hyattsville, Md, USA, 2004.

[11] R. B. Ervin, J. D. Wright, C. Y. Wang, and J. Kennedy-Stephens, *Dietary Intake of Selected Vitamins for the United States Population: 1999-2000*, Advance Data from Vital Health Statistics, no. 339, National Center for Health Statistics, Hyattsville, Md, USA, 2004.

[12] C. T. Price, J. R. Langford, and F. A. Liporace, "Essential nutrients for bone health and a review of their availability in the average North American diet," *Open Orthopaedics Journal*, vol. 6, pp. 143–149, 2012.

[13] W. M. C. Chumlea, "Silica, a mineral of unknown but emerging health importance," *Journal of Nutrition, Health and Aging*, vol. 11, no. 2, p. 93, 2007.

[14] R. Jugdaohsingh, "Silicon and bone health," *Journal of Nutrition, Health and Aging*, vol. 11, no. 2, pp. 99–110, 2007.

[15] E. M. Carlisle, "Silicon: a requirement in bone formation independent of vitamin D_1," *Calcified Tissue International*, vol. 33, no. 1, pp. 27–34, 1981.

[16] F. H. Nielsen and H. H. Sandstead, "Are nickel, vanadium, silicon, fluorine, and tin essential for man? A review," *American Journal of Clinical Nutrition*, vol. 27, no. 5, pp. 515–520, 1974.

[17] E. M. Carlisle, "Silicon: a possible factor in bone calcification," *Science*, vol. 167, no. 3916, pp. 279–280, 1970.

[18] E. M. Carlisle, "A relationship between silicon and calcium in bone formation," *Federal Proceedings*, vol. 29, p. 565, 1970.

[19] K. Schwarz and D. B. Milne, "Growth-promoting effects of silicon in rats," *Nature*, vol. 239, no. 5371, pp. 333–334, 1972.

[20] M. Hott, C. de Pollak, D. Modrowski, and P. J. Marie, "Short-term effects of organic silicon on trabecular bone in mature ovariectomized rats," *Calcified Tissue International*, vol. 53, no. 3, pp. 174–179, 1993.

[21] H. M. Macdonald, A. C. Hardcastle, R. Jugdaosingh, W. D. Fraser, D. M. Reid, and J. J. Powell, "Dietary silicon interacts with oestrogen to influence bone health: evidence from the Aberdeen Prospective Osteoporosis Screening Study," *Bone*, vol. 50, no. 3, pp. 681–687, 2012.

[22] K. R. Martin, "The chemistry of silica and its potential health benefits," *Journal of Nutrition, Health and Aging*, vol. 11, no. 2, pp. 94–98, 2007.

[23] W. Peters, D. Smith, and S. Lugowski, "Silicon assays in women with and without silicone gel breast implants—a review," *Annals of Plastic Surgery*, vol. 43, no. 3, pp. 324–330, 1999.

[24] S. Sripanyakorn, R. Jugdaohsingh, W. Dissayabutr, S. H. C. Anderson, R. P. H. Thompson, and J. J. Powell, "The comparative absorption of silicon from different foods and food supplements," *British Journal of Nutrition*, vol. 102, no. 6, pp. 825–834, 2009.

[25] S. Giammarioli, M. Mosca, and E. Sanzini, "Silicon content of Italian mineral waters and its contribution to daily intake," *Journal of Food Science*, vol. 70, no. 8, pp. S509–S512, 2005.

[26] F. Aguilar, B. Dusemund, P. Galtier et al., "Scientific opinion: monomethylsilanetriol added for nutritional purposes to food supplements," *European Food Safety Authority Journal*, vol. 950, pp. 1–12, 2009.

[27] H. J. Gitelman, F. R. Alderman, and S. J. Perry, "Silicon accumulation in dialysis patients," *American Journal of Kidney Diseases*, vol. 19, no. 2, pp. 140–143, 1992.

[28] Y. Fujino, Y. Inoue, M. Onodera et al., "Case of concrete hardener poisoning complicated with acute renal failure treated by hemodialysis," *Chudoku Kenkyu*, vol. 20, no. 3, pp. 263–268, 2007 (Japanese).

[29] M. H. Lee, Y. H. Lee, T. H. Hsu, M. T. Chen, and L. S. Chang, "Silica stone—development due to long time oral trisilicate intake. Case report," *Scandinavian Journal of Urology and Nephrology*, vol. 27, no. 2, pp. 267–269, 1993.

[30] E. M. Carlisle, "In vivo requirement for silicon in articular cartilage and connective tissue formation in the chick," *Journal of Nutrition*, vol. 106, no. 4, pp. 478–484, 1976.

[31] K. Schwarz, "A bound form of silicon in glycosaminoglycans and polyuronides," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 70, no. 5, pp. 1608–1612, 1973.

[32] D. M. Reffitt, N. Ogston, R. Jugdaohsingh et al., "Orthosilicic acid stimulates collagen type 1 synthesis and osteoblastic differentiation in human osteoblast-like cells in vitro," *Bone*, vol. 32, no. 2, pp. 127–135, 2003.

[33] C. D. Seaborn and F. H. Nielsen, "Dietary silicon and arginine affect mineral element composition of rat femur and vertebra," *Biological Trace Element Research*, vol. 89, no. 3, pp. 239–250, 2002.

[34] C. D. Seaborn and F. H. Nielsen, "Dietary silicon affects acid and alkaline phosphatase and ^{45}calcium uptake in bone of rats,"

Journal of Trace Elements in Experimental Medicine, vol. 7, no. 1, pp. 11–18, 1994.

[35] Y. Kanda, "Piezpresistance effect of silicon," *Sensors and Actuators A*, vol. 28, no. 2, pp. 83–91, 1991.

[36] T. Miclau, K. J. Bozic, B. Tay et al., "Bone injury, regeneration, and repair," in *Orthopedic Basic Science: Foundations of Cllnical Practice*, T. A. Einhorn, R. J. O'Keefe, and J. A. Buckwalter, Eds., pp. 331–348, American Adacemy of Orthopedic Surgeons, Rosemont, Ill, USA, 2007.

[37] K. Sahin, M. Onderci, N. Sahin et al., "Dietary arginine silicate inositol complex improves bone mineralization in quail," *Poultry Science*, vol. 85, no. 3, pp. 486–492, 2006.

[38] F. Z. Küçükbay, H. Yazlak, N. Sahin et al., "Effects of dietary arginine silicate inositol complex on mineral status in rainbow trout (Oncorhynchus mykiss)," *Aquaculture Nutrition*, vol. 14, no. 3, pp. 257–262, 2008.

[39] J. W. Merkley and E. R. Miller, "The effect of sodium fluoride and sodium silicate on growth and bone strength of broilers," *Poultry Science*, vol. 62, no. 5, pp. 798–804, 1983.

[40] B. D. Nielsen, G. D. Potter, E. L. Morris et al., "Training distance to failure in young racing quarter horses fed sodium zeolite A," *Journal of Equine Veterinary Science*, vol. 13, no. 10, pp. 562–567, 1993.

[41] H. Rico, J. L. Gallego-Lago, E. R. Hernandez et al., "Effect of silicon supplement on osteopenia induced by ovariectomy in rats," *Calcified Tissue International*, vol. 66, no. 1, pp. 53–55, 2000.

[42] Y. J. Bae, J. Y. Kim, M. K. Choi, Y. S. Chung, and M. H. Kim, "Short-term administration of water-soluble silicon improves mineral density of the femur and tibia in ovariectomized rats," *Biological Trace Element Research*, vol. 124, no. 2, pp. 157–163, 2008.

[43] M. H. Kim, Y. J. Bae, M. K. Choi, and Y. S. Chung, "Silicon supplementation improves the bone mineral density of calcium-deficient ovariectomized rats by reducing bone resorption," *Biological Trace Element Research*, vol. 128, no. 3, pp. 239–247, 2009.

[44] D. N. Kalu, "The ovariectomized rat model of postmenopausal bone loss," *Bone and Mineral*, vol. 15, no. 3, pp. 175–191, 1991.

[45] M. Calomme, P. Geusens, N. Demeester et al., "Partial prevention of long-term femoral bone loss in aged ovariectomized rats supplemented with choline-stabilized orthosilicic acid," *Calcified Tissue International*, vol. 78, no. 4, pp. 227–232, 2006.

[46] T. D. Spector, M. R. Calomme, S. H. Anderson et al., "Choline-stabilized orthosilicic acid supplementation as an adjunct to calcium/vitamin D3 stimulates markers of bone formation in osteopenic females: a randomized, placebo-controlled trial," *BMC Musculoskeletal Disorders*, vol. 9, article 85, 2008.

[47] H. Kayongo-Male and J. L. Julson, "Effects of high levels of dietary silicon on bone development of growing rats and turkeys fed semi-purified diets," *Biological Trace Element Research*, vol. 123, no. 1–3, pp. 191–201, 2008.

[48] M. J. Coathup, S. Samizadeh, Y. S. Fang, T. Buckland, K. A. Hing, and G. W. Blunn, "The osteoinductivity of silicate-substututed calcium phosphate," *Journal of Bone and Joint Surgery. American*, vol. 93, no. 23, pp. 2219–2226, 2011.

[49] A. M. Pietak, J. W. Reid, M. J. Stott, and M. Sayer, "Silicon substitution in the calcium phosphate bioceramics," *Biomaterials*, vol. 28, no. 28, pp. 4023–4032, 2007.

[50] W. Waked and J. Grauer, "Silicates and bone fusion," *Orthopedics*, vol. 31, no. 6, pp. 591–597, 2008.

[51] M. Bohner, "Silicon-substituted calcium phosphates—a critical view," *Biomaterials*, vol. 30, no. 32, pp. 6403–6406, 2009.

[52] F. Chen, P. Cole, L. Wen, Z. Mi, and E. J. Trapido, "Estimates of trace element intakes in Chinese farmers," *Journal of Nutrition*, vol. 124, no. 2, pp. 196–201, 1994.

[53] A. Anasuya, S. Bapurao, and P. K. Paranjape, "Fluoride and silicon intake in normal and endemic fluorotic areas," *Journal of Trace Elements in Medicine and Biology*, vol. 10, no. 3, pp. 149–155, 1996.

[54] O. Johnell and J. A. Kanis, "An estimate of the worldwide prevalence, mortality and disability associated with hip fracture," *Osteoporosis International*, vol. 15, no. 11, pp. 897–902, 2004.

[55] R. Jugdaohsingh, K. L. Tucker, N. Qiao, L. A. Cupples, D. P. Kiel, and J. J. Powell, "Dietary silicon intake is positively associated with bone mineral density in men and premenopausal women of the Framingham Offspring cohort," *Journal of Bone and Mineral Research*, vol. 19, no. 2, pp. 297–307, 2004.

[56] S. A. McNaughton, C. Bolton-Smith, G. D. Mishra, R. Jugdaohsingh, and J. J. Powell, "Dietary silicon intake in postmenopausal women," *British Journal of Nutrition*, vol. 94, no. 5, pp. 813–817, 2005.

[57] H. J. Bowen and A. Peggs, "Determination of the silicon content of food," *Journal of the Science of Food and Agriculture*, vol. 35, no. 11, pp. 1225–1229, 1984.

[58] R. Jugdaohsingh, S. H. C. Anderson, K. L. Tucker et al., "Dietary silicon intake and absorption," *American Journal of Clinical Nutrition*, vol. 75, no. 5, pp. 887–893, 2002.

[59] H. M. Macdonald, A. E. Hardcastle, R. Jugdaohsingh, and D. M. Reid, "Dietary silicon intake is associated with bone mineral density in premenopausal women and postmenopausal women taking HRT," *Journal of Bone and Mineral Research*, vol. 20, p. S393, 2005.

[60] M. Ten Bolscher, J. C. Netelenbos, R. Barto, L. M. van Buuren, and W. J. F. van der Vijgh, "Estrogen regulation of intestinal calcium absorption in the intact and ovariectomized adult rat," *Journal of Bone and Mineral Research*, vol. 14, no. 7, pp. 1197–1202, 1999.

[61] J. Eisinger and D. Clairet, "Effects of silicon, fluoride, etidronate and magnesium on bone mineral density: a retrospective study," *Magnesium Research*, vol. 6, no. 3, pp. 247–249, 1993.

[62] A. Schiano, F. Eisinger, P. Detolle, A. M. Laponche, B. Brisou, and J. Eisinger, "Silicium, bone tissue and immunity," *Revue du Rhumatisme et des Maladies Ostéo-Articulaires*, vol. 46, no. 7–9, pp. 483–486, 1979.

[63] Z. Li, H. Karp, A. Zerlin, T. Y. A. Lee, C. Carpenter, and D. Heber, "Absorption of silicon from artesian aquifer water and its impact on bone health in postmenopausal women: a 12 week pilot study," *Nutrition Journal*, vol. 9, no. 1, article 44, 2010.

[64] J. J. Powell, S. A. McNaughton, R. Jugdaohsingh et al., "A provisional database for the silicon content of foods in the United Kingdom," *British Journal of Nutrition*, vol. 94, no. 5, pp. 804–812, 2005.

[65] K. L. Tucker, R. Jugdaohsingh, J. J. Powell et al., "Effects of beer, wine, and liquor intakes on bone mineral density in older men and women," *American Journal of Clinical Nutrition*, vol. 89, no. 4, pp. 1188–1196, 2009.

[66] S. Sripanyakorn, R. Jugdaohsingh, A. Mander, S. L. Davidson, R. P. H. Thompson, and J. J. Powell, "Moderate ingestion of alcohol is associated with acute ethanol-induced suppression of circulating CTX in a PTH-independent fashion," *Journal of Bone and Mineral Research*, vol. 24, no. 8, pp. 1380–1388, 2009.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 768579, 9 pages
http://dx.doi.org/10.1155/2013/768579

Research Article

Glucocorticoid-Induced Bone Loss Is Associated with Abnormal Intravertebral Areal Bone Mineral Density Distribution

Louise I. Manning,[1,2] Andrew M. Briggs,[1,3,4] Sharon Van Doornum,[1] Ashwini Kale,[1,2] Susan Kantor,[1,2] and John D. Wark[1,2]

[1] *University of Melbourne, Department of Medicine, Royal Melbourne Hospital, Parkville, VIC 3050, Australia*
[2] *Bone and Mineral Service, Royal Melbourne Hospital, Parkville, VIC 3050, Australia*
[3] *Curtin Health Innovation Research Institute, Curtin University, Bentley, WA 6102, Australia*
[4] *Arthritis Victoria and Osteoporosis Victoria, Elsternwick, VIC 3185, Australia*

Correspondence should be addressed to John D. Wark; jdwark@unimelb.edu.au

Received 3 January 2013; Accepted 16 April 2013

Academic Editor: Cory Xian

Individuals with glucocorticoid-induced osteoporosis experience vertebral fractures at an increased rate and at higher vertebral areal bone mineral density (aBMD) than individuals with primary osteoporosis. Standard posteroanterior- (PA-) projection dual energy X-ray absorptiometry (DXA) lacks the diagnostic sensitivity required for reliable estimation of vertebral fracture risk in individuals. Assessment of subregional vertebral aBMD using lateral-projection DXA may improve the predictive value of DXA parameters for fracture. One hundred and four individuals were recruited and grouped for this study: primary osteoporosis with no history of vertebral fracture ($n = 43$), glucocorticoid-induced bone loss ($n = 13$), and healthy controls ($n = 48$). Standard PA-projection and supine-lateral scans were performed, and lateral scans were analysed according to an established protocol to measure aBMD within 6 subregions. Main effects for subregion and group were assessed and observed, by ANCOVA. Ratios were calculated between subregions and compared between groups, to overcome the potentially confounding influence of variability in subregional geometry. Significantly lower values were observed in the glucocorticoid group for the ratios of (i) anterior subregion: whole vertebral body and (ii) posterior: whole vertebral body when compared to the primary osteoporosis and control groups ($P < 0.05$). Lower anterior subregional aBMD in individuals on glucocorticoid therapy may help to explain the increased vertebral fracture risk in this patient group.

1. Introduction

Glucocorticoid therapy is widely used for the management of inflammatory and allergic conditions. While being effective in ameliorating these conditions, glucocorticoids can adversely affect bone quality and bone strength [1], thereby leading to an increased propensity to fracture. Secondary bone fragility or osteoporosis due to glucocorticoid therapy, termed glucocorticoid-induced osteoporosis (GIO), is a clinically important phenomenon. This is primarily because of reduced skeletal integrity predisposing to fragility fracture, but also because patients with GIO experience vertebral fractures at a rate which exceeds individuals with primary osteoporosis, despite a comparable areal BMD (aBMD) [2–7]. Identifying possible mechanisms underlying this observation is therefore important.

The effects of glucocorticoids are mediated by the cytosolic glucocorticoid receptor [8] (cGCR), which is expressed on a variety of skeletal and extraskeletal cells [9]. The adverse effects of glucocorticoids on bone are complex and are both direct and indirect. Important direct effects include an early transient increase in osteoclastic bone resorption, a reduction in osteoblast differentiation and function, and an increase in osteocyte apoptosis [10]. There may also be indirect effects leading to increased fracture risk including glucocorticoid-induced myopathy [11].

This may explain the rapid early phase of bone loss upon commencement of therapy. Glucocorticoids similarly affect

bone loss due to their proapoptotic effects on osteoblasts and osteocytes [8, 12, 13]. Defects in the osteocyte network within bone negatively affect bone mineralisation and microarchitecture and may explain the exaggerated trabecular bone loss [3, 7, 14].

Vertebral fractures are the most common type of osteoporosis-related fracture [15–17] and often present without obvious acute symptoms [18], leading to lack of recognition and treatment. Vertebral fractures frequently occur spontaneously or from minor trauma [18] and are associated with morbidity including decreased physical function (e.g., balance and muscle function), loss of height, compromised pulmonary capacity, increased thoracic kyphosis, and acute and chronic back pain [6, 15, 17–21]. These sequelae become more significant with increasing numbers of vertebral fractures. Vertebral fractures strongly predict the risk of future fracture [22–25], in particular subsequent vertebral and, to a lesser extent, appendicular fractures.

Possible explanations for the increased vertebral fracture risk among individuals with GIO compared to those with primary osteoporosis may lie in the pathophysiology of bone loss with underlying systemic inflammatory disease in these individuals, in particular rheumatoid arthritis, which has been found to be associated with increased fracture risk [26, 27], the pharmacokinetics of glucocorticoids, or perhaps poor measurement specificity of bone parameters like aBMD in these individuals.

Posteroanterior- (PA-) projection dual energy X-ray absorptiometry (DXA) is the most commonly used modality for the measurement of aBMD at the hip and lumbar spine [28]. A strong relationship exists between aBMD and bone mineral content (BMC) derived from PA-projection DXA scans [29] and these variables are used ubiquitously to diagnose and monitor skeletal integrity. However, they cannot be used to reliably predict an individual's vertebral fracture risk, particularly in the context of glucocorticoid-induced bone loss [30–32]. Lateral-projection DXA has better specificity and sensitivity when detecting vertebral osteoporosis and age-related bone loss and is better able to detect vertebral fractures than PA-projection scanning [30, 32–35]. These attributes of lateral-projection scanning are most likely related to the ability to isolate the vertebral body from the cortical bone-rich posterior elements of the vertebra, thus limiting the analysis to the metabolically-active trabecular bone of interest.

Lateral-projection scans also enable measurement of BMC and aBMD in intravertebral subregions. Variation in aBMD between intravertebral subregions could explain why one individual may sustain a vertebral fracture whilst another may not, due to subregional variation in bone compressive strength; in essence, a chain is only as strong as its weakest link. *Ex vivo* research undertaken by Wegrzyn et al. [36] supported this hypothesis and suggested that trabecular microarchitecture and its associated regional heterogeneity contribute to vertebral fracture risk prediction. Older studies utilising histomorphometry, QCT, and DXA techniques have consistently shown relatively lower bone volume and trabecular thickness in both central and anterior vertebral subregions [36–40]. These *ex vivo* studies suggest that there could be a difference in intravertebral aBMD between individuals with and without vertebral fracture and potentially in those with glucocorticoid-induced bone loss.

A technique to measure *in vivo* vertebral subregional aBMD has been developed by our research team with a matched supine-lateral DXA scanning technique using a Hologic QDR4500A densitometer [41]. Subsequent studies have further validated the subregional methodology, establishing high short-term *in vivo* precision [41], moderate to high intrarater and interrater precision [41], and concurrent validity when compared with pQCT and micro-CT [42]. Validation findings were later replicated using a much larger sample size [43], demonstrating that lateral DXA is valid when measuring heterogeneity in aBMD between intravertebral subregions. Lateral DXA measurement of aBMD has also been shown to be a better predictor of vertebral failure load at both the whole vertebral body [44] and within subregions [45], compared with standard PA DXA.

BMC derived from DXA is directly influenced by bone size; thus, comparing BMC between large and small stature people becomes problematic. While normalising BMC to projection area, creating aBMD (g/cm^2), corrects somewhat for variability in bone size, aBMD is still influenced by bone size as it cannot account for bone depth and thus cannot measure true (volumetric) bone density, which remains independent of bone size. Consequently, comparing subregional aBMD (srBMD) between individuals is not valid, due to inevitable heterogeneity in subregional bone geometry among individuals. To minimise the potentially confounding effect of variable subregional bone geometry on srBMD differences, ratios of srBMD may be compared between individuals. Deriving a ratio index of srBMD from an individual and comparing it to the same ratio in other individuals eliminate the potentially confounding effect of differences in subregional geometry between individuals. Therefore, the aim of this study was to compare srBMD ratios between individuals with primary and secondary osteoporosis as a means to explain a possible mechanism underlying the increased rate of vertebral fractures observed in GIO populations.

2. Materials and Methods

2.1. Design. A cross-sectional cohort study was undertaken in a tertiary hospital-based bone densitometry unit (BDU).

2.2. Participants. A convenience sample of 104 individuals was recruited sequentially for this study to form three participant groups. These were (i) individuals diagnosed with primary osteoporosis who had no history of vertebral fracture ($n = 43$), (ii) individuals diagnosed with glucocorticoid-induced osteoporosis (GIO) or glucocorticoid-induced osteopenia who had no history of vertebral fracture ($n = 13$), and (iii) healthy controls ($n = 48$). Participants in the GIO and primary osteoporosis groups were referred to the BDU for clinical scanning purposes, while control participants were recruited from the community through advertisements in local media. The study protocol was approved by the Human Research Ethics Committee of Melbourne Health

(2009.085). All participants provided written, informed consent to participate.

General inclusion criteria were that all participants were required to be aged ≥50 years old; female participants had to have been at least 5-year postmenopausal, defined as 5 years or more, since their last menstrual period; and all individuals were required to be independent with ambulation and activities of daily living. General exclusion criteria included a history of spinal surgery, any vertebral fracture classified as Grade 1 or higher according to the Genant criteria [46], a body mass index (BMI) greater than 33 or less than 18 kg/m^2, any formally diagnosed musculoskeletal conditions in the spine that may affect the accuracy of a spinal DXA scan, and the current use of bone-active therapies. To be included in the glucocorticoid group, participants needed to have had a history of glucocorticoid use of ≥5 mg/day of prednisolone continuously for at least 6 months within the last 3 years along with the diagnosis of either osteoporosis or osteopenia, based on WHO criteria, using DXA-derived T-scores (T-score ≤ −1.5) from either the total hip, total spine, or L3 vertebral body. Individuals in the primary osteoporosis group were required to have a diagnosis of osteoporosis based on WHO criteria (T-score ≤ −2.5) at the total hip, total spine, or L3 vertebral body from the PA-projection DXA scan. Individuals in the control group were required to have normal BMD based on WHO criteria (T-score > −1.0) at all of these sites. Eligibility to participate was determined from a telephone interview and a review of DXA data from formal clinical scans (GIO and primary osteoporosis groups) or voluntary research scans (control group).

2.3. Protocol. Each participant attended the BDU where demographic and clinical data were collected via questionnaire and height (cm) and mass (kg) measured to ensure their BMI fell within the required range of 18–33 kg/m^2. After DXA scanning, a 10-year risk of sustaining a hip or major osteoporotic fracture was calculated using the FRAX tool using Australian reference data [47].

2.4. Bone Densitometry

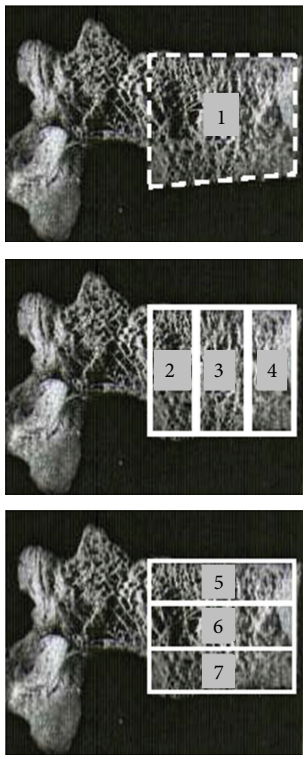
2.4.1. DXA Scanning. All participants were scanned on a Hologic QDR4500A fan beam densitometer (Bedford, MA, USA), using Hologic software version 9.10D. To monitor the reproducibility of the machine's results, a quality control spine phantom with known BMC was scanned daily prior to any patient scans. Over the data collection period, the mean coefficient of variation (%CV) was 0.35%, indicating excellent temporal stability of the machine.

All participants underwent standard PA-projection scanning of the hip and spine, following standard Hologic protocols to derive aBMD, BMC, T-scores, and Z-scores at the right hip and spine, either for clinical (GIO and primary osteoporosis groups) or research (control group) purposes. These standard scans were used to determine group eligibility according to WHO classification criteria. In order to exclude the presence of vertebral fracture, Instant Vertebral Assessment (IVA) scans were acquired to assess vertebral morphometry between T5 and L4 (Hologic software 9.10D). IVA has been reported to have high specificity (>90%), high sensitivity (70–86%), negative predictive values (93.6–99.4%), and good reliability between raters [16, 17, 25, 48, 49]. A matched supine-lateral scan of the lumbar spine was then acquired using the array scanning mode and this scan set was used for measurement of srBMD.

2.4.2. DXA Analysis. Standard PA DXA scans of the hip and spine were analysed according to the manufacturer's instructions [50]. A combination of qualitative and quantitative methods was used to identify and diagnose vertebral fracture(s) on the basis of images gained from IVA scanning. Vertebral fractures were defined as grade 2 deformity or higher according to the semiquantitative method described by Genant et al. [46]; a method with excellent reproducibility [51, 52]. This was achieved by visual inspection of vertebral morphometry, from the IVA scan, and a measured reduction in anterior, middle, or posterior vertebral height of at least 25–40% or a 20–40% reduction in vertebral area, as calculated by Hologic software 9.10D. Adjustment factors calculated from normal vertebral height ratios, established by Diacinti et al. [53] for T5-L4, were included in the calculation of height ratios, serving as reference data to limit overestimation of fracture incidence. The reliability of IVA scans to diagnose vertebral fractures has been established previously by Chapurlat et al. [49].

Lumbar spine images acquired from the lateral DXA scans were used to calculate aBMD in seven regions of interest (ROIs) at the L3 vertebral body (Figure 1), consistent with established protocols for which reliability and validity have been established [38, 41–45, 54]. L3 was used as the target vertebra as it is less affected by overlapping ribs than L2 [55]. Prior to the subregional ROI analysis, the global ROI window used during the Hologic analysis was set with a maximum height of 151 pixels and width of 141 pixels to ensure consistent software-defined analysis parameters between images. ROI 1 was defined as the entire vertebral body area and hence was demarcated by the four borders of the L3 vertebral body (superior and inferior endplates and anterior/posterior edges of the centrum). ROI 1 was therefore comparable to the vertebral area used to calculate aBMD in the standard Hologic analysis of lateral scan data. ROIs 2–7 consisted of six intravertebral subregions, of which the size and shape were selected manually. ROIs 2–4 were sagittally orientated, equally dividing ROI 1, by width, into thirds. ROIs 5–7 were oriented transversely, again dividing the total area of ROI 1 into equal thirds, by height. A consistent pixel width and height for sagittal and transverse subregions were employed to maintain the uniformity of dimensions. After the size and shape of the subregions were defined, individual aBMD measurements were calculated for each ROI.

2.5. Data Analysis. Descriptive and clinical characteristics were compared between the three groups using a one-way ANOVA. Differences in srBMD between groups were examined with two 3 × 4 ANCOVAs with one repeated measure. For each model, "group" was set as the between-subject factor ($k = 3$) and ROI as the within-subject repeated measure ($k = 4$) *a priori*, while L3 vertebral body area (ROI 1



Region 1: whole area (integrated cortical and trabecular bone) of the vertebral body, defined by the four corners of the vertebral body in the sagittal plane

Subregion 2: posterior third of ROI 1

Subregion 3: middle third of ROI 1

Subregion 4: anterior third of ROI 1

Subregion 5: superior third of ROI 1

Subregion 6: central third of ROI 1

Subregion 7: interior third of ROI 1

Figure 1: DXA-derived vertebral subregions defined using Hologic software. ROI 1 (whole) was defined by the four corners of the vertebra. ROIs 2–4 (posterior, middle, and anterior) formed equal thirds in the area of ROI 1, oriented sagittally. ROIs 5–7 (superior, central, and inferior) formed equal thirds in area of ROI 1, oriented transversely. Reprinted from Briggs et al. [42] with permission from Elsevier Copyright Clearance Center.

area), derived from the lateral-projection scan, was used as a covariate to account for observed variability in bone size between the groups. To ensure that no overlapping subregions were compared *post hoc*, ANCOVA 1 included whole vertebral area (ROI 1) and the three subregions orientated sagittally (ROIs 2–4), whilst ANCOVA 2 included ROI 1 and the three subregions orientated transversely (ROIs 5–7).

To minimise the potentially confounding influence of subregional geometry on subregional BMD values between groups, ratios of srBMD were compared between groups with a one-way ANOVA. Twelve ratios were derived: six comparing subregional values (ROIs 2–7) to the whole vertebral area (ROI 1) and six comparing non-overlapping subregions, consistent with an earlier analysis [54].

The critical alpha level of significance was set at 0.05 (2-tailed) and Bonferroni corrections were made for multiple *post hoc* comparisons in all statistical models. Statistical analysis was undertaken using SPSS version 19.1 for Windows.

3. Results

3.1. Descriptive Measures. While there was no difference in age and height between the groups, there was a significantly greater proportion of males in the GIO group compared to the other groups (Table 1). Significant differences were also observed between the groups for mass, BMI, FRAX score, *T*-scores for the spine and hip, and L3 vertebral body area (ROI 1). The GIO group had significantly higher mass (mean difference (MD) = 13.77 kg, 95% CI = 4.96–22.58), BMI (MD = 3.00 kg/m^2, 95% CI = 0.48–5.53), *T*-scores at the PA total spine (MD = 0.99 SD, 95% CI = 0.16–1.82) and the total hip (MD = 0.69 SD, 95% CI = 0.06–1.32), and a significantly larger L3 vertebral body area (MD = 2.27 cm^2, 95% CI = 0.46–4.07) compared to the primary osteoporosis group. When compared to controls, the primary osteoporosis group was observed to have significantly lower mass (MD = 10.06 kg, 95% CI = 4.33–15.91), lower BMI (MD = 2.65, 95% CI = 0.97–4.33) and lower *T*-scores at the PA total spine (MD = 2.66, 95% CI = 2.11–3.21), lateral spine (MD = 1.40, 95% CI = 0.73–2.06), and total hip (MD = 1.86, 95% CI = 1.44–2.28). The GIO group also had significantly lower *T*-scores at the PA total spine (MD = 1.67, 95% CI = 0.85–2.48) and significantly higher FRAX hip scores (MD = 1.67, 95% CI = 0.42–2.43) compared to controls. Both the GIO and primary osteoporosis groups had significantly higher FRAX major osteoporotic fracture scores than controls (MD = 3.42, 95% CI = 0.89–5.95 and MD = 1.96, 95% CI = 0.13–2.80, resp.).

3.2. Differences in Mean Areal srBMD between Groups

3.2.1. Sagittal Subregions. A main effect for subregion was observed ($F = 4.29$, $P < 0.01$), with mean adjusted aBMD in all subregions being significantly different from each other ($P < 0.0001$). All analyses were adjusted for ROI 1 area, to account for differences in vertebral size between groups. One exception was observed for the whole vertebral body (ROI 1) mean adjusted aBMD versus middle subregional (ROI 3) mean adjusted aBMD ($P = 1.00$). The lowest adjusted mean (±SD) aBMD was observed in the anterior subregion (0.411 ± 0.126 g/cm^2) (ROI 4) and the highest in the posterior subregion (0.583 ± 0.137 g/cm^2) (ROI 2), and importantly, these adjusted mean srBMD values varied significantly from the adjusted mean srBMD of the whole vertebral body (Figure 2). A main effect for group ($F = 15.75$, $P < 0.0001$) was observed, reflecting lower adjusted mean srBMD in both the GIO and primary osteoporosis groups compared to controls (MD = 0.14 g/cm^2, 95% CI = 0.05–0.24 and MD = 0.12 g/cm^2, 95% CI = 0.05–0.18, resp.) (Figure 2). No significant difference was observed between the GIO and primary osteoporosis groups.

No significant group × subregion interaction main effect was observed ($F = 1.44$, $P = 0.169$); however, adjusted mean (±SD) aBMD in the anterior subregion (ROI 4) was lower in the GIO group (0.355 ± 0.148 g/cm^2) compared to the primary osteoporosis group (0.416 ± 0.079 g/cm^2, MD = 0.06 g/cm^2, 95% CI = 0.031–0.09) ($P = 0.017$), a result not observed at any other subregion. This finding represented an effect size (Cohen's *d*) of $d = 0.51$.

3.2.2. Transverse Subregions. A main effect for subregion was observed ($F = 2.80$, $P = 0.04$), with mean adjusted aBMD in all subregions being significantly different from each other ($P < 0.0001$), with the exception of the whole vertebral

Table 1: Descriptive measures for each group and pooled data, expressed as the mean (SD) for age, height, mass, and BMI and as the mean (95% CI) for FRAX data, *T*-scores, and L3 area.

Descriptive measure	Group			*Pooled*
	Primary osteoporosis	GIO	Control	
N (% female)	43 (95.3)	13 (30.8)	48 (83.3)	*104 (81.7)*
Age (years)	61.1 (5.0)	63.7 (7.8)	61.5 (5.6)	*62.1 (6.1)*
Height (cm)	163.1 (7.6)	169.1 (8.7)	166.8 (6.5)	*166.3 (7.6)*
Mass (kg)	61.8 (10.0)[b,c]	75.6 (16.1)[a]	71.9 (8.8)	*69.8 (11.6)*
BMI (kg/m^2)	23.2 (3.0)[b,c]	26.2 (3.7)[a]	25.8 (2.8)	*25.1 (3.2)*
FRAX major osteoporotic (%)	4.5 (3.7 to 5.4)[c]	6.0 (3.3 to 8.7)[c]	2.6 (2.0 to 3.2)	*4.4 (3.0 to 5.8)*
FRAX hip (%)	1.1 (0.8 to 1.4)	2.1 (0.8 to 3.4)[c]	0.4 (0.1 to 0.7)	*1.2 (0.6 to 1.8)*
T-score PA spine (L1–4)	−2.6 (−2.9 to −2.4)[b,c]	−1.6 (−2.4 to −0.9)[a,c]	0.0 (−0.2 to 0.3)	*−1.4 (−1.8 to −1.0)*
T-score total hip	−2.0 (−2.2 to −1.8)[b,c]	−1.3 (−1.9 to −0.8)[a,c]	−0.2 (−0.4 to 0.1)	*−1.8 (−1.5 to −0.8)*
L3 area (ROI 1) (cm^2)	8.8 (8.1 to 9.5)[b]	11.1 (10.1 to 12.0)[a]	9.9 (9.1 to 10.0)	*9.9 (9.1 to 10.5)*

[a]Significant difference compared to the primary osteoporosis group ($P < 0.01$); [b]significant difference compared to the GIO group ($P < 0.01$); [c]significant difference compared to control group ($P < 0.01$).

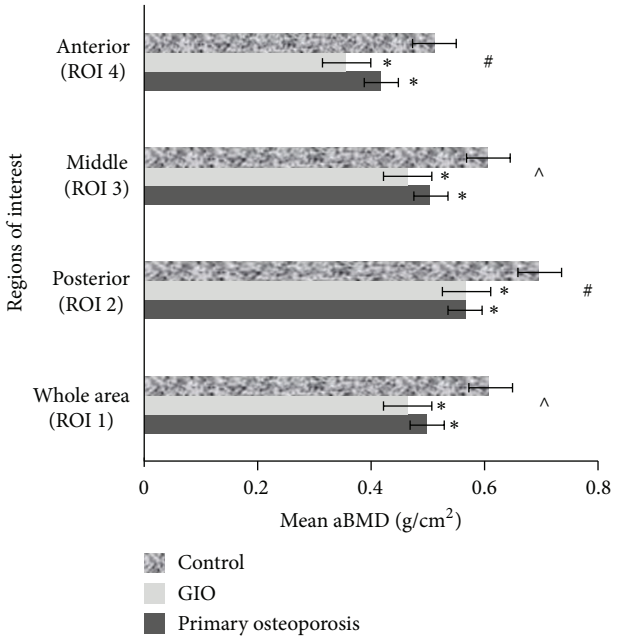


Figure 2: ANOVA 1, mean areal BMD for sagittal regions of interest between groups, adjusted for ROI 1 area. Error bars are SEM. *Significantly different to controls ($P < 0.0001$). #Significantly different compared to all other ROIs. ^Significantly different to ROIs 2 and 4.

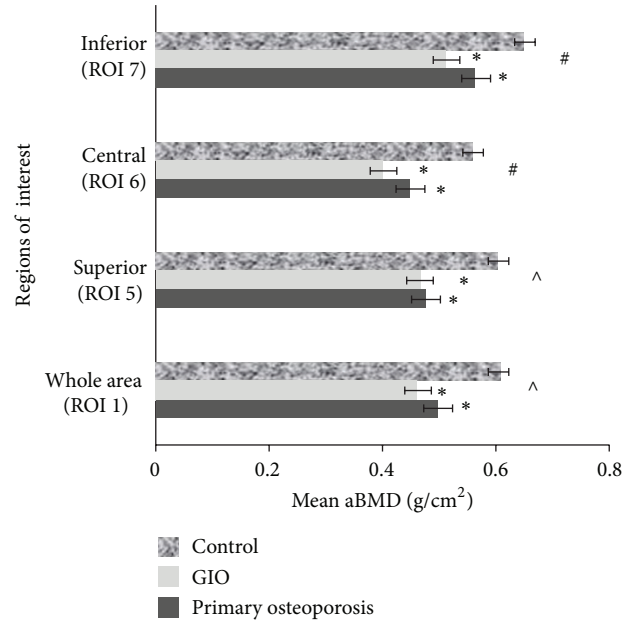


Figure 3: ANOVA 2, mean areal BMD for transverse regions of interest between groups, adjusted for ROI 1 area. Error bars are SEM. *Significantly different to controls ($P < 0.0001$). #Significantly different compared to all other ROIs. ^Significantly different to ROIs 6 and 7.

body (ROI 1) adjusted mean aBMD versus superior subregional (ROI 5) adjusted mean aBMD ($P = 1.00$). The lowest adjusted aBMD was observed in the central subregion (0.451 ± 0.133 g/cm^2) (ROI 6) and the highest in the inferior subregion (0.559 ± 0.122 g/cm^2) (ROI 7), and importantly, these adjusted mean srBMD varied significantly from the adjusted mean srBMD of the whole vertebral body (Figure 3). A significant main effect for group ($F = 15.96$, $P < 0.0001$) was observed, with lower adjusted mean srBMD in both the primary osteoporosis and GIO groups compared to controls (MD = 0.108 g/cm^2, 95% CI = 0.05–0.17 and MD = 0.14 g/cm^2, 95% CI = 0.06–0.23, resp.) (Figure 3). No significant group × subregion interaction was observed ($P = 0.88$).

3.3. Ratios of srBMD. Table 2 displays srBMD ratio comparisons between groups. Comparing subregions to the whole vertebral area, the GIO group demonstrated a significantly higher ratio for ROI 2 : ROI 1 (1.27) when compared with the primary osteoporosis (1.13; MD = 0.13, 95% CI = 0.04–0.23) and control groups (1.16; MD = 0.12, 95% CI = 0.01–0.20) ($P < 0.05$) and a significantly lower ratio for ROI 4 : ROI 1 (0.72) when compared to the primary osteoporosis

Table 2: Mean (95% CI) ratio values of srBMD for all groups.

Ratio for subregions	Primary osteoporosis	GIO	Control
Posterior : whole (2 : 1)	1.13 (1.09–1.17)	**1.27 (1.15–1.38)**[a]	1.16 (1.12–1.19)
Middle : whole (3 : 1)	1.01 (0.99–1.03)	1.01 (0.92–1.10)	1.00 (0.98–1.01)
Anterior : whole (4 : 1)	0.84 (0.81–0.88)	**0.72 (0.58–0.86)**[a]	0.83 (0.80–0.86)
Superior : whole (5 : 1)	0.95 (0.93–0.98)	1.01 (0.94–1.10)	1.00 (0.96–1.04)
Central : whole (6 : 1)	0.90 (0.87–0.92)	0.85 (0.78–0.92)	0.92 (0.89–0.95)
Inferior : whole (7 : 1)	1.14 (1.12–1.18)	1.13 (1.02–1.24)	1.08 (1.05–1.11)
Middle : posterior (3 : 2)	0.90 (0.86–0.95)	0.81 (0.71–0.91)	0.87 (0.84–0.90)
Anterior : posterior (4 : 2)	0.76 (0.70–0.82)	**0.59 (0.45–0.74)**[a]	0.73 (0.69–0.77)
Anterior : middle (4 : 3)	0.84 (0.80–0.88)	0.75 (0.56–0.93)	0.84 (0.80–0.88)
Central : superior (6 : 5)	0.95 (0.91–1.00)	0.85 (0.75–0.95)	0.94 (0.89–0.99)
Superior : inferior (5 : 7)	0.85 (0.80–0.90)	0.93 (0.77–1.09)	0.95 (0.88–1.01)
Central : inferior (6 : 7)	0.80 (0.76–0.84)	0.78 (0.65–0.91)	0.86 (0.81–0.92)

[a]Significant difference compared to both primary osteoporosis and control groups ($P < 0.05$).

(0.84; MD = 0.13, 95% CI = 0.02–0.23) and control (0.83; MD = 0.12, 95% CI = 0.01–0.22) groups ($P < 0.05$). Comparing between subregions, the GIO group demonstrated a significantly lower ratio for ROI 4 : ROI 2 (0.59), compared to the primary osteoporosis (0.76; MD = 0.17, 95% CI = 0.03–0.31) and control (0.73; MD = 0.14, 95% CI = 0.00–0.27) groups ($P < 0.05$). No significant differences in srBMD ratios were observed between the primary osteoporosis and control groups for any ratio.

4. Discussion

4.1. Main Finding. Primarily, this study has shown that DXA can identify differences in srBMD within a vertebra, as displayed by the significant main effect for subregion ($P < 0.05$), consistent with earlier data [38, 42, 43]. Secondly, it was found that the GIO group demonstrated significantly lower results for the anterior subregion : whole vertebra ratio (ROI 4 : ROI 1) and the anterior subregion : posterior subregion ratio (ROI 4 : ROI 2) when compared to both the primary osteoporosis and control groups. These data may help explain why vertebral fractures occur at relatively higher PA aBMD values than what is seen in typical primary osteoporosis, since the intravertebral distribution of bone appears to be abnormal in glucocorticoid-treated patients but not in patients with primary osteoporosis. The findings indicate that the anterior subregion may be of particular interest in a GIO population for assessing individuals' risk of vertebral fracture and that the ratio technique may prove useful in a clinical setting as a means of determining bone distribution without needing to adjust for vertebral size.

4.2. Sagittal Subregional Intravertebral aBMD Profile (ROIs 2–4). Main effects for both subregion and group were observed, demonstrating that this study, along with the earlier pilot work [56], has been able to show differences in subregional aBMD *in vivo*. The lowest mean srBMD was observed in the anterior subregion (ROI 4), a result consistent with previous *ex vivo* histomorphometry, QCT, and DXA studies [36–40, 42, 43] and the highest was in the posterior subregion (ROI 2). This distribution pattern is probably explained by a combination of a higher cortical bone component in the posterior subregion compared to the anterior subregion and a result of changes in vertebral loading profiles, in particular intervertebral disc degeneration and changes in lumbar spine posture [21, 57]. When disc degeneration occurs, force loading increases on the neural arch, hence decreasing loading on the anterior region and causing regional demineralisation [57–59]. Although no significant group × subregion interaction effect was observed, we detected significantly lower srBMD in the anterior subregion (ROI 4) for the GIO group compared to the primary osteoporosis group. Despite a significant finding with a medium effect size, this result should be considered in the context of a relatively small sample size and disproportionate sex distribution. Nonetheless, this result occurred despite the larger proportion of male patients in the GIO group which intuitively would have increased the mean aBMD in the GIO group, relative to a female-dominated GIO group. If all confounding factors were removed and sample sizes increased and balanced between the groups, this difference in aBMD may have been even more marked. We observed a power of 44% for this two-tailed analysis, suggesting that with a larger sample size, in the order of 60 per group, a power of 80% could be achieved.

4.3. Transverse Subregional Intravertebral aBMD Profile (ROIs 5–7). As with the sagittal subregions, main effects were observed for both subregion and group. The central subregion (ROI 6) exhibited the lowest aBMD, a result consistent with previous *ex vivo* studies [36–40, 42, 43]. The highest observed aBMD was in the inferior subregion (ROI 7) and may be explained due to this region's proximity to the chondral endplate, an area rich in cortical bone [60]. There was no significant subregion × group interaction observed nor were there any significant differences in supero-inferior ratios. This was not consistent with previous findings suggesting lower bone content at the central subregion.

4.4. Ratios. While it is possible to compare srBMD between groups, caution is required due to variability in subregional

bone geometry. The comparison of ratios is therefore a feasible approach to account for differences in vertebral size and may reduce gender effects caused by differences in bone size. Ratios also provide information on the distribution of bone within the subregions; hence the lower observed ROI 4 : ROI 2 and ROI 4 : ROI 1 ratios suggest less bone is distributed into the anterior subregion compared to the posterior subregion and vertebral body as a whole. This may suggest that the anterior subregion is at an increased risk of fracture (particularly anterior wedge fracture) within individuals exposed to glucocorticoid therapies, compared to other subregions, whilst the posterior subregion appears to be less affected.

4.5. Strengths and Limitations. Strengths of this study include the use of the widely employed Genant semiquantitative approach [46] along with vertebral height reference data [53] for fracture diagnosis and the use of our group's established, reliable, and validated subregional analysis protocol [41–43, 45, 54]. The relatively small sample in the GIO group led to a sex imbalance between groups. Despite the GIO group being predominantly male compared to the other groups which were predominantly female, statistically and clinically-significant deficits were found in the glucocorticoid group. Nonetheless, we cannot exclude the possibility that differences in intravertebral bone distribution were related to sex differences between groups. The use of a cross-sectional study design does not allow conclusions about causal relationships and thus future longitudinal work would be beneficial in strengthening this study's findings.

4.6. Future Directions. The use of intravertebral aBMD ratios to indicate abnormalities in bone distribution has the potential for application in a clinical setting, especially for men, where limited lateral reference data are available. The subregional approach may enhance the ability to monitor the effects of bone-active medications on bone architecture. Intervention studies would lend further support to this application of the technique.

5. Conclusions

A robust connection exists between aBMD and vertebral strength, hence indicating the important role of BMD in reflecting fracture risk. This study has shown that a lateral subregional approach to aBMD measurement provides greater information than standard PA-projection DXA about differences in aBMD between individuals with GIO and individuals with primary osteoporosis. In particular, glucocorticoid-treated patients had abnormal intravertebral distribution of bone, with relatively low aBMD in the anterior subregion. Glucocorticoid-treated individuals at high risk of sustaining vertebral fractures may be better identified with the clinical application of this technique.

Authors' Contribution

The authors note with great sadness that Susan Kantor passed away while this paper was in preparation. She made a major original contribution to this research.

Acknowledgments

Heath Bachli is acknowledged for data collection early in the study. Dr. Andrew Briggs was supported by a fellowship awarded by the Australian National Health and Medical Research Council (NHMRC).

References

[1] M. Takahata, J. R. Maher, S. C. Juneja et al., "Mechanisms of bone fragility in a mouse model of glucocorticoid-treated rheumatoid arthritis: implications for insufficiency fracture risk," *Arthritis & Rheumatism*, vol. 64, no. 11, pp. 3649–3659, 2012.

[2] T. P. van Staa, R. F. Laan, I. P. Barton, S. Cohen, D. M. Reid, and C. Cooper, "Bone density threshold and other predictors of vertebral fracture in patients receiving oral glucocorticoid therapy," *Arthritis and Rheumatism*, vol. 48, no. 11, pp. 3224–3229, 2003.

[3] T. P. van Staa, H. G. M. Leufkens, L. Abenhaim, B. Zhang, and C. Cooper, "Use of oral corticosteroids and risk of fractures," *Journal of Bone and Mineral Research*, vol. 15, no. 6, pp. 993–1000, 2000.

[4] K. Natsui, K. Tanaka, M. Suda et al., "High-dose glucocorticoid treatment induces rapid loss of trabecular bone mineral density and lean body mass," *Osteoporosis International*, vol. 17, no. 1, pp. 105–108, 2006.

[5] R. N. J. De Nijs, J. W. G. Jacobs, J. W. J. Bijlsma et al., "Prevalence of vertebral deformities and symptomatic vertebral fractures in corticosteroid treated patients with rheumatoid arthritis," *Rheumatology*, vol. 40, no. 12, pp. 1375–1383, 2001.

[6] M. S. van Brussel and W. F. Lems, "Clinical relevance of diagnosing vertebral fractures by vertebral fracture assessment," *Current Osteoporosis Reports*, vol. 7, no. 3, pp. 103–106, 2009.

[7] M. Steinbuch, T. E. Youket, and S. Cohen, "Oral glucocorticoid use is associated with an increased risk of fracture," *Osteoporosis International*, vol. 15, no. 4, pp. 323–328, 2004.

[8] B. Bouvard, M. Audran, E. Legrand, and D. Chappard, "Ultrastructural characteristics of glucocorticoid-induced osteoporosis," *Osteoporosis International*, vol. 20, no. 6, pp. 1089–1092, 2009.

[9] S. Lekamwasam, J. Adachi, D. Agnusdei et al., "A framework for the development of guidelines for the management of glucocorticoid-induced osteoporosis," *Osteoporosis International*, vol. 23, no. 9, pp. 2257–2276, 2012.

[10] R. S. Weinstein, "Glucocorticoid-induced osteoporosis," *Reviews in Endocrine and Metabolic Disorders*, vol. 2, no. 1, pp. 65–73, 2001.

[11] R. M. R. Pereira and J. Freire de Carvalho, "Glucocorticoid-induced myopathy," *Joint Bone Spine*, vol. 78, no. 1, pp. 41–44, 2011.

[12] S. C. Manolagas, "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis," *Endocrine Reviews*, vol. 21, no. 2, pp. 115–137, 2000.

[13] C. Roux, "Are glucocorticoids really deleterious to bone health?" *Joint Bone Spine*, vol. 78, supplement 2, pp. S211–S213, 2011.

[14] N. E. Lane, "Epidemiology, etiology, and diagnosis of osteoporosis," *American Journal of Obstetrics and Gynecology*, vol. 194, no. 2, Supplement, pp. S3–S11, 2006.

[15] K. D. Schuiling, K. Robinia, and R. Nye, "Osteoporosis update," *Journal of Midwifery & Women'S Health*, vol. 56, no. 6, pp. 615–627, 2011.

[16] H. K. Genant, J. Li, C. Y. Wu, and J. A. Shepherd, "Vertebral fractures in osteoporosis: a new method for clinical assessment," *Journal of Clinical Densitometry*, vol. 3, no. 3, pp. 281–290, 2000.

[17] B. Buehring, D. Krueger, M. Checovich et al., "Vertebral fracture assessment: impact of instrument and reader," *Osteoporosis International*, vol. 21, no. 3, pp. 487–494, 2010.

[18] M. A. C. van der Weijden, I. E. van der Horst-Bruinsma, J. C. van Denderen et al., "High frequency of vertebral fractures in early spondylarthropathies," *Osteoporosis International*, vol. 23, no. 6, pp. 1683–1690, 2012.

[19] A. El Maghraoui, A. Mounach, A. Rezqi et al., "Vertebral fracture assessment in asymptomatic men and its impact on management," *Bone*, vol. 50, no. 4, pp. 853–857, 2012.

[20] A. M. Greig, K. L. Bennell, A. M. Briggs, J. D. Wark, and P. W. Hodges, "Balance impairment is related to vertebral fracture rather than thoracic kyphosis in individuals with osteoporosis," *Osteoporosis International*, vol. 18, no. 4, pp. 543–551, 2007.

[21] A. M. Briggs, A. M. Greig, K. L. Bennell, and P. W. Hodges, "Paraspinal muscle control in people with osteoporotic vertebral fracture," *European Spine Journal*, vol. 16, no. 8, pp. 1137–1144, 2007.

[22] J. A. Kanis, O. Johnell, C. De Laet et al., "A meta-analysis of previous fracture and subsequent fracture risk," *Bone*, vol. 35, no. 2, pp. 375–382, 2004.

[23] J. A. Kanis, A. Oden, O. Johnell et al., "The use of clinical risk factors enhances the performance of BMD in the prediction of hip and osteoporotic fractures in men and women," *Osteoporosis International*, vol. 18, no. 8, pp. 1033–1046, 2007.

[24] P. D. Delmas, L. D. van Langerijt, N. B. Watts et al., "Underdiagnosis of vertebral fractures is a worldwide problem: the IMPACT study," *Journal of Bone and Mineral Research*, vol. 20, no. 4, pp. 557–563, 2005.

[25] A. Bazzocchi, P. Spinnato, F. Fuzzi et al., "Vertebral fracture assessment by new dual-energy X-ray absorptiometry," *Bone*, vol. 50, no. 4, pp. 836–841, 2012.

[26] M. Ghazi, S. Kolta, K. Briot, J. Fechtenbaum, S. Paternotte, and C. Roux, "Prevalence of vertebral fractures in patients with rheumatoid arthritis: revisiting the role of glucocorticoids," *Osteoporosis International*, vol. 23, no. 2, pp. 581–587, 2012.

[27] A. E. Maghraoui, A. Rezqi, A. Mounach, L. Achemlal, A. Bezza, and I. Ghozlani, "Prevalence and risk factors of vertebral fractures in women with rheumatoid arthritis using vertebral fracture assessment," *Rheumatology*, vol. 49, no. 7, Article ID keq084, pp. 1303–1310, 2010.

[28] A. Singer, "Osteoporosis diagnosis and screening," *Clinical Cornerstone*, vol. 8, no. 1, pp. 9–18, 2006.

[29] D. Bürklein, E. M. Lochmüller, V. Kuhn et al., "Correlation of thoracic and lumbar vertebral failure loads with in situ vs. ex situ dual energy X-ray absorptiometry," *Journal of Biomechanics*, vol. 34, no. 5, pp. 579–587, 2001.

[30] J. M. Zmuda, J. A. Cauley, N. W. Glynn, and J. S. Finkelstein, "Posterior-anterior and lateral dual-energy x-ray absorptiometry for the assessment of vertebral osteoporosis and bone loss among older men," *Journal of Bone and Mineral Research*, vol. 15, no. 7, pp. 1417–1424, 2000.

[31] I. R. Reid, M. C. Evans, and J. Stapleton, "Lateral spine densitometry is a more sensitive indicator of glucocorticoid-induced bone loss," *Journal of Bone and Mineral Research*, vol. 7, no. 10, pp. 1221–1225, 1992.

[32] J. S. Finkelstein, R. L. Cleary, J. P. Butler et al., "A comparison of lateral versus anterior-posterior spine dual energy x-ray absorptiometry for the diagnosis of osteopenia," *Journal of Clinical Endocrinology and Metabolism*, vol. 78, no. 3, pp. 724–730, 1994.

[33] M. Jergas, M. Breitenseher, C. C. Gluer et al., "Which vertebrae should be assessed using lateral dual-energy X-ray absorptiometry of the lumbar spine," *Osteoporosis International*, vol. 5, no. 3, pp. 196–204, 1995.

[34] G. Guglielmi, S. K. Grimston, K. C. Fischer, and R. Pacifici, "Osteoporosis: diagnosis with lateral and posteroanterior dual x-ray absorptiometry compared with quantitative CT," *Radiology*, vol. 192, no. 3, pp. 845–850, 1994.

[35] F. W. Lafferty and D. Y. Rowland, "Correlations of dual-energy X-ray absorptiometry, quantitative computed tomography, and single photon absorptiometry with spinal and non-spinal fractures," *Osteoporosis International*, vol. 6, no. 5, pp. 407–415, 1996.

[36] J. Wegrzyn, J. P. Roux, M. E. Arlot et al., "Role of trabecular microarchitecture and its heterogeneity parameters in the mechanical behavior of ex vivo human L3 vertebrae," *Journal of Bone and Mineral Research*, vol. 25, no. 11, pp. 2324–2331, 2010.

[37] A. M. Briggs, A. M. Greig, and J. D. Wark, "The vertebral fracture cascade in osteoporosis: a review of aetiopathogenesis," *Osteoporosis International*, vol. 18, no. 5, pp. 575–584, 2007.

[38] A. M. Briggs, J. D. Wark, S. Kantor, N. L. Fazzalari, A. M. Greig, and K. L. Bennell, "Bone mineral density distribution in thoracic and lumbar vertebrae: an ex vivo study using dual energy X-ray absorptiometry," *Bone*, vol. 38, no. 2, pp. 286–288, 2006.

[39] O. Cvijanovic, D. Bobinac, S. Zoricic et al., "Age- and region-dependent changes in human lumbar vertebral bone a histomorphometric study," *Spine*, vol. 29, no. 21, pp. 2370–2375, 2004.

[40] P. Pollintine, J. H. Tobias, D. S. McNally et al., "Intervertebral disc degeneration increases load-bearing by the neural arch and reduces BMD in the anterior vertebral body," *Journal of Bone and Mineral Research*, vol. 17, pp. S178–S178, 2002.

[41] A. M. Briggs, J. D. Wark, S. Kantor et al., "In vivo intrarater and interrater precision of measuring apparent bone mineral density in vertebral subregions using supine lateral dual-energy X-ray absorptiometry," *Journal of Clinical Densitometry*, vol. 8, no. 3, pp. 314–319, 2005.

[42] A. M. Briggs, E. Perilli, I. H. Parkinson et al., "Novel assessment of subregional bone mineral density using DXA and pQCT and subregional microarchitecture using micro-CT in whole human vertebrae: applications, methods, and correspondence between Technologies," *Journal of Clinical Densitometry*, vol. 13, no. 2, pp. 161–174, 2010.

[43] A. Briggs, E. Perilli, I. Parkinson et al., "Measurement of subregional vertebral bone mineral density in vitro using lateral projection dual-energy X-ray absorptiometry: validation with peripheral quantitative computed tomography," *Journal of Bone & Mineral Metabolism*, vol. 30, no. 2, pp. 222–231, 2012.

[44] E. Perilli, A. M. Briggs, S. Kantor et al., "Failure strength of human vertebrae: prediction using bone mineral density measured by DXA and bone volume by micro-CT," *Bone*, vol. 50, pp. 1416–1425, 2012.

[45] E. Perilli, A. M. Briggs, J. D. Codrington et al., "Vertebral body strength: prediction using subregional areal bone mineral density from DXA compared to subregional bone microarchitecture from micro-CT," in *Proceedings of the Australian and New Zealand Bone and Mineral Society Annual Scientific Meeting*, Perth, Australia, 2012.

[46] H. K. Genant, C. Y. Wu, C. van Kuijk, and M. C. Nevitt, "Vertebral fracture assessment using a semiquantitative technique,"

Journal of Bone and Mineral Research, vol. 8, no. 9, pp. 1137–1148, 1993.

[47] World Health Organization Collaborating Centre for Metabolic Bone Diseases, "Welcome to FRAX," in *WHO Fracture Risk Assessment Tool*, University of Sheffield, Sheffield, UK, http://www.shef.ac.uk/FRAX/.

[48] J. A. Rea, J. Li, G. M. Blake, P. Steiger, H. K. Genant, and I. Fogelman, "Visual assessment of vertebral deformity by X-ray absorptiometry: a highly predictive method to exclude vertebral deformity," *Osteoporosis International*, vol. 11, no. 8, pp. 660–668, 2000.

[49] R. D. Chapurlat, F. Duboeuf, H. O. Marion-Audibert, B. Kalpakçioglu, B. H. Mitlak, and P. D. Delmas, "Effectiveness of instant vertebral assessment to detect prevalent vertebral fracture," *Osteoporosis International*, vol. 17, no. 8, pp. 1189–1195, 2006.

[50] Hologic-Inc, *QDR 4500 Fan Beam X-ray Bone Densitometer: User's Guide*, Waltham, Mass, USA, 1996.

[51] H. K. Genant and M. Jergas, "Assessment of prevalent and incident vertebral fractures in osteoporosis research," *Osteoporosis International*, vol. 14, pp. S43–S55, 2003.

[52] J. T. Schousboe, T. Vokes, S. B. Broy et al., "Vertebral fracture assessment: the 2007 ISCD official positions," *Journal of Clinical Densitometry*, vol. 11, no. 1, pp. 92–108, 2008.

[53] D. Diacinti, D. Pisani, R. Del Fiacco et al., "Vertebral morphometry by X-ray absorptiometry: which reference data for vertebral heights?" *Bone*, vol. 49, no. 3, pp. 526–536, 2011.

[54] A. M. Briggs, *Pathomechanics of spinal osteoporosis: subregional bone mineral density and physiological loading [Ph.D. thesis]*, School of Physiotherapy and Department of Medicine, The University of Melbourne, Melbourne, Australia, 2006.

[55] R. C. Rupich, M. G. Griffin, R. Pacifici, L. V. Avioli, and N. Susman, "Lateral dual-energy radiography: artifact error from rib and pelvic bone," *Journal of Bone and Mineral Research*, vol. 7, no. 1, pp. 97–101, 1992.

[56] A. M. Briggs, J. D. Wark, A. M. Greig et al., "Subregional bone mineral density measurement in the lumbar spine using DXA: potential for the application to osteoporosis and vertebral fractures," in *Osteoporosis: Etiology, Diagnosis and Treatment*, B. E. Mattingly and A. C. Pillare, Eds., pp. 1–50, Nova Publishers, New York, NY, USA, 2009.

[57] M. A. Adams, "Basic science of spinal degeneration," *Surgery*, vol. 30, pp. 347–350, 2012.

[58] J. Homminga, R. Aquarius, V. E. Bulsink et al., "Can vertebral density changes be explained by intervertebral disc degeneration?" *Medical Engineering & Physics*, vol. 34, pp. 453–458, 2012.

[59] M. A. Adams, P. Pollintine, J. H. Tobias, G. K. Wakley, and P. Dolan, "Intervertebral disc degeneration can predispose to anterior vertebral fractures in the thoracolumbar spine," *Journal of Bone and Mineral Research*, vol. 21, no. 9, pp. 1409–1416, 2006.

[60] J. P. Grant, T. R. Oxland, and M. F. Dvorak, "Mapping the structural properties of the lumbosacral vertebral endplates," *Spine*, vol. 26, no. 8, pp. 889–896, 2001.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 754328, 8 pages
http://dx.doi.org/10.1155/2013/754328

Research Article

Dietary Calcium Intake and Calcium Supplementation in Hungarian Patients with Osteoporosis

Gábor Speer,[1,2] Pál Szamosujvári Jr.,[3] Péter Dombai,[4] Katalin Csóré,[3] Kinga Mikófalvi,[3] Tímea Steindl,[3] Ildikó Streicher,[3] Júlia Tarsoly,[3] Gergely Zajzon,[5] Péter Somogyi,[6] Pál Szamosújvári,[3] and Péter Lakatos[1]

[1] *Semmelweis University, 1st Department of Medicine, 1083 Budapest, Hungary*
[2] *Pálmai-Med Private Medical Praxis, Margit körút 44, 1027 Budapest, Hungary*
[3] *Pharma Patent Ltd, 1138 Budapest, Hungary*
[4] *Soldra International Ltd, 1024 Budapest, Hungary*
[5] *Semmelweis University, Institute of Health Informatics Development and Further Training, 1082 Budapest, Hungary*
[6] *National Institute for Sport Medicine, 1123 Budapest, Hungary*

Correspondence should be addressed to Gábor Speer; vitamindspeer@gmail.com

Received 2 November 2012; Revised 22 February 2013; Accepted 13 March 2013

Academic Editor: Mei Li

Purpose. Adequate calcium intake is the basis of osteoporosis therapy—when this proves insufficient, even specific antiosteoporotic agents cannot exert their actions properly. *Methods*. Our representative survey analyzed the dietary intake and supplementation of calcium in 8033 Hungarian female and male (mean age: 68 years) (68.01 (CI95: 67.81–68.21)) patients with osteoporosis. *Results*. Mean intake from dietary sources was 665 ± 7.9 mg (68.01 (CI95: 67.81–68.21)) daily. A significant positive relationship could be detected between total dietary calcium intake and lumbar spine BMD ($P = 0.045$), whereas such correlation could not be demonstrated with femoral T-score. Milk consumption positively correlated with femur ($P = 0.041$), but not with lumbar BMD. The ingestion of one liter of milk daily increased the T-score by 0.133. Average intake from supplementation was 558 ± 6.2 mg (68.01 (CI95: 67.81–68.21)) daily. The cumulative dose of calcium—from both dietary intake and supplementation—was significantly associated with lumbar ($r = 0.024$, $P = 0.049$), but not with femur BMD ($r = 0.021$, $P = 0.107$). The currently recommended 1000–1500 mg total daily calcium intake was achieved in 34.5% of patients only. It was lower than recommended in 47.8% of the cases and substantially higher in 17.7% of subjects. *Conclusions*. We conclude that calcium intake in Hungarian osteoporotic patients is much lower than the current recommendation, while routinely applied calcium supplementation will result in inappropriately high calcium intake in numerous patients.

1. Introduction

As suggested by abundant data, calcium supplementation by itself—although to a modest extent only—can decrease bone loss, and thereby increase bone mineral density (BMD) in osteoporotic patients [1]. According to evidence from the Cochrane database, at least two years of calcium supplementation will result in a BMD increase of 1.66% in the lumbar spine, 1.60% in the hip region, and 1.91% in the distal radius, respectively [2]. However, for the prevention of osteoporotic fractures, calcium supplementation is effective only in combination with vitamin D, although there are data available to refute the importance of this intervention [3].

The Decaylos I study, conducted on 3270 elderly French women, aged 84 years on average, evaluated the role of concomitantly administered calcium and vitamin D in reducing fracture risk [4]. The subjects received 1200 mg calcium with 800 IU vitamin D or placebo daily, over three years. By the end of the third year, the number of hip fractures, as well as of all nonvertebral fractures decreased by 29% and 24%, respectively. In a Danish study [5] of 9605 elderly patients, combination therapy with 1000 mg calcium and

800 IU vitamin D daily reduced the number of fractures significantly, by 27%. These findings were confirmed by Tang et al. [6]. In their meta-analysis of 29 trials conducted on 64,000 individuals over the age of 50 years, calcium supplementation was associated with a 12% reduction of the total number of fractures.

Nevertheless, contradicting results have also been reported. In a Dutch study of 2578 women [7], mean dietary calcium intake, which was considered high (868 mg/day on average), was supplemented with 400 IU/day vitamin D for 3.5 years, but even this failed to mitigate fracture risk. In the secondary prevention RECORD study [8], 5292 osteoporotic patients aged over 70 years and with prevalent fractures received 1000 mg calcium with 800 IU vitamin D daily, or placebo. This regimen failed to achieve any significant reduction of fracture risk in the treatment groups. However, only 60% of the subjects were compliant to the therapy (who have taken their study medications according to the intended dosage), which seriously challenges the validity of this finding.

Currently, supplementation with vitamin D and calcium should be regarded as the basic therapy of osteoporosis [9, 10]. In osteoporotic patients, the daily intake of at least 800 to 1000 IU vitamin D and of 1000 to 1500 mg calcium is necessary to increase BMD and to reduce fracture risk [11, 12]. Although calcium and vitamin D are similarly effective whether they are from dietary sources or from supplementation; international and Hungarian data suggest that the intake of both needs an—occasionally substantial—increase [13].

Other potential explanations for the study findings, seemingly challenging the effectiveness of calcium and vitamin D replacement, include the use of nonstandard methods for estimating the daily intake of subjects, and supplementation with various calcium preparations, which produced differing increases of serum calcium levels [14]. Together, these might sometimes have led to suboptimal efficacy of the daily supplementation with calcium and vitamin D.

The aim of our study was to appraise the dietary intake as well as the supplementation of calcium in osteoporotic patients in Hungary.

2. Material and Methods

2.1. Participants and Study Protocol. We conducted a survey using a validated questionnaire (source: modified from Groupe de Recherche et d'Information sur les Ostéoporoses, http://www.grio.org/calcul-apport-calcique-quotidien.php) [15], completed by medical professionals at the osteoporosis centers, following an interview with study subjects. One hundred and eighty-one physicians of 110 study sites, evenly distributed over Hungary, co-operated in this survey. The completed questionnaires accumulated information on osteoporotic 9215 patients.

The questionnaires were dispensed in batches of fifty, in a form similar to that of copybooks comprising sheets with preprinted serial numbers. Using simple questions (most of which required a numerical entry as a response only), the questionnaire attempted to estimate the intake of calcium-containing foods over a given period (The questionnaire (amended version)). These responses were then used to gauge daily calcium intake based on the frequency of ingestions and of the known calcium content of individual foodstuffs. Additional questions, pertaining to calcium supplementation with medicinal products, served to determine daily intake from this source. The severity of osteoporosis was characterized by bone density measured on the total femur and the lumbar (L_{2-4}) spine (BMD was expressed as g/cm^2 and as T-score). Bone mineral density was measured at the total femur and at the lumbar spine by dual X-ray absorptiometry by Lunar Prodigy (GE Medical Systems, Diegem, Belgium), Norland pDEXA (CooperSurgical Inc., Trumbull, CT, USA), and Hologic QDR 4500 (Hologic Inc., Bedford, MA, USA). Coefficient of variation was below 1% at both sites. Osteoporosis was defined according to the WHO criteria, as a T-score less than −2.5 at any measured site.

The questionnaire (amended version)

Serial N°:

Ca-intake.hu Study

version 2.0 of the questionnaire for the appraisal of daily calcium intake in osteoporotic patients

Gender: female/male

Patient's initials:

Age:

Date of birth:

Body weight:

Body height:

(Please do measure the weight/height of the patient!)

Lumbar T-score:

Region: L_{1-5}/L_{2-4}/L_{2-5}

Femoral T-score:

Densitometer make & model:

Antiosteoporotic agent:

Dosage:
Daily/Weekly/Monthly/Every 3 months/Annually

Vitamin D supplementation:
tablets/capsules/solution

Dosage: x Daily/Weekly/Other

Calcium supplementation: Yes/No

If yes, tablets.
Dosage: x tablets daily.

OTC preparation? Yes/No

If POM:

Is it prescribed by a specialist? Yes/No
Is it prescribed by the family practitioner? Yes/No

(1) *Do you drink milk every day?*

If yes, how many glasses (2 deciliters)?
If not every day, then how many glasses of milk do you drink *per week*?

(2) *Do you eat yogurt, kefir, or curd?*

If yes, then how many servings of 100 mL do you eat *per week*?

(3) *Do you eat sour cream?*

If yes, then how many servings of 100 mL do you eat *per week?*

(4) *How much bread do you eat daily?*

Whole slice: . . . pieces

(5) *How much pasta do you eat per week?*

· · · × 100-g servings

(6) *How much meat do you eat per week?*

(a) chicken (100-g servings): . . ./week
(b) pork (100-g servings): . . ./week
(c) Parisian/saveloy sausages (100-g servings): . . ./week

(7) *Do you eat canned fish in oil?*

If yes, how many cans *per week?*

(8) *How much vegetable do you eat per week?*

(300-g servings)
(a) spinach, sorrel: . . . servings
(b) green peas, kale, French beans: . . . servings

(9) *Do you eat cheese regularly?*

If yes, how many 20-g servings *per week* (see the photo)?

(10) *How much potato do you eat per week?*

· · · × 100-g servings

(11) *How many eggs do you eat per week?*

(12) *How many medium-sized apples do you eat per week?*

(13) *How many glasses (200-mL) of mineral water do you drink daily?*

(a) branded products: Szentkirályi, Natur Aqua
(b) branded products: Theodora Quelle, Margitszigeti, Visegrádi
(c) other unbranded mineral water:

How long have you been adhering to these eating habits?

(a) all my life
(b) for . . . years

If you have changed your diet long ago, you had done this on:

(a) advice from your doctor
(b) other advice

TO BE COMPLETED BY THE INVESTIGATOR!

Calcium content of the daily menu:

(1) . . .
(2) . . .
(3) . . .
(4) . . .
(5) . . .
(6.a) . . .
(6.b) . . .
(6.c) . . .
(7) . . .
(8.a) . . .
(8.b) . . .
(9) . . .
(10) . . .
(11) . . .
(12) . . .
(13.a) . . .
(13.b) . . .
(13.c) . . .

Daily calcium intake:

Questionnaires from 8033 of the 9215 surveyed osteoporotic patients were complete and thus suitable for evaluation. This means that considering the approximately 330,000 patients (including treated and untreated ones, ESKI, 2009) as reckoned by the collaborating study sites, population coverage exceeded 2.5% altogether. Within the evaluated population (n = 8033), the proportion of males was 7.1% (n = 569) compared to the dominant 92.9% share of females (n = 7464). Mean age of the subjects was 68 years (range 41 to 98). The age distribution of the study population was similar to that exhibited by the national age distribution chart, even for males and females analyzed separately. The body mass index (BMI) was normal in 38.8% of participants (BMI = 18.5-24.9 kg/m^2); the proportion of underweight subjects was 6.2% (BMI < 18.5 kg/m^2), whereas 55.0% were overweight (BMI = 25.0–29.9 kg/m^2) or obese (≥30 kg/m^2). Mean lumbar and femoral T-scores were −2.836 ± 0.03 and −2.434 ± 0.03,

Table 1: The baseline characteristics of our patients.

Gender (%)	Male = 569 (7.1) Female = 7464 (92.9) Total = 8033 (100)
Age (years)	41–98 Mean: 68.01
Lumbar *T*-score	−9.37–1.5 Mean: −2.836 ± 0.03
Femoral *T*-score	−8.66–0.9 Mean: −2.434 ± 0.03
Weight (kg)	32–130 Mean: 66.78
Height (cm)	120–194 Mean: 159.93
BMI (kg/m^2)	13.62–60.26 Mean: 26.11
Vitamin D supplementation (%)	*n* = 5773 (71.7)
Calcium supplementation (%)	*n* = 5813 (72.6)
Mean dietary calcium intake (mg)	665 ± 7.9 Female = 599 ± 22.7 Male = 673 ± 8.4

respectively. Lumbar and femoral BMD values were related also to age (Table 1).

We did not impose quotas on the selection of responders, in order to accomplish the widest possible population coverage. The course of the survey had two stages, during which a slightly different (amended) version of the same questionnaire was used. Modification was necessary because the original version contained ambiguous questions on cheese consumption. Specifically, 50 grams were defined as a serving, but this proved excessive, resulting in unrealistically high cheese consumption; this was changed to 20 grams in the second version. Additionally, the first version contained two questions on two cheese categories. In the second version, this was reduced to a single, comprehensive question not distinguishing between different types of cheese. Finally, the question on meat consumption reckoned with daily intake in the original version. All these changes had no influence on the standardized analysis of results.

2.2. Statistics. The initial step of data processing consisted of digitizing the questionnaires, performing the digitally assisted recording of data in duplicate, and finally, correcting errors. Handwritten notes modifying the frequency entries made on the questionnaires were also recorded (e.g., when the investigator invalidated the "daily" specification by striking it through and entered "weekly" in replacement before completing the entry on bread consumption). Error correction involved checking for records containing logical inconsistencies, outlier values, and skipped entries. Questionnaires containing uncorrectable errors were excluded from the analysis along with those that were deemed unusable for other reasons. The latter included duplicates (identified by matching patient's initials, date of birth, and gender on questionnaires from the same or from a nearby study site); sheets without the age or gender of the subject (if retrieving these data was not feasible, for example, owing to the absence of the date of birth or the information on marital status). Sheets containing data from subjects without osteoporosis (as evidenced by *T*-scores of the lumbar spine and of the femur) were also discarded. As no selection quotas were imposed during the survey, weighing was used to ensure the representativeness of the sample. This was necessary, because the proportions of patients included in the individual counties were at variance with nationwide data on patient distribution. For example, the proportion of patient data accumulated from Tolna County was larger than that obtained from Vas County, whereas national data show a different distribution. Therefore, the results from both counties were corrected with an appropriate multiplier—using a method known as element number stabilization weighing, to obtain results matching their nationwide weights. We recruited the entire Hungarian population of osteoporotic patients as reference (data source: National Institute for Quality and Organizational Development in Healthcare and Medicine—Informatics and System Analysis Directorate/ESKI/, 2009). It is defined as the total number of individuals (specified according to county, age, and gender) receiving in-/outpatient care within the Hungarian health care system, as well as any form of osteoporosis is specified in their primary care reports as an underlying or accompanying disorder. Patients were reckoned by using the data on their age and residence (by county), as recorded at the first presentation during the actual year. The numbers of patients per counties and male-to-female ratios were approximated to the national average. As—among others—age was taken into account during weighing, this method we consider element number stabilization weighing for three dimensions (age, gender, and region).

During the calculations, in the knowledge of the calcium content of selected foodstuffs, we performed the multiplication corresponding with the given frequency period to obtain per patient calcium intake in total and for each foodstuff. Data accumulated with the two versions of the questionnaire were pooled, in order to increase sample size. As regards to the original version of the questionnaire, calcium intake calculated from responses to the question on cheese consumption was 2.5 times higher than that reckoned from the entries of the corresponding item of the amended version. Considering the amendment of the original version after the preliminary analysis (changes included attaching a photo make estimating the quantity of consumed cheese easier), we implemented the following correction. Calcium intake from cheese consumption, as estimated with the original version, was adjusted with the quotient of the means determined with both versions.

These data on dietary calcium intake were used to exclude unrealistically low or high values, and the same procedure was then performed on calcium supplementation data. Disease severity and the appropriateness and efficacy of the diet and medication were compared with each other, as well as by regions. No sample size calculation was performed prior

to conduct of the study. For the data analysis, we performed ANOVA tests and linear regression models, respectively. The significance level (alpha) was used 0.05. The given values at the numeric results are the 95% confidence intervals (CI) of the results. The analyses were performed using the SPSS (SPSS Inc., Chicago, IL, USA) software package (IBM SPSS Statistics 20.0.0).

3. Results

Of the 8033 evaluated osteoporotic subjects, 5733 (71.7%) received vitamin D supplementation with a mean dose of 756 ± 8.8 IU (68.01 [CI95: 67.81–68.21]), which is essentially in agreement with the recommendations of current professional protocols. Hydroxylated vitamin D was administered to 13.3% of patients. We did not detect any relationship between vitamin D supplementation and BMD values.

The mean dietary calcium intake was 665 ± 7.9 mg (68.01 [CI95: 67.81–68.21]). It was influenced by several factors. These include gender, as the daily intake was significantly lower in males than in females −599 ± 22.7 versus 673 ± 8.4 mg (68.01 [CI95: 67.81–68.21]) ($P < 0.001$). Additional influencing factors included the presence as well as the volume of milk consumption (Figure 1). The higher was the daily milk consumption, the greater became dietary calcium intake. Using this parameter, we assigned the study population into the following four subsets: (1) patients abstaining from milk, (2) subjects with low consumption (less than one glass—200 mL—of milk per week), (3) moderate consumption (less than a half glass—100 mL—of milk a day), or (4) regular milk drinkers (more than a half glass—100 mL—of milk daily). The proportions of subjects in these categories were 20.1%, 3.8%, 12.5%, and 63.6%, respectively. According to this stratification, dietary calcium intake was significantly ($P < 0.001$) greater in regular milk drinkers than in other subjects. The daily intake of calcium from dietary source was not related to BMI.

T-scores correlated with dietary calcium intake, according to a multiple relationship. The quantity of calcium from food sources was not related to the BMD of the femur ($P > 0.1$), whereas a positive correlation could be demonstrated with lumbar T-score ($P = 0.045$). Analyzing BMD exclusively from the aspect of milk consumption, a positive correlation could be shown with femoral BMD, but not for lumbar BMD (Figure 2). In other words, a daily milk consumption of one liter (providing 1200 mg calcium) increased the T-score of the total femur by 0.133.

Of the 8033 subjects, 5813 received some form of calcium supplementation, which proved adequate for 72.6%. The mean daily dose of the supplemented calcium was 558 ± 6.2 mg. Analysis by age did not reveal any significant difference, whereas comparison by gender did: it was on average 560 ± 6.6 mg (68.01 (CI95: 67.81–68.21)) in females and significantly smaller 534 ± 18.9 mg (68.01 (CI95: 67.81–68.21)) in males ($P = 0.012$).

The cumulative dose of calcium from dietary intake and from supplementation was significantly related to lumbar BMD ($r = 0.024$, $P = 0.049$), while no relationship could be detected with femoral BMD ($r = 0.021$, $P = 0.107$).

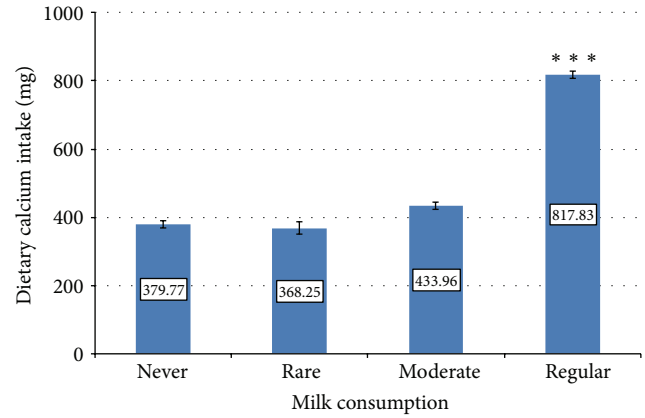


Figure 1: The relationship between the volume of milk consumption and daily calcium intake ($P < 0.001$): the higher was the daily milk consumption, the greater became dietary calcium intake. *Note*. Low milk consumption: never or less than a glass (two deciliters) of milk per week (rare); moderate milk consumption: less than half a glass (one deciliter) of milk a day; regular milk drinkers: more than half a glass (one deciliter) of milk daily. $^{***}P < 0.001$ versus abstainers and subjects with low or moderate milk consumption.

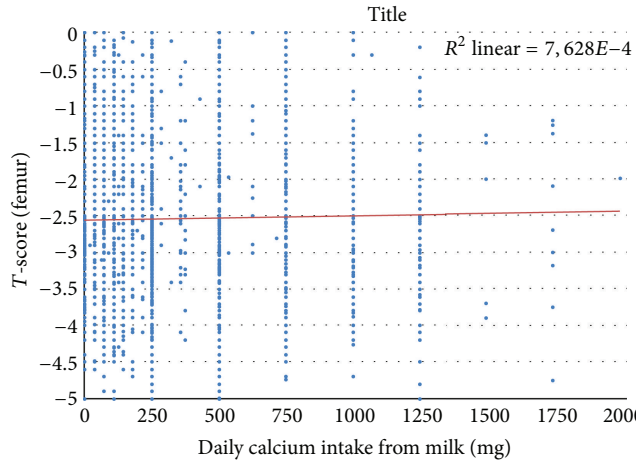


Figure 2: The relationship between the presence of milk consumption and the T-score of the femur ($P = 0.041$).

Figure 3 illustrates calcium supplementation in relation to dietary calcium intake. The recommended daily allowance of 1000 to 1500 mg calcium was fulfilled in as low as 34.5% of treated patients only. In the majority of osteoporotic patients, total daily calcium intake was below this target or exceeded it substantially.

4. Discussion

The Ca-Intake.hu study was the first Hungarian project to evaluate daily calcium intake in osteoporotic patients. Earlier, only Biró et al. have published data on Hungarian healthy adolescents [16]. According to their findings, daily calcium intake is as low as 800 mg in boys and 700 mg in girls. In France, for example, it is 754 mg—that is, half of the rec-

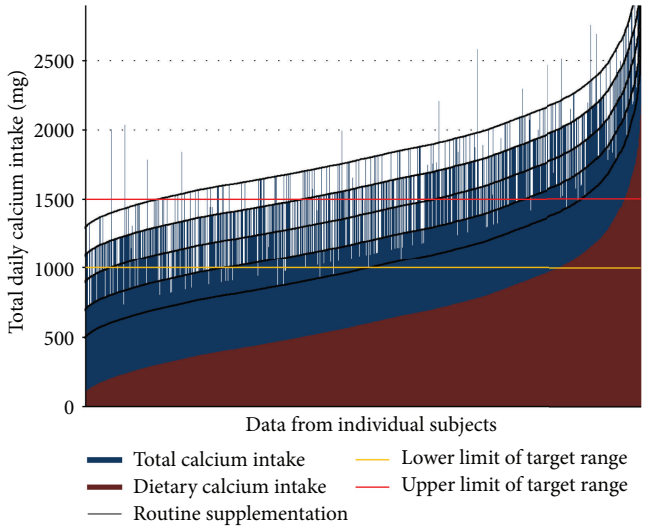


Figure 3: The relationship between calcium intake from supplementation and from dietary sources. *Note*. The recommended RDA of 1000 to 1500 mg total calcium intake is met in approximately 34.5% of patients only. In the majority of subjects, total daily calcium intake is either below or above the recommended range.

ommended RDA—among urban dweller women with postmenopausal osteoporosis [17]. Our analysis of dietary calcium intake in more than 8000 patients, which is an outstandingly large population even in international comparison, has supplied evidence confirming that calcium intake among osteoporotic patients in Hungary is even lower than most of the published data.

Additionally, the Ca-Intake.hu study supplied information on average daily calcium intake from supplements, which was 558 mg. In view of the dietary intake, this amount of calcium results in an adequate total calcium intake in the lesser proportion (34.5%) of patients only, and it is below or well above the recommended in the majority of osteoporotic patients. This finding is probably a consequence of routinely handled calcium supplementation doses. This practice can be seen even in clinical pharmacology trials conducted in osteoporosis. The study protocol only rarely prescribes the appraisal of calcium intake along with the consequential adjustment of supplementation. Not withstanding the conflicting data, 1000 to 1500 mg/day calcium and 800 to 1000 IU vitamin D in combination are likely to increase bone mass and mitigate fracture risk effectively [18–21]. Actually, suboptimal dosing with calcium could be a potential explanation for the findings from the international studies that failed to demonstrate the beneficial effect of calcium and vitamin D supplementation either on bone mass or on of fracture prevention. In the RECORD study [8], the unfavorable results are attributable to poor patient compliance; however, the underdosing of calcium supplementation is just as deleterious. Moreover, it should be kept in mind that excessive calcium intake can increase the number of bone fractures [22] as well as the incidence of prostate cancer in males [23].

In our study, milk consumption correlated with BMD of the total femur, whereas calcium from other foods or total daily calcium intake had no such relationship. The opposite holds true for the bone density of lumbar vertebrae which was not related to milk consumption but showed a positive correlation with the cumulative dose of calcium from dietary intake and from supplementation. Others reported contrary findings in adolescents: bone densitometry of the lumbar spine (without appraisal of the femur) showed a significant relationship (mediated by insulin-like growth factor 1/IGF-1/) between BMD and milk consumption, but not with calcium from other dietary sources [24]. Potential explanations for these findings include the concomitant intake of additional nutrients (such as magnesium) and proteins present in milk. IGF-1, as well as milk proteins have been shown to exert an anabolic effect on bone. IGF-1 increases the number and enhances the activity of osteoblasts, while stimulating collagen synthesis by these cells [25]. The beneficial effect of milk consumption as well as of treatment with the extract of the milk basic protein fraction on bone turnover (both in cortical and in trabecular substance) in adolescents as well as in adult or elderly women have been reported by others [24, 26, 27]—although contradicting data also exist [28]. The STRAMBO study, for example, showed in males under the age of 65 a harmful effect of a daily calcium intake less than 600 mg both on cortical and on trabecular bone [29].

Our study also has several limitations. The study results inherited a limitation from the questionnaire distribution. At the beginning of the study, no sample size calculation was made, so this resulted in an unbalanced distribution of patient numbers from each Hungarian region. Though these limitations apply, the relatively high sample size strengthens the power of the study. The questionnaire design itself leads to concerns about our results. Data entry and cleaning efforts had been made to identify and clean the strike throughs of weekly/daily frequencies. The one-time questionnaire method should be replaced by digital questionnaire or a week-long food and drink logging in the future to avoid biases resulting from the patient's situation being interviewed personally by their physician. Another limitation is caused by the exact definition of food quantities for that we have to define more robust method to ensure that all patients understand the objective food amounts. Strait methods of measuring calcium intake via water and other drinks should also be determined for the future. In conclusion, these limitations do not affect our main finding about the inappropriate methodology in the field of calcium supplementation.

As confirmed by the Ca-Intake.hu study, the bone density of lumbar vertebrae positively correlated with the total daily intake of calcium from food and from supplementation. Boonen et al. [18] processed the results of six randomized clinical studies involving 45,000 patients and found an 18% reduction of hip fractures with calcium and vitamin D supplementation. The ICARO study [30] was the first to demonstrate that the lack of compliance with calcium and vitamin D regimens reduces the antifracture efficacy of antiosteoporotic therapies, compared to that seen in controlled studies (and in combination with adequate calcium and vitamin D supplementation). Thus, any of the tested antiosteoporotic agents can achieve its maximum effect only in the presence of calcium and vitamin

D sufficiency. According to the findings of the Ca-Intake.hu study, the level of vitamin D supplementation in Hungary is on par with that recommended by current international guidelines.

5. Conclusion

In conclusion, calcium intake in Hungarian osteoporotic patients is much lower than the current recommendation, while routinely applied calcium supplementation will result in inappropriately high calcium intake in numerous patients.

Conflict of Interests

K. Csóré, K. Mikófalvi, T. Steindl, I. Streicher, and J. Tarsoly are full-time employees of Pharma Patent Pharmaceuticals Corporation. P. Szamosujvári jr. and P. Szamosújvári are full-time employees and having an equity interest in Pharma Patent Pharmaceuticals Corporation. P. Dombai, G. Zajzon, and P. Somogyi have no conflict of interests.

Acknowledgments

P. Lakatos received consulting or advisory board fees from Amgen, Eli Lilly, Merck, Novartis, and Seriver; lecture fees from Pharma Patent, Amgen, Eli Lilly, Merck, Novartis, Roche, and Servier; and grant support from Amgen, Roche, and GlaxoSmithKline. G. Speer received lecture fees from Pharma Patent, Novartis, Roche, Gedeon Richter, Novo-Nordisk, and Servier and received a grant support from TEVA-Biogal. The Ca-Intake.hu study has been implemented with the sponsorship and logistical support of Pharma Patent Ltd. The Ca-Intake.hu study group are as follows: Krisztina Anton, László Aradvári, Zsuzsanna Bakonyi, Beáta Baksay, Attila Balog, Ádám Balogh, Ágnes Balogh, Judit Balogh, Zsuzsanna Balogh, Ildikó Baranyai, Katalin Barkóczi, Zsuzsanna Ildikó Barta, Renáta Bartal, Enikő Benkei, László Beranek, Enikő Berkes, Katalin Bezzegh, Zsuzsanna Bihari, Judit Bíró, Erzsébet Boros, Emese Borsányi, Gabriella Böcz, Ágnes Böröcz, Rita Buck, Judit Chende, András Czinege, Valéria Csala, Gábor Csengei, Éva Csernátony, Lajos Csurilla, Éva Deme, Attila Dombi, Gyula Dömötöri, Edit Drescher, Ildikó Elek, Erzsébet Erős, Erzsébet Farkas, József Fatér, Fiorenza Ferrari, Mária Figler, Mária Fliszár, Zita Fükő, Katalin Gácsi, Csaba Gálfi, István Gara, Erzsébet Gáspár, Erika Gulyás, Marina Hacsunc, Andrea Hajas, Csaba Halász, Judit Halász, Fuad Hamadeh, Ágnes Hárságyi, Péter Hatházi, Judit Haulik, Mária Hermann, Éva Homoki, József Horváth, Katalin Horváth, Kinga Horváth, Krisztina Horváth, Mária Hunyadi, Gábor Hutás, Zsuzsa Hutkai, Zsuzsanna Ila, Orsolya Imre, Zsuzsanna Imre, Judit Jász, Bernadett Juhász, Péter Kadlót, Éva Kardos, Sándor Kárpáti, Zsuzsanna Kerekes, Mónika Kertész, Sándor Kis, Mária Kisgyörgy, Erika Kiss, Ildikó Kiss, László Kiss, Mária Kiss-Antal, Ágnes Klemencsics, Tímea Kocsy, András Korányi, János Kornafeld, András Koroknai, Attila Kovács, Mária Kovács, Anita Kozma, László Kozma, Blanka Körmöczy, Mária Köteles, Klára Kucsera, Enikő Kulcsár, Péter Lakatos, Anikó Lanszki, Irén László, Márta László, Zoltán Lazsádi, Botond Literáti-Nagy, Mária Edit Magdics, Péter Makay, Zsófia Markovits, Magdolna Maros, Borbála Miskolczi, Ágnes Molnár, Györgyi Molnár, Myrtill Molnár, Katalin Nádor, Ágota Nagy, Filoména Nagy, Katalin Nagy, Magdolna Nagy, Péter József Nagy, Zsolt Nagy, Lóránd Németh, Noémi Nógrádi, Rita Orosz, Kinga Palotás, Katalin Palov, Tibor Patócs, Anna Mária Pávich, Mária Pelsőczy, Irén Péntek, Lászlóné Péter, Ágota Podlovics, László Popdán, Anna Pornói, Annamária Pós, Judit Pulai, Ildikó Pusztai, Katalin Rába, Anita Rabóczki, Éva Rácz, Judit Radics, István Raffay, Teréz Róth, Kriszta Sárosi, Tímea Schöbel, Lajos Schreithofer, Katalin Seregély, Annamária Silló, Adelina Siminischi, Péter Somogyi, Gábor Speer, Katalin Szabó, Ibolya Szakács, Magda Szakmári, Szilvia Szamosi, János Szász, Zoltán Szekanecz, Gabriella Székely, Margit Székely, László Szekeres, Ágnes Szemenyei, Mónika Szemenyei, Zita Szemők, György Szénási, Csilla Szentiványi, László Tamási, Zsuzsanna Tantó, Irén Tarkó, Gyöngyi Tatár, György Tatár, Ildikó Tefner, Erzsébet Toronyi, Edit Tóth, Margit Tóth, Mariett Tóth, Edit Törös, Klára Tugyi, Adrienne Újváry, Emese Valco, Tamás Vámosi, Andrea Váncsa, Éva Váradi, Boglárka Varga, Margit Varga, Cecilia Varjú, Mária Vincze, Sznezska Zdravkova, and Imre Zsohár.

References

[1] B. Shea, G. Wells, A. Cranney et al., "VII. Meta-analysis of calcium supplementation for the prevention of postmenopausal osteoporosis," *Endocrine Reviews*, vol. 23, no. 4, pp. 552–559, 2002.

[2] B. Shea, G. Wells, A. Cranney et al., "Calcium supplementation on bone loss in postmenopausal women," *Cochrane Database of Systematic Reviews*, no. 1, Article ID CD004526, 2004.

[3] R. L. Prentice, M. B. Pettinger, R. D. Jackson et al., "Health risks and benefits from calcium and vitamin D supplementation: women's health initiative clinical trial and cohort study," *Osteoporosis International*, vol. 24, no. 2, pp. 567–580, 2013.

[4] M. C. Chapuy, M. E. Arlot, P. D. Delmas, and P. J. Meunier, "Effect of calcium and cholecalciferol treatment for three years on hip fractures in elderly women," *British Medical Journal*, vol. 308, no. 6936, pp. 1081–1082, 1994.

[5] E. R. Larsen, L. Mosekilde, and A. Foldspang, "Vitamin D and calcium supplementation prevents osteoporotic fractures in elderly community dwelling residents: a pragmatic population-based 3-year intervention study," *Journal of Bone and Mineral Research*, vol. 19, no. 3, pp. 370–378, 2004.

[6] B. M. Tang, G. D. Eslick, C. Nowson, C. Smith, and A. Bensoussan, "Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: a meta-analysis," *The Lancet*, vol. 370, no. 9588, pp. 657–666, 2007.

[7] P. Lips, W. C. Graafmans, M. E. Ooms, P. D. Bezemer, and L. M. Bouter, "Vitamin D supplementation and fracture incidence in elderly persons: a randomized, placebo-controlled clinical trial," *Annals of Internal Medicine*, vol. 124, no. 4, pp. 400–406, 1996.

[8] A. M. Grant, "Oral vitamin D3 and calcium for secondary prevention of low-trauma fractures in elderly people (Randomised Evaluation of Calcium or vitamin D, RECORD): a randomised

placebo-controlled trial," *The Lancet*, vol. 365, no. 9471, pp. 1621–1628, 2005.

[9] J. J. Body, P. Bergmann, S. Boonen et al., "Evidence-based guidelines for the pharmacological treatment of postmenopausal osteoporosis: a consensus document by the Belgian Bone Club," *Osteoporosis International*, vol. 21, no. 10, pp. 1657–1680, 2010.

[10] R. M. Francis, "Calcium, vitamin D and involutional osteoporosis," *Current Opinion in Clinical Nutrition & Metabolic Care*, vol. 9, pp. 13–17, 2006.

[11] P. Lips, R. Bouillon, N. M. Van Schoor et al., "Reducing fracture risk with calcium and vitamin D," *Clinical Endocrinology*, vol. 73, no. 3, pp. 277–285, 2010.

[12] "Eisenberg Center at Oregon Health & Science University, Fracture Prevention Treatments for Postmenopausal Women with Osteoporosis. Comparative Effectiveness Review Summary Guides for Clinicians [Internet]. Rockville (MD): Agency for Healthcare Research and Quality (US) (2007-2008)".

[13] H. Lilliu, R. Pamphile, M. C. Chapuy, J. Schulten, M. Arlot, and P. J. Meunier, "Calcium-vitamin D3 supplementation is cost-effective in hip fractures prevention," *Maturitas*, vol. 44, no. 4, pp. 299–305, 2003.

[14] H. J. Heller, L. G. Greer, S. D. Haynes, J. R. Poindexter, and C. Y. C. Pak, "Pharmacokinetic and pharmacodynamic comparison of two calcium supplements in postmenopausal women," *Journal of Clinical Pharmacology*, vol. 40, no. 11, pp. 1237–1244, 2000.

[15] P. Fardellone, J. L. Sebert, M. Bouraya et al., "Evaluation of dietary calcium content by frequential self-questionnaire," *Revue du Rhumatisme et des Maladies Osteo-Articulaires*, vol. 58, no. 2, pp. 99–103, 1991.

[16] L. Biró, A. Regöly-Mérei, K. Nagy et al., "Dietary habits of school children: representative survey in metropolitan elementary schools—Part two," *Annals of Nutrition and Metabolism*, vol. 51, pp. 454–460, 2007.

[17] P. Fardellone, F. E. Cotté, C. Roux, E. Lespessailles, F. Mercier, and A. F. Gaudin, "Calcium intake and the risk of osteoporosis and fractures in French women," *Joint Bone Spine*, vol. 77, no. 2, pp. 154–158, 2010.

[18] S. Boonen, P. Lips, R. Bouillon, H. A. Bischoff-Ferrari, D. Vanderschueren, and P. Haentjens, "Need for additional calcium to reduce the risk of hip fracture with vitamin D supplementation: evidence from a comparative metaanalysis of randomized controlled trials," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 4, pp. 1415–1423, 2007.

[19] M. Kärkkäinen, M. Tuppurainen, K. Salovaara et al., "Effect of calcium and vitamin D supplementation on bone mineral density in women aged 65–71 years: a 3-year randomized population-based trial (OSTPRE-FPS)," *Osteoporosis International*, vol. 21, no. 12, pp. 2047–2055, 2010.

[20] K. Salovaara, M. Tuppurainen, M. Kärkkäinen et al., "Effect of vitamin D3 and calcium on fracture risk in 65- to 71-year-old women: a population-based 3-year randomized, controlled trial—the OSTPRE-FPS," *Journal of Bone and Mineral Research*, vol. 25, no. 7, pp. 1487–1495, 2010.

[21] H. A. Bischoff-Ferrari, D. P. Kiel, B. Dawson-Hughes et al., "Dietary calcium and serum 25-hydroxyvitamin D status in relation to BMD among U.S. adults," *Journal of Bone and Mineral Research*, vol. 24, no. 5, pp. 935–942, 2009.

[22] T. R. Klompmaker, "Lifetime high calcium intake increases osteoporotic fracture risk in old age," *Medical Hypotheses*, vol. 65, no. 3, pp. 552–558, 2005.

[23] C. Rodriguez, M. L. McCullough, A. M. Mondul et al., "Calcium, dairy products, and risk of prostate cancer in a prospective cohort of United States men," *Cancer Epidemiology Biomarkers and Prevention*, vol. 12, no. 7, pp. 597–603, 2003.

[24] L. Esterle, J. P. Sabatier, F. Guillon-Metz et al., "Milk, rather than other foods, is associated with vertebral bone mass and circulating IGF-1 in female adolescents," *Osteoporosis International*, vol. 20, no. 4, pp. 567–575, 2009.

[25] E. F. Carney, "Modulation of IGF-I might prevent osteoporosis," *Nature Reviews Rheumatology*, vol. 8, article 440, 2012.

[26] S. Aoe, T. Koyama, Y. Toba, A. Itabashi, and Y. Takada, "A controlled trial of the effect of milk basic protein (MBP) supplementation on bone metabolism in healthy menopausal women," *Osteoporosis International*, vol. 16, no. 12, pp. 2123–2128, 2005.

[27] S. Soroko, T. L. Holbrook, S. Edelstein, and E. Barrett-Connor, "Lifetime milk consumption and bone mineral density in older women," *American Journal of Public Health*, vol. 84, no. 8, pp. 1319–1322, 1994.

[28] D. Jesudason and P. Clifton, "The interaction between dietary protein and bone health," *Journal of Bone and Mineral Metabolism*, vol. 29, no. 1, pp. 1–14, 2011.

[29] A. Chaitou, S. Boutroy, N. Vilayphiou et al., "Association of bone microarchitecture with parathyroid hormone concentration and calcium intake in men: the STRAMBO study," *European Journal of Endocrinology*, vol. 165, pp. 151–159, 2011.

[30] S. Adami, G. Isaia, G. Luisetto et al., "Fracture incidence and characterization in patients on osteoporosis treatment: the ICARO study," *Journal of Bone and Mineral Research*, vol. 21, no. 10, pp. 1565–1570, 2006.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 534352, 7 pages
http://dx.doi.org/10.1155/2013/534352

Clinical Study

Serum β-Catenin Levels Associated with the Ratio of RANKL/OPG in Patients with Postmenopausal Osteoporosis

Xiao-Juan Xu, Lin Shen, Yan-Ping Yang, Rui Zhu, Bo Shuai, Cheng-Gang Li, and Man-Xiang Wu

Department of Integrated Traditional Chinese Medicine and Western Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, China

Correspondence should be addressed to Lin Shen; shenlinhb@yahoo.com.cn and Yan-Ping Yang; yanpingy11@yahoo.com.cn

Received 27 December 2012; Revised 22 March 2013; Accepted 1 April 2013

Academic Editor: Peng-Fei Shan

Objective. To demonstrate the role of Wnt/β-catenin canonical pathway in postmenopausal osteoporosis by evaluating serum β-catenin levels in patients with postmenopausal osteoporosis and analyzing their possible relationship with serum OPG, RANKL, the ratio of RANKL/OPG, sclerostin, and bone turnover markers. *Methods*. 480 patients with postmenopausal osteoporosis and 170 healthy postmenopausal women were enrolled in the study. Serum β-catenin, OPG, RANKL, and sclerostin levels were measured by enzyme-linked immunosorbent assay. Bone status was assessed by measuring bone mineral density and bone turnover markers. Estradiol levels were also detected. *Results*. Serum β-catenin levels were lower in postmenopausal osteoporotic women compared to nonosteoporotic postmenopausal women (26.26 ± 14.81 versus 39.33 ± 5.47 pg/mL, $P < 0.001$). Serum β-catenin was positively correlated with osteoprotegerin ($r = 0.232$, $P < 0.001$) and negatively correlated with the ratio of RANKL/OPG, body mass index, and sclerostin ($r = -0.128$, $P = 0.005$; $r = -0.117$, $P = 0.010$; $r = -0.400$, $P < 0.001$, resp.) in patients with postmenopausal osteoporosis. *Conclusion*. The results indicate that lower serum β-catenin and concomitantly higher ratio of RANKL/OPG may be involved in the pathogenesis of postmenopausal osteoporosis. Functional communication between RANKL/RANK/OPG system and Wnt pathways plays an important role in postmenopausal osteoporosis.

1. Introduction

Postmenopausal osteoporosis (PMOP) is a highly prevalent disease, characterized by reduced bone mass, leading to increased bone fragility and fracture risk, caused by estrogen deficiency. A lot of recent reports provide evidence that the Wnt/β-catenin (canonical) signaling, one of the three known pathways of Wnt signaling, may be implicated in pathogenesis of postmenopausal osteoporosis. The Wnt/β-catenin pathway is essential for normal osteogenesis [1–3]. The Wnt/β-catenin canonical pathway is modulated by a number of factors that include Dickkopf (Dkk-1) and sclerostin, which compete with the Wnt/β-catenin for binding to LRP5/6, disrupting (Dkk-1) or antagonizing (sclerostin) LRP5/6 mediated Wnt signaling [4]. Receptor activator of NF-κB ligand (RANKL) is highly expressed on the surface of bone marrow stromal cells (BMSCs) and preosteoblasts [5]. When RANKL binds to RANK, osteoclast differentiation and function are enhanced [6]. Osteoprotegerin (OPG), produced by BMSCs and osteoblasts, is a soluble decoy receptor to inhibit RANK-RANKL-mediated osteoclastogenesis [7, 8]. de Toni et al. reported that OPG expression is regulated by β-catenin in colon cancer cells [9]. Recent evidence implicated that sclerostin, a major Wnt/β-catenin antagonist, stimulates expression of RANKL [10, 11]. Based on recent studies, we hypothesize that some relationships may exist between Wnt/β-catenin signaling and RANKL/RANK/OPG system in PMOP.

To demonstrate the role of Wnt/β-catenin canonical pathway in postmenopausal osteoporosis, we evaluated the levels of serum β-catenin, OPG, RANKL, sclerostin, and bone turnover markers in postmenopausal osteoporotic patients and compared them to those in postmenopausal nonosteoporotic women. In addition, we analyzed the relationships of β-catenin with OPG, RANKL, the ratio of RANKL/OPG, sclerostin, and bone turnover markers.

2. Subjects and Methods

2.1. Study Population. Our cross-sectional study included 480 postmenopausal women with osteoporosis and 170 healthy postmenopausal women as a control group. According to the principle of statistics, the PMOP patients were enrolled from 4 hospitals (2 in Hubei province, 1 in Jiangxi province, and 1 in Jilin province in China) by advertisement recruiting during the period of December 2009 and March 2012 in China. Eligible PMOP participants were required to have a natural menopausal history of 2–10 years and a BMD T-score of <−2.5 at the lumbar spine using dual-energy X-ray absorptiometry (DXA). Exclusion criteria were as follows: (1) treatment with calcitonin, bisphosphonates, raloxifene, estrogen, or estrogen/progestogens within 12 months, (2) coexistence of any other metabolic bone disease except for osteoporosis, (3) severe chronic disease, including malignancy, (4) medication that could affect bone metabolism, (5) previous radiation therapy, and (6) abnormal liver and kidney function tests. Healthy postmenopausal women were enrolled from physical examination center in Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. After a medical examination, they were excluded from osteoporosis and other diseases affecting bone metabolism. The protocol was approved by the responsible Clinical Trial Ethics Committee. Written informed consent was obtained from each participant.

2.2. Clinical Evaluation. In all subjects, we measured height and weight and calculated body mass index (BMI) using the Quetelet formula (weight in kilograms divided by the square of height in meters). Bone mineral density (BMD) was measured for the anteroposterior lumbar spine (L1–L4) by dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance; GE Healthcare, Madison, WI, USA). A control phantom was scanned every day, and all DXA measurements were performed by experienced operators in every hospital. Osteoporosis was defined as a T-score of <−2.5 at the lumbar spine.

2.3. Laboratory Data. Venous blood samples were taken in the morning between 8:00 AM and 9:00 AM after an overnight fast. The samples were centrifuged for 10 minutes at approximately 3000 r/min within 30 minutes, and the serum was separated and stored at −80°C prior to analysis.

Serum β-catenin, RANKL, OPG, and sclerostin levels were measured by enzyme-linked immunosorbent assay (ELISA, Yanhui biotechnology Co., Ltd., Shanghai, China). According to the manufacturer's instructions, the minimum detectable amount of human β-catenin and OPG kit was less than 1.0 pg/mL. The minimum detectable amount of human sclerostin and RANKL kit was less than 1.0 pmol/L. Intra- and interassay coefficients of variation were less than 15%. No significant cross-reactivity or interference was observed. Serum β-isomerized C-terminal crosslinking of type I collagen (CTX), intact N-terminal propeptide of type I collagen (PINP), N-mid fragment of osteocalcin (N-MID-OT), and 25-hydroxyvitamin D (25(OH)D) levels were measured using automated Roche electrochemiluminescence system. Serum estradiol levels were measured by electrochemiluminescence system in the Department of Nuclear Medicine. Intra- and interassay variations were <6% in our laboratory.

2.4. Statistical Analysis. All data for continuous variables were described as mean ± SD. Serum levels of β-catenin and other parameters between patients and controls were compared by independent-samples t-test. Spearman's coefficient of correlation was used for correlation between serum levels of β-catenin and other parameters in both groups. Multiple regression analysis was used to determine the influence of one independent variable after correcting for others. All statistics were analyzed using SPSS 16.0 software. A P value of less than 0.05 was considered statistically significant in all tests.

3. Results

The main characteristics and laboratory data of the study population were listed in Table 1. There was no statistically significant difference between patients and controls for age, weight, height, and BMI. CTX and PINP serum concentrations were higher in postmenopausal osteoporotic women than in nonosteoporotic postmenopausal women. There was no significant difference between patients and controls for serum estradiol levels, N-MID-OT levels, 25(OH)D levels, and OPG levels. The differences for β-catenin, sclerostin, and RANKL levels between two groups were significant, with $P < 0.001$.

We examined the correlations between serum β-catenin levels and various parameters in patients and controls. As shown in Table 2, serum β-catenin levels were positively correlated with OPG and negatively correlated with sclerostin, the ratio of RANKL/OPG, BMI, and BMD. No correlation between serum β-catenin and age, estradiol, N-MID-OT, 25(OH)D, CTX, PINP, and RANKL was found. No correlation between serum β-catenin and other parameters was observed in control group. A multiple regression analysis was performed to check correlations among the potential determinant variables. β-catenin was designated as the dependent variable, whereas age, BMI, BMD, estradiol, N-MID-OT, 25(OH)D, CTX, PINP, sclerostin, OPG, and RANKL were included as independent variables. In this analysis, BMD, PINP, sclerostin, and OPG were found to be independent predictors of serum β-catenin levels in PMOP, after adjusting for age, BMI, estradiol, N-MID-OT, 25(OH)D, CTX, and RANKL (Table 3). Scattered dots (Figures 1, 2, 3, and 4) showed the correlations of serum β-catenin levels with OPG, RANKL, the ratio of RANKL/OPG, and sclerostin, respectively.

4. Discussion

The adult skeleton undergoes continuous remodeling through tight coupling of opposing bone-resorbing osteoclasts and bone-forming osteoblasts. The contributing elements to the function of bone homeostasis are regulated hierarchically through a series of cell signals, cross-talk, and

Table 1: The characteristics and laboratory data of patients and controls.

	PMOP group	Control group	P
Number	480	170	
Age (years)	58.55 ± 3.55	58.47 ± 3.52	0.813
Weight (kg)	59.43 ± 4.92	59.33 ± 4.63	0.823
Height (m)	1.54 ± 0.05	1.55 ± 0.05	0.277
BMI (kg/m^2)	24.92 ± 1.66	24.73 ± 1.63	0.190
BMD (g/m^2)	0.817 ± 0.073	0.997 ± 0.073	<0.001
T-score	−3.196 ± 0.599	−1.638 ± 0.542	<0.001
Estradiol (pmol/mL)	36.63 ± 15.23	38.59 ± 16.08	0.157
N-MID-OT (ng/mL)	16.49 ± 5.96	16.19 ± 5.81	0.564
25(OH)D (ng/mL)	14.68 ± 4.93	14.74 ± 4.76	0.893
CTX (ng/mL)	0.410 ± 0.086	0.323 ± 0.065	<0.001
PINP (ng/mL)	51.69 ± 9.05	46.03 ± 10.10	0.005
β-catenin (pg/mL)	26.26 ± 14.81	39.33 ± 5.47	<0.001
Sclerostin (pmol/L)	38.79 ± 7.43	52.86 ± 6.69	<0.001
OPG (pg/mL)	155.07 ± 91.06	157.92 ± 71.67	0.679
RANKL (pmol/L)	158.10 ± 94.53	116.03 ± 54.89	<0.001
Ratio of RANKL/OPG	1.60 ± 1.76	0.87 ± 0.45	<0.001

Data for continuous variables are presented as mean ± SD.

Table 2: Associations of serum β-catenin with other parameters in PMOP group and control group.

	PMOP group		Control group	
	R	P	R	P
Age	−0.029	0.525	−0.080	0.301
Weight	−0.038	0.400	−0.048	0.536
Height	0.038	0.402	−0.078	0.312
BMI	−0.117	0.010	0.036	0.641
BMD	−0.207	<0.001	−0.081	0.294
T-score	−0.195	<0.001	−0.085	0.272
Estradiol	0.033	0.470	−0.085	0.269
N-MID-OT	0.070	0.125	−0.047	0.542
25(OH)D	−0.031	0.493	−0.004	0.956
CTX	0.050	0.277	0.013	0.862
PINP	0.033	0.469	−0.018	0.811
Sclerostin	−0.400	<0.001	0.145	0.060
OPG	0.232	<0.001	0.112	0.145
RANKL	0.067	0.143	0.134	0.081
Ratio of RANKL/OPG	−0.128	0.005	0.032	0.674

The table shows Spearman's correlation coefficients (R) and associated P values (P) in PMOP group and in control group.

cascades, essentially focused on members of the tumour necrosis factor superfamily RANKL and its receptors, RANK and OPG [12–14]. During normal bone remodeling, RANKL binds to the RANK transmembrane receptor on osteoclast precursors and induces differentiation and activation. OPG, also produced by BMSCs and osteoblasts, is a soluble member of the tumor necrosis factor receptor family (TNFR family), inhibits the differentiation and fusion of the osteoclastic precursor cells, and blocks the activation of mature osteoclasts [15]. When RANKL binds to RANK, osteoclast differentiation and function are enhanced [6]. Thus, targeting the RANKL/RANK/OPG system should produce potent effects on osteoclast differentiation and function [6]. Recent findings have shown that the Wnt/β-catenin canonical pathway in osteoblasts/stromal cells suppresses osteoclastogenesis through the upregulation of OPG expression and the downregulation of RANKL expression [16, 17].

The cross-sectional study confirms that β-catenin is detectable in human serum, as observed very recently by Gaudio et al. in patients with type 2 diabetes mellitus (T2DM) [18], and indicates that patients with PMOP have β-catenin serum levels lower than controls. β-catenin, a pivotal signaling molecule of the Wnt pathway, has been shown to be important in osteoblast differentiation, proliferation, and apoptosis [19]. Overexpression of β-catenin increased Wnt signaling activity [20]. Lower β-catenin levels may reflect the lower Wnt signaling activity in our PMOP cohort. Recent evidence has indicated that the Wnt/β-catenin pathway plays an important role in skeletal development and growth [21, 22], particularly in bone mass acquisition, remodeling, differentiation, and maintenance [23, 24]. The mechanisms are still unclear and being explored. The importance of the canonical pathway in bone biology has been emphasized by the identification of a link between bone mass and mutations in the LRP5 gene [25]. Loss-of-function mutations in LRP5 reduce the number of osteoblasts and cause osteoporosis [25, 26]. Canonical Wnts (Wnt3a) bind to the receptor complex of Frizzled and LRP5 or LRP6, inhibit GSK-3β, and promote the accumulation of β-catenin in osteoblasts [27]. The accumulated β-catenin translocates into the nucleus and together with TCF/LEF induces the expression of OPG to inhibit RANK-RANKL-mediated osteoclastogenesis [28]. In our study, we detected higher RANKL serum concentration and concomitantly similar OPG serum concentration at the protein levels in PMOP patients compared to controls. This suggested that there was a more seriously impaired balance between osteoblastic bone formation and osteoclastic bone resorption in patients with PMOP. In our study, we found a significant negative correlation between β-catenin and the ratio of RANKL/OPG. It suggested that some cross-links were present between Wnt/β-catenin signaling pathway and RANKL/RANK/OPG system and provided evidence that increased RANKL/OPG expression was related to reduction of Wnt/β-catenin signaling activity in PMOP.

We also found a significant negative correlation between β-catenin and sclerostin, which in fact agrees with a major contribution of sclerostin to the impairment of the Wnt/β-catenin signaling pathway in this setting. The pattern was unchanged in a multiple regression model. Sclerostin, an osteocyte-derived and -secreted glycoprotein, has been shown to influence the activity of Wnt signaling pathways [29]. The mechanism is not completely understood. According to the current knowledge, sclerostin antagonizes the canonical Wnt pathway by preventing the formation of the Wnt-Frizzled-LRP5 complex by competitively binding to LRP6 and LRP5, transmembrane proteins that together with Frizzled receptors mediate the actions of Wnts [4, 30–33].

Table 3: Result for multiple regressions in PMOP group and in control group.

	PMOP group					Control group				
	B	SE	*β*	*t*	Sig.	*B*	SE	*β*	*t*	Sig.
Age	−0.059	0.171	−0.014	−0.348	0.728	−0.097	0.126	−0.063	−0.775	0.439
BMI	−0.312	0.364	−0.035	−0.859	0.391	0.331	0.269	0.098	1.229	0.221
BMD	−18.870	9.533	−0.093	−1.979	0.048	−3.708	5.920	−0.050	−0.626	0.532
Estradiol	−0.011	0.040	−0.011	−0.279	0.780	−0.030	0.029	−0.087	−1.014	0.312
N-MID-OT	0.173	0.103	0.070	1.682	0.093	0.010	0.083	0.011	0.126	0.900
25(OH)D	0.024	0.122	0.008	0.197	0.844	−0.004	0.099	−0.003	−0.039	0.969
CTX	−3.369	7.680	−0.020	−0.439	0.661	−7.431	8.829	−0.088	−0.842	0.401
PINP	−0.169	0.069	−0.103	−2.451	0.015	−0.025	0.047	−0.046	−0.526	0.600
Sclerostin	−0.684	0.089	−0.343	−7.721	<0.001	0.106	0.067	0.130	1.599	0.112
OPG	0.047	0.007	0.291	7.174	<0.001	0.003	0.008	0.036	0.349	0.727
RANKL	0.011	0.006	0.073	1.836	0.067	0.014	0.008	0.136	1.676	0.096

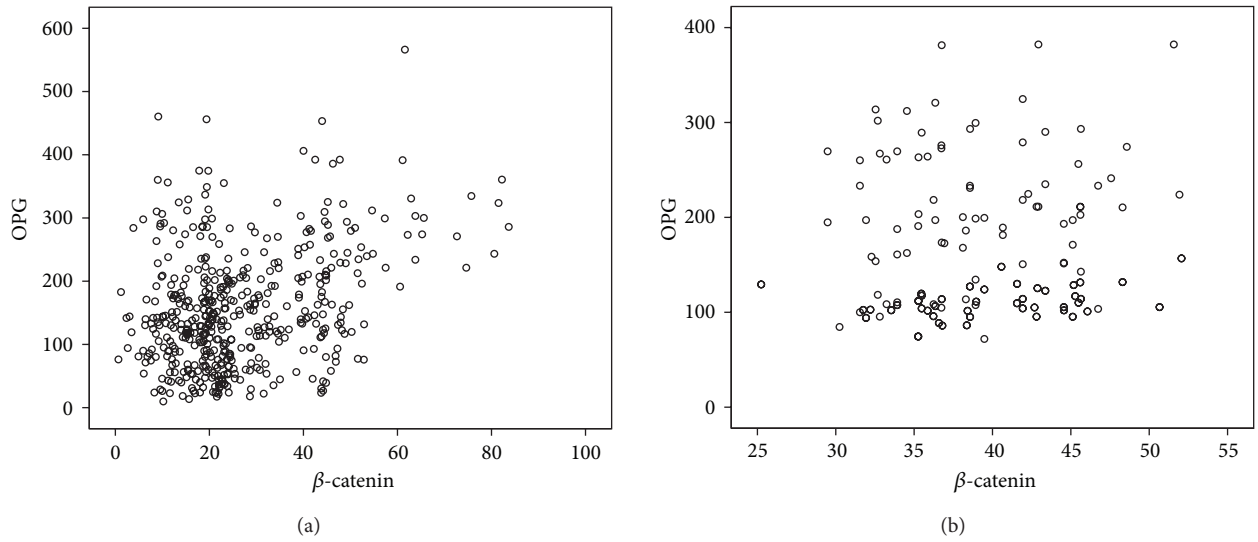


Figure 1: Univariate correlation (Spearman analysis) between *β*-catenin and OPG serum levels in PMOP patients ((a) $r = 0.232$, $P < 0.001$) and controls ((b) $r = 0.112$, $P = 0.145$).

Beyond that, sclerostin was found to interact with several other Wnt pathway regulatory molecules such as secreted Frizzled related protein 4 (sFRP4), casein kinase II, and TRAF2- and NCK-interacting kinase [34–37].

Both CTX and PINP significantly increased in PMOP cohort, confirming that higher bone turnover took place in this population compared to controls. However, no association between the *β*-catenin and CTX and the PINP was observed in both the PMOP group and the control group. Furthermore, we observed no correlation of serum *β*-catenin levels with age. Different from our findings, the recent report by Gaudio et al. [18] showed that there were significant correlations of serum *β*-catenin levels with age and serum sclerostin levels in T2DM patients and with age in controls. These discrepancies may be related to differences in the population characteristics. Our cohort in the study was limited to postmenopausal women with the age of 50.5–65.5 years old.

In our PMOP cohort, it is difficult to explain the negative correlation of serum *β*-catenin levels with lumbar spine BMD. For the contradictory phenomenon, the rational explanation would be that *β*-catenin is increased in a compensatory manner. Another interesting thing was that we observed a negative correlation between serum *β*-catenin and BMI. The result may be related to the role of Wnt/*β*-catenin signaling on inhibiting adipogenesis of mesenchymal precursors [38, 39].

In our control group, we found no associations of *β*-catenin with OPG and the ratio of RANKL/OPG. We hypothesized that the function communication between Wnt/*β*-catenin signaling and RANKL/RANK/OPG system was not established in postmenopausal non-osteoporosis women.

The study has some limitations. It was a cross-sectional design, and the causative nature of the associations between *β*-catenin, RANKL/OPG, and other variables cannot be established. We analyzed serum *β*-catenin, RANKL/OPG

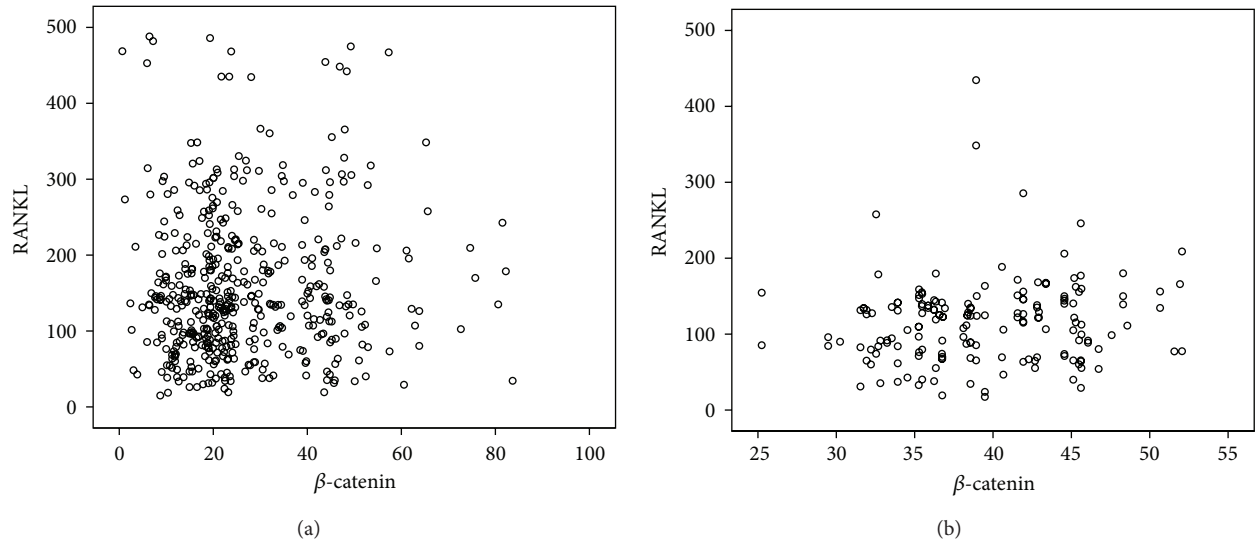


Figure 2: Univariate correlation (Spearman analysis) between β-catenin and RANKL serum levels in PMOP patients ((a) $r = 0.067$, $P = 0.143$) and controls ((b) $r = 0.134$, $P = 0.081$).

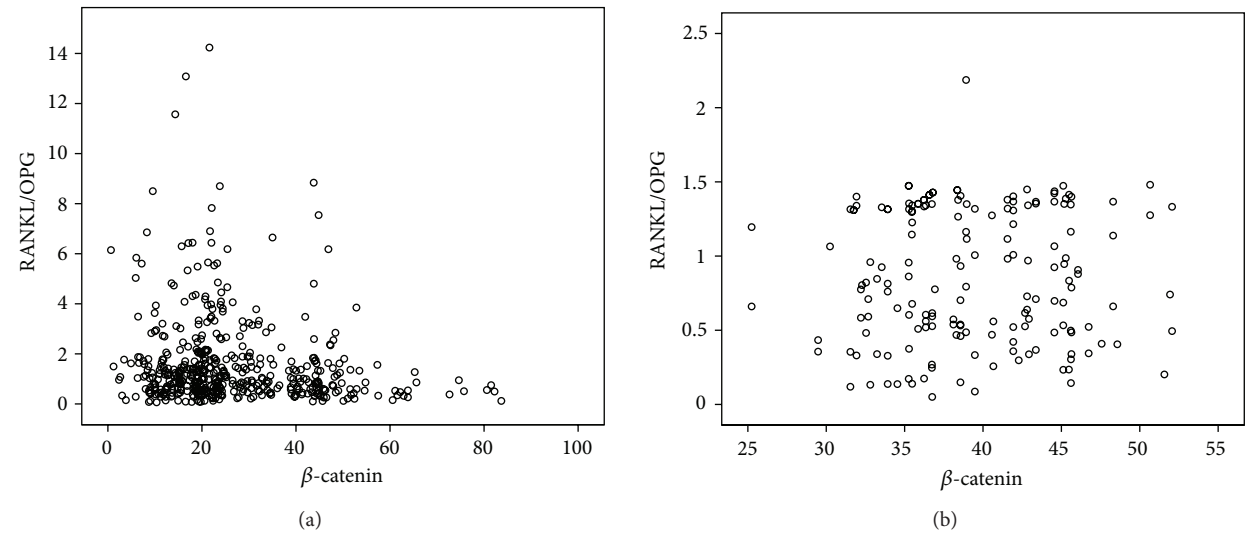


Figure 3: Univariate correlation (Spearman analysis) between β-catenin and RANKL/OPG serum levels in PMOP patients ((a) $r = -0.128$, $P = 0.005$) and controls ((b) $r = 0.032$, $P = 0.674$).

levels which may not be sensitive enough to reflect their expression in bone cells.

5. Conclusions

Given the existence of multiple pathogeneses of PMOP, the recognition of the role of Wnt/β-catenin signaling on bone metabolism has stimulated a number of studies on the effects of this signaling on treatment of PMOP. However, the effects of Wnt signaling on bone metabolism and the involved molecular mechanisms remain unclear. According to our findings, functional communication between RANKL/RANK/OPG system and Wnt/β-catenin signaling pathway plays an important role in postmenopausal osteoporosis. Targeting the Wnt/β-catenin signaling to change the ratio of RANKL/OPG to alter bone turnover can potentially provide an approach for postmenopausal osteoporosis therapy.

Conflict of Interests

There is no conflict of interests.

Acknowledgments

The authors thank all the subjects who participated in the present study and the staff at Department of Nuclear

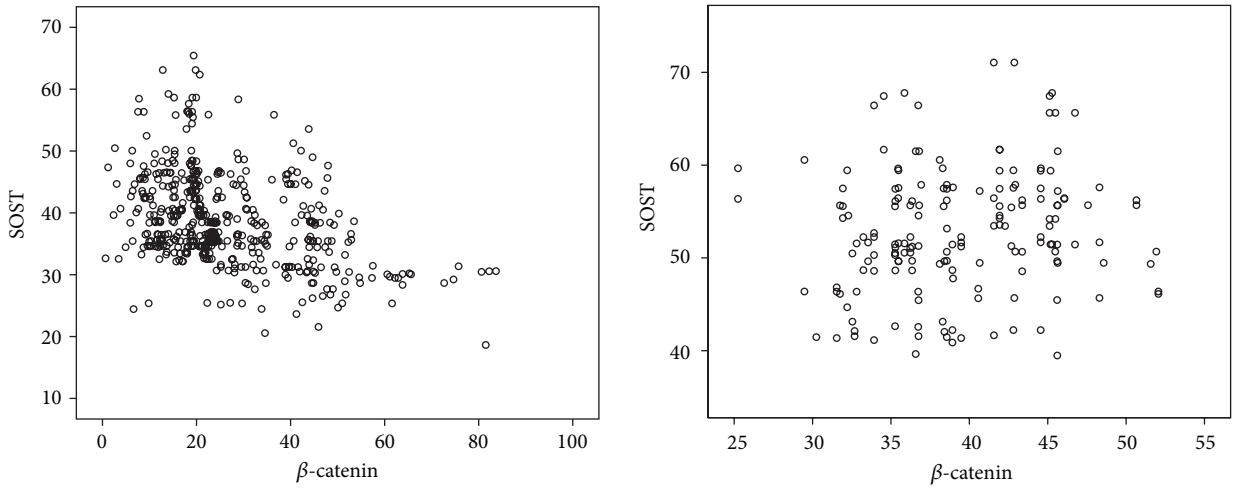


Figure 4: Univariate correlation (Spearman analysis) between β-catenin and sclerostin serum levels in PMOP patients ((a) $r = -0.400$, $P < 0.001$) and controls ((b) $r = 0.145$, $P = 0.060$).

Medicine of Union Hospital in Wuhan for providing automated electrochemiluminescence system and Lunar Prodigy. They thank Vidhi Heerah, M.D. and Ph.D., from the Republic of Mauritius for helping with the paper revision. This study was supported by NSFC (Natural Science Foundation of China) (Grant no. 81072943) and Health Department of Hubei province (Grant no. 2012Z-Z01).

References

[1] T. F. Day, X. Guo, L. Garrett-Beal, and Y. Yang, "Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis," *Developmental Cell*, vol. 8, no. 5, pp. 739–750, 2005.

[2] H. Jian, X. Shen, I. Liu, M. Semenov, X. He, and X. F. Wang, "Smad3-dependent nuclear translocation of β-catenin is required for TGF-β1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells," *Genes and Development*, vol. 20, no. 6, pp. 666–674, 2006.

[3] X. L. Liu, C. C. Li, K. J. Liu, C. Y. Cui, Y. Z. Zhang, and Y. Liu, "The influence of fluoride on the expression of inhibitors of Wnt/β-catenin signaling pathway in rat skin fibroblast cells," *Biological Trace Element Research*, vol. 148, no. 1, pp. 117–121, 2012.

[4] M. Semënov, K. Tamai, and X. He, "SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor," *The Journal of Biological Chemistry*, vol. 280, no. 29, pp. 26770–26775, 2005.

[5] T. Ikeda, M. Utsuyama, and K. Hirokawa, "Expression profiles of receptor activator of nuclear factor κB ligand, receptor activator of nuclear factor κB, and osteoprotegerin messenger RNA in aged and ovariectomized rat bones," *Journal of Bone and Mineral Research*, vol. 16, no. 8, pp. 1416–1425, 2001.

[6] L. C. Hofbauer, S. Khosla, C. R. Dunstan, D. L. Lacey, W. J. Boyle, and B. L. Riggs, "The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption," *Journal of Bone and Mineral Research*, vol. 15, no. 1, pp. 2–12, 2000.

[7] J. R. Arron and Y. Choi, "Bone versus immune system," *Nature*, vol. 408, no. 6812, pp. 535–536, 2000.

[8] Y. Kobayashi, N. Udagawa, and N. Takahashi, "Action of RANKL and OPG for osteoclastogenesis," *Critical Reviews in Eukaryotic Gene Expression*, vol. 19, no. 1, pp. 61–72, 2009.

[9] E. N. de Toni, S. E. Thieme, A. Herbst et al., "OPG is regulated by β-catenin and mediates resistance to TRAIL-induced apoptosis in colon cancer," *Clinical Cancer Research*, vol. 14, no. 15, pp. 4713–4718, 2008.

[10] J. Xiong and C. A. O'Brien, "Osteocyte RANKL: new insights into the control of bone remodeling," *Journal of Bone and Mineral Research*, vol. 27, no. 3, pp. 499–505, 2012.

[11] A. R. Wijenayaka, M. Kogawa, H. P. Lim, L. F. Bonewald, D. M. Findlay, and G. J. Atkins, "Sclerostin stimulates osteocyte support of osteoclast activity by a RANKL-dependent pathway," *PloS ONE*, vol. 6, no. 10, Article ID e25900, 2011.

[12] D. L. Lacey, E. Timms, H. L. Tan et al., "Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation," *Cell*, vol. 93, no. 2, pp. 165–176, 1998.

[13] W. S. Simonet, D. L. Lacey, C. R. Dunstan et al., "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density," *Cell*, vol. 89, no. 2, pp. 309–319, 1997.

[14] L. E. Mulcahy, D. Taylor, T. C. Lee, and G. P. Duffy, "RANKL and OPG activity is regulated by injury size in networks of osteocyte-like cells," *Bone*, vol. 48, no. 2, pp. 182–188, 2011.

[15] B. Chen, X. D. Li, D. X. Liu et al., "Canonical Wnt signaling is required for Panax notoginseng saponin-mediated attenuation of the RANKL/OPG ratio in bone marrow stromal cells during osteogenic differentiation," *Phytomedicine*, vol. 19, no. 11, pp. 1029–1034, 2012.

[16] D. A. Glass II, P. Bialek, J. D. Ahn et al., "Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation," *Developmental Cell*, vol. 8, no. 5, pp. 751–764, 2005.

[17] G. J. Spencer, J. C. Utting, S. L. Etheridge, T. R. Arnett, and P. G. Genever, "Wnt signalling in osteoblasts regulates expression of the receptor activator of NFκB ligand and inhibits osteoclastogenesis in vitro," *Journal of Cell Science*, vol. 119, no. 7, pp. 1283–1296, 2006.

[18] A. Gaudio, F. Privitera, K. Battaglia et al., "Sclerostin levels associated with inhibition of the Wnt/β-catenin signaling and reduced bone turnover in type 2 diabetes mellitus," *Journal of Clinical Endocrinology and Metabolism*, vol. 97, no. 10, pp. 3744–3750, 2012.

[19] M. L. Johnson and M. A. Kamel, "The Wnt signaling pathway and bone metabolism," *Current Opinion in Rheumatology*, vol. 19, no. 4, pp. 376–382, 2007.

[20] R. Zhang, B. O. Oyajobi, S. E. Harris et al., "Wnt/β-catenin signaling activates bone morphogenetic protein 2 expression in osteoblasts," *Bone*, vol. 52, no. 1, pp. 145–156, 2013.

[21] R. Baron, G. Rawadi, and S. Roman-Roman, "Wnt signaling: a key regulator of bone mass," *Current Topics in Developmental Biology*, vol. 76, pp. 103–127, 2006.

[22] C. Hartmann, "A Wnt canon orchestrating osteoblastogenesis," *Trends in Cell Biology*, vol. 16, no. 3, pp. 151–158, 2006.

[23] S. L. Holmen, C. R. Zylstra, A. Mukherjee et al., "Essential role of β-catenin in postnatal bone acquisition," *The Journal of Biological Chemistry*, vol. 280, no. 22, pp. 21162–21168, 2005.

[24] V. Krishnan, H. U. Bryant, and O. A. MacDougald, "Regulation of bone mass by Wnt signaling," *Journal of Clinical Investigation*, vol. 116, no. 5, pp. 1202–1209, 2006.

[25] Y. Gong, R. B. Slee, N. Fukai et al., "LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development," *Cell*, vol. 107, no. 4, pp. 513–523, 2001.

[26] M. Kato, M. S. Patel, R. Levasseur et al., "Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor," *Journal of Cell Biology*, vol. 157, no. 2, pp. 303–314, 2002.

[27] N. Takahashi, K. Maeda, A. Ishihara, S. Uehara, and Y. Kobayashi, "Regulatory mechanism of osteoclastogenesis by RANKL and Wnt signals," *Frontiers in Bioscience*, vol. 16, no. 1, pp. 21–30, 2011.

[28] C. V. Gurban and O. Mederle, "The OPG/RANKL system and zinc ions are promoters of bone remodeling by osteoblast proliferation in postmenopausal osteoporosis," *Romanian Journal of Morphology and Embryology*, vol. 52, no. 3, supplement, pp. 1113–1119, 2011.

[29] D. G. Winkler, M. K. Sutherland, J. C. Geoghegan et al., "Osteocyte control of bone formation via sclerostin, a novel BMP antagonist," *EMBO Journal*, vol. 22, no. 23, pp. 6267–6276, 2003.

[30] X. Li, Y. Zhang, H. Kang et al., "Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling," *The Journal of Biological Chemistry*, vol. 280, no. 20, pp. 19883–19887, 2005.

[31] B. Mao, W. Wu, G. Davidson et al., "Kremen proteins are Dickkopf receptors that regulate Wnt/β-catenin signalling," *Nature*, vol. 417, no. 6889, pp. 664–667, 2002.

[32] R. L. van Bezooijen, B. A. J. Roelen, A. Visser et al., "Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist," *Journal of Experimental Medicine*, vol. 199, no. 6, pp. 805–814, 2004.

[33] D. L. Ellies, B. Viviano, J. McCarthy et al., "Bone density ligand, sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity," *Journal of Bone and Mineral Research*, vol. 21, no. 11, pp. 1738–1749, 2006.

[34] Y. Gao and H. Y. Wang, "Casein kinase 2 is activated and essential for Wnt/β-catenin signaling," *The Journal of Biological Chemistry*, vol. 281, no. 27, pp. 18394–18400, 2006.

[35] R. Nusse, "Relays at the membrane," *Nature*, vol. 438, no. 7069, pp. 747–749, 2005.

[36] R. Satow, M. Shitashige, T. Jigami et al., "Traf2- and Nck-interacting kinase is essential for canonical Wnt signaling in Xenopus axis formation," *The Journal of Biological Chemistry*, vol. 285, no. 34, pp. 26289–26294, 2010.

[37] M. Shitashige, R. Satow, T. Jigami et al., "Traf2- and Nck-interacting kinase is essential for Wnt signaling and colorectal cancer growth," *Cancer Research*, vol. 70, no. 12, pp. 5024–5033, 2010.

[38] S. E. Ross, N. Hemati, K. A. Longo et al., "Inhibition of adipogenesis by Wnt signaling," *Science*, vol. 289, no. 5481, pp. 950–953, 2000.

[39] C. N. Bennett, S. E. Ross, K. A. Longo et al., "Regulation of Wnt signaling during adipogenesis," *The Journal of Biological Chemistry*, vol. 277, no. 34, pp. 30998–31004, 2002.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 167138, 7 pages
http://dx.doi.org/10.1155/2013/167138

Review Article

Osteoporosis Associated with Antipsychotic Treatment in Schizophrenia

Haishan Wu,[1] Lu Deng,[2] Lipin Zhao,[2] Jingping Zhao,[1] Lehua Li,[1] and Jindong Chen[1]

[1] *Institute of Mental Health, Second Xiangya Hospital, Central South University, Changsha 410011, China*
[2] *Department of Nursing, Second Xiangya Hospital, Central South University, Changsha 410011, China*

Correspondence should be addressed to Jindong Chen; chenjd269@163.com

Received 19 December 2012; Revised 20 February 2013; Accepted 18 March 2013

Academic Editor: Guang-Da Xiang

Schizophrenia is one of the most common global mental diseases, with prevalence of 1%. Patients with schizophrenia are predisposed to diabetes, coronary heart disease, hypertension, and osteoporosis, than the normal. In comparison with the metabolic syndrome, for instance, there are little reports about osteoporosis which occurs secondary to antipsychotic-induced hyperprolactinaemia. There are extensive recent works of literature indicating that osteoporosis is associated with schizophrenia particularly in patients under psychotropic medication therapy. As osteoporotic fractures cause significantly increased morbidity and mortality, it is quite necessary to raise the awareness and understanding of the impact of antipsychotic-induced hyperprolactinaemia on physical health in schizophrenia. In this paper, we will review the relationship between schizophrenia, antipsychotic medication, hyperprolactinaemia, and osteoporosis.

1. Introduction

Schizophrenia is one of the most common global mental diseases, with prevalence of 1%. It is a major cause of disability and affects patients in the quality of life and work as well as interpersonal and self-care functioning. Moreover, the schizophrenic are under an increased threefold risk of premature death and shortened life expectancy of 10–20 years [1–3]. As the improvement psychosis treatments, there has been an increasing awareness of the need for high quality physical health care for the schizophrenic [4].

Compared to the increasingly significant recognition and management of obesity and metabolic problems, the appreciation of bone health has lagged behind. Osteoporosis is characterized by decreased bone stiffness, as signified by low bone mineral density (BMD), vertebral or nonvertebral fragility fractures, and disruption of bone microarchitecture. It is a significant health problem afflicting the global people [5, 6], and the female cases were more than male ones (5:2) older than 50 years of age, which brings about a disease burden of around £1.8 billion in the UK and £30 billion in whole Europe [7]. Although the high incidence rate of osteoporosis and osteoporotic fractures in the schizophrenic patients was first reported about 20 years ago [8–10], related reports about the increased risk of osteoporotic fracture and earlier onset of osteoporosis in the schizophrenic patients are seldom published [11]. Recently, many papers have presented convincing evidence that decrease of bone mineral density is related to schizophrenia particularly in patients treated with psychotropic medication [12–15].

In this paper, we will review the osteoporosis epidemiology and risk factors of schizophrenia to investigate whether antipsychotics can contribute to the development of osteoporosis. Our discussion focuses on the possible mechanisms involved and the clinical implications of such a relationship. And some prevention measures for osteoporosis in the schizophrenic will bring forth.

2. Epidemiology of Osteoporosis in Schizophrenic Patients

Comparing to normal people, patients with chronic schizophrenia actually show an exceedingly high prevalence of osteoporosis and bone fracture, and there have been a large

amount of reports which indicate that bone mineral density decreased markedly in them [16, 17]. For instance, a study comparing ultrasound bone mass in 73 patients with schizophrenia on antipsychotic therapy with a matched number of healthy controls demonstrated increased bone loss in the former [18]. In addition, a UK General Practice Research Database study including 29,889 matched controls reported a statistically significant association between prolactin-raising antipsychotics and fractures; it showed that the relative risk of fracture at any site was increased 2.5-fold in premenopausal women with psychotic disorders, while hip fracture rates were increased 5.1-fold and 6.4-fold in older women and men, respectively, [19]. A Danish study also found a 1.2-fold increased fracture risk in those taking antipsychotics [20], while a Dutch population-based case-control study reported a 1.68-fold and 1.33-fold increased risk for hip or femur fracture for current and past users of antipsychotics, respectively [12]. Furthermore, a large case control analysis, including 22,250 hip/femur fractures with an equal number of controls, provided evidence that patients on antipsychotics were at an increased risk of hip/femur fractures, regardless of the antipsychotic drug prescribed [16].

3. Risk Factors for Osteoporosis in Schizophrenic Patients

Since such a high incidence of osteoporosis in schizophrenia, we should consider the possible risk factors involved and the clinical implications of such a relationship. The risk factors can be grouped into genetic and modifiable risk factors.

3.1. Genetic Risk Factors. Genetic risk factors include female sex, old age, White or Asian race, or family history of osteoporosis [21]. In a study carried out in 2006 with schizophrenic patients treated with haloperidol, Jung et al. obtained results that female patients, instead of the male, showed significantly lower BMD, using densitometry techniques by DEXA (dual-energy X-ray absorptiometry), than the normal controls in all bone regions studied. Therefore, BMD loss in in schizophrenic patients tended to differ by gender [22]. But the result is in disagreement with several studies of psychiatric patients, which significantly found lower bone mineral density in men than in women associated with neuroleptic use [23, 24]. These gender differences may owe to the age differences in onset of schizophrenia [25]; that is to say, men have an age at onset approximately 5 years younger than that in women, and illness-related factors including medication will therefore have a longer-lasting impact on male patients. An alternative explanation suggested by Hummer and Huber is that women with schizophrenia take better care of themselves with regard to adequate nutrition and exercise than men and therefore have less osteoporosis [26]. Bone density in elderly persons is highly relevant to the risk of osteoporotic fracture was recognized by many years ago [27]. Ethnicity and family history of osteoporosis are important factors influencing the incidence of osteoporosis; also, Cauley reported rates of fragility fracture differ depending on race/ethnicity and are typically higher among those of White race [28]; Rybakowski et al. improved the functional polymorphism −1149 G/T (rs1341239) of the prolactin gene, and the G allele was associated with a diagnosis of schizophrenia in antipsychotic-induced osteoporosis [29].

3.2. Modifiable Risk Factors. Modifiable risk factors include low body mass index (<20–25 m/kg^2), smoking, physical inactivity, poor dietary calcium intake, vitamin D deficiency, symptoms of the disease, and certain psychotropic medications [21, 30]. Liu et al. recently conducted a systematic review on osteoporosis risk factors in men and determined that the most important risk factors include age over 70 years old and low body mass which was in the general population [31]. The similar result was found by Hummer et al. in schizophrenia patients [23]. Smoking is particularly another important risk factor in schizophrenia, as much as 64% of patients with it have been known to smoke on a daily basis [32]. It was first recognized as a risk factor for osteoporosis in the mid-1970s [33], Law and Hackshaw conducted a meta-analysis and report a strong correlation between cigarette smoking and low BMD or hip fracture in postmenopausal women; they reported that one in eight fractures is accountable to smoking and that smoking increases lifetime risk of osteoporotic fractures from 12% to 19% in women up to the age of 85 years and from 22% to 37% to the age of 90 years [34]. The influence of physical inactivity on bone mineral density might be a consequence of the behavior of patients caused by negative, depressive. Patients with negative and depressive symptoms are likely to be physically inactive and show less tendency to go outside, which could in turn lead to lower 25-hydroxy-vitamin D3 levels while 25-hydroxy-vitamin D3 is a determinant of BMD in children and adolescents [35]. Positive symptoms such as paranoid delusions, on the other hand, can lead to an erratic intake of food, leading to nutritional deficits, as well as poor dietary calcium intake, vitamin D deficiency. Both types of behavior, therefore, could have a negative impact on bone mineral density.

4. Antipsychotic-Induced Osteoporosis and the Mechanism

Except an increased prevalence of traditional risk factors, such as reduced physical activity, increased smoking, reduced calcium and vitamin D intake, as well as disease-specific factors, and certain psychotropic medications (Figure 1).

4.1. The Physiological of Antipsychotic-Induced Hyperprolactinemia. The mechanisms of antipsychotic-induced osteoporosis are complex, and the most possible one may be the hyperprolactinemia. Prolactin (PRL) is a 23 kDa polypeptide hormone secreted by the lactotroph cells of the anterior pituitary gland. Prolactin homeostasis is the result of a complex balance between positive and negative stimuli, deviating from both external and endogenous environments. Antipsychotics are the most common cause of pharmacologic hyperprolactinemia, and the majority of antipsychotic agents cause hyperprolactinemia [36]. There have been many studies

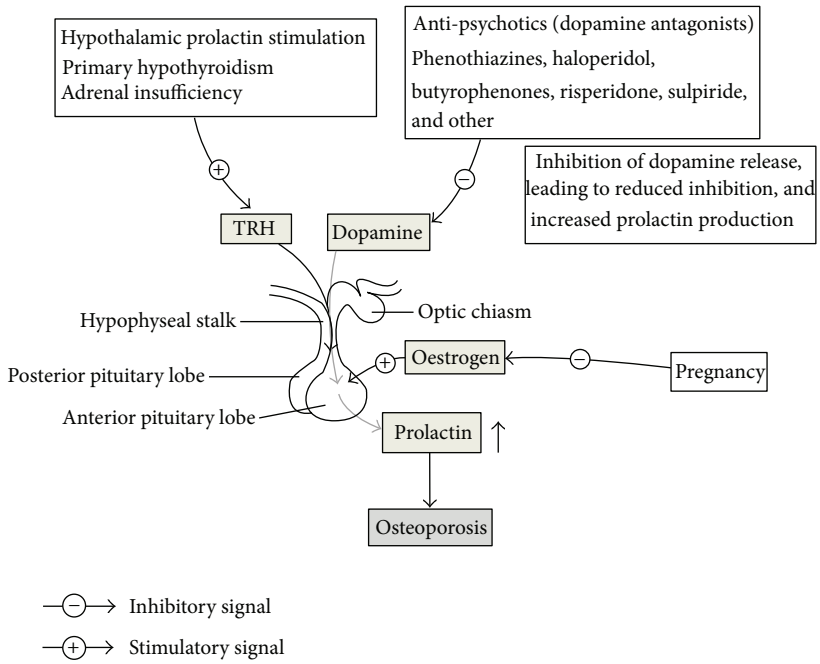


Figure 1: The physiological of antipsychotic-induced osteoporosis.

that presumed a correlation between the decrease of bone mineral density found in patients with schizophrenia and hyperprolactinemia caused by long-term medication with antipsychotics also [26, 37, 38]. Cross-sectional studies have indicated that the prevalence of hyperprolactinaemia ranges from 42–93% in women and 18–72% in men [37]. Most of conventional antipsychotics can cause prolactin elevation, Marken reported treatment with conventional antipsychotics in patients with schizophrenia has been shown to increase serum prolactin concentrations 5–10 times above that of healthy control subjects in 1992 [39]. While among atypical antipsychotics, hyperprolactinaemia is well pronounced with by risperidone and paliperidone, followed by amisulpride [40, 41]. In the USA, Montgomery et al. report that hyperprolactinaemia occurred with all antipsychotics in their trial (risperidone 91%, olanzapine 40%, quetiapine 22%, and clozapine 11%) [42].

The mechanism of hyperprolactinemia is that antipsychotics block the dopamine D2 receptor of lactotrophs in the anterior pituitary and the prolactin secretion inhibiting function and consequently causes hyperprolactinemia [43]. Dopamine, secreted in hypothalamic periventricular zone (periventricular nucleus and arcuate nucleus) and released from neuronal projections in the median eminence, reaches the anterior pituitary gland through portal vessels (system known as "tuberoinfundibular dopamine pathway" or "TIDA"). The dopamine-mediated inhibition of prolactin secretion occurs through the binding of D2 receptors on the membrane of lactotroph cells and involves several signal transduction systems, resulting in inhibition of prolactin gene transcription and reduction of prolactin synthesis and release.

It appears that risperidone and amisulpride are the main atypical antipsychotics associated with statistically significant increases in serum prolactin. As mentioned previously, amisulpride and risperidone penetrate the blood brain barrier poorly and, therefore, reach much higher concentrations in plasma than the CNS [44]. One study demonstrated that in amisulpride- and risperidone-treated rats, D2 receptor occupancy was higher in the pituitary (peripheral) than the striatum (central), with doses of amisulpride sufficient to induce 25% D2 receptor occupancy at the striatum inducing 100% D2 receptor occupancy at the pituitary gland. This may explain why amisulpride and risperidone seem to be associated with increased risk of hyperprolactinemia compared with other antipsychotic drugs such as clozapine, olanzapine, and quetiapine [45–47]. A positron emission tomography examination of D2 occupancy in the pituitary and temporal cortex supported this explanation and suggested that the greater rise in prolactin with risperidone may be due to the drug elimination from the brain by P-glycoprotein [48].

4.2. The Consequences of Hyperprolactinaemia. With the advent of prolactin sparing antipsychotics, ample consideration needs to be given to the physiological consequences of hyperprolactinaemia in schizophrenic patients. Hyperprolactinaemia has direct effects on the brain and on other organs. Direct consequences include galactorrhoea. Indirect consequences of hyperprolactinaemia include oligomenorrhoea or amenorrhoea, erratic or absent ovulation, sexual dysfunction, reduced bone mineral density, and cardiovascular disease [49].

Antipsychotics-induced hyperprolactinemia may influence bone metabolism in two ways. On one hand, hyperprolactinemia might directly affect bone turnover by stimulating bone resorption relative to bone formation [50, 51]. When recombinant prolactin was administered to pregnant rats, there was a 30% decrease of alkaline phosphatase in the newborn pups, despite normal parathyroid hormone and calcium concentrations. This appears to result from a direct suppressive effect of prolactin on rat osteoblast as demonstrated in primary cell cultures. Histology of the newborn pup bone showed reduced calvarial bone and reduced endochondral ossification. By contrast, the increase ratio of prolactin, which mirrored the increase of lactation in animal models, is associated with an increase of intestinal calcium absorption in animal models [52]. As a result, the calcium homeostasis may be improven. The importance of prolactin has been further illustrated in the prolactin receptor mouse knockout, which has marked hyperprolactinaemia and a decrease in bone formation rate and reduced bone mineral density, as measured by DXA [53]. The molecular mechanisms for these direct effects are not fully understood but may involve RANKL as it has been shown that prolactin can enhance production of mRNA for RANKL [54].

On the other hand, prolonged hyperprolactinemia may cause hypogonadotropic hypogonadism [55], resulting in suppression of gonadotropin-releasing hormone (GnRH) secretion in the hypothalamus and diminished secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary gland, resulting in a diminished secretion of sex hormones and ultimately in changes in bone metabolism [56]. Oestrogen is a well-known and prominent factor in bone metabolism. Hypoestrogenism leads to an increased risk for osteoporosis. Oestrogen inhibits osteoclastic activity while increasing gene expression in osteoblasts and increasing the level of type I collagen produced by osteoblast cells. Furthermore, oestrogen affects the synthesis of 25-OH-D and the absorption of calcium in the intestine. By contrast, there are fewer studies of testosterone in the context of bone metabolism. However, due to its effect on osteoblastic activity, low levels, of testosterone are correlated with osteopenia and/or osteoporosis. In the same way, dehydroepiandrosterone (DHEA) and its sulfate (DHEAS), important androgen as well as oestrogen precursor, are known to correlate with BMD [57].

5. Prevention and Treatment

Despite the established evidence of antipsychotic-induced hyperprolactinaemia, and hyperprolactinaemia associated with osteoporosis, published guidance on the monitoring and management of elevated prolactin levels in patients receiving antipsychotic treatment is lacking. The efficacy of preventive measures and treatment strategies to avoid or treat osteoporosis and reduce the risk in patients on antipsychotic medications could be the subject of health strategy studies.

5.1. Recommendations for the Prevention of Osteoporosis. It should be in line with established preventive measures, although there are many risk factors for osteoporosis—some of which can not be changed, including being female, being Caucasian or Asian, and having a direct relative who has had an osteoporotic fracture. However, there are many risk factors can be addressed, which can allow patients with schizophrenia to take control of their bone health and help prevent osteoporosis, including having a well-balanced diet, making lifestyle changes like stopping smoking, and minimising alcohol and caffeine intake. Vitamin D therapy is a recommended clinical practice in patients suffering from a decrease of bone mineral density [58, 59]. A prophylactic addition of vitamin D to the treatment of patients with schizophrenia who suffer from vitamin D deficiency would avoid loss of bone mineral density [60].

Clinicians should ask questions to detect risk factors before treatment start and then give patients relevant information. For prolactin-raising antipsychotics, it has been recommended that patients are questioned on possible prolactin-related effects until a stabilized dose is achieved [61]. In addition to the above trial data, Peveler et al. recommended that all patients prescribed antipsychotics should undergo prolactin screening at initiation of a three-month treatment [62]. In patients with elevated prolactin levels, other potential causes of hyperprolactinaemia should be ruled out [45, 61], several management options are available to counteract the effects of antipsychotic-induced hyperprolactinaemia [45], Such as dose reduction, switching drug or adding the partial agonist aripiprazole, that include the use of dopamine agonists. The addition of a D2 receptor agonist to an existing antipsychotic treatment is another management option. The dopamine agonist, bromocriptine, corrects elevated prolactin levels and has been shown to increase mean bone mineral density [63]. However, bromocriptine may be associated with adverse effects such as postural hypotension and gastrointestinal symptoms [45]. Adjunctive aripiprazole, which is a partial agonist, concomitant treatment may correct prolactin levels reduced prolactin [64]. Within 12 weeks, prolactin levels had fallen, and there was improvement in libido after switched or added aripiprazole to the medication of 27 patients. In males, both erectile and ejaculatory difficulties improved. In females, menstrual dysfunction was also significantly improved [65].

5.2. Recommendations for the Treatment of Osteoporosis. Once diagnosed, treatment of osteoporosis should be initiated in close cooperation with other multidisciplinary teams in order to reduce falls and prevent fractures. Except safer exercise options and falls prevention, there are a number of medications to treat osteoporosis and help reduce the risk of fractures. Drugs to treat osteoporosis can be grouped into two categories. The first ones are comprised of agents that limit the rate of bone loss. These drugs, also known as "anti-resorption drugs," may decrease the rate at which osteoclasts reabsorb bone. The other category of drugs or the so-called "bone forming drugs" may promote bone formation. Recently, only antiresorbers are approved in the United States by the FDA for treating osteoporosis, and none of the drugs in this group has proven to be effctive yet.

Bisphosphonates are medications that slow the breakdown and removal of bone (i.e., resorption). This kind of drugs is widely used for the prevention and treatment of osteoporosis in postmenopausal women. It is also recommended for men or postmenopausal women with severe hip or spine osteoporosis. Zoledronic acid or raloxifene may be suggested for patients who cannot tolerate oral bisphosphonates, or who have difficulty in taking the medication, including an inability to sit upright for 30 to 60 minutes. Parathyroid hormone is another medication that can be used to treat osteoporosis. Oestrogen therapy for the development and maintenance of bone health has been well documented. The positive role of oestrogen replacement in prevention and treatment of osteoporosis among postmenopausal women is widely accepted, while oestrogen use among premenopausal females is not yet established as an effective treatment. Many researchers have identified increases in BMD and slightly increases the risk of breast cancer, stroke, and blood clots in premenopausal amenorrheic females treated with hormone replacement [66, 67]. Due to the incidence of breast cancer, strokes, blood clots, and heart attacks may increase in the women who take estrogen; the FDA recommends that women should take the lowest effective dose for the shortest period possible. Estrogen should be considered only in the situation that the patient is at a significant risk for osteoporosis, while patients who do not have any estrogen should be considered to take osteoporosis medications as the first choice.

6. Conclusions

In conclusion, the combined use of typical antipsychotics including atypical risperidone and amisulpride increases the incidence of hyperprolactinemia in patients receiving treatment, which in turn causes a reduction in BMD but an increased risk of fracture. Evidence suggests that this mechanism of action results through a "direct pathway" in osteoblast cells independently and more prominently through an "indirect pathway" via hypothalamic-pituitary-gonadal axis [68]. Prolactin screening programs in patients receiving long-term treatment (>6 months) with these types of antipsychotics may be necessary, even in the absence of clinical symptoms relating to hyperprolactinemia, in order to identify those with the highest risk of developing medication-induced osteopenia and osteoporosis. Further controlled studies and adequate guidance are essential to increase awareness and understanding of the impact of antipsychotic-induced hyperprolactinaemia on physical health in schizophrenia.

References

[1] J. H. Renn, N. P. Yang, C. M. Chueh, C. Y. Lin, T. H. Lan, and P. Chou, "Bone mass in schizophrenia and normal populations across different decades of life," *BMC Musculoskeletal Disorders*, vol. 10, article 1, 2009.

[2] J. Tiihonen, J. Lönnqvist, K. Wahlbeck et al., "11-year follow-up of mortality in patients with schizophrenia: a population-based cohort study (FIN11 study)," *The Lancet*, vol. 374, no. 9690, pp. 620–627, 2009.

[3] S. Kisely, N. Preston, J. Xiao et al., "Reducing all-cause mortality among patients with psychiatric disorders: a population-based study," *Canadian Medical Association Journal*, vol. 185, no. 1, pp. 50–56, 2013.

[4] D. P. J. Osborn, G. Levy, I. Nazareth, I. Petersen, A. Islam, and M. B. King, "Relative risk of cardiovascular and cancer mortality in people with severe mental illness from the United Kingdom's General Practice Research Database," *Archives of General Psychiatry*, vol. 64, no. 2, pp. 242–249, 2007.

[5] J. Y. Reginster and N. Burlet, "Osteoporosis: a still increasing prevalence," *Bone*, vol. 38, no. 2, supplement 1, pp. S4–S9, 2006.

[6] L. Nshimyumukiza, A. Durand, M. Gagnon et al., "An economic evaluation: simulation of the cost/effectiveness and cost/utility of universal prevention strategies against osteoporosis-related fractures," *Journal of Bone and Mineral Research*, vol. 28, no. 2, pp. 383–394, 2013.

[7] S. R. Cummings and L. J. Melton, "Osteoporosis I: epidemiology and outcomes of osteoporotic fractures," *Lancet*, vol. 359, no. 9319, pp. 1761–1767, 2002.

[8] T. Higuchi, T. Komoda, M. Sugishita et al., "Certain neuroleptics reduce bone mineralization in schizophrenic patients," *Neuropsychobiology*, vol. 18, no. 4, pp. 185–188, 1987.

[9] N. J. Delva, J. L. Crammer, S. V. Jarzylo et al., "Osteopenia, pathological fractures, and increased urinary calcium excretion in schizophrenic patients with polydipsia," *Biological Psychiatry*, vol. 26, no. 8, pp. 781–793, 1989.

[10] G. Abraham, R. H. Friedman, C. Verghese, and J. de Leon, "Osteoporosis and schizophrenia: can we limit known risk factors?" *Biological Psychiatry*, vol. 38, no. 2, pp. 131–132, 1995.

[11] G. Abraham, U. Halbreich, R. H. Friedman, and R. C. Josiassen, "Bone mineral density and prolactin associations in patients with chronic schizophrenia," *Schizophrenia Research*, vol. 59, no. 1, pp. 17–18, 2003.

[12] S. Pouwels, T. P. Van Staa, A. C. G. Egberts, H. G. M. Leufkens, C. Cooper, and F. de Vries, "Antipsychotic use and the risk of hip/femur fracture: a population-based case-control study," *Osteoporosis International*, vol. 20, no. 9, pp. 1499–1506, 2009.

[13] G. W. K. Hugenholtz, E. R. Heerdink, T. P. Van Staa, W. A. Nolen, and A. C. G. Egberts, "Risk of hip/femur fractures in patients using antipsychotics," *Bone*, vol. 37, no. 6, pp. 864–870, 2005.

[14] V. O'Keane and A. M. Meaney, "Antipsychotic drugs: a new risk factor for osteoporosis in young women with schizophrenia?" *Journal of Clinical Psychopharmacology*, vol. 25, no. 1, pp. 26–31, 2005.

[15] T. Kishimoto, M. de Hert, H. E. Carlson, P. Manu, and C. U. Correll, "Osteoporosis and fracture risk in people with schizophrenia," *Current Opinion in Psychiatry*, vol. 25, no. 5, pp. 415–429, 2012.

[16] L. Howard, G. Kirkwood, and M. Leese, "Risk of hip fracture in patients with a history of schizophrenia," *British Journal of Psychiatry*, vol. 190, pp. 129–134, 2007.

[17] D. U. Jung, D. L. Kelly, M. K. Oh et al., "Bone mineral density and osteoporosis risk in older patients with schizophrenia," *Journal of Clinical Psychopharmacology*, vol. 31, no. 4, pp. 406–410, 2011.

[18] P. Rey-Sanchez, J. M. Lavado-García, M. L. Canal-Macías, M. A. Gómez-Zubeldia, R. Roncero-Martín, and J. D. Pedrera-Zamorano, "Ultrasound bone mass in schizophrenic patients on antipsychotic therapy," *Human Psychopharmacology*, vol. 24, no. 1, pp. 49–54, 2009.

[19] K. M. Abel, H. F. Heatlie, L. M. Howard, and R. T. Webb, "Sex- and age-specific incidence of fractures in mental illness: a historical, population-based cohort study," *Journal of Clinical Psychiatry*, vol. 69, no. 9, pp. 1398–1403, 2008.

[20] P. Vestergaard, L. Rejnmark, and L. Mosekilde, "Anxiolytics and sedatives and risk of fractures: effects of half-life," *Calcified Tissue International*, vol. 82, no. 1, pp. 34–43, 2008.

[21] C. V. Tyler Jr., C. W. Snyder, and S. Zyzanski, "Screening for osteoporosis in community-dwelling adults with mental retardation," *Mental Retardation*, vol. 38, no. 4, pp. 316–321, 2000.

[22] D. U. Jung, R. R. Conley, D. L. Kelly et al., "Prevalence of bone mineral density loss in Korean patients with schizophrenia: a cross-sectional study," *Journal of Clinical Psychiatry*, vol. 67, no. 9, pp. 1391–1396, 2006.

[23] M. Hummer, P. Malik, R. W. Gasser et al., "Osteoporosis in patients with schizophrenia," *American Journal of Psychiatry*, vol. 162, no. 1, pp. 162–167, 2005.

[24] U. Halbreich, N. Rojansky, S. Palter et al., "Decreased bone mineral density in medicated psychiatric patients," *Psychosomatic Medicine*, vol. 57, no. 5, pp. 485–491, 1995.

[25] H. Hafner, W. an der Heiden, S. Behrens et al., "Causes and consequences of the gender difference in age at onset of schizophrenia," *Schizophrenia Bulletin*, vol. 24, no. 1, pp. 99–113, 1998.

[26] M. Hummer and J. Huber, "Hyperprolactinaemia and antipsychotic therapy in schizophrenia," *Current Medical Research and Opinion*, vol. 20, no. 2, pp. 189–197, 2004.

[27] S. L. Hui, C. W. Slemenda, and C. C. Johnston, "Age and bone mass as predictors of fracture in a prospective study," *Journal of Clinical Investigation*, vol. 81, no. 6, pp. 1804–1809, 1988.

[28] J. A. Cauley, "Defining ethnic and racial differences in osteoporosis and fragility fractures," *Clinical Orthopaedics and Related Research*, vol. 469, no. 7, pp. 1891–1899, 2011.

[29] J. K. Rybakowski, M. Dmitrzak-Weglarz, P. Kapelski, and J. Hauser, "Functional-1149 g/t polymorphism of the prolactin gene in schizophrenia," *Neuropsychobiology*, vol. 65, no. 1, pp. 41–44, 2012.

[30] J. C. Shim, D. U. Jung, S. S. Jung et al., "Adjunctive varenicline treatment with antipsychotic medications for cognitive impairments in people with schizophrenia: a randomized double-blind placebo-controlled trial," *Neuropsychopharmacology*, vol. 37, no. 3, pp. 660–668, 2012.

[31] H. Liu, N. M. Paige, C. L. Goldzweig et al., "Screening for osteoporosis in men: a systematic review for an american college of physicians guideline," *Annals of Internal Medicine*, vol. 148, no. 9, pp. 685–701, 2008.

[32] F. Dickerson, C. R. Stallings, A. E. Origoni et al., "Cigarette smoking among persons with schizophrenia or bipolar disorder in routine clinical settings, 1999–2011," *Psychiatric Services*, vol. 64, no. 1, pp. 44–50, 2013.

[33] H. W. Daniell, "Osteoporosis of the slender smoker. Vertebral compression fractures and loss of metacarpal cortex in relation to postmenopausal cigarette smoking and lack of obesity," *Archives of Internal Medicine*, vol. 136, no. 3, pp. 298–304, 1976.

[34] M. R. Law and A. K. Hackshaw, "A meta-analysis of cigarette smoking, bone mineral density and risk of hip fracture: recognition of a major effect," *British Medical Journal*, vol. 315, no. 7112, pp. 841–846, 1997.

[35] M. Pekkinen, H. Viljakainen, E. Saarnio, C. Lamberg-Allardt, and O. Mäkitie, "Vitamin D is a major determinant of bone mineral density at school age," *PLoS One*, vol. 7, no. 7, Article ID e40090, 2012.

[36] M. E. Molitch, "Medication-induced hyperprolactinemia," *Mayo Clinic Proceedings*, vol. 80, no. 8, pp. 1050–1057, 2005.

[37] C. Bushe, M. Shaw, and R. C. Peveler, "A review of the association between antipsychotic use and hyperprolactinaemia.," *Journal of Psychopharmacology*, vol. 22, supplement 2, pp. 46–55, 2008.

[38] M. M. Carvalho and C. Gois, "Hyperprolactinemia in mentally ill patients," *Acta Médica Portuguesa*, vol. 24, no. 6, pp. 1005–1012, 2011.

[39] P. A. Marken, R. F. Haykal, and J. N. Fisher, "Management of psychotropic-induced hyperprolactinemia," *Clinical Pharmacy*, vol. 11, no. 10, pp. 851–856, 1992.

[40] J. M. Jerrell, J. Bacon, J. T. Burgis, and S. Menon, "Hyperprolactinemia-related adverse events associated with antipsychotic treatment in children and adolescents," *Journal of Adolescent Health*, vol. 45, no. 1, pp. 70–76, 2009.

[41] Y. Roke, P. N. Van Harten, A. M. Boot, and J. K. Buitelaar, "Antipsychotic medication in children and adolescents: a descriptive review of the effects on prolactin level and associated side effects," *Journal of Child and Adolescent Psychopharmacology*, vol. 19, no. 4, pp. 403–414, 2009.

[42] J. Montgomery, E. Winterbottom, M. Jessani et al., "Prevalence of hyperprolactinemia in schizophrenia: association with typical and atypical antipsychotic treatment," *Journal of Clinical Psychiatry*, vol. 65, no. 11, pp. 1491–1498, 2004.

[43] I. R. Falconer, J. V. Langley, and A. T. Vacek, "Effect of prolactin on 86Rb+ uptake, potassium content and [G-3H]ouabain binding of lactating rabbit mammary tissue," *Journal of Physiology*, vol. 334, pp. 1–17, 1983.

[44] M. Jaber, S. W. Robinson, C. Missale, and M. G. Caron, "Dopamine receptors and brain function," *Neuropharmacology*, vol. 35, no. 11, pp. 1503–1519, 1996.

[45] P. M. Haddad and A. Wieck, "Antipsychotic-induced hyperprolactinaemia: mechanisms, clinical features and management," *Drugs*, vol. 64, no. 20, pp. 2291–2314, 2004.

[46] R. A. Bressan, K. Erlandsson, E. P. Spencer, P. J. Ell, and L. S. Pilowsky, "Prolactinemia is uncoupled from central D2/D3 dopamine receptor occupancy in amisulpride treated patients," *Psychopharmacology*, vol. 175, no. 3, pp. 367–373, 2004.

[47] M. Markianos, J. Hatzimanolis, and L. Lykouras, "Neuroendocrine responsivities of the pituitary dopamine system in male schizophrenic patients during treatment with clozapine, olanzapine, risperidone, sulpiride, or haloperidol," *European Archives of Psychiatry and Clinical Neuroscience*, vol. 251, no. 3, pp. 141–146, 2001.

[48] R. Arakawa, M. Okumura, H. Ito et al., "Positron emission tomography measurement of dopamine D2 receptor occupancy in the pituitary and cerebral cortex: relation to antipsychotic-induced hyperprolactinemia," *Journal of Clinical Psychiatry*, vol. 71, no. 9, pp. 1131–1137, 2010.

[49] T. Baptista, A. Lacruz, T. Meza et al., "Antipsychotic drugs and obesity: is prolactin involved?" *Canadian Journal of Psychiatry*, vol. 46, no. 9, pp. 829–834, 2001.

[50] D. Seriwatanachai, K. Thongchote, N. Charoenphandhu et al., "Prolactin directly enhances bone turnover by raising osteoblast-expressed receptor activator of nuclear factor κB ligand/osteoprotegerin ratio," *Bone*, vol. 42, no. 3, pp. 535–546, 2008.

[51] K. J. Motyl, I. Dick-de-Paula, A. E. Maloney et al., "Trabecular bone loss after administration of the second-generation antipsychotic risperidone is independent of weight gain," *Bone*, vol. 50, no. 2, pp. 490–498, 2012.

[52] S. Puntheeranurak, N. Charoenphandhu, and N. Krishnamra, "Enhanced trabecular-bone calcium deposition in female rats with a high physiological dose of prolactin diminishes after ovariectomy," *Canadian Journal of Physiology and Pharmacology*, vol. 84, no. 10, pp. 993–1001, 2006.

[53] P. Clément-Lacroix, C. Ormandy, L. Lepescheux et al., "Osteoblasts are a new target for prolactin: analysis of bone formation in prolactin receptor knockout mice," *Endocrinology*, vol. 140, no. 1, pp. 96–105, 1999.

[54] T. J. Martin and M. T. Gillespie, "Receptor activator of nuclear factor κB ligand (RANKL): another link between breast and bone," *Trends in Endocrinology and Metabolism*, vol. 12, no. 1, pp. 2–4, 2001.

[55] S. M. Graham, D. Howgate, W. Anderson et al., "Risk of osteoporosis and fracture incidence in patients on antipsychotic medication," *Expert Opinion on Drug Safety*, vol. 10, no. 4, pp. 575–602, 2011.

[56] A. M. Meaney and V. O'Keane, "Prolactin and schizophrenia: clinical consequences of hyperprolactinaemia," *Life Sciences*, vol. 71, no. 9, pp. 979–992, 2002.

[57] K. Bácsi, J. P. Kósa, G. Borgulya et al., "CYP3A7∗1C polymorphism, serum dehydroepiandrosterone sulfate level, and bone mineral density in postmenopausal women," *Calcified Tissue International*, vol. 80, no. 3, pp. 154–159, 2007.

[58] S. Sherman, "Preventing and treating osteoporosis: strategies at the millennium," *Annals of the New York Academy of Sciences*, vol. 949, pp. 188–197, 2001.

[59] N. B. Watts, "Therapies to improve bone mineral density and reduce the risk of fracture: clinical trial results," *Journal of Reproductive Medicine for the Obstetrician and Gynecologist*, vol. 47, supplement 1, pp. 82–92, 2002.

[60] M. A. Rodriguez-Martinez and E. C. Garcia-Cohen, "Role of Ca(2+) and vitamin D in the prevention and treatment of osteoporosis," *Pharmacology & Therapeutics*, vol. 93, no. 1, pp. 37–49, 2002.

[61] S. R. Marder, S. M. Essock, A. L. Miller et al., "Physical health monitoring of patients with schizophrenia," *American Journal of Psychiatry*, vol. 161, no. 8, pp. 1334–1349, 2004.

[62] R. C. Peveler, D. Branford, L. Citrome et al., "Antipsychotics and hyperprolactinaemia: clinical recommendations," *Journal of Psychopharmacology*, vol. 22, supplement 2, pp. 98–103, 2008.

[63] M. J. Stampfer, G. A. Colditz, and W. C. Willett, "Menopause and heart disease: a review," *Annals of the New York Academy of Sciences*, vol. 592, pp. 193–203, 1990.

[64] J. C. Shim, J. G. K. Shin, D. L. Kelly et al., "Adjunctive treatment with a dopamine partial agonist, aripiprazole, for antipsychotic-induced hyperprolactinemia: a placebo-controlled trial," *American Journal of Psychiatry*, vol. 164, no. 9, pp. 1404–1410, 2007.

[65] A. Mir, K. Shivakumar, R. J. Williamson, V. McAllister, V. O'Keane, and K. J. Aitchison, "Change in sexual dysfunction with aripiprazole: a switching or add-on study," *Journal of Psychopharmacology*, vol. 22, no. 3, pp. 244–253, 2008.

[66] A. C. Hergenroeder, "Bone mineralization, hypothalamic amenorrhea, and sex steroid therapy in female adolescents and young adults," *Journal of Pediatrics*, vol. 126, no. 5, part 1, pp. 683–689, 1995.

[67] B. Gulekli, M. C. Davies, and H. S. Jacobs, "Effect of treatment on established osteoporosis in young women with amenorrhoea," *Clinical Endocrinology*, vol. 41, no. 3, pp. 275–281, 1994.

[68] D. Seriwatanachai, N. Krishnamra, and J. P. T. M. Van Leeuwen, "Evidence for direct effects of prolactin on human osteoblasts: inhibition of cell growth and mineralization," *Journal of Cellular Biochemistry*, vol. 107, no. 4, pp. 677–685, 2009.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 216107, 13 pages
http://dx.doi.org/10.1155/2013/216107

Research Article

Effects of Growth Hormone Replacement Therapy on Bone Mineral Density in Growth Hormone Deficient Adults: A Meta-Analysis

Peng Xue,[1] Yan Wang,[1] Jie Yang,[2] and Yukun Li[1]

[1] *Department of Endocrinology, The Third Hospital of Hebei Medical University, 139 Ziqiang Road, Shijiazhuang, Hebei 050000, China*
[2] *Department of Epidemiology and Biostatistics, School of Public Health, Hebei Medical University, 361 East Zhongshan Road, Shijiazhuang, Hebei 050000, China*

Correspondence should be addressed to Yukun Li; liyukun@medmail.com.cn

Received 30 December 2012; Revised 8 March 2013; Accepted 13 March 2013

Academic Editor: Ling-Qing Yuan

Objectives. Growth hormone deficiency patients exhibited reduced bone mineral density compared with healthy controls, but previous researches demonstrated uncertainty about the effect of growth hormone replacement therapy on bone in growth hormone deficient adults. The aim of this study was to determine whether the growth hormone replacement therapy could elevate bone mineral density in growth hormone deficient adults. *Methods*. In this meta-analysis, searches of Medline, Embase, and The Cochrane Library were undertaken to identify studies in humans of the association between growth hormone treatment and bone mineral density in growth hormone deficient adults. Random effects model was used for this meta-analysis. *Results*. A total of 20 studies (including one outlier study) with 936 subjects were included in our research. We detected significant overall association of growth hormone treatment with increased bone mineral density of spine, femoral neck, and total body, but some results of subgroup analyses were not consistent with the overall analyses. *Conclusions*. Our meta-analysis suggested that growth hormone replacement therapy could have beneficial influence on bone mineral density in growth hormone deficient adults, but, in some subject populations, the influence was not evident.

1. Introduction

The major role of growth hormone (GH) during childhood is to promote bone growth and linear growth, but GH continues to have important metabolic actions throughout life. Besides growth, GH is known to affect body composition, bone mineralization, and lipid and glucose metabolism [1]. For instance, GH can accelerate bone turnover, which is supported by several lines of evidence. In vitro studies show that GH and its major effector, insulin-like growth factor-1 (IGF-1), are both mitogens for osteoblasts [2, 3].

The condition of GH deficiency (GHD) has been accepted as a definite syndrome, and the clinical and biochemical abnormalities in GHD patients are also well known. They involve mainly the cardiovascular system, lipid metabolism, body composition, mineral metabolism, and quality of life [4, 5]. For example, adult patients with childhood-onset or adult-onset GHD exhibit reduced bone mineral density (BMD) compared with healthy controls [6, 7]. Moreover, clinical studies have shown that the prevalence of fractures is 2.7–3 times higher in GHD patients than in age-matched controls. Data from these studies suggest that the increased risk may be due to GHD rather than other pituitary hormone deficiencies [8, 9].

Many studies have demonstrated that the abnormalities of GHD patients may be reversed by GH replacement therapy, but the evidence is not all conclusive. In particular, the effect of GH treatment on BMD is less clear, though it is well established that GH promotes longitudinal bone growth. Some studies suggest an improvement in BMD [10], some show no effect [11], and others suggest a decrease in BMD related to GH treatment [12]. Moreover, the association

between GH treatment and BMD may be influenced by other factors such as gender, treatment time, GH dosage, or geographic location.

We, therefore, undertook a meta-analysis on the effects of GH replacement therapy on BMD based on available studies.

2. Methods

2.1. Search Strategy and Inclusion Criteria. We systematically searched Medline, Embase, and Cochrane Library for studies written in English (from their commencements to December 2012). The search used the following terms: "growth hormone," "GH," "somatotropin," "bone," "bone mineral density" and "BMD." The following three sites of BMD were included in this meta-analysis: spine, femoral neck (FN), and total body (TB).

Studies in humans of the effects of GH treatment on BMD, regardless of sample size, were included if they met the following criteria: (1) data were reported on at least one of the three sites (spine, FN, and TB) of BMD; (2) BMD was measured by dual-energy X-ray absorptiometry (DXA); (3) we only included studies in which mean BMD and standard deviations (SDs), or standard errors (SEs) were available; (4) adult subjects (>16 years old); (5) subjects were patients with GHD who received GH treatment. The excluded studies included reviews, editorials, comments, letters, and abstracts.

2.2. Data Extraction. Two investigators independently reviewed the articles and selected eligible studies according to the inclusion criteria for eligible studies. Irrelevant studies were excluded. For studies with the same population resources or overlapping datasets, the most complete one was included. Study details and data were extracted independently and to a standardized electronic form by two investigators, and discrepancies were adjudicated by a third reviewer until consensus was achieved on every item. The following information was extracted from each study: last name of first author, year of publication, country, subject population, mean, BMD and SDs (or SEs) of subjects at baseline and after GH treatment.

2.3. Statistical Analysis. For this meta-analysis, all data should be given as mean and SDs. In those studies, where values of SEs were originally reported, the values of SDs were calculated. When information was reported for more than one subpopulation in one study, each subpopulation was treated as a separate comparison in our meta-analysis. BMDs in the three sites (spine, FN, and TB) were continuous outcomes presented on different scales (g/cm^2, z-scores or t-scores), so we used a pooled standardized mean difference (SMD) with 95% confidence intervals (CI) calculated using the final follow-up P values to analyze the effects of GH treatment on BMD. All data were initially analyzed with a fixed effects model. If heterogeneity was found, the analysis should be redone using a random effects model. A P value of 0.05 was considered statistically significant.

Heterogeneity of the effect across studies was assessed by Q statistics, which is distributed as χ^2 statistics. I^2 statistics were provided to quantify the percentage of total variation across studies that was attributable to heterogeneity rather than to chance. An I^2 value >50% represented substantial variability, and heterogeneity was considered to be significant at $P < 0.10$, a conservative standard for meta-analyses. In the presence of heterogeneity, sensitivity analyses were performed to identify the outlier studies. The influence of outliers was also assessed to evaluate the impact of their removal. Moreover, there might be effective modificationcaused by study-level characteristics including treatment time, GH dosage, manufacturer of DXA scanner, and geographic location. Thus, subgroup analyses were further conducted to detect the source of heterogeneity. Treatment time subgroups were defined as ≤2 years or >2 years. GH dosage subgroups were defined as fixed dosage and dosage depending on serum IGF-1 values. Manufacturer of DXA scanner subgroups was defined as GE-Lunar or Hologic Inc, since the studies using other manufacturers of DXA scanner (Norland) are too few. Geographic location subgroups were defined as Europe, America, or Oceania.

We performed a visual inspection of the funnel plot for publication bias. The funnel plot should be asymmetric when there is publication bias and symmetric in the case of no publication bias. We performed Egger and Begg tests to measure the funnel plot asymmetry using a significance level of $P < 0.05$.

All statistical analyses were performed by using STATA 11.0 (Stata Corporation, College Station, TX, USA). The results of our research were reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

3. Results

3.1. Studies Included in the Meta-Analysis. Our literature search produced 657 citations written in English, of which we selected 69 for further review of the full text. A total of 49 studies were excluded for unavailable or incomplete data. Finally, 20 unique studies were available for this meta-analysis [10, 11, 13–30]. Of these, 18 studies (included 20 comparisons), 16 studies (included 18 comparisons), and 11 studies (included 12 comparisons) presented data on BMD of spine, FN, and TB, respectively. Tables 1 and 2 summarized the characteristics and the data of the included studies.

In all eligible studies, there were 3 studies separately providing the information on more than one subpopulation. Each subpopulation was treated as a separate comparison. A total of 936 subjects were included in this meta-analysis.

3.2. Association between GH Treatment and BMD of Spine. We initially performedthe meta-analysis on all 18 studies (including 20 comparisons) with a fixed effects model. For the presence of significant heterogeneity ($I^2 = 82.9\%$), the analysis wasredone using a random effects model. The results suggested significant association between GH treatment and increased BMD of spine (SMD = 0.540, 95% CI [0.272, 0.808], $P < 0.001$; $I^2 = 82.9\%$, $P < 0.001$ for Q test).

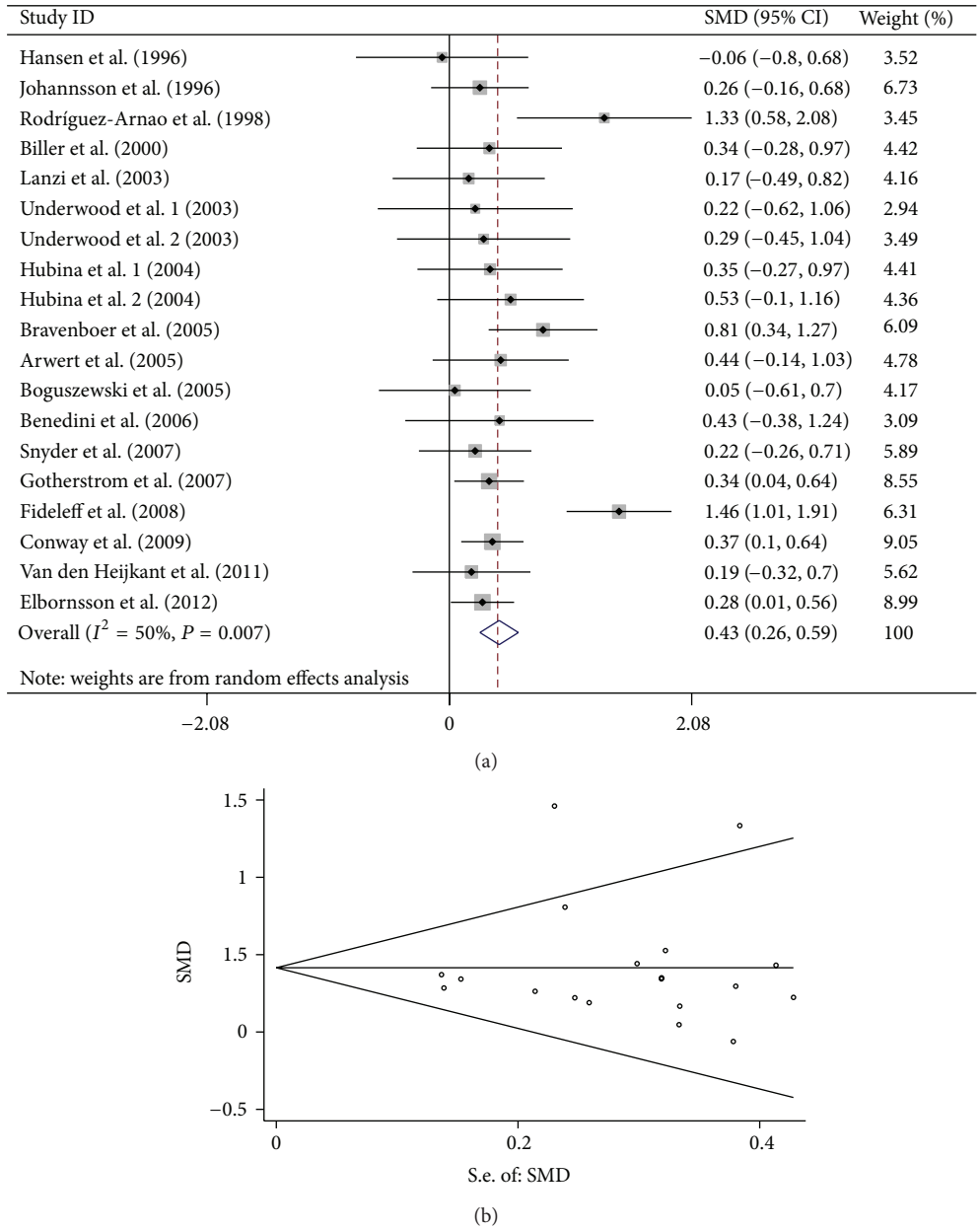


Figure 1: Forest plot and funnel plot for the association between GH treatment and BMD of spine. (a) Forest plot using a random effects model. (b) Funnel plot using Begg methods.

Sensitivity analyses showed that there was an outlier study (study ID: Rota et al.). When the outlier study was omitted, 17 studies (including 19 comparisons) were included in the meta-analysis. The heterogeneity was decreased and the results also suggested significant association between GH treatment and increased BMD of spine (SMD = 0.429, 95% CI [0.263, 0.594], $P < 0.001$; $I^2 = 50.0\%$, $P = 0.007$ for Q test) (Figure 1(a)).

To further detect the source of heterogeneity, we performed subgroup analyses stratified by the characteristics (treatment time, GH dosage, manufacturer of DXA scanner, and geographic location) of the subjects. The results did not suggest significant association between GH treatment and BMD of spine in American subjects (SMD = 0.461, 95% CI [−0.049, 0.971], $P = 0.076$; $I^2 = 76.3\%$, $P = 0.001$ for Q test). But a significant association between GH treatment and increased BMD of spine in the other subgroups was found. Significant heterogeneity was removed or decreased in some subgroups. Table 3 summarizes the subgroup analyses results.

3.3. Association between GH Treatment and BMD of FN. Similarly, we performed the meta-analysis on all 16 studies (including 18 comparisons) with arandom effects model. The results suggested significant association between GH treatment and increased BMD of FN (SMD = 0.476, 95% CI [0.190, 0.761], $P = 0.001$; $I^2 = 83.0\%$, $P < 0.001$ for Q test).

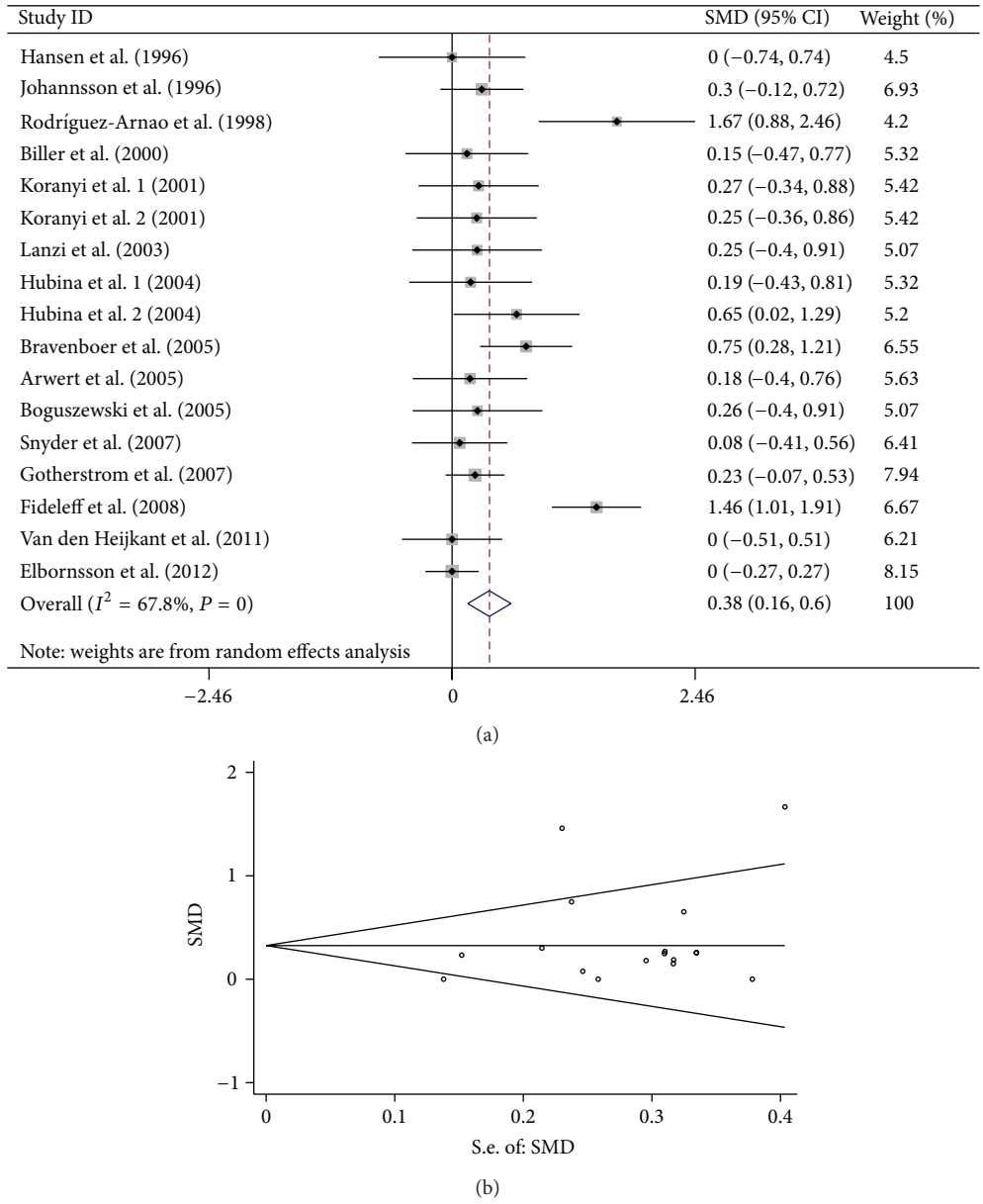


Figure 2: Forest plot and funnel plot for the association between GH treatment and BMD of FN. (a) Forest plot using a random effects model. (b) Funnel plot using Begg methods.

Sensitivity analyses showed that there was also an outlier study (study ID: Rota et al.). When the outlier study was omitted, 15 studies (including 17 comparisons) were included in the meta-analysis. The heterogeneity was decreased and the results also suggested significant association between GH treatment and increased BMD of FN (SMD = 0.377, 95% CI [0.158, 0.595], P = 0.001; I^2 = 67.8%, P < 0.001 for Q test) (Figure 2(a)).

We also performed subgroup analyses to further detect the source of heterogeneity. The results did not suggest significant association between GH treatment and BMD of FN in subjects treated by GH for ≤2 years (SMD = 0.289, 95% CI [−0.009, 0.587], P = 0.057; I^2 = 51.2%, P = 0.045 for Q test) and American subjects (SMD = 0.501, 95% CI [−0.227, 1.229], P = 0.177; I^2 = 86.1%, P < 0.001 for Q test). But significant association between GH treatment and increased BMD of FN in the other subgroups were found. Moreover, the significant heterogeneity was removed or decreased in some subgroups. Table 4 summarizes the subgroup analyses results.

3.4. Association between GH Treatment and BMD of TB. Analogously, we performed the meta-analysis on all 11 studies (including 12 comparisons) with a random effects model. The results suggested significant association between GH treatment and increased BMD of TB (SMD = 0.242, 95% CI [0.019, 0.466], P = 0.034; I^2 = 69.6%, P < 0.001 for Q test) (Figure 3(a)).

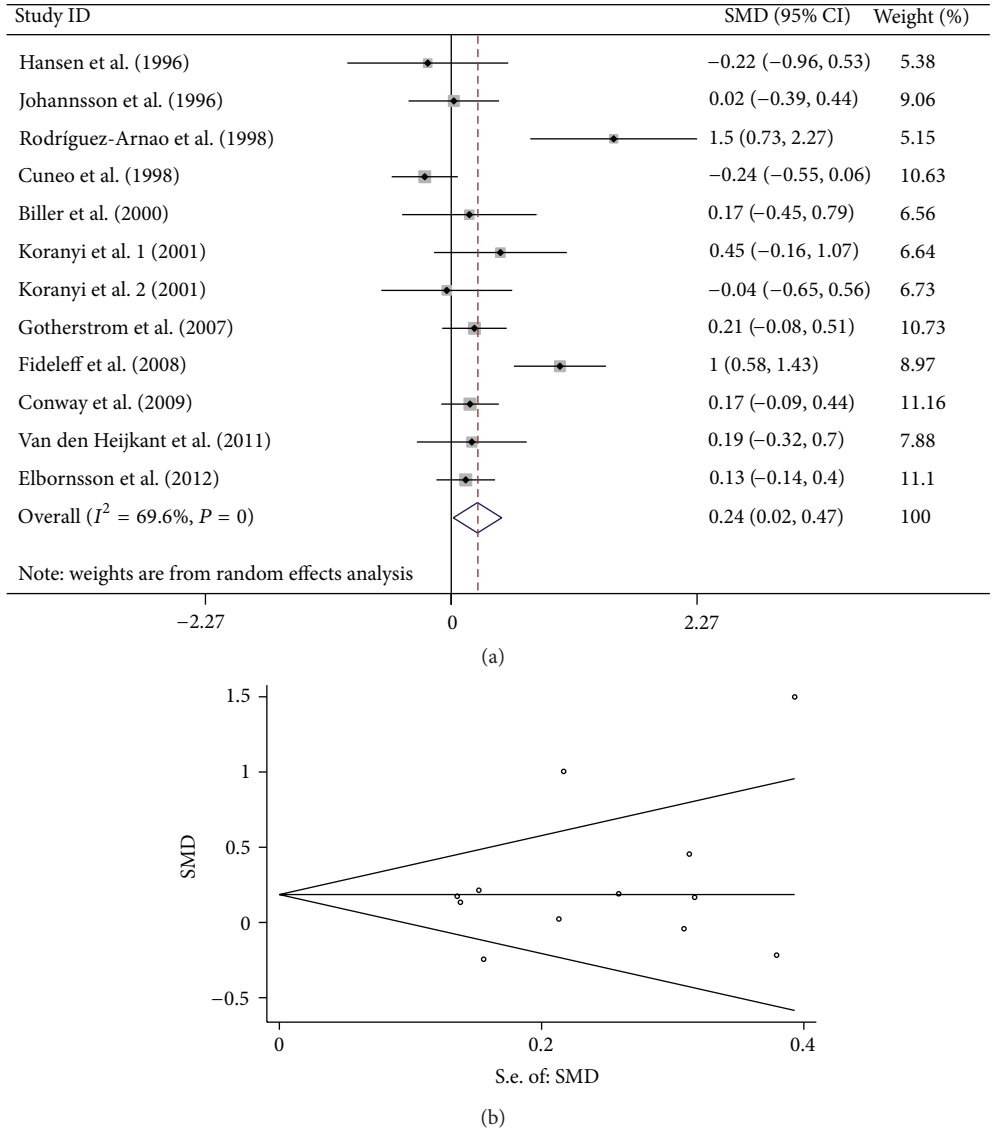


Figure 3: Forest plot and funnel plot for the association between GH treatment and BMD of TB. (a) Forest plot using a random effects model. (b) Funnel plot using Begg methods.

Sensitivity analyses showed that there was no outlier study.

We also performed subgroup analyses to further detect the source of heterogeneity. The results did not suggest significant association between GH treatment and BMD of TB in subjects with treatment time ≤2 years (SMD = 0.159, 95% CI [−0.148, 0.466], P = 0.311; I^2 = 68.1%, P = 0.004 for Q test), subjects who received fixed GH dosage (SMD = 0.205, 95% CI [−0.406, 0.816], P = 0.512; I^2 = 82.7%, P = 0.001 for Q test), subjects whose BMD was measured by DXA scanner manufactured by Hologic Inc (SMD = 0.317, 95% CI [−0.101, 0.736], P = 0.137; I^2 = 66.8%, P = 0.017 for Q test), subjects whose BMD was measured by DXA scanner manufactured by GE-Lunar Inc (SMD = 0.207, 95% CI [−0.083, 0.497], P = 0.162; I^2 = 74.8%, P = 0.001 for Q test), European subjects (SMD = 0.224, 95% CI [−0.015, 0.463], P = 0.066; I^2 = 51.2%, P = 0.045 for Q test), American subjects (SMD = 0.618, 95% CI [−0.200, 1.435], P = 0.139; I^2 = 78.9%, P < 0.029 for Q test,) and Oceanian subjects (SMD = −0.028, 95% CI [−0.438, 0.381], P = 0.892; I^2 = 75.6%, P = 0.043 for Q test), but as significant association between GH treatment and increased BMD of TB in the other subgroups was found. Moreover, the significant heterogeneity was removed or decreased in some subgroups. Table 5 summarizes the subgroup analyses results.

3.5. Heterogeneity and Publication Bias. Significant heterogeneity was separately observed among the available studies on BMD of spine, FN, and TB. To detect the source of heterogeneity, we performed subgroup analyses stratified by the characteristics of the subjects. Significant heterogeneity was removed or decreased in some subgroups but still existed in other subgroups.

Table 1: Patient characteristics in included studies.

References	Countries	Study subjects	Criteria of GHD	Treatment time	GH usage
Hansen et al., 1996 [13]	Denmark	9 males and 5 females aged 31–57	<10 mU/L in ITT[a]	1 yr	2.0 IU/m^2·day
Johannsson et al., 1996 [14]	Sweden	24 males and 20 females aged 23–66	<5 mU/L in ITT	2 yr	First 4 weeks: 0.1 IU/kg·week Thereafter: 0.25 IU/kg·week
Rodríguez-Arnao et al., 1998 [15]	UK	18 males and 17 females aged 21.1–59.9	<10 mU/L in ITT	1 yr	First 4 weeks: 0.125 IU/kg·week Thereafter: 0.25 IU/kg·week
Cuneo et al., 1998 [16]	Australia	50 males and 33 females aged 41.2 ± 1.5	<5 mU/L in ITT	1 yr	First month: 0.125 IU/kg·week Thereafter: 0.25 IU/kg·week
Biller et al., 2000 [17]	USA	38 males aged 48.9 ± 2.0	NA[b]	1.5 yr	Initial dose: 10 μg/kg·day Then adjusted accordingly to the serum IGF-I values
Koranyi et al., 2001 [18]	Sweden	28 males and 14 females aged 17–61	NA	5 yr	Initial dose: 0.25 IU/kg·week Then adjusted accordingly to the serum IGF-I values
Lanzi et al., 2003 [19]	Italy	10 males and 8 females aged 17–50	<9 μg/L in ARG[c]	0.5 yr	Initial dose: 4 μg/kg·day Then adjusted accordingly to the serum IGF-I values
Underwood et al., 2003 [20]	USA	39 males and 25 females aged 23.8 ± 4.2	<5 μg/L in clonidine and L-dopa stimulation tests	2 yr	25 μg/kg·day or 12.5 μg/kg·day
Hubina et al., 2004 [21]	Hungary	11 males and 9 females aged 22–67	<3 μg/L in ITT or ARG	3 yr	1.2 IU/day (average dose)
Bravenboer et al., 2005 [22]	The Netherlands	38 males aged 20–35	<7 μg/L in ITT or GHRH[d]	5 yr	0.67 mg/m^2·day
Arwert et al., 2005 [23]	The Netherlands	23 males aged 20–40	<7 μg/L in ITT or GHRH	10 yr	First 0.5 yr: 1.2 or 3 IU/m^2·day 0.5–2 yr: 2 IU/m^2·day Then adjusted accordingly to the serum IGF-I values
Boguszewski et al., 2005 [24]	Brazil	7 males and 11 females aged 21–58	<3 μg/L in ITT	1 yr	0.6 IU/day
Benedini et al., 2006 [25]	Italy	6 males and 6 females aged 29–54	<9 μg/L in ARG and GHRH	1 yr	Initial dose: 0.25 mg/day (for men) or 0.4 mg/day (for women) Then adjusted accordingly to the serum IGF-I values
Snyder et al., 2007 [26]	USA	20 males and 13 females aged 29–54	<2.4 μg/L in ITT or ARG	2 yr	Initial dose: 2 μg/kg·day Then adjusted accordingly to the serum IGF-I values
Gotherstrom et al., 2007 [27]	Sweden	52 males and 35 females aged 22–74	<3 μg/L in ITT	10 yr	64 patients: the initial dose was 0.25 IU/kg·week and then individualized 23 patients: the dose was individualized from the start of the treatment
Rota et al., 2008 [28]	Italy	35 males and 29 females aged 30–50	<9 μg/L in ARG and GHRH	2 yr	Initial dose: 4 μg/kg·day Then adjusted accordingly to the serum IGF-I values
Fideleff et al., 2008 [10]	Argentina	22 males and 26 females aged 18–66	<0.14 pmol/L in ITT	4 yr	Initial dose: 0.1 mg/day Then adjusted accordingly to the serum IGF-I values
Conway et al., 2009 [29]	Australia	65 males and 44 females aged 21.1 ± 2.3	<3 μg/L in ITT	2 yr	Initial dose: 0.2 mg/day (for men) or 0.4 mg/day (for women) Then adjusted accordingly to the serum IGF-I values

Table 1: Continued.

References	Countries	Study subjects	Criteria of GHD	Treatment time	GH usage
van den Heijkant et al., 2011 [11]	The Netherlands	12 males and 8 females aged 23.9 ± 3.0	<3 μg/L in ITT	2 yr	Initial dose: 0.1 mg/m^2·day Then adjusted accordingly to the serum IGF-I values
Elbornsson et al., 2012 [30]	Sweden	72 males and 54 females aged 22–74	<3 μg/L in ITT or GHRH	15 yr	64 patients: the initial dose was 0.25 IU/kg·week and then individualized 62 patients: the dose was individualized from the start of the treatment

[a]Insulin tolerance test; [b]not available; [c]arginine test; [d]GH-releasing hormone.

For the 17 studies (with an outlier study excluded) focusing on BMD of spine, both Egger's regression ($P = 0.789$) and Begg methods ($P = 0.889$) did not show publication bias (Figure 1(b)). For the 15 studies (with an outlier study excluded) focusing on BMD of FN, both Egger's regression ($P = 0.285$) and Begg methods ($P = 0.303$) did not show publication bias (Figure 2(b)). For the 11 studies focusing on BMD of TB, both Egger's regression ($P = 0.309$) and Begg methods ($P = 0.631$) did not show publication bias (Figure 3(b)).

4. Discussion

In our meta-analysis, we detected an outlier study (study ID: Rota 2008) through sensitivity analyses when we performed the meta-analysis on the association of GH treatment and BMD of spine and FN. In the study mentioned above, patients aged below 30 years and above 50 years were excluded, which might make it an outlier study.

We detected significant overall association between GH treatment and increased BMD of spine, FN, and TB. GH could exert both direct and indirect effects on bone. (1) For direct effects on bone, there was increasing evidence that the GH-IGF axis played a vital role in determining BMD and maintaining bone health and that perturbations in this axis might predispose to the development of osteoporosis. Although GH could act on cells directly through specific receptors [31, 32], most of its anabolic actions were mediated through IGF-1 [33–35]. GH stimulated the secretion of IGF-1, largely from the liver, which then acted in an endocrine fashion. GH also stimulated IGF-1 locally in target tissues such as bone, where it might act in a paracrine or autocrine fashion [36, 37]. Thus, the effect of GH on bone was mediated, at least in part by IGF-1, and bone mass was known to be linked to circulating levels of IGF-1 [38]. In vitro studies had shown that GH-IGF-1 bound to preosteoblasts or mature osteoblasts to induce differentiation and proliferation while also regulating osteoclastic differentiation and activity providing a mechanism to couple bone resorption and formation [39, 40]. In addition, GH also increased biomarkers of bone turnover in normal subjects as well as adults and children with GHD [13, 41]. In almost all of the included studies in our meta-analysis, the serum IGF1 levels of adult GHD patients were significantly increased by the GH treatment, which were listed in Table 2. (2) For indirect effects on bone, It was known that GH had an anabolic effect on skeletal muscle, and it particularly seemed to increase muscle mass and isometric muscle strength when given in physiologically therapeutic doses to GHD patients. Klefter and Feldt-Rasmussen analyzed many trials measuring effects of GH on both muscle and bones [42], and then suggested that there could be a connection between increases in muscle mass and strength and changes in BMD in GHD patients treated with GH. This supported the present physiological concept that the mass and strength of bones were primarily determined by dynamic loads from the skeletal muscles [43, 44].

Significant heterogeneity was found in our meta-analysis. Several study-level variables leading to heterogeneity were defined by subgroup analyses including treatment time, GH dosage, manufacturer of DXA scanner and geographic location. Some results of subgroup analyses were not consistent with the overall analyses.

Firstly, we did not detect significant association between GH treatment and BMD of FN and TB in subjects with treatment time ≤2 yr. GH-IGF1 stimulated bone remodeling which occurred as a biphasic process, dominated initially by bone resorption and only later by bone formation. This biphasic sequence might also explain the initial decrease in BMD reported in several clinical trials [45, 46]. Thus, significant increases in BMD did not usually occur until 12–24 months of treatment, and clinical trials with duration of 24 months or less might not be expected to find significant increases in bone parameters.

Secondly, we did not detect significant association between GH treatment and BMD of TB in subjects received fixed GH dosage (weight- or surface-area-based dosing regimens). Early studies used weight- or surface-area-based dosing regimens that resulted in a higher GH dose than titrating GH dose to normalize the serum IGF-1 level in subsequent years [47]. And the use of dose titration means that it takes longer to establish the patient on a maintenance GH dose. Thus, known differences in the time until response of BMD to GH are recognized. In our meta-analysis, GH treatment time in the studies which determined the GH dosage depending on serum IGF-1 level is mostly longer than that isthe studies which used fixed GH dosage. So, the effect of the former dosing regimens on BMD might be more evident.

Thirdly, we did not detect significant association between GH treatment and BMD of TB in the subjects whose BMD

Table 2: BMD measurements and outcomes.

References	DXA scanner manufacturer	BMD sites	CV[a] of BMD measurements	Baseline BMD (mean ± SD)	Posttreatment BMD (mean ± SD)	BMD scales	Serum IGF1 changes
Hansen et al., 1996 [13]	Hologic Inc.	Spine FN TB	0.6% 1.4% 1.6%	0.960 ± 0.170 0.920 ± 0.150 1.100 ± 0.070	0.950 ± 0.150 0.920 ± 0.150 1.080 ± 0.110	g/cm^2	Increased 263 ± 98%
Johannsson et al., 1996 [14]	GE-Lunar	Spine FN TB	0.5% 1.6% 0.4%	1.170 ± 0.186 0.944 ± 0.133 1.164 ± 0.133	1.218 ± 0.179 0.986 ± 0.146 1.167 ± 0.119	g/cm^2	Increased from 77 ± 7 to 302 ± 15 μg/L
Rodríguez-Arnao et al., 1998 [15]	Hologic Inc.	Spine FN TB	1.0% 1.8% NA[b]	1.000 ± 0.030 0.790 ± 0.030 1.150 ± 0.020	1.040 ± 0.030 0.840 ± 0.030 1.180 ± 0.020	g/cm^2	Increased significantly with 31% of the total patients showing IGF1 levels above the age-corrected limit of normal
Cuneo et al., 1998 [16]	GE-Lunar	TB	<3.8%	1.149 ± 0.155	1.120 ± 0.092	g/cm^2	Increased from 100 to 280 ± 20 μg/L
Biller et al., 2000 [17]	Hologic Inc.	Spine FN TB	NA NA NA	0.700 ± 0.134 0.920 ± 0.165 1.189 ± 0.134	0.751 ± 0.161 0.948 ± 0.206 1.219 ± 0.215	g/cm^2	NA
Koranyi et al., 2001 (1) [18]	GE-Lunar	FN TB	NA NA	0.919 ± 0.206 1.111 ± 0.110	0.975 ± 0.211 1.161 ± 0.110	g/cm^2	Increased from 85.8 ± 15.9 to 295.1 ± 36.3 μg/L
Koranyi et al., 2001 (2) [18]	GE-Lunar	FN TB	NA NA	1.012 ± 0.142 1.214 ± 0.101	1.048 ± 0.147 1.210 ± 0.092	g/cm^2	Increased from 135.4 ± 15.5 to 283.3 ± 28.9 μg/L
Lanzi et al., 2003 [19]	Hologic Inc.	Spine FN	0.5% NA	−1.080 ± 0.180 −0.900 ± 0.370	−1.050 ± 0.180 −0.810 ± 0.340	t-score	Increased from 102.94 ± 16.93 to 226.17 ± 17.10 μg/L
Underwood et al., 2003 (1) [20]	GE-Lunar	Spine	NA	−1.340 ± 1.360	−1.050 ± 0.180	z-score	IGF1 SDS increased from −5.2 ± 2.6 to −0.6 ± 1.5
Underwood et al., 2003 (2) [20]	GE-Lunar	Spine	NA	−1.010 ± 1.410	−0.610 ± 1.300	z-score	IGF1 SDS increased from −3.8 ± 1.5 to 1.2 ± 1.5
Hubina et al., 2004 (1) [21]	Hologic Inc.	Spine FN	0.35% NA	−1.690 ± 1.480 −0.750 ± 2.147	−1.210 ± 1.252 −0.380 ± 1.789	t-score	IGF1 SDS increased from −2.53 ± 0.85 to 0.12 ± 0.41
Hubina et al., 2004 (2) [21]	Hologic Inc.	Spine FN	0.35% NA	−1.200 ± 2.147 −0.450 ± 0.894	−0.210 ± 1.565 0.210 ± 1.118	t-score	IGF1 SDS increased from −3.61 ± 0.96 to 0.74 ± 0.28
Bravenboer et al., 2005 [22]	Norland	Spine FN	2.4% 2.3%	0.920 ± 0.140 0.820 ± 0.150	1.050 ± 0.180 0.940 ± 0.170	g/cm^2	Increased from 9.9 ± 5.7 to 27.2 ± 11.8 nM

Table 2: Continued.

References	DXA scanner manufacturer	BMD sites	CV[a] of BMD measurements	Baseline BMD (mean ± SD)	Posttreatment BMD (mean ± SD)	BMD scales	Serum IGF1 changes
Arwert et al., 2005 [23]	Hologic Inc.	Spine FN	NA 2.1%	0.900 ± 0.150 0.780 ± 0.120	0.960 ± 0.120 0.800 ± 0.100	g/cm^2	Increased from 9.7 ± 2.1 to 26.6 ± 6.1 nM
Boguszewski et al., 2005 [24]	GE-Lunar	Spine FN	1.2% 1.5%	1.121 ± 0.210 0.903 ± 0.170	1.131 ± 0.210 0.948 ± 0.180	g/cm^2	Increased from 76.9 ± 70.4 to 133.7 ± 134.1 μg/L
Benedini et al., 2006 [25]	Hologic Inc.	Spine	NA	0.950 ± 0.130	1.000 ± 0.100	g/cm^2	Increased from 60 ± 29 to 151 ± 49 μg/L
Snyder et al., 2007 [26]	Hologic Inc.	Spine FN	0.37–0.51%	1.050 ± 0.130 0.820 ± 0.130	1.080 ± 0.140 0.830 ± 0.130	g/cm^2	IGF1 SDS increased from −1.65 ± 0.92 to 0.20 ± 1.40
Gotherstrom et al., 2007 [27]	GE-Lunar	Spine FN TB	0.5% 0.6% 0.4%	1.161 ± 0.205 0.939 ± 0.159 1.163 ± 0.140	1.243 ± 0.270 0.976 ± 0.159 1.194 ± 0.149	g/cm^2	Increased from 99.5 ± 6.6 to 223.3 ± 9.8 μg/L
Rota et al., 2008 [28]	Hologic Inc.	Spine FN	1.0% 1.5%	−1.700 ± 0.200 −0.700 ± 0.200	−1.300 ± 0.100 −0.400 ± 0.100	z-score	Increased 174.1 ± 31.2% in men and 301.7 ± 97.1% in women
Fideleff et al., 2008 [10]	GE-Lunar	Spine FN TB	NA NA NA	−1.300 ± 1.386 −1.200 ± 1.386 −1.000 ± 1.386	0.300 ± 0.693 0.400 ± 0.693 0.100 ± 0.693	z-score	IGF1 SDS increased from −4.54 ± 0.42 to 0.36 ± 0.25
Conway et al., 2009 [29]	Hologic Inc.	Spine TB	NA NA	0.910 ± 0.130 0.980 ± 0.110	0.960 ± 0.140 1.000 ± 0.120	g/cm^2	Increased from 132.9 ± 128.1 to 361.6 ± 259.5 μg/L
Van den Heijkant et al., 2011 [11]	Hologic Inc.	Spine FN TB	NA NA NA	0.960 ± 0.110 0.840 ± 0.130 1.010 ± 0.100	0.980 ± 0.100 0.840 ± 0.130 1.030 ± 0.110	g/cm^2	NA
Elbornsson et al., 2012 [30]	GE-Lunar	Spine FN TB	<1.5%	1.170 ± 0.224 0.940 ± 0.112 1.170 ± 0.112	1.230 ± 0.190 0.940 ± 0.190 1.190 ± 0.190	g/cm^2	Increased from 103 ± 6 to 183 ± 7 μg/L

[a]Coefficient of variation; [b]not available.

Table 3: Subgroup analyses results of the association between GH treatment and BMD of spine

Subgroups	Effects of GH treatment on BMD			Heterogeneity		
	SMD	95% CI	P	I^2 (%)	χ^2	P
Treatment time						
≤2 yr	0.311	0.159–0.463	0.000	0.0	9.62	0.565
>2 yr	0.597	0.275–0.919	0.000	73.6	22.69	0.001
GH dosage						
Fixed dosage	0.429	0.172–0.686	0.001	34.2	12.16	0.144
Dosage depending on serum IGF-1 values	0.429	0.203–0.655	0.000	62.2	23.79	0.005
Manufacturer of DXA scanner						
Hologic Inc	0.362	0.204–0.520	0.000	0.0	9.15	0.518
GE-lunar	0.440	0.097–0.783	0.012	74.7	23.72	0.001
Geographic location[a]						
Europe	0.385	0.232–0.537	0.000	13.9	12.78	0.308
America	0.461	−0.049–0.971	0.076	76.3	21.07	0.001

[a]There was only one study including Oceanian subjects.

Table 4: Subgroup analyses results of the association between GH treatment and BMD of FN.

Subgroups	Effects of GH treatment on BMD			Heterogeneity		
	SMD	95% CI	P	I^2 (%)	χ^2	P
Treatment time						
≤2 yr	0.289	−0.009–0.587	0.057	51.2	14.36	0.045
>2 yr	0.440	0.119–0.761	0.007	76.9	34.70	0.000
GH usage						
Fixed dosage	0.520	0.178–0.861	0.003	56.5	13.79	0.032
Dosage depending on serum IGF-1 values	0.289	0.007–0.571	0.045	72.3	32.51	0.000
Manufacturer of DXA scanner						
Hologic Inc	0.306	0.018–0.595	0.037	49.7	15.89	0.044
GE-lunar	0.392	0.026–0.758	0.036	80.2	30.34	0.000
Geographic location[a]						
Europe	0.313	0.117–0.508	0.002	48.0	23.08	0.027
America	0.501	−0.227–1.229	0.177	86.1	21.54	0.000

[a]There was no study including Oceanian subjects.

Table 5: Subgroup analyses results of the association between GH treatment and BMD of TB.

Subgroups	Effects of GH treatment on BMD			Heterogeneity		
	SMD	95% CI	P	I^2 (%)	χ^2	P
Treatment time						
≤2 yr	0.159	−0.148–0.466	0.311	68.1	18.81	0.004
>2 yr	0.352	0.015–0.688	0.041	70.9	13.76	0.008
GH usage						
Fixed dosage	0.205	−0.406–0.816	0.512	82.7	17.35	0.001
Dosage depending on serum IGF-1 values	0.283	0.076–0.491	0.007	52.0	14.59	0.042
Manufacturer of DXA scanner						
Hologic Inc	0.317	−0.101–0.736	0.137	66.8	12.06	0.017
GE-lunar	0.207	−0.083–0.497	0.162	74.8	23.77	0.001
Geographic location						
Europe	0.224	−0.015–0.463	0.066	51.2	14.34	0.045
America	0.618	−0.200–1.435	0.139	78.9	4.75	0.029
Oceania	−0.028	−0.438–0.381	0.892	75.6	4.10	0.043

was measured by DXA scanner manufactured by Hologic Inc or GE-lunar but got a significant overall association between GH treatment and increased BMD of TB. Absolute values of BMD, using DXA, might differ between instruments from different manufacturers. previous study performed a comparison of longitudinal measurements in the spine and proximal femur using lunar and Hologic instruments [48]. Despite the significant correlations, the agreement between the two densitometers was not high and there might be significant errors in individual subjects if one uses measurements from one densitometer to predict the change in BMD using the scanner of the other manufacturer. Furthermore, there were three studies (study ID: Arwert 2005, Gotherstrom 2007, Elbornsson 2012) which lasted for more than 10 years included in our meta-analysis. In the three studies, the operating criterion of BMD measurements changed partly during the GH treatment. In the study reported by Arwert et al., BMD measurements were performed with Norland XR-26 at the beginning of the study, and with Hologic QDR-4500 at the end of the study, but they tried to resolve this problem. They measured the European Spine Phantom (ESP) on both devices, and the results of ESP measurements showed very similar BMD values. In the study reported by Gotherstrom et al., the software versions of Lunar DPX-L were changed several times (from 1.1 to finally 1.35) during the study, but the version 1.33 was generally used during the large period of the study. In the study reported by Elbornsson et al., BMD measurements were performed with LUNAR DPX-L scanner at the beginning of the study and with LUNAR Prodigy scanner at the end of the study. They measured 31 subjects' BMD with both scanners on the same day, and the BMD values of the subjects were not significantly different between scanners.

Fourthly, we did not detect significant association between GH treatment and BMD of spine, F, and TB in American subjects. In addition, we did not detect significant association between GH treatment and BMD of TB in European and Oceanian subjects but got a significant overall association between GH treatment and increased BMD of TB. Although GH was a major regulator of IGF-1 concentrations, other factors such as nutrition and insulin concentration were also important in its regulation. In different countries or geographic location, the nutrition or insulin concentration of GHD patients might also be different.

Heterogeneity was removed or decreased in some subgroups but still existed in other subgroups. Thus, in addition to treatment time, GH dosage, manufacturer of DXA scanner and geographic location, there might be other factors leading to heterogeneity. For instance, previous study indicated that gender might influence the association between GH treatment and BMD, and several studies suggested that men had a greater treatment response to GH replacement than women. The mechanisms underlying these gender differences were not fully understood, but sex hormones might play a role [49, 50]. However, there were too few studies analyzing the results of GH treatment separately for males and females in the studies included in our meta-analysis, which made it impossible to undertake subgroup analyses stratified by gender. For the studies focus on BMD of spine, FN and TB, both Egger's regression and Begg methods did not show publication bias.

In our study, we mainly investigated the effects of GH treatment on BMD in adult GHD patients. Besides its beneficial effects on bone, GH treatment is also suggested to alleviate at least some of the aspects of the reduced physical and psycHological health associated with GHD in adult life [51, 52]. Severe quality of life (QoL) impairment is evident in a significant proportion of adults with GHD, and the beneficial effects of physiological GH replacement on QoL in affected individuals are well documented [53–55]. However, the risk of adverse effects may increase in the GHD adults treated with GH, such as oedema, joint stiffness, and carpal tunnel syndrome [54]. Moreover, serum IGF1 levels increase in the GHD patients with GH treatment. In the general population, higher circulating IGF1 levels are associated with increased incidence of prostate, colorectal, and premenopausal breast cancer [56]. To date, however, there have been no published long-term studies in adults with GHD treated with GH with respect to the development of nonpituitary malignancies.

The present study has some limitations that should be considered. Firstly, because only studies that were indexed by the selected databases were included for data analysis, some relevant published studies or unpublished studies might be missed, which might have biased our results. Secondly, our meta-analysis only included adult subjects since it was inappropriate to pool data from studies in children, where growth had a major effect of GH therapy; with studies in adults, this was not the case.

5. Conclusions

Considered together, these studies seem to indicate that GH treatment had beneficial influence on BMD in GHD adults, but in some subject populations, the influence was not evident.

Conflict of Interests

None of the authors has any conflict of interests to declare.

Authors' Contributions

P. Xue and Y. Wang contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of Hebei Province (no. C2009001179) and Grant from Hebei Provincial Health Bureau (no. 20090316).

References

[1] I. Shimon, "Growth hormone replacement for adult growth hormone deficiency," *Expert Opinion on Pharmacotherapy*, vol. 4, no. 11, pp. 1977–1983, 2003.

[2] M. Ernst and E. R. Froesch, "Growth hormone dependent stimulation of osteoblast-like cells in serum-free cultures via

local synthesis of insulin-like growth factor I," *Biochemical and Biophysical Research Communications*, vol. 151, no. 1, pp. 142–147, 1988.

[3] Y. H. Joung, E. J. Lim, P. Darvin et al., "MSM enhances GH signaling via the Jak2/STAT5b pathway in osteoblast-like cells and osteoblast differentiation through the activation of STAT5b in MSCs," *PLoS One*, vol. 7, no. 10, Article ID e47477, 2012.

[4] P. V. Carroll, E. R. Christ, B. Å. Bengtsson et al., "Growth hormone deficiency in adulthood and the effects of growth hormone replacement: a review," *Journal of Clinical Endocrinology and Metabolism*, vol. 83, no. 2, pp. 382–395, 1998.

[5] A. Geisler, N. Lass, N. Reinsch et al., "Quality of life in children and adolescents with growth hormone deficiency: association with growth hormone treatment," *Hormone Research in Paediatrics*, vol. 78, no. 2, pp. 94–99, 2012.

[6] P. Kann, B. Piepkorn, B. Schehler et al., "Effect of long-term treatment with GH on bone metabolism, bone mineral density and bone elasticity in GH-deficient adults," *Clinical Endocrinology*, vol. 48, no. 5, pp. 561–568, 1998.

[7] N. A. Tritos, A. H. Hamrahian, D. King et al., "A longer interval without GH replacement and female gender are associated with lower bone mineral density in adults with childhood-onset GH deficiency: a KIMS database analysis," *European Journal of Endocrinology*, vol. 167, no. 3, pp. 343–351, 2012.

[8] C. Wüster, R. Abs, B. A. Bengtsson et al., "The influence of growth hormone deficiency, growth hormone replacement therapy, and other aspects of hypopituitarism on fracture rate and bone mineral density," *Journal of Bone and Mineral Research*, vol. 16, no. 2, pp. 398–405, 2001.

[9] T. Rosén, L. Wilhelmsen, K. Landin-Wilhelmsen, G. Lappas, and B. Å. Bengtsson, "Increased fracture frequency in adult patients with hypopituitarism and GH deficiency," *European Journal of Endocrinology*, vol. 137, no. 3, pp. 240–245, 1997.

[10] H. L. Fideleff, H. R. Boquete, G. Stalldecker, A. V. Giaccio, and P. G. V. Sobrado, "Comparative results of a 4-year study on cardiovascular parameters, lipid metabolism, body composition and bone mass between untreated and treated adult growth hormone deficient patients," *Growth Hormone and IGF Research*, vol. 18, no. 4, pp. 318–324, 2008.

[11] S. van den Heijkant, G. Hoorweg-Nijman, J. Huisman et al., "Effects of growth hormone therapy on bone mass, metabolic balance, and well-being in young adult survivors of childhood acute lymphoblastic leukemia," *Journal of Pediatric Hematology/Oncology*, vol. 33, no. 6, pp. e231–e238, 2011.

[12] N. Suganuma, M. Furuhashi, T. Hirooka et al., "Bone mineral density in adult patients with Turner's syndrome: analyses of the effectiveness of GH and ovarian steroid hormone replacement therapies," *Endocrine Journal*, vol. 50, no. 3, pp. 263–269, 2003.

[13] T. B. Hansen, K. Brixen, N. Vahl et al., "Effects of 12 months of growth hormone (GH) treatment on calciotropic hormones, calcium homeostasis, and bone metabolism in adults with acquired GH deficiency: a double blind, randomized, placebo-controlled study," *Journal of Clinical Endocrinology and Metabolism*, vol. 81, no. 9, pp. 3352–3359, 1996.

[14] G. Johannsson, T. Rosén, I. Bosaeus, L. Sjöström, and B. Å. Bengtsson, "Two years of growth hormone (GH) treatment increases bone mineral content and density in hypopituitary patients with adult-onset GH deficiency," *Journal of Clinical Endocrinology and Metabolism*, vol. 81, no. 8, pp. 2865–2873, 1996.

[15] J. Rodríguez-Arnao, I. James, A. Jabbar et al., "Serum collagen crosslinks as markers of bone turn-over during GH replacement therapy in growth hormone deficient adults," *Clinical Endocrinology*, vol. 48, no. 4, pp. 455–462, 1998.

[16] R. C. Cuneo, S. Judd, J. D. Wallace et al., "The Australian multicenter trial of growth hormone (GH) treatment in GH- deficient adults," *Journal of Clinical Endocrinology and Metabolism*, vol. 83, no. 1, pp. 107–116, 1998.

[17] B. M. K. Biller, G. Sesmilo, H. B. A. Baum, D. Hayden, D. Schoenfeld, and A. Klibanski, "Withdrawal of long-term physiological growth hormone (GH) administration: differential effects on bone density and body composition in men with adult-onset GH deficiency," *Journal of Clinical Endocrinology and Metabolism*, vol. 85, no. 3, pp. 970–976, 2000.

[18] J. Koranyi, J. Svensson, G. Götherström, K. S. Sunnerhagen, B. Å. Bengtsson, and G. Johannsson, "Baseline characteristics and the effects of five years of GH replacement therapy in adults with GH deficiency of childhood or adulthood onset: a comparative, prospective study," *Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 10, pp. 4693–4699, 2001.

[19] R. Lanzi, M. Losa, I. Villa et al., "GH replacement therapy increases plasma osteoprotegerin levels in GH-deficient adults," *European Journal of Endocrinology*, vol. 148, no. 2, pp. 185–191, 2003.

[20] L. E. Underwood, K. M. Attie, and J. Baptista, "Growth hormone (GH) dose-response in young adults with childhood-onset GH deficiency: a two-year, multicenter, multiple-dose, Placebo-controlled study," *Journal of Clinical Endocrinology and Metabolism*, vol. 88, no. 11, pp. 5273–5280, 2003.

[21] E. Hubina, L. Kovacs, I. Szabolcs et al., "The effect of gender and age on growth hormone replacement in growth hormone-deficient patients," *Hormone and Metabolic Research*, vol. 36, no. 4, pp. 247–253, 2004.

[22] N. Bravenboer, P. J. Holzmann, J. C. Ter Maaten, L. M. Stuurman, J. C. Roos, and P. Lips, "Effect of long-term growth hormone treatment on bone mass and bone metabolism in growth hormone-deficient men," *Journal of Bone and Mineral Research*, vol. 20, no. 10, pp. 1778–1784, 2005.

[23] L. I. Arwert, J. C. Roos, P. Lips, J. W. R. Twisk, R. A. Manoliu, and M. L. Drent, "Effects of 10 years of growth hormone (GH) replacement therapy in adult GH-deficient men," *Clinical Endocrinology*, vol. 63, no. 3, pp. 310–316, 2005.

[24] C. L. Boguszewski, L. H. F. Meister, D. C. T. Zaninelli, and R. B. Radominski, "One year of GH replacement therapy with a fixed low-dose regimen improves body composition, bone mineral density and lipid profile of GH-deficient adults," *European Journal of Endocrinology*, vol. 152, no. 1, pp. 67–75, 2005.

[25] S. Benedini, L. Dalle Carbonare, N. Albiger et al., "Effect of short-term therapy with recombinant human growth hormone (GH) on metabolic parameters and preclinical atherosclerotic markers in hypopituitary patients with growth hormone deficiency," *Hormone and Metabolic Research*, vol. 38, no. 1, pp. 16–21, 2006.

[26] P. J. Snyder, B. M. K. Biller, A. Zagar et al., "Effect of growth hormone replacement on BMD in adult-onset growth hormone deficiency," *Journal of Bone and Mineral Research*, vol. 22, no. 5, pp. 762–770, 2007.

[27] G. Gotherstrom, B. A. Bengtsson, I. Bossæus, G. Johansson, and J. Svensson, "Ten-year GH replacement increase bone mineral density in hypopituitary patients with adult onset GH deficiency," *European Journal of Endocrinology*, vol. 156, no. 1, pp. 55–64, 2007.

[28] F. Rota, M. C. Savanelli, L. Tauchmanova et al., "Bone density and turnover in young adult patients with growth hormone deficiency after 2-year growth hormone replacement according with gender," *Journal of Endocrinological Investigation*, vol. 31, no. 2, pp. 94–103, 2008.

[29] G. S. Conway, M. Szarras-Czapnik, K. Racz et al., "Treatment for 24 months with recombinant human GH has a beneficial effect on bone mineral density in young adults with childhood-onset GH deficiency," *European Journal of Endocrinology*, vol. 160, no. 6, pp. 899–907, 2009.

[30] M. Elbornsson, G. Gotherstrom, I. Bosaeus, B. A. Bengtsson, G. Johannsson, and J. Svensson, "Fifteen years of GH replacement increases bone mineral density in hypopituitary patients with adult-onset GH deficiency," *European Journal of Endocrinology*, vol. 166, no. 5, pp. 787–795, 2012.

[31] D. W. Leung, S. A. Spencer, G. Cachianes et al., "Growth hormone receptor and serum binding protein: purification, cloning and expression," *Nature*, vol. 330, no. 6148, pp. 537–543, 1987.

[32] M. Andreassen, J. Frystyk, J. Faber, and L. O. Kristensen, "GH activity and markers of inflammation: a crossover study in healthy volunteers treated with GH and a GH receptor antagonist," *European Journal of Endocrinology*, vol. 166, no. 5, pp. 811–819, 2012.

[33] L. S. Mathews, R. E. Hammer, R. L. Brinster, and R. D. Palmiter, "Expression of insulin-like growth factor I in transgenic mice with elevated levels of growth hormone is correlated with growth," *Endocrinology*, vol. 123, no. 1, pp. 433–437, 1988.

[34] N. S. Becker, P. Verdu, M. Georges et al., "The role of GHR and IGF1 genes in the genetic determination of African pygmies' short stature," *European Journal of Human Genetics*, 2012.

[35] C. Barner, M. Petersson, B. Eden Engstrom, Hoybye, and C. :, "Effects on insulin sensitivity and body composition of combination therapy with GH and IGF1 in GH deficient adults with type 2 diabetes," *European Journal of Endocrinology*, vol. 167, no. 5, pp. 697–703, 2012.

[36] J. M. P. Holly and J. A. H. Wass, "Insulin-like growth factors; autocrine, paracrine or endocrine? New perspectives of the somatomedin hypothesis in the light of recent developments," *Journal of Endocrinology*, vol. 122, no. 3, pp. 611–618, 1989.

[37] F. I. Arnaldez and L. J. Helman, "Targeting the insulin growth factor receptor 1," *Hematology/Oncology Clinics of North America*, vol. 26, no. 3, pp. 527–542, 2012.

[38] S. Yakar, C. J. Rosen, W. G. Beamer et al., "Circulating levels of IGF-1 directly regulate bone growth and density," *Journal of Clinical Investigation*, vol. 110, no. 6, pp. 771–781, 2002.

[39] M. Kassem, L. Mosekilde, and E. F. Eriksen, "Growth hormone stimulates proliferation of normal human bone marrow stromal osteoblast precursor cells in vitro," *Growth Regulation*, vol. 4, no. 3, pp. 131–135, 1994.

[40] J. M. Wit and C. Camacho-Hubner, "Endocrine regulation of longitudinal bone growth," *Endocrine Development*, vol. 21, pp. 30–41, 2011.

[41] G. I. Baroncelli, S. Bertelloni, C. Ceccarelli, D. Cupelli, and G. Saggese, "Dynamics of bone turnover in children with GH deficiency treated with GH until final height," *European Journal of Endocrinology*, vol. 142, no. 6, pp. 549–556, 2000.

[42] O. Klefter and U. Feldt-Rasmussen, "Is increase in bone mineral content caused by increase in skeletal muscle mass/strength in adult patients with GH-treated GH deficiency? A systematic literature analysis," *European Journal of Endocrinology*, vol. 161, no. 2, pp. 213–221, 2009.

[43] D. N. Proctor, L. J. Melton, S. Khosla, C. S. Crowson, M. K. O'Connor, and B. L. Riggs, "Relative influence of physical activity, muscle mass and strength on bone density," *Osteoporosis International*, vol. 11, no. 11, pp. 944–952, 2000.

[44] H. M. Frost, "The Utah paradigm of skeletal physiology: an overview of its insights for bone, cartilage and collagenous tissue organs," *Journal of Bone and Mineral Metabolism*, vol. 18, no. 6, pp. 305–316, 2000.

[45] A. Giustina, G. Mazziotti, and E. Canalis, "Growth hormone, insulin-like growth factors, and the skeleton," *Endocrine Reviews*, vol. 29, no. 5, pp. 535–559, 2008.

[46] A. Kaur and S. R. Phadke, "Analysis of short stature cases referred for genetic evaluation," *The Indian Journal of Pediatrics*, vol. 79, no. 12, pp. 1597–1600, 2012.

[47] R. D. Murray, C. J. Skillicorn, S. J. Howell, C. A. Lissett, A. Rahim, and S. M. Shalet, "Dose titration and patient selection increases the efficacy of GH replacement in severely GH deficient adults," *Clinical Endocrinology*, vol. 50, no. 6, pp. 749–757, 1999.

[48] N. A. Pocock, K. A. Noakes, M. Griffiths et al., "A comparison of longitudinal measurements in the spine and proximal femur using lunar and hologic instruments," *Journal of Bone and Mineral Research*, vol. 12, no. 12, pp. 2113–2118, 1997.

[49] W. M. Drake, J. Rodríguez-Arnao, J. U. Weaver et al., "The influence of gender on the short and long-term effects of growth hormone replacement on bone metabolism and bone mineral density in hypopituitary adults: a 5-year study," *Clinical Endocrinology*, vol. 54, no. 4, pp. 525–532, 2001.

[50] L. E. Olson, C. Ohlsson, and S. Mohan, "The role of GH/IGF-I-mediated mechanisms in sex differences in cortical bone size in mice," *Calcified Tissue International*, vol. 88, no. 1, pp. 1–8, 2011.

[51] P. V. Carroll, W. M. Drake, K. T. Maher et al., "Comparison of continuation or cessation of growth hormone (GH) therapy on body composition and metabolic status in adolescents with severe GH deficiency at completion of linear growth," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 8, pp. 3890–3895, 2004.

[52] V. K. B. Prabhakar and S. M. Shalet, "Aetiology, diagnosis, and management of hypopituitarism in adult life," *Postgraduate Medical Journal*, vol. 82, no. 966, pp. 259–266, 2006.

[53] L. J. Woodhouse, A. Mukherjee, S. M. Shalet, and S. Ezzat, "The influence of growth hormone status on physical impairments, functional limitations, and health-related quality of life in adults," *Endocrine Reviews*, vol. 27, no. 3, pp. 287–317, 2006.

[54] A. Hazem, M. B. Elamin, I. Bancos et al., "Body composition and quality of life in adults treated with GH therapy: a systematic review and meta-analysis," *European Journal of Endocrinology*, vol. 166, no. 1, pp. 13–20, 2012.

[55] J. B. Deijen, L. I. Arwert, J. Witlox, and M. L. Drent, "Differential effect sizes of growth hormone replacement on quality of life, well-being and health status in growth hormone deficient patients: a meta-analysis," *Health and Quality of Life Outcomes*, vol. 3, p. 63, 2005.

[56] A. G. Renehan, M. Zwahlen, C. Minder, S. T. O'Dwyer, S. M. Shalet, and M. Egger, "Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis," *The Lancet*, vol. 363, no. 9418, pp. 1346–1353, 2004.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 902513, 7 pages
http://dx.doi.org/10.1155/2013/902513

Review Article

Bone Mineral Density and Osteoporosis after Preterm Birth: The Role of Early Life Factors and Nutrition

Claire L. Wood,[1] Alexander M. Wood,[2] Caroline Harker,[3] and Nicholas D. Embleton[1,4,5]

[1] *Child Health, Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne NE1 4LP, UK*
[2] *Orthopaedic Department, Wansbeck General Hospital, Woodhorn Lane, Ashington, Northumberland NE63 9JJ, UK*
[3] *Newcastle University, Framlington Place, Newcastle Upon Tyne NE2 4HH, UK*
[4] *Newcastle Neonatal Service, Newcastle Hospitals NHS Foundation Trust, Newcastle upon Tyne NE1 4LP, UK*
[5] *Institute of Health and Society, Newcastle University, Framlington Place, Newcastle Upon Tyne NE2 4HH, UK*

Correspondence should be addressed to Nicholas D. Embleton; nicholas.embleton@ncl.ac.uk

Received 30 December 2012; Accepted 24 March 2013

Academic Editor: Ling-Qing Yuan

The effects of preterm birth and perinatal events on bone health in later life remain largely unknown. Bone mineral density (BMD) and osteoporosis risk may be programmed by early life factors. We summarise the existing literature relating to the effects of prematurity on adult BMD and the Developmental Origins of Health and Disease hypothesis and programming of bone growth. Metabolic bone disease of prematurity and the influence of epigenetics on bone metabolism are discussed and current evidence regarding the effects of breastfeeding and aluminium exposure on bone metabolism is summarised. This review highlights the need for further research into modifiable early life factors and their effect on long-term bone health after preterm birth.

1. Introduction

Preterm birth accounts for 5–10% of births in the UK. Worldwide almost 10% of babies are born preterm, representing more than 15 million births every year [1]. Preterm birth is defined by the World Health Organisation (WHO) as all live births before 37 completed weeks gestation. Preterm can be further subdivided into extremely preterm (<28 weeks), very preterm (28–32 weeks), and moderate preterm (32–<37 completed weeks) [1]. A preterm baby faces many challenges. Feeding problems are almost inevitable in the very preterm group as a coordinated suck and swallow is not established until around 34 weeks corrected gestation. Extremely preterm infants and those who are unwell may require IV fluids or a period of total parenteral nutrition before full feeds can be established. Many preterms born at less than 32 weeks will have some degree of respiratory distress syndrome (RDS), due to lung immaturity, and may require ventilatory support. Giving antenatal steroids reduces the incidence and severity of respiratory and other complications. The use of supplemental oxygen increases the risk of retinopathy of prematurity and may exacerbate oxidant damage in many organs and tissues but is vital for improved survival.

Despite these challenges, survival has improved dramatically in the last few years, especially in developed countries. More than 50% of babies born at 24 weeks gestation regularly survive long term with improved nutrition being one potential factor contributing to these improvements. As this cohort of survivors reaches middle age the impact of preterm birth on long-term metabolic outcomes such as bone mineral density will become increasingly important.

Osteoporosis is characterised by the depletion of bone mineral mass, combined with bone microarchitecture deterioration and a resultant increased fracture risk [2]. It is one of the most prevalent skeletal disorders; with estimates that up to 30% of women and 12% of men over the age of 50 are affected [3], it has a similar lifetime risk to coronary heart disease [4]. Bone mineral density in

adulthood depends predominantly on growth and mineralisation of the skeleton and the resultant peak bone mass achieved and then, to a lesser extent, on the subsequent loss. Longitudinal studies of girls suggests that this peak is reached about 30 years of age [5]. For each standard deviation decrease in bone mineral density, fracture risk doubles in girls, similar to the risk in postmenopausal women [6].

It is estimated that osteoporosis affects 3 million people in the UK and results in 250,000 fractures annually [2]. It has vast public health consequences due to the morbidity and mortality of the resulting fractures and the associated healthcare expenditure. As there is no cure, it is important to identify early life influences on later bone mineral density, which may aid the development of interventions to optimise bone health and reduce osteoporosis risk.

We present a review of the current literature regarding early life factors and the impact of nutrition on bone mineral density and bone health after preterm birth, in order to inform further research and highlight current challenges facing the clinicians responsible for this cohort.

2. Bone Mineral Density (BMD) Programming in Term Infants

There is strong evidence linking early life exposures and later peak bone mass in childhood, for example, the contributions of physical exercise both in utero and childhood, cigarette smoking during pregnancy, and diet and endocrine status in childhood [7, 8]. Bone mineral density shows strong tracking during childhood and adolescence growth and into adulthood. A reduced peak BMD in childhood is associated with increased fracture risk and has been proposed as one of the best predictors of later life fracture risk in females [9]. Gender also influences neonatal bone composition at term with males attaining greater bone area, bone mineral content (BMC), and BMD than females after adjustment for gestation [10]. In addition to factors influencing peak bone mass during childhood and adolescence, evidence is growing that bone mineral density and thus osteoporosis risk can be modulated during intrauterine and infant life [11]. A retrospective study involving term infants demonstrated independent effects of birth weight and weight at one year on bone size and strength during the sixth and seventh decades after adjustment for confounding lifestyle factors [12]. These associations may reflect the intrauterine programming of skeletal development [13] and its subsequent tracking throughout the lifecourse.

Research also suggests that some of the predisposition for osteoporosis can be attributed to polygenic genetic inheritance. For example, polymorphisms in vitamin D and oestrogen receptor genes and collagen coding genes have been implicated [14]. It is likely that the genes that determine an increased risk of osteoporosis will vary among people of different ethnic backgrounds. In the future, genomic studies may provide information regarding the susceptibility of osteoporosis and likely treatment response and may become an adjunct to clinical management.

3. The Developmental Origins of Health and Disease (DOHaD) Hypothesis and Programming of Bone Growth

The Developmental Origins of Health and Disease (DOHaD) hypothesis suggest that nutritional imbalance during critical windows in early life can permanently influence or "programme" long-term development and disease in later life [15]. Much of the original work was by Barker who reported the relationship with low birthweight, used as a proxy for fetal growth, with coronary heart disease [16, 17]. It became apparent, however, that these mechanisms and effects were not restricted to fetal life and that nutrition and growth in infancy (and perhaps in later childhood) were also crucial, leading to the incorporation of elements of evolutionary biology and the adoption of the term DOHaD.

Recently, research has linked birth weight, birth length, and placental weight to later osteoporosis risk [18–20]. Known predictors of osteoporosis risk comprise genetic predisposition and environmental influences such as diet and exercise. However, a significant portion of BMD variance remains unexplained [19]. It is proposed that this remaining variation results from the programming of systems controlling skeletal growth trajectory during critical growth periods [13].

4. Epigenetics and Bone Metabolism

Many of the programming effects may be modulated by epigenetic mechanisms. Epigenetics is the study of mitotically heritable alterations in gene expression potential that are not caused by changes in DNA sequence. The classic examples are DNA methylation and histone acetylation [21]. These processes do not alter the nucleotide sequence in DNA but result in differences in gene expression and transcription and may also involve post-transcriptional effects on other processes such as protein translation. Early life growth and nutritional exposures appear to affect the "cellular memory" and result in variation in later life phenotypes. Much of this work is still in the early stages but initial data suggest that epigenetic mechanisms may underlie the process of developmental plasticity and its effect on the risk of osteoporosis. One of the models that have been postulated is the role of maternal vitamin D status and postnatal calcium transfer. Calcium and vitamin D are vital nutrients in bone development. Early work concerning methylation and vitamin D receptors and placental calcium transporters suggests that epigenetic regulation might explain how maternal vitamin D levels affect bone mineralisation in the neonate [21]. Much of the current research is in animal models, but if the changes can be replicated in humans, epigenetic or other biomarkers may provide risk assessment tools to enable targeted intervention to those at greatest risk of osteoporosis.

5. Metabolic Bone Disease of Prematurity

The preterm population is particularly susceptible to metabolic bone disease for two key reasons: firstly, 80% of fetal bone mineral accumulation occurs during the last

trimester of pregnancy, with a surge in placental transfer of calcium, magnesium, and phosphorus to the neonate [22]. A preterm infant who spends this period without the placenta and the associated regulatory maternal environment will therefore have lower BMD and significantly lower bone mineral content than an infant born at term.

Secondly, providing adequate nutrition to preterm infants can be extremely challenging. Most extremely preterm infants (<32 weeks gestation) require support with parenteral nutrition (PN) because of complex factors including metabolic "immaturity" (that may limit nutrient intake) and a delay in establishing enteral feeds. In addition, solubility issues with PN solutions mean it is impossible to provide sufficient mineral via the parenteral route alone. Maternal breast milk is associated with a range of benefits in the short-term (e.g., reduction in the incidence of necrotising enterocolitis, a potentially fatal illness associated with milk feeds) and long-term (e.g., improved cognitive outcome) but alone will not meet nutrient requirements without fortification. Therefore as well as being born with a mineral deficit, the often stormy neonatal course and nutritional practicalities of providing adequate mineral intake means that many preterm infants develop osteopenia of prematurity.

As preterm infants grow, mineral uptake is compromised through the low content in unfortified breast milk (especially phosphate) and inefficient absorption due to an underdeveloped gastrointestinal tract [9]. This results in a greater loss of long bone density than observed in term infants and further increases the risk of metabolic bone disease [9]. Ex utero living conditions also mean it is more difficult for infants to move and stress their bones as they would have done in-utero [23, 24]. As well as mineral compromise, lower BMD in preterms is also a consequence of other factors such as steroid use [25], respiratory compromise [25], and infection [18] which may damage bone trabeculae. Although metabolic bone disease of prematurity is often asymptomatic and self-limiting [9], concern remains that under-mineralisation during such a critical period could increase the risk of childhood fracture and cause reduced peak bone mass [26] and therefore an increased risk of future osteoporosis.

6. The Effects of Prematurity on Adult BMD

There is conflicting data regarding the long-term consequences of preterm birth on the skeleton and the potential for peak BMD compared to their term counterparts. Preterm infants are known to have a lower bone mass [27], BMD [26] and BMC [25] at the corrected age of term, as well as a lower weight and ponderal index [26]. A study of 7-year-old boys showed greater measures of cortical thickness, whole body BMC, and hip BMD in term compared to preterm boys after adjustment for weight, height and age. These differences remained after adjustment for birth weight, length of neonatal hospital stay, and current activity level [28]. A study by Fewtrell et al. in 2000 [29] found former preterm infants who were followed up at around 10 years of age were shorter, lighter, and had lower BMC than controls. These differences continue through childhood and possibly persist until puberty [25, 28], although results are difficult to interpret due to the confounding effects of puberty and the interaction with bone size and later BMD. In a study by Backström et al., individuals who were born preterm were assessed with computerized tomography as young adults. Lower bone strength was demonstrated at the distal tibia and radius compared to age and sex matched controls [30]. This effect was more pronounced in males and remained after adjustment for potential confounders.

Several studies have failed to demonstrate an association between preterm birth and later bone strength, although all of these [28, 31, 32] were undertaken in small populations. A possible explanation for the variation in study results may be in the timing of follow-up as catch up in bone mineralisation may occur primarily in late childhood and adolescence. Other studies have found that although preterms were smaller, their BMD was appropriate for size. Adults who were born preterm may be shorter than their term born counterparts. As some studies may not have made appropriate adjustments for current size it is difficult to determine whether BMD is appropriate for current size or not [28].

7. Early Nutrition and Growth Influences on Bone Metabolism after Preterm Birth

Several maternal factors are known to have a negative impact on neonatal growth and skeletal mineralisation in term infants. Although not discussed in detail here, examples are shorter maternal height, low parity, smoking during pregnancy, low fat stores [33, 34], and low vitamin D exposure [9, 22].

There is conflicting data regarding the influence of birthweight on later BMD. Low birthweight (LBW) is defined by WHO as <2500 g [1]. LBW is usually a consequence of being preterm or small for gestational age (i.e., born with a birthweight on less than the 10th centile). Some studies suggests that very low birth weight (VLBW, <1500 g birthweight) infants, whether preterm or not, attain a suboptimal peak bone mass in part due to their small size and subnormal skeletal mineralisation [31]. A recent study by Callreus et al. highlighted the long-term influence of birthweight on bone mineral content but found an absence of association of birthweight with bone density once adult body weight was also taken into consideration [39]. The Hertfordshire cohort study involving over 600 subjects showed that birthweight was independently associated with bone density at 60–75 years of age. Although another study found no association with preterm birth and peak bone mass [35], an effect of being small for gestational age was apparent, suggesting a proportion of later bone mass is determined by fetal growth. Further research has also shown a significant association between shorter gestation and adverse skeletal outcomes [31].

Several studies in infants have shown the influence of early growth on later bone health in those born preterm. In a study by Cooper et al., those who were lightest at 1 year of age had the lowest BMC [22]. In a further study, weight gain during the first two years of life predicted BMD at age 9–14 [40]. Fewtrell et al. also showed a positive association of body

weight and height at both premature birth and 18 months with bone size, BMC, and BMD at aged 8–12 years [36]. It was hypothesised that those with the most substantial increase in height between birth and follow-up showed the greatest bone mass. They also demonstrated that birth length alone was a strong predictor of later bone mass, and it was suggested that optimising linear growth early may be beneficial to later bone health. Although conducted with a large cohort ($n = 201$), few measurements were taken after discharge and dual-energy X-ray absorptiometry (DXA) analysis was only taken at 8–12 years. As a result, changes in growth and corresponding bone mass at potentially critical epochs of infancy were not measured.

Optimising early growth through nutritional interventions generates positive and lasting effects on bone mineralisation [28] and it is hypothesised that this may partially counteract preterm bone deficits. A systematic review by Kuschel and Harding in 2009 showed that fortifying the nutrition of preterm babies improves growth and bone mineral aggregation [41].

Lieben et al. [42] and Kanazawa et al. [43] discuss an interaction between bone and glucose metabolism involving adipocyte-originated leptin and osteoblast-derived osteocalcin. They postulate that healthy bone matrix protein increases insulin sensitivity in other tissues and that people with metabolic syndrome who are insulin resistant also have poorer bone quality and increased risk of osteoporotic fracture. The "metabolic syndrome" involves many biological systems, but insulin sensitivity or resistance is perhaps the area subject to the most detail study in later life. This interaction is potentially a very important one; those who were born preterm appear to be at a higher risk for metabolic syndrome in later life and studies examining the influence of birth weight on later health consistently show that in low birth weight born adults, there is decreased insulin sensitivity. The critical period in the preterm determining later insulin resistance is unclear at present. Bazaes et al. found that low preterm birthweight was associated with impaired insulin sensitivity [44], which supports Barker's hypothesis. Singhal et al. [45] showed that preterm infants who received higher nutrient intakes during the first 2 weeks of life had higher levels of insulin resistance in adolescence. These studies may be showing a potential adipocyte-osteocalcin interaction and suggest that the relationship between nutrition and later bone and metabolic health is complex, and this is an area that clearly warrants further research.

8. Aluminium and Bone Mineral Density

Aluminium has no active role in the human body but is inadvertently ingested in the preterm for several reasons. Firstly preterms are exposed to high levels of aluminium in standard parenteral nutrition (PN) regimes. The current trend is for early PN to optimise early growth and associated neurocognitive function. Most aluminium is accumulated through unavoidable contamination via calcium gluconate stored in glass vials. In adults, this aluminium load is probably adequately dealt with by the kidneys, but the premature infant's renal system is relatively immature so accumulation occurs. Adverse effects of aluminium in bone have been seen in uraemic adults and there are now studies showing that infants who received aluminium-depleted PN had significantly higher BMC of the lumbar spine [46]. A direct effect on bone structure is unlikely as bone will have been remodeled several times by adulthood, but it is thought that the presence of aluminium may modify the response of bone cells to stimuli such as when loading forces are applied through exercise.

9. Effects of Breastfeeding on Bone Metabolism

There is conflicting evidence as to whether breastfeeding has a protective role in the primary prevention of osteoporosis. In some studies, such as that of Fewtrell et al., breast milk consumption was found to result in higher adult BMD [37] despite the milk being unfortified and having a lower mineral content than formula. This suggests a possible role for beneficial non-nutrient components such as growth factors. In another study, bone mass at follow-up age of approximately 10 years was positively associated with the duration of breastfeeding [47], yet other studies have shown no benefits at a similar age [48, 49]. Other studies have not demonstrated an ongoing relationship in adulthood between breastfeeding and bone mass [22]. Given the known benefits of breastfeeding and the lack of proven negative association, it seems prudent to strongly encourage breastfeeding, despite slower infant growth trajectories.

10. Vitamin D and Bone Mineral Density

It is difficult for the preterm infant to match the in-utero accretion of minerals. Calcium absorption depends on calcium and vitamin D intakes and phosphorus levels, which affect calcium retention. In clinical practice, very few babies need calcium supplementation if they receive either a preterm formula or breastmilk along with breastmilk fortifier [50]. Suboptimal maternal vitamin D levels have been reported from many sources [51]. There are few studies in the preterm population but data from term infants clearly show maternal vitamin D insufficiency to be associated with adverse BMD both in infancy and later follow-up [52]. Considering the prevalence of vitamin D deficiency in pregnant mothers, the European Society of Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) committee recommends vitamin D supplementation in the region of 800–1000 IU per day to preterm infants to rapidly correct low fetal plasma levels and that they should be continued through infancy [53].

11. Limitations of the Current Evidence

Little is known concerning the early life control mechanisms for bone development [26] and the lack of prospective research in this area has been highlighted [30]. The potential for confounding in observational studies is also an important consideration. Poor nutrition is often an inevitable consequence in the sickest neonate who in turn will be more likely

Table 1: Summary of key papers on BMD and osteoporosis after preterm birth.

Author	Year	Cohort type	Study design	Findings
Rigo et al. [9]	2007	Preterm and term	Review	Greater loss of BMD in preterms than in terms during neonatal period. Maternal vitamin D exposure affects bone health in the newborn.
Bowden et al. [25]	1999	Preterm and term	Retrospective cross-sectional	Preterm infants have reduced bone mineral mass in conjunction with reduced growth and hip BMD aged 8 years.
Hovi et al. [31]	2009	LBW infants	Cohort	VLBW young adults have reduced peak BMD than their term peers.
Ahmad et al. [26]	2010	Preterm and term	Prospective	Preterms had lower body weight, length and BMD at term compared to term-born infants.
Abou Samra et al. [28]	2009	Preterm and term	Cross-sectional	Term males have greater bone size and mass than preterm males at follow-up aged 7 years.
Backström et al. [30]	2005	Preterm and term	Cross-sectional	Preterms have smaller cross-sectional bone dimensions in adulthood than terms.
Dalziel et al. [35]	2006	Preterm	RCT with longitudinal follow-up	Antenatal steroids did not affect peak bone mass. LBW and short gestation predicted reduced adult height. Slow fetal growth predicted lower bone mass.
Fewtrell et al. [36]	2000	Preterm	Longitudinal	Bone mass at 8–12 years is related to current size. Linear growth important in maximising bone mass.
Fewtrell et al. [37]	2009	Preterm	Longitudinal	Infant diet does not affect peak bone mass.
Breukhoven et al. [38]	2011	Preterm	Cross-sectional	Preterm birth does not affect BMD in young adults.

to have a poorer metabolic outcome. A 2011 meta-analysis stated that research from a variety of populations may clarify inconsistencies concerning the relationship between early life events and subsequent bone health [19], and there are few studies relating gestational length to adolescent BMD [9]. There is a need for longitudinal studies utilising randomised controlled trials of preterm infants where possible, and providing detailed information on early life exposures as well as bone measurement data.

One of the greatest challenges of longitudinal cohort studies, especially in children, is the attritional losses over time. In addition, much of the current data available is from preterm infants recruited to studies in the 1980s, an era predating the widespread use of antenatal steroids and surfactant therapy; two of the key practices that have had the most dramatic effects on long term survival. As cohorts of preterm born adults reach middle age, their risk of osteoporosis and their antecedent risks factors will become increasingly apparent. Table 1 summarises some of the key research on BMD and osteoporosis after preterm birth.

12. Conclusion

As survival rates continue to improve, the long term effects of premature birth become increasingly important. Only decades of future follow-up will truly ascertain the risk of osteoporosis and fracture after preterm birth. Because there is no cure for osteoporosis, preventative measures are important to minimise risk in this susceptible population. Genetic and intrauterine environmental factors that influence fetal growth trajectory have long-term consequences on body composition. Clearer identification of risk factors and refinement of biomarkers for later bone health will enable earlier preventative strategies. Reduction of the exposure of preterm infants to aluminium is an urgent research and clinical priority. Breastfeeding along with appropriately formulated breastmilk fortifiers to ensure adequate mineral intake and optimal growth should be strongly encouraged. As early mineral deficiency and metabolic bone disease are often asymptomatic during neonatal period, careful follow-up is required to identify at risk groups. Targeted prevention, early diagnosis and appropriate timely treatment may then significantly reduce the individual, health service, and societal burden of osteoporosis in the future.

Key learning points are as follows.

(i) There are conflicting data regarding the effects of preterm birth and/or LBW on later BMD.

(ii) Need for further research into modifiable early life factors and long-term bone health.

(iii) Breastfeeding (with appropriate fortifiers) and vitamin D supplementation may have long term benefits on BMD in preterm infants.

(iv) Reduction of aluminium exposure in preterm infants is an urgent priority.

References

[1] Born Too Soon, The Global Action Report on Preterm Birth, Switzerland, 2012.

[2] H. Blencowe, S. Cousens, M. Oestergaard, D. Chou, A. Moller, and R. Narwal, "National, regional and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications," *The Lancet*, vol. 379, no. 9832, pp. 2162–2172, 2012.

[3] L. J. Melton, "Perspectives: how many women have osteoporosis now?" *Journal of Bone and Mineral Research*, vol. 10, no. 2, pp. 175–177, 1995.

[4] WHO, "WHO scientific group on the assessment of osteoporosis at primary health care level," Summary Meeting Report, World Health Organization, Brussels, Belgium, 2004.

[5] R. R. Recker, K. M. Davies, S. M. Hinders, R. P. Heaney, M. R. Stegman, and D. B. Kimmel, "Bone gain in young adult women," *Journal of the American Medical Association*, vol. 268, no. 17, pp. 2403–2408, 1992.

[6] B. L. Riggs and L. J. Melton, "The worldwide problem of osteoporosis: insights afforded by epidemiology," *Bone*, vol. 17, supplement 5, pp. 505–511, 1995.

[7] J. P. Bonjour, G. Theintz, B. Buchs, D. Slosman, and R. Rizzoli, "Critical years and stages of puberty for spinal and femoral bone mass accumulation during adolescence," *The Journal of Clinical Endocrinology and Metabolism*, vol. 73, no. 3, pp. 555–563, 1991.

[8] M. J. Valimaki, M. Karkkainen, C. Lamberg-Allardt et al., "Exercise, smoking, and calcium intake during adolescence and early adulthood as determinants of peak bone mass," *British Medical Journal*, vol. 309, no. 6949, pp. 230–235, 1994.

[9] J. Rigo, C. Pieltain, B. Salle, and J. Senterre, "Enteral calcium, phosphate and vitamin D requirements and bone mineralization in preterm infants," *Acta Paediatrica*, vol. 96, no. 7, pp. 969–974, 2007.

[10] I. Ahmad, D. Nemet, A. Eliakim et al., "Body composition and its components in preterm and term newborns: a cross-sectional, multimodal investigation," *American Journal of Human Biology*, vol. 22, no. 1, pp. 69–75, 2010.

[11] E. Dennison, C. Cooper, and Z. Cole, "Early development and osteoporosis and bone health," *Journal of Developmental Origins of Health and Disease*, vol. 1, no. 3, pp. 142–149, 2010.

[12] H. Oliver, K. A. Jameson, A. A. Sayer, C. Cooper, and E. M. Dennison, "Growth in early life predicts bone strength in late adulthood: The Hertfordshire Cohort Study," *Bone*, vol. 41, no. 3, pp. 400–405, 2007.

[13] C. R. Gale, C. N. Martyn, S. Kellingray, R. Eastell, and C. Cooper, "Intrauterine programming of adult body composition," *The Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 1, pp. 267–272, 2001.

[14] M. Peacock, C. H. Turner, M. J. Econs, and T. Foroud, "Genetics of osteoporosis," *Endocrine Reviews*, vol. 23, no. 3, pp. 303–326, 2002.

[15] R. L. Goldenberg, J. F. Culhane, J. D. Iams, and R. Romero, "Epidemiology and causes of preterm birth," *The Lancet*, vol. 371, no. 9606, pp. 75–84, 2008.

[16] "The Barker Theory: New insights into ending chronic disease: Sprout Creative," 2012, http://www.thebarkertheory.org/index.php.

[17] D. J. P. Barker, P. D. Winter, C. Osmond, B. Margetts, and S. J. Simmonds, "Weight in infancy and death from ischaemic heart disease," *The Lancet*, vol. 2, no. 8663, pp. 577–580, 1989.

[18] P. Hovi, S. Andersson, A.-L. Jarvenpaa et al., "Decreased bone mineral density in adults born with very low birth weight: a cohort study," *PLoS Medicine*, vol. 6, no. 8, Article ID e1000135, 2009.

[19] J. Baird, M. A. Kurshid, M. Kim, N. Harvey, E. Dennison, and C. Cooper, "Does birthweight predict bone mass in adulthood? A systematic review and meta-analysis," *Osteoporosis International*, vol. 22, no. 5, pp. 1323–1334, 2011.

[20] J. Harvey, M. Javaid, N. Arden, J. Poole, S. Crozier, and S. Robinson, "Maternal predictors of neonatal bone size and geometry: the Southampton Women's survey," *Journal of Developmental Origins of Health and Disease*, vol. 1, no. 1, pp. 35–41, 2010.

[21] S. Earl, N. Harvey, and C. Cooper, "The epigenetic regulation of bone mass," *IBMS BoneKey*, vol. 7, no. 2, pp. 54–62, 2010.

[22] C. Cooper, S. Westlake, N. Harvey, K. Javaid, E. Dennison, and M. Hanson, "Review: developmental origins of osteoporotic fracture," *Osteoporosis International*, vol. 17, no. 3, pp. 337–347, 2006.

[23] M. E. Miller and T. N. Hangartner, "Temporary brittle bone disease: association with decreased fetal movement and osteopenia," *Calcified Tissue International*, vol. 64, no. 2, pp. 137–143, 1999.

[24] D. Nemet, T. Dolfin, I. Litmanowitz, R. Shainkin-Kestenbaum, M. Lis, and A. Eliakim, "Evidence for exercise-induced bone formation in premature infants," *International Journal of Sports Medicine*, vol. 23, no. 2, pp. 82–85, 2002.

[25] L. S. Bowden, C. J. Jones, and S. W. Ryan, "Bone mineralisation in ex-preterm infants aged 8 years," *European Journal of Pediatrics*, vol. 158, no. 8, pp. 658–661, 1999.

[26] I. Ahmad, D. Nemet, A. Eliakim et al., "Body composition and its components in preterm and term newborns: a cross-sectional, multimodal investigation," *American Journal of Human Biology*, vol. 22, no. 1, pp. 69–75, 2010.

[27] J. De Schepper, F. Cools, Y. Vandenplas, and O. Louis, "Whole body bone mineral content is similar at discharge from the hospital in premature infants receiving fortified breast milk or preterm formula," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 41, no. 2, pp. 230–234, 2005.

[28] H. Abou Samra, D. Stevens, T. Binkley, and B. Specker, "Determinants of bone mass and size in 7-year-old former term, late-preterm, and preterm boys," *Osteoporosis International*, vol. 20, no. 11, pp. 1903–1910, 2009.

[29] M. S. Fewtrell, A. Prentice, T. J. Cole, and A. Lucas, "Effects of growth during infancy and childhood on bone mineralization and turnover in preterm children aged 8-12 years," *Acta Paediatrica*, vol. 89, no. 2, pp. 148–153, 2000.

[30] M. C. Backström, A. L. Kuusela, A. M. Koivisto, and H. Sievänen, "Bone structure and volumetric density in young adults born prematurely: a peripheral quantitative computed tomography study," *Bone*, vol. 36, no. 4, pp. 688–693, 2005.

[31] P. Hovi, S. Andersson, A. L. Jarvenpaac et al., "Decreased bone mineral density in adults born with very low birth weight: a cohort study," *PLoS Medicine*, vol. 6, no. 8, Article ID e1000135, 2009.

[32] A. M. Boot, M. A. J. De Ridder, H. A. P. Pols, E. P. Krenning, and S. M. P. F. De Muinck Keizer-Schrama, "Bone mineral density in children and adolescents: relation to puberty, calcium intake, and physical activity," *The Journal of Clinical Endocrinology and Metabolism*, vol. 82, no. 1, pp. 57–62, 1997.

[33] N. C. Harvey, M. K. Javaid, N. K. Arden et al., "Maternal predictors of neonatal bone size and geometry: the Southampton Women's Survey," *Journal of Developmental Origins of Health and Disease*, vol. 1, no. 1, pp. 35–41, 2010.

[34] C. S. Yajnik and U. S. Deshmukh, "Maternal nutrition, intrauterine programming and consequential risks in the offspring," *Reviews in Endocrine and Metabolic Disorders*, vol. 9, no. 3, pp. 203–211, 2008.

[35] S. R. Dalziel, S. Fenwick, T. Cundy et al., "Peak bone mass after exposure to antenatal betamethasone and prematurity: follow-up of a randomized controlled trial," *Journal of Bone and Mineral Research*, vol. 21, no. 8, pp. 1175–1186, 2006.

[36] M. S. Fewtrell, A. Prentice, T. J. Cole, and A. Lucas, "Effects of growth during infancy and childhood on bone mineralization and turnover in preterm children aged 8-12 years," *Acta Paediatrica*, vol. 89, no. 2, pp. 148–153, 2000.

[37] M. S. Fewtrell, J. E. Williams, A. Singhal, P. R. Murgatroyd, N. Fuller, and A. Lucas, "Early diet and peak bone mass: 20 year follow-up of a randomized trial of early diet in infants born preterm," *Bone*, vol. 45, no. 1, pp. 142–149, 2009.

[38] P. E. Breukhoven, R. W. J. Leunissen, S. W. K. de Kort, R. H. Willemsen, and A. C. S. Hokken-Koelega, "Preterm birth does not affect bone mineral density in young adults," *European Journal of Endocrinology*, vol. 164, no. 1, pp. 133–138, 2011.

[39] M. Callreus, F. McGuigan, and K. Akesson, "Birthweight is more important for peak bone mineral content than for bone density: the PEAK-25 study of 1061 young adult women," *Osteoporosis International*, vol. 24, no. 4, pp. 1347–1355, 2013.

[40] S. Bhopal, K. Mann, N. Embleton, M. Korada, T. Cheetham, and M. Pearce, "The influence of early growth on bone mineral density at age 9-14 years in children born preterm," in *Journal of Developmental Origins of Health and Disease: 7th World Congress on Developmental Origins of Health and Disease*, Cambridge University Press, 2011.

[41] C. A. Kuschel and J. E. Harding, "Protein supplementation of human milk for promoting growth in preterm infants," *Cochrane Database of Systematic Reviews*, no. 2, Article ID CD000433, 2000.

[42] L. Lieben, F. Callewaert, and R. Bouillon, "Bone and metabolism: a complex crosstalk," *Hormone Research*, vol. 71, supplement 1, pp. 134–138, 2009.

[43] I. Kanazawa, T. Yamaguchi, M. Yamauchi et al., "Adiponectin is associated with changes in bone markers during glycemic control in type 2 diabetes mellitus," *The Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 8, pp. 3031–3037, 2009.

[44] R. Bazaes, A. Alegria, E. Pittaluga, and E. Avila, "Determinants of insulin sensitivity and secretion in very low birthweight children," *The Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 3, pp. 1267–1272, 2004.

[45] A. Singhal, M. Fewtrell, T. J. Cole, and A. Lucas, "Low nutrient intake and early growth for later insulin resistance in adolescents born preterm," *The Lancet*, vol. 361, no. 9363, pp. 1089–1097, 2003.

[46] M. Fewtrell, N. Bishop, and C. J. Edmonds, "Aluminium exposure from parenteral nutrition in preterm infants: bone health at 15 year follow-up," *Pediatrics*, vol. 124, pp. 1372–1379, 2009.

[47] G. Jones, M. Riley, and T. Dwyer, "Breastfeeding in early life and bone mass in prepubertal children: a longitudinal study," *Osteoporosis International*, vol. 11, no. 2, pp. 146–152, 2000.

[48] N. C. Harvey, S. M. Robinson, S. R. Crozier et al., "Breastfeeding and adherence to infant feeding guidelines do not influence bone mass at age 4 years," *British Journal of Nutrition*, vol. 102, no. 6, pp. 915–920, 2009.

[49] R. J. Young, D. L. Antonson, P. W. Ferguson, N. D. Murray, K. Merkel, and T. E. Moore, "Neonatal and infant feeding: effect on bone density at 4 years," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 41, no. 1, pp. 88–93, 2005.

[50] R. Tinnion and N. E. Embleton, "How to use... alkaline phosphatase in neonatology," *Archives of Disease in Childhood*, vol. 97, pp. 157–163, 2012.

[51] D. K. Dror and L. H. Allen, "Vitamin D inadequacy in pregnancy: biology, outcomes, and interventions," *Nutrition Reviews*, vol. 68, no. 8, pp. 465–477, 2010.

[52] J. Rigo, C. Pieltain, B. Salle, and J. Senterre, "Enteral calcium, phosphate and vitamin D requirements and bone mineralization in preterm infants," *Acta Paediatrica*, vol. 96, no. 7, pp. 969–974, 2007.

[53] C. Agostoni, G. Buonocore, V. P. Carnielli et al., "Enteral nutrient supply for preterm infants: commentary from the european society of paediatric gastroenterology, hepatology and nutrition committee on nutrition," *Journal of Pediatric Gastroenterology and Nutrition*, vol. 50, no. 1, pp. 85–91, 2010.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 321074, 8 pages
http://dx.doi.org/10.1155/2013/321074

Research Article

Self-Repair of Rat Cortical Bone Microdamage after Fatigue Loading In Vivo

Bo Wu,[1] Chan Zhang,[1] Bo Chen,[1] Ling Zhang,[1] Ruchun Dai,[1] Xianping Wu,[1] Yebin Jiang,[2] and Eryuan Liao[1]

[1] *Institute of Metabolism and Endocrinology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China*
[2] *Department of Radiology, University of Michigan Hospitals, 1500 E Medical Center Drive, Ann Arbor, MI 48109, USA*

Correspondence should be addressed to Ruchun Dai; dairuchun@yahoo.com.cn

Received 3 January 2013; Accepted 24 March 2013

Academic Editor: Peng-Fei Shan

Bone microdamage can be repaired through bone remodeling induced by loading. In this study, a loading device was developed for improved efficiency and the self-repair process of bone microdamage was studied in ovariectomized rats. First, four-point bending fixtures capable of holding two live rats simultaneously were designed. Rats were loaded and subjected to a sinusoidal wave for 10,000 cycles. They were then divided into four groups to evaluate time points from 1 to 4 weeks in the microdamage repair process. The loaded right ulna was used for microdamage parameter analysis, and the loaded right radius was tested for mechanical properties. In all groups, microdamage consisted primarily of microcracks, which were observed in bone surrounding the force-bearing point. The values of the microdamage parameters were significantly lower at 3 weeks than at 2 weeks. However, none of the differences in mechanical properties between any four groups were statistically significant. This study shows that the improved application of loading in the form of bending for double-rat simultaneous administration was practical and efficient. These results suggest that microdamage was repaired between 2 weeks to 3 weeks after fatigue damage and microdamage is a more sensitive index of bone quality than mechanical properties.

1. Introduction

From an engineering materials perspective, fatigue loading conditions can cause the formation and accumulation of microdamage. In bone, microdamage causes a gradual loss of stiffness, which may be considered an indicator of impending failure. Bone develops microdamage after cyclic loading. This triggers remodeling processes in order to maintain skeletal integrity. Animal models have shown that microdamage defects tend to be elliptical in shape tend emerging between osteons, and growing parallel to them [1]. Microcrack parameters can be considered alternative parameters in the evaluation of bone biomechanical quality but diffuse damage cannot [2]. Microcrack length plays an important role in fatigue damage. Microcracks can also be a barrier to microdamage propagation [3]. If the microcracks are $<100\,\mu$m in length, they may stop at osteonal boundaries, but longer microcracks (length $>400\,\mu$m) pass through cement lines [4, 5]. Microdamage accumulation is often accompanied by reduced fracture resistance. Three-point bending tests have shown that bone stiffness may drop by 41%, and nanoindentation testing indicated that elastic modulus tissue material properties can be reduced by 26%. The differentials are mainly related to the effects of microcracks [6].

A number of animal microdamage models have used fatigue testing to replicate fatigue damage. Some researchers apply fatigue testing ex vivo to distinguish between different changes in mechanical properties after potential and possible drug therapy. In vivo microdamage models are also used. In these studies, rat ulnae are loaded until 30–40% loss of stiffness is attained [6, 7]. Most models use an axial fatigue loading pattern [7–10]. However, it is difficult to locate the microdamage and force bearing points in these cases. The solution proposed involves using 60% of the total bone length of the ulna or 50% of the humerus [7]. This is not ideal for studies of the relationship between microdamage and stress.

Fatigue loading in vivo has been used to study microdamage-targeted remodeling research. Bone is a living biological material that can repair microdamage and consists of basic multicellular units (BMU). Osteocytes are thought to sense bone damage and mechanical changes induced by intermittent loading and signal to osteoclasts to remove microdamage, and to osteoblasts to form new bone [11]. If the damage is under a certain microdamage threshold, it is repaired by focal bone remodeling. Otherwise, it may lead to a possible fatigue fracture. Microdamage has been shown to trigger the activation of microdamage repair-targeted remodeling [6]. However, linear microcracks, but not diffuse microdamage, drive focal injury induced apoptosis and activates resorption [12]. It has been postulated that mechanical loading may be necessary for microdamage repair and remodeling [13]. Despite extensive work, there has been a limited description of the microdamage repair process induced by intermittent loading of bone. Researchers often apply axial loading, which does not facilitate location of the force-bearing point or the exact position of microdamage. Four-point bending loading fatigue testing has also been conducted in some studies, which circumvents these problems with axial loading. Unfortunately, this type of loading can be time-consuming. The fixtures in these studies allow loading of only one rat at a time. In the present study, we attempt to explain the self-repair process of bone microdamage in ovariectomized (OVX) Sprague-Dawley rats, using an in vivo four-point bending fatigue loading method. Specifically, our research group developed a four-point bending fatigue loading apparatus that can be used on two rats simultaneously. This can produce horizontal fatigue loading and make force-bearing points easy to locate.

2. Methods

2.1. Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Second Xiangya Hospital of Central South University. Forty Sprague-Dawley rats (female, 7 months old, 350 ± 55 g) were used for this study. The ovariectomy was performed by making an incision on the back to expose the ovaries. The ovaries were clamped and removed with the fallopian tubes being ligated, and the skin was then sutured. Then rats were subjected to loading 3 weeks later after operation using the pressure ended method. Rats were anaesthetized using pentobarbitone injection (2% pentobarbitone, 1.5 mL/Kg) during loading. Before and after loading, the rats were allowed unrestricted cage activity and unlimited access to food and water. At the end of the experiment, animals were euthanized with injection of pentobarbitone into the peritoneal cavity.

2.2. Development of Loading Fixture for Two Rats In Vivo. Using the four-point bending principle, we developed a fixture that would allow us to subject two rats to sustained bending fatigue loading simultaneously (China patent number: 201110060409.9). This apparatus is compatible with a PLD-5010 fatigue damage electronic machine (Changchun Research Institute of Test Machines, Changchun, China, Patent number: ZL00225310.0). This machine converts the rotary motion of a motor into rectilinear motion of the mechanical component.

The fixture was composed of the main loading assembly and loading plates. The anesthetized rats were placed on the loading plates, with room for two rats. The plate position was easily adjusted to accommodate different sizes of rats (Figure 1). The main loading assembly contains the upper connecting rod, linear compression spring, lower connecting rod, upper indenter, and lower supports. The spring was provided with a connecting shaft to the upper connecting rod with a groove connection, facilitating quick and easy disassembly. The maximum load of the spring was 100 N, exceeding the force needed to model the microdamage. This allowed the user to control the load placed on rats' limbs by controlling the deformation and testing force, which were shown in real time on the screen of the measurement system. A T-shaped slide was connected to the upper indenter. This slide contained a rolling bearing in order to reduce the lateral force that may be generated during the testing process and ensure accuracy. The upper indenter could also be moved to accommodate differently sized rats.

This device can produce horizontal fatigue loading and facilitate the precise location of the force bearing point using a mechanical sensor connected to the lower supports, ensuring loading is conducted on the forearms of the rat until a pressure endpoint is reached.

2.3. Fatigue Loading In Vivo and Establishment of Self-Repair Model. The rats were anesthetized through intraperitoneal injection of 2% pentobarbitone prior to loading. The right antebrachium of each rat was placed on the lower support bars, and the force bearing point was marked. Cyclic fatigue loading was performed at loads of 0.058 N/g (4 Hz, sinusoidal wave, 10,000 cycles, once every other day for 2 weeks). Each rat's left forearm served as a control. The rats were randomly divided into four groups, sacrificed at 1 week (1st week group), 2 weeks (2nd week group), 3 weeks (3rd week group), and 4 weeks (4th week group 4) after fatigue loading. In order to evaluate bone formation, tetracycline (30 mg/kg, 13 and 14 days before euthanasia) and calcein (5 mg/kg, 3 and 4 days before sacrifice) were given to all rats by intraperitoneal injection.

2.4. Basic Fuchsin Staining and Optical Microscopy. After excision of the ulnae, we defined the force-bearing point on the distal ulna as point 1, the point on the proximal ulna as point 4 (upward force), and the middle force-bearing points on the ulna as points 2 and 3 (downward force), and then marked the force-bearing point using thin wire. Ulnae were dehydrated and stained in ascending series of alcohols containing 1% basic fuchsin. They were then subjected to hyalinization and dimethylbenzene and embedded in poly-methylmethacrylate until polymerization was complete. Thick sections (80–100 μm) were then cut transversely and sequentially using a diamond saw. Sections were observed using a Leica DMLA polarized light microscopy (Leica Corporation, Wetzla, Germany). A charge coupled device (CCD) Leica DFC500 (Leica Corporation, Wetzla, Germany) camera

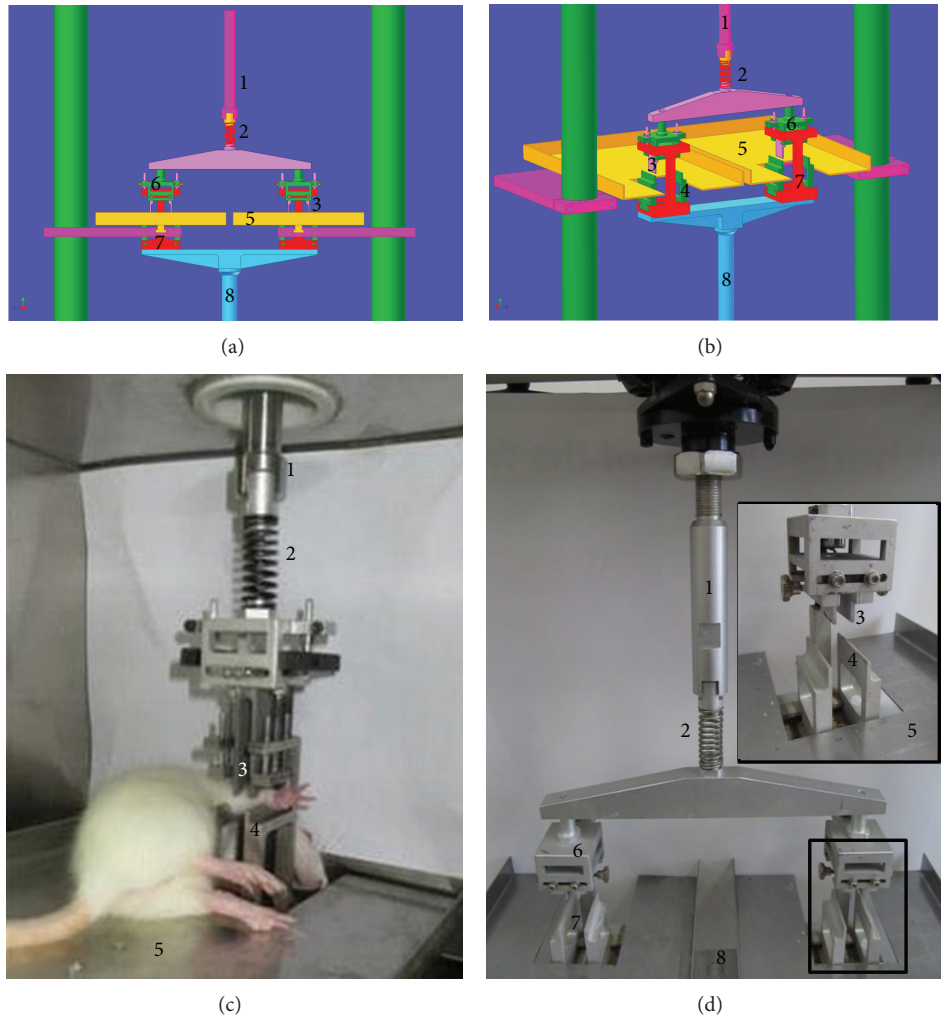


FIGURE 1: The four-point bending fatigue test apparatus is an open loop system consisting of the following components: (1) upper connecting rod, (2) linear compression spring, (3) upper indenter, (4) lower supports, (5) loading plates where the rat is placed, (6) T-shaped upper indenter slide, (7) C-shaped lower support slide, and (8) lower connecting rod. The picture shows the following. (a) Front view of the apparatus. (b) Lateral view. (c) Fixture with a single rat. (d) Fixture system for two rats.

was used to capture images of areas where microdamage was found.

Image-analysis software (Leica Qwin image-analysis system, Leica Corporation, Wetzla, Germany) was used to calculate the values of microdamage parameters, such as average microcrack length (Cr.Le), number of microcracks (Cr.N), microcrack surface density (Cr.S.Dn), microcrack density (Cr.Dn), and resorptive space density (Rs.Sp.Dn). These values may indicate the resorptive activity of the remodeling process. Data from each ulna from several sections (4–6/ulna) are summarized [2].

2.5. Evaluation of the Mechanical Properties of the Radius. Three-point bending tests to failure were performed under displacement control conditions (electronic universal testing machine WDW3100 (Changchun Research Institute of Test Machines, Changchun, China)). Loading was conducted with the loaded right radius lying on two supports with a span of 12 mm. The cross-head speed was 0.2 mm/s. Matched image analysis software was used to draw the load-deformation curve. Software was used to calculate the linear region between 30% and 50% peak load.

2.6. Statistical Analysis. To determine whether fatigue loading caused any decreases in the average length of the microdamage defects, Cr.Le and Cr.S.Dn were averaged and compared to values in the corresponding time groups using independent t-tests. The Kruskal-Wallis ANOVA test was used to analyze the loss of mechanical properties, and the Mann-Whitney U test was use to analyze Rs.Sp.Dn and Cr.Dn. Significance was set at $P < 0.05$, and data are reported as mean ± standard error of the mean.

3. Results and Discussion

3.1. Results. Microdamage was observed in all of the loaded ulnae. Microcracks were found to emerge in the region between force-bearing points 1-2 and points 3-4 (on both

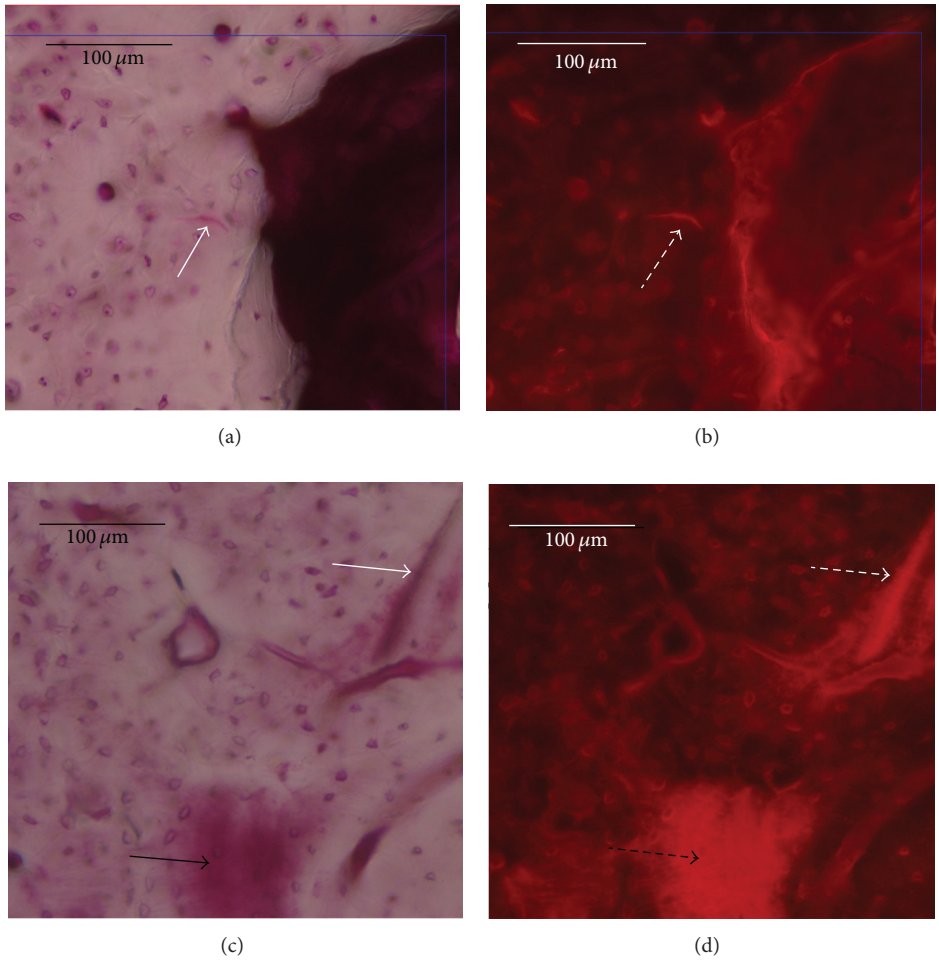


Figure 2: Basic fuchsin staining showing microcracks (white arrow) in (a) OVX 2nd week group rats and (c) OVX 1st week group rats. (b, d) Red epifluorescent light microscopy showing the same microcrack (white dashed arrow) in the identical view. Diffused microdamage (black arrow) was detected in (c) cortical bone (black arrow) and under (d) red epifluorescent light microscopy (black dashed arrow). Scale bars = 100 μm.

sides of the bone). Diffuse microdamage was observed in the region between points 2 and 3 (middle of the bone). The greatest amount of microdamage was observed around the force-bearing point, primarily in the form of microcracks (Figure 2). No signs of soft tissue trauma, hematoma, disruption to the periosteum, or intramedullary vasculature were observed.

3.1.1. Microdamage Parameters. The values of microdamage parameters, such as average microcrack length (Cr.Le), number of microcracks (Cr.N), microcrack surface density (Cr.S.Dn), and microcrack density (Cr.Dn), were significantly lower in group 2 than in group 3 ($P < 0.05$), but no statistically significant difference was observed between groups 1 and 2 or between groups 3 and 4 ($P > 0.05$) (Figure 3).

3.1.2. Mechanical Properties. Peak loading and elasticity modulus were used as indexes to determine the mechanical properties of the fatigue-loaded radius bones of rats. No statistically significantly differences in peak loading or elasticity modulus were observed among the four groups ($P > 0.05$) (Figure 3, Table 1).

3.1.3. Variations in Absorptive Space. Absorptive spaces were observed primarily during the first week after fatigue damage. They remained visible during the second and third weeks, but were almost undetectable during the fourth week. The process of osteogenesis can be observed through the two fluorescent lines produced by tetracycline and calcein (Figure 4). The differences in the density of the resorptive spaces (Rs.Sp.Dn) between groups 1 and 2 and between groups 2 and 3 were found to be statistically significant ($P < 0.05$). However, the difference between groups 3 and 4 groups was not statistically significant ($P > 0.05$) (Figure 3, Table 1).

3.2. Discussion. To our knowledge, the present study is one of the only studies to develop a fixture capable of loading two rats at a time and identify the window of time during which the self-repair of microdamage takes place after fatigue loading in vivo. Rat ulna bone microdamage was morphologically

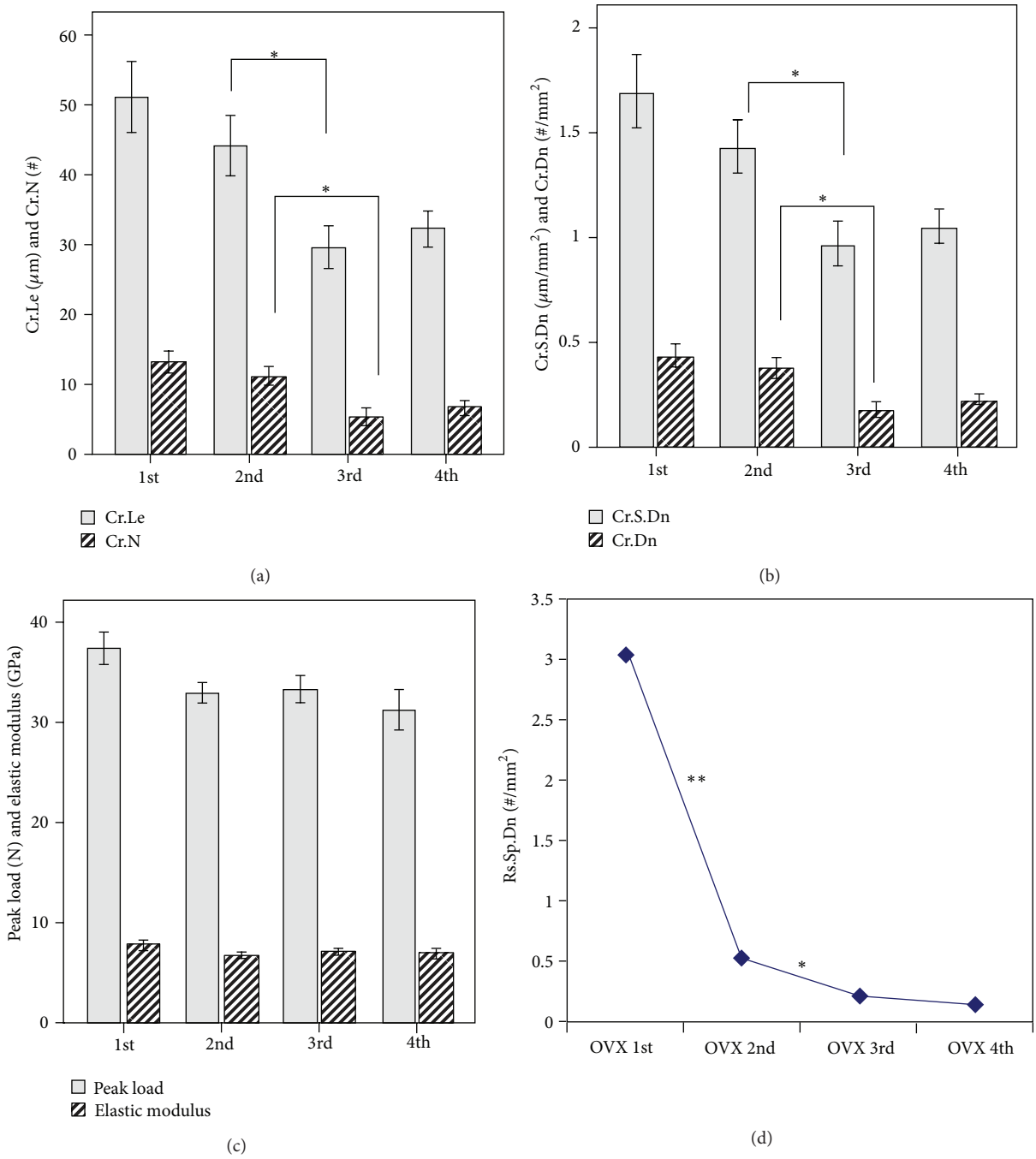


Figure 3: Parameters and mechanical properties of microcracks and absorptive spaces versus time. Specific values are listed in Table 1. (a) Average microcrack length (Cr.Le) and average number of microcracks (Cr.N), (b) microcrack surface density (Cr.S.Dn) and microcrack density (Cr.Dn), (c) peak load and modulus of elasticity, and (d) absorptive lacunar density. $^{*}P < 0.05$, $^{**}P < 0.01$.

self-repaired 2-3 weeks after fatigue damage. Microdamage was detected after 4 weeks of fatigue loading. One limitation of this study is that grouping should be added and maintained until microdamage is completely repaired. Microdamage may be a more sensitive index of bone quality than mechanical properties.

Microdamage events are varied, and have different mechanical effects. At least 3 different types of microdamage should be considered. Microcracks and diffuse microdamage have been identified as two kinds of microdamage. Microfractures may or wispy microdamage also be a third, but is not as established. These different forms of damage not only show distinct morphology but are also repaired through different mechanisms. Microcracks and diffuse damage are repaired by damage-targeted remodeling [14]. Linear microcracks can induce self-repair through osteocyte-activated

Table 1: Microdamage and absorptive space parameters ($\overline{x} \pm s$).

	OVX 1st ($n = 9$)	OVX 2nd ($n = 9$)	OVX 3rd ($n = 9$)	OVX 4th ($n = 8$)
Cr.Le (μm)	51.20 ± 15.17	44.21 ± 13.00[a]	31.53 ± 7.96	29.71 ± 8.98
Cr.S.Dn (μm/mm^2)	1.70 ± 0.54	1.41 ± 0.300[a]	1.01 ± 0.26	0.96 ± 0.29
Cr.Dn (#/mm^2)	0.43 ± 0.15	0.36 ± 0.15[a]	0.200 ± 0.12	0.18 ± 0.09
Rs.Sp.Dn (#/mm^2)	3.032[b]	0.530[a]	0.208	0.143

Values are expressed as means ± SD.
Cr.Le: mean microcrack length; Cr.Dn: microcrack density; Cr.S.Dn: microcrack surface density; Rs.Sp.Dn: absorptive space density.
[a]$P < 0.05$, relative to with the OVX 3rd week groups; [b]$P < 0.05$, relative to the OVX 2nd week group.

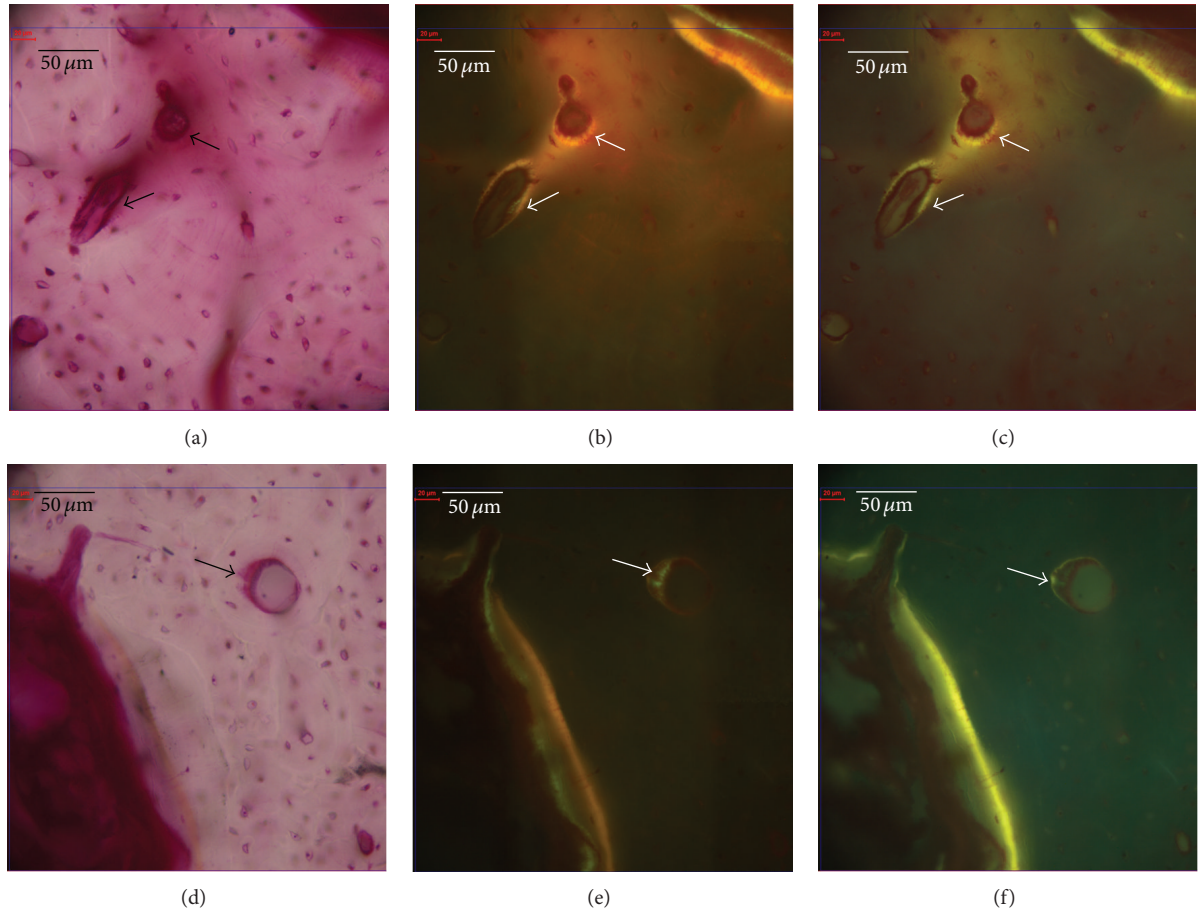


Figure 4: (a, d) Basic fuchsin staining showed resorption spaces (black arrow) in the cortical bone. Blue-violet epifluorescent light microscopy revealed area of osteogenesis (white arrow) and (b, e) green fluorescence attributable to calcein showing osteogenesis during the 4 days preceding euthanasia, (b, e) orange fluorescence and (c, f) yellow fluorescence attributed to acheomycin, which indicated osteogenesis in the 2 weeks before euthanasia. Scale bars = 50 μm.

resorption, but diffuse microdamage cannot. This may be due to a lack of apoptotic responses [12]. However, microfractures are repaired in the same manner as normal fractures. This process involves endochondral ossification [15]. Calluses form over fractures in the damaged area. These are eventually remodeled into normal trabecular structures. In the present study, the microdamage detected was mostly microcracks. Other types of damage include diffuse microdamage. No wispy microdamage was found. Microfractures tend to occur only in cancellous bone. We observed only 2 kinds of microdamage in the cortical bones of rats. Loading can cause microdamage, but many studies apply axial fatigue loading, which may limit research meant to explore the relationship between microdamage and stress. Four-point bending loading may address this problem somewhat by allowing the user to more accurately position of the force-bearing point and the microdamage. Some studies have involved bending fatigue loading [16–18]. However, this method requires repeat loading, and existing fixtures can load only one rat at a time, which is inefficient. For this reason, we designed a new type of fixture that can accommodate two rats simultaneously to will improve the efficiency of fatigue testing.

In the bending fatigue test, the force-bearing side experiences pressure load, and the opposite side experiences tensile load with fractures occurring on the tensile side. In our study with four-point bending fatigue testing, microcracks were more common in the pressure side of the bone. This conclusion is similar to the results of another bending fatigue test applied to the long bones of cows [19]. There is an obvious difference between the two loading methods. During axial loading, microdamage, including microcracks and diffused microdamage, were concentrated on ulnar side of the ulna. This may have been because the ulnar side of the bone experienced stronger compressive and tensile force during axial loading. In our study, microcracks emerged on both sides of the bone. Diffused microdamage appeared in the middle of the bone. Distinct categories of force may explain the reason for the diversity observed with respect to microdamage. Shear stress was found to be related to fatigue microcracks and not to diffused microdamage [20]. There was pronounced shear stress between both side of the bone and the areas between two force-bearing points, where one point received upward force while the other point received downward force (point 1 to 2 and point 3 to 4). But no obvious shear stress appeared in the middle of bone (point 2 to 3). This may because it was located between two homodromous pressure points.

To the best of our knowledge, no studies have focused on the time window during which targeted remodeling of microcracks take place. Periosteal woven bone formation can be detected 3–7 days after stress fracture [21]. More pronounced callus formation can be observed 2–4 weeks after osteoporotic fractures. This was demonstrated by callus width and area measurement [22]. Our research indicated that microdamage on rat ulna was mainly repaired between 2-3 weeks of fatigue loading.

It has become clear that microdamage-induced resorption occurs mechanistically via osteocyte apoptosis [14]. Fatigue loading induces microdamage, which may occur in parallel with osteocyte apoptosis and bone resorption [6]. Treatment with apoptotic inhibitors can prevent apoptosis and resorption, with a possible dose-response relationship between the two processes [14]. In the present study, we observed resorption space after one week of fatigue loading. The number of resorption spaces decreased markedly after two weeks of loading. The number of resorption spaces on the ulna was about 6 times greater in cancellous bone than in cortical bone. This may be because the osteoclasts migrate more easily in cancellous bone and because the osteoclast must migrate a larger distance to reach the microdamaged areas in the cortical bone. This also explains why the resorption spaces were more commonly found to localize with regions near the periosteum. It was recently proven that the activation of resorption can be induced by enhanced expression of RANKL [15]. The response may depend on the size of the microcrack. It has been thought that larger cracks may involve greater increases in RANKL and greater decreases in OPG than smaller cracks [23].

Only a few studies have focused on microdamage parameters or considered them as indexes of the curative effects of drugs. Our results indicate that microdamage is a more sensitive index than mechanical properties from our research results. Microdamage parameters may reveal the efficacy of drugs from the microdamage repair field. For example, bisphosphonate may reduce the risk of fracture, but could also impair the repair of microdamage by targeted remodeling. This may be due to oversuppression of bone turnover [24]. Neither estrogen nor raloxifene decreases microcrack surface density or improves mechanical properties of the bone [24, 25]. The effects and limits of many different kinds of drugs may be examined using these techniques.

4. Conclusion

In conclusion, we developed a fatigue loading fixture and improved its efficiency. Our data support the conclusion that rat ulna bone microdamage is morphologically self-repaired between week 2 and week 3. Bone microdamage can be visualized and may be a more sensitive index than mechanical properties. Future work based on this research could confirm these results and involve the use of this fixture for the study of bone fatigue loading physiology and for the testing of drug efficacy.

Acknowledgments

This work was supported by the Natural Science Foundation of China (NSFC, 81070695, and 81270960) and the Program for New Century Excellent Talents in University (NCET-11-0507). The authors would also like to thank engineer Xuehua Liang for assistance in the manufacturing of the loading fixture.

References

[1] D. Taylor and T. Clive Lee, "Measuring the shape and size of microcracks in bone," *Journal of Biomechanics*, vol. 31, no. 12, pp. 1177–1180, 1998.

[2] R. C. Dai, E. Y. Liao, C. Yang, X. P. Wu, and Y. Jiang, "Microcracks: an alternative index for evaluating bone biomechanical quality," *Journal of Bone and Mineral Metabolism*, vol. 22, no. 3, pp. 215–223, 2004.

[3] A. M. Parfitt, "Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression," *Bone*, vol. 30, no. 1, pp. 5–7, 2002.

[4] S. Mohsin, F. J. O'Brien, and T. C. Lee, "Osteonal crack barriers in ovine compact bone," *Journal of Anatomy*, vol. 208, no. 1, pp. 81–89, 2006.

[5] T. Diab and D. Vashishth, "Morphology, localization and accumulation of in vivo microdamage in human cortical bone," *Bone*, vol. 40, no. 3, pp. 612–618, 2007.

[6] O. Verborgt, G. J. Gibson, and M. B. Schaffler, "Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo," *Journal of Bone and Mineral Research*, vol. 15, no. 1, pp. 60–67, 2000.

[7] S. J. Sample, Z. Hao, A. P. Wilson, and P. Muir, "Role of calcitonin gene-related peptide in bone repair after cyclic fatigue loading," *PLoS ONE*, vol. 6, no. 6, Article ID e20386, 2011.

[8] J. Macione, N. B. Kavukcuoglu, R. S. A. Nesbitt, A. B. Mann, N. Guzelsu, and S. P. Kotha, "Hierarchies of damage induced

loss of mechanical properties in calcified bone after in vivo fatigue loading of rat ulnae," *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 4, no. 6, pp. 841–848, 2011.

[9] A. E. Tami, P. Nasser, M. B. Schaffler, and M. L. Knothe Tate, "Noninvasive fatigue fracture model of the rat ulna," *Journal of Orthopaedic Research*, vol. 21, no. 6, pp. 1018–1024, 2003.

[10] S. J. Sample, R. J. Collins, A. P. Wilson et al., "Systemic effects of ulna loading in male rats during functional adaptation," *Journal of Bone and Mineral Research*, vol. 25, no. 9, pp. 2016–2028, 2010.

[11] R. D. Chapurlat and P. D. Delmas, "Bone microdamage: a clinical perspective," *Osteoporosis International*, vol. 20, no. 8, pp. 1299–1308, 2009.

[12] B. C. Herman, L. Cardoso, R. J. Majeska, K. J. Jepsen, and M. B. Schaffler, "Activation of bone remodeling after fatigue: differential response to linear microcracks and diffuse damage," *Bone*, vol. 47, no. 4, pp. 766–772, 2010.

[13] E. I. Waldorff, K. B. Christenson, L. A. Cooney, and S. A. Goldstein, "Microdamage repair and remodeling requires mechanical loading," *Journal of Bone and Mineral Research*, vol. 25, no. 4, pp. 734–745, 2010.

[14] L. Cardoso, B. C. Herman, O. Verborgt, D. Laudier, R. J. Majeska, and M. B. Schaffler, "Osteocyte apoptosis controls activation of intracortical resorption in response to bone fatigue," *Journal of Bone and Mineral Research*, vol. 24, no. 4, pp. 597–605, 2009.

[15] D. B. Burr, "Why bones bend but don't break," *Journal of Musculoskeletal and Neuronal Interactions*, vol. 11, no. 4, pp. 270–285, 2011.

[16] M. D. Landrigan and R. K. Roeder, "Systematic error in mechanical measures of damage during four-point bending fatigue of cortical bone," *Journal of Biomechanics*, vol. 42, no. 9, pp. 1212–1217, 2009.

[17] M. D. Roberts, T. J. Santner, and R. T. Hart, "Local bone formation due to combined mechanical loading and intermittent hPTH-(1-34) treatment and its correlation to mechanical signal distributions," *Journal of Biomechanics*, vol. 42, no. 15, pp. 2431–2438, 2009.

[18] C. Arregui-Dalmases, J. H. Ash, E. Del Pozo, J. R. Kerrigan, and J. Crandall, "Failure of the lumbar pedicles under bending loading—biomed 2010," *Biomedical Sciences Instrumentation*, vol. 46, pp. 148–153, 2010.

[19] T. Diab and D. Vashishth, "Effects of damage morphology on cortical bone fragility," *Bone*, vol. 37, no. 1, pp. 96–102, 2005.

[20] Y. N. Yeni, F. J. Hou, T. Ciarelli, D. Vashishth, and D. P. Fyhrie, "Trabecular shear stresses predict in vivo linear microcrack density but not diffuse damage in human vertebral cancellous bone," *Annals of Biomedical Engineering*, vol. 31, no. 6, pp. 726–732, 2003.

[21] G. R. Wohl, D. A. Towler, and M. J. Silva, "Stress fracture healing: fatigue loading of the rat ulna induces upregulation in expression of osteogenic and angiogenic genes that mimic the intramembranous portion of fracture repair," *Bone*, vol. 44, no. 2, pp. 320–330, 2009.

[22] W. H. Cheung, S. K. H. Chow, M. H. Sun, L. Qin, and K. S. Leung, "Low-intensity pulsed ultrasound accelerated callus formation, angiogenesis and callus remodeling in osteoporotic fracture healing," *Ultrasound in Medicine and Biology*, vol. 37, no. 2, pp. 231–238, 2011.

[23] L. E. Mulcahy, D. Taylor, T. C. Lee, and G. P. Duffy, "RANKL and OPG activity is regulated by injury size in networks of osteocyte-like cells," *Bone*, vol. 48, no. 2, pp. 182–188, 2011.

[24] J. M. O'Neal, T. Diab, M. R. Allen, B. Vidakovic, D. B. Burr, and R. E. Guldberg, "One year of alendronate treatment lowers microstructural stresses associated with trabecular microdamage initiation," *Bone*, vol. 47, no. 2, pp. 241–247, 2010.

[25] O. Brennan, O. D. Kennedy, T. C. Lee, S. M. Rackard, and F. J. Obrien, "Effects of estrogen deficiency and bisphosphonate therapy on osteocyte viability and microdamage accumulation in an ovine model of osteoporosis," *Journal of Orthopaedic Research*, vol. 29, no. 3, pp. 419–424, 2011.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 715025, 9 pages
http://dx.doi.org/10.1155/2013/715025

Research Article

Prevalence of Fracture Risk Factors in Postmenopausal Women Enrolled in the POSSIBLE US Treatment Cohort

Nicole Yurgin,[1] Sally Wade,[2] Sacha Satram-Hoang,[3] David Macarios,[1] and Marc Hochberg[4]

[1] *Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320-1799, USA*
[2] *Wade Outcomes Research and Consulting, 358 South 700 East, Suite B432, Salt Lake City, UT 84102, USA*
[3] *Q.D. Research, Inc., 8789 Auburn Folsom Road, Suite C501, Granite Bay, CA 95746, USA*
[4] *Division of Rheumatology and Clinical Immunology, University of Maryland School of Medicine, 10 S. Pine Street, MSTF 8-34, Baltimore, MD 21201, USA*

Correspondence should be addressed to Nicole Yurgin; nyurgin@amgen.com

Received 21 December 2012; Revised 21 February 2013; Accepted 7 March 2013

Academic Editor: Cory Xian

Subject- and physician-reported data from 4,429 postmenopausal women receiving osteoporosis treatment in the Prospective Observational Scientific Study Investigating Bone Loss Experience (POSSIBLE US) were used to assess the prevalence of risk factors (RFs) and on-study fracture. RFs assessed at study entry were age >70 years; fracture since age 50; minimum T-score (hip/spine) ≤ -2.5 at diagnosis; body mass index $<18.5\,kg/m^2$; rheumatoid arthritis; parental history of hip fracture; current smoking; and recent oral glucocorticoid use. Data were collected with semiannual self-administered questionnaires. Results were stratified by physician-reported osteoporosis/osteopenia diagnosis. Low T-score and age >70 years were the most common RFs in the osteoporosis group, and age >70 years and prior fracture were the most common risk factors in the osteopenia group. Multiple RFs were more common than a single RF in osteoporotic women (54.2% versus 34.6%; $P < 0.0001$) but not osteopenic women (13.8% versus 33.6%; $P < 0.0001$). Women with multiple RFs had more on-study osteoporosis-related fractures than women with a single RF (osteoporosis group: 9.9% versus 6.2%; $P = 0.0092$; osteopenia group: 11.2% versus 4.7%; $P < 0.0001$). In postmenopausal women receiving osteoporosis treatment, multiple RFs increased fracture risk. RFs, in addition to bone mineral density, can help identify candidates for osteoporosis treatment.

1. Introduction

The WHO fracture risk assessment tool (FRAX) is a computer-based algorithm that assesses fracture probability of men and women [1]. FRAX was developed using clinical risk factor data from population-based cohorts with 250,000 person-years of followup from Europe, North America, and Japan [2]. This approach uses easily obtained information on clinical risk factors to estimate the 10-year risk of hip fracture and major osteoporotic fracture (spine, forearm, hip, or shoulder) and was incorporated into the United States (U.S.) Preventive Services Task Force osteoporosis screening recommendations in 2011 [3]. The National Osteoporosis Foundation (NOF) also recommends that physicians use FRAX when possible, along with a detailed medical history, physical examination, and bone mineral density (BMD) assessment to diagnose osteoporosis and guide treatment decisions [4]. However, little is known about the prevalence of specific fracture risk factors in postmenopausal women who are currently being treated for osteoporosis.

In the current study, the prevalence of risk factors for fracture in a treated population was assessed using data from the Prospective Observational Scientific Study Investigating Bone Loss Experience in the US (POSSIBLE US), which was a large, longitudinal cohort study of postmenopausal women who were prescribed osteoporosis therapy in a primary care setting [5]. We also examined the associations between the number of risk factors per subject at study entry and the incidence of on-study fracture. We computed FRAX scores using the online calculation tool, assessed the distribution of these scores, and examined the on-study fracture experience of women with different risk strata.

2. Methods

2.1. Data Source and Study Population. From October 2005 to January 2007, 134 primary care physicians in the US enrolled 5,015 postmenopausal women who were receiving treatment for bone loss into the POSSIBLE US treatment cohort. The study design and subject characteristics have been previously described [5]. Briefly, all subjects in this Institutional Review Board-approved study had been identified for osteoporosis therapy by their primary care physician and prescribed ≥1 of the following osteoporosis medications: oral bisphosphonate (i.e., alendronate/alendronate sodium with cholecalciferol, risedronate/risedronate with calcium, and ibandronate); oral or transdermal postmenopausal estrogen; parathyroid hormone; calcitonin; raloxifene; or calcium; and/or vitamin D. Calcium and vitamin D supplements were classified as nonpharmacological therapy. Since this was an observational study of routine care, prescribing decisions were based on the judgment of the enrolling physicians. After providing informed consent at a routine visit with the enrolling primary care physician, each subject completed a self-administered baseline questionnaire to report demographic characteristics and lifestyle behaviors, osteoporosis medication use, satisfaction and side effects related to these osteoporosis therapies, and health-related quality of life. Followup questionnaires (mailed to subjects every 6 months after entry for up to 3 years) also included questions about the occurrence of on-study fracture. For each subject, the enrolling physician provided relevant medical history at both study entry and for routine followup visits.

Women from the POSSIBLE US cohort who had a physician-reported diagnosis of osteoporosis or osteopenia on the study enrollment form ($N = 4{,}429$) were included in these analyses. Risk factors were identified using physician- and subject-reported data collected at the subject's enrollment into the study and included: age >70 years; history of fracture since age 50; minimum reported hip or spine T-score ≤ -2.5 at diagnosis; body mass index $<18.5\,kg/m^2$; rheumatoid arthritis; parental history of hip fracture; current cigarette smoking; and oral glucocorticoid use in the 6 months prior to study entry. BMD assessments were not required for study enrollment; however, diagnostic T-scores were available for 89% of subjects with osteoporosis and 92% of subjects with osteopenia.

2.2. Statistical Analysis. Descriptive statistics (counts and percentages) quantified the prevalence of risk factors, with results reported separately for subjects diagnosed by their enrolling physician with either osteoporosis (osteoporosis group) or osteopenia (osteopenia group). Statistical differences between groups were assessed using chi-square tests for categorical data.

Available data were used to calculate the 10-year probability of hip fracture and major osteoporosis-related fracture using the US version of the online FRAX calculation tool [1]. FRAX uses race-specific norms; therefore, FRAX scores were calculated only for POSSIBLE US subjects who reported their race as Caucasian, Hispanic, African American, or Asian. Diagnoses of secondary osteoporosis and relevant alcohol use (≥3 units per day) were defaulted to "no" in the calculation tool for all subjects as these data were not collected in the POSSIBLE US cohort. The distribution of FRAX scores was assessed categorically, and the mean (95% confidence intervals) and median FRAX scores were computed.

Incident fractures reported by subjects throughout follow up were classified as either osteoporosis-related or not osteoporosis-related using a published classification schema [6]. Specifically, fractures occurring at locations listed by Warriner et al. [6] as "more likely to be because of osteoporosis" were considered osteoporosis-related for the purposes of this analysis. The number and percentage of subjects with on-study fracture were reported for all fractures and osteoporosis-related fractures and by fracture location. Fracture incidence was computed separately for subjects with osteoporosis and osteopenia, and results were stratified by the number of risk factors at study entry identified for each subject (0, 1, ≥2).

Chi-square tests for categorical data were used to compare fracture incidence for women who met different risk thresholds (i.e., ≥3% 10-year predicted risk for hip fracture or ≥20% 10-year predicted risk for major osteoporotic fracture) based on NOF classification of FRAX scores. Statistical significance was assessed using pairwise comparisons of the percentage of subjects with on-study fracture for subjects with scores above and below the risk thresholds. Since FRAX was originally developed and validated in untreated populations, we analyzed data for subjects who were not using pharmacologic therapy on entry into POSSIBLE US (i.e., subjects who reported either not using any osteoporosis agent or taking only calcium/vitamin D at or within 2 months of study entry). An additional analysis was conducted using data from pharmacologically treated subjects (i.e., subjects who reported using pharmacological therapy at or within 2 months of entering the study). Missing data were not imputed, and all statistical analyses were conducted using SAS version 9.1 software (SAS Institute Inc., Cary, NC, USA).

3. Results

Data were analyzed for 1,916 women in the osteoporosis group (age: mean 67.8 years; median 61.0 years) and 2,513 women in the osteopenia group (age: mean 62.2 years; median 61.0 years). The majority of women in each group were Caucasian: 87% in the osteoporosis group and 90% in the osteopenia group. Mean followup was 869 days (median: 959 days) for the osteoporosis group and 873 days (median: 932 days) for the osteopenia group. Among the pharmacologically treated patients in the study population, the probability of persisting with the osteoporosis therapy used at study entry was 66% (95% confidence interval: 64%, 68%) at 12 months after study entry [7].

The number of reported risk factors per subject ranged from 0 to 5 (Figure 1). Subjects in the osteopenia group were more likely to have 0 risk factors compared with subjects in the osteoporosis group (52.6% versus 11.2%, $P < 0.0001$). Multiple risk factors were more common than a single risk factor in the osteoporosis group (54.2% versus 34.6%, $P <$

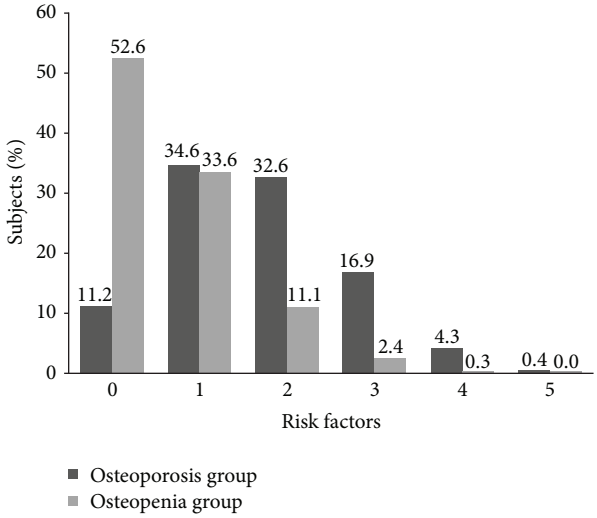


Figure 1: Percentage of subjects with osteoporosis or osteopenia by number of fracture risk factors.

Table 1: Subject-reported on-study fracture.

Subjects with	Osteoporosis group (N = 1,916) n (%)	Osteopenia group (N = 2,513) n (%)
No on-study fracture	1,692 (88.3)	2,284 (90.9)
Any on-study fracture	224 (11.7)	229 (9.1)
Any osteoporosis-related fracture	153 (8.0)	138 (5.5)
Hip fracture	35 (1.8)	22 (0.9)
Spine fracture	35 (1.8)	29 (1.2)
Nonhip/nonspine fracture	104 (5.4)	99 (3.9)
Multiple osteoporosis-related fractures	46 (2.4)	37 (1.5)

0.0001) but not in the osteopenia group (13.8% versus 33.6%, $P < 0.0001$).

Among subjects in the osteoporosis group, most common fracture risk factors (singly and in combination) were hip or spine T-score ≤ −2.5 at diagnosis, age >70 years, and history of fracture since age 50 (Figure 2(a)). These 3 risk factors were reported in 2.8% to 24.1% of osteoporosis subjects with a single risk factor and in 25.4% to 49.6% of subjects with multiple risk factors.

The most common fracture risk factors among subjects in the osteopenia group who had a single risk factor reported were age >70 years, history of fracture since age 50, and current cigarette smoking, occurring in 11.1%, 7.0%, and 7.0% of subjects, respectively (Figure 2(b)). These 3 risk factors along with rheumatoid arthritis occurred in 3.3% to 9.1% of osteopenia subjects with multiple risk factors.

Table 1 summarizes subject-reported on-study fracture. At least 1 on-study fracture of any type was reported by 11.7% of women in the osteoporosis group and 9.1% of women in the osteopenia group ($P = 0.0059$). Overall, 8.0% of the osteoporosis group and 5.5% of the osteopenia group ($P < 0.0001$) reported an osteoporosis-related fracture. The highest incidence of on-study fracture and osteoporosis-related on-study fracture occurred in women with multiple risk factors (Figure 3). The percentage of women in the osteoporosis group reporting any on-study fracture ranged from 7.9% for those with 0 risk factors to 13.4% for those with multiple risk factors, and the percentage with osteoporosis-related fractures ranged from 4.2% for those with 0 risk factors to 9.9% for women with multiple risk factors. Corresponding results for the osteopenia group were from 7.9% to 14.7% for any on-study fracture and from 4.5% to 11.2% for osteoporosis-related fracture. On-study osteoporosis-related fractures were more common in women with multiple risk factors compared with women with a single risk factor in both the osteoporosis group (9.9% versus 6.2%, $P = 0.0092$) and the osteopenia group (11.2% versus 4.8%, $P < 0.0001$, Table 2). Osteoporosis-related fractures at locations other than the hip or spine were the most common in both the osteoporosis and osteopenia groups (Table 2). The percentage of subjects with a nonhip, nonspine osteoporosis-related fracture ranged from 3.3% to 6.2% in the osteoporosis group and from 3.6% to 6.9% in the osteopenia group, depending on the number of risk factors. Similarly, the percentage of subjects with multiple osteoporosis-related fractures ranged from 0.5% to 3.4% in the osteoporosis group and from 1.1% to 3.7% in the osteopenia group.

FRAX scores were computed for 4,295 (97%) subjects in the POSSIBLE US cohort. The mean (95% confidence intervals) 10-year predicted hip fracture risk was 3.8% (3.6%, 4.0%) for subjects pharmacologically treated for bone loss (n = 2,996) and 3.3% (3.1%, 3.6%) for nonpharmacologically treated subjects (n = 1,299). The median scores for these 2 subject groups were 1.8% and 1.4%, respectively. The mean (95% confidence intervals) 10-year predicted risk for any major osteoporotic fracture was 13.6% (13.3%, 14.0%) for pharmacologically treated subjects and 12.4% (11.9%, 12.9%) for nonpharmacologically treated subjects.

A greater percentage of the pharmacologically treated subjects met or exceeded the NOF threshold for major osteoporosis fracture risk compared with nonpharmacologically treated subjects (20.4% versus 15.9%, $P < 0.001$; Figure 4), and this finding also held for the hip fracture risk threshold (34.7% versus 31.3%, $P = 0.033$). On-study fracture incidence was similar for nonpharmacologically treated subjects whose FRAX scores met or exceeded the NOF thresholds for hip fracture or major osteoporotic fracture compared with subjects whose scores were below these thresholds (Table 3). However, similar analyses were conducted for the 2,996 pharmacologically treated subjects with FRAX scores (Table 4), and the incidence of on-study fracture (any and osteoporotic) was significantly higher among subjects whose risk scores met or exceeded the NOF thresholds ($P < 0.0001$). Overall, 5.7% of the 407 subjects who met or exceeded the 3% hip fracture risk threshold experienced an on-study fracture compared with 5.5% of the 892 subjects with scores below the threshold ($P = 0.95$). Of the 206 subjects who met or exceeded the 20% threshold for major osteoporotic fracture risk, 6.8% experienced an on-study fracture compared with

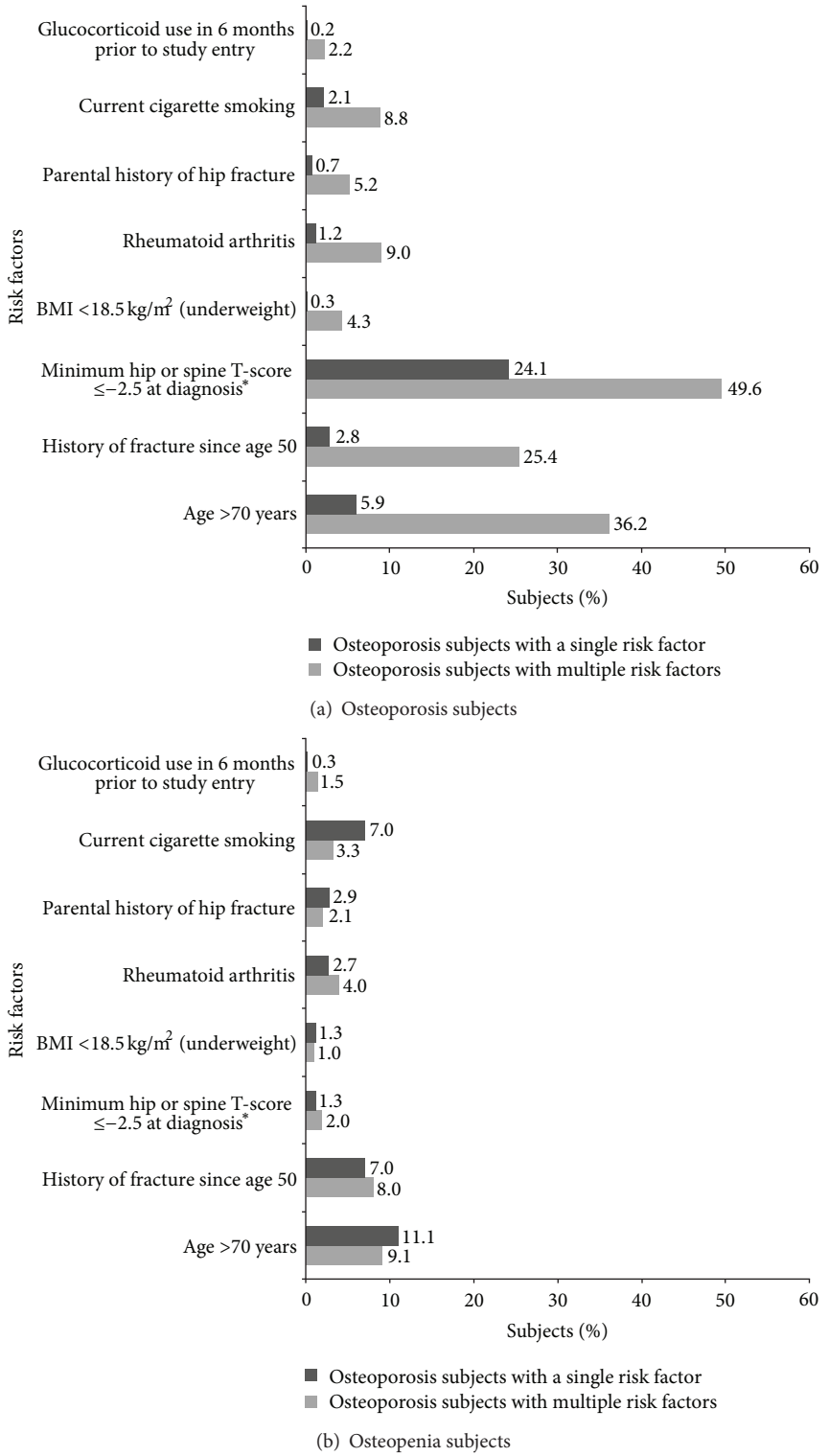
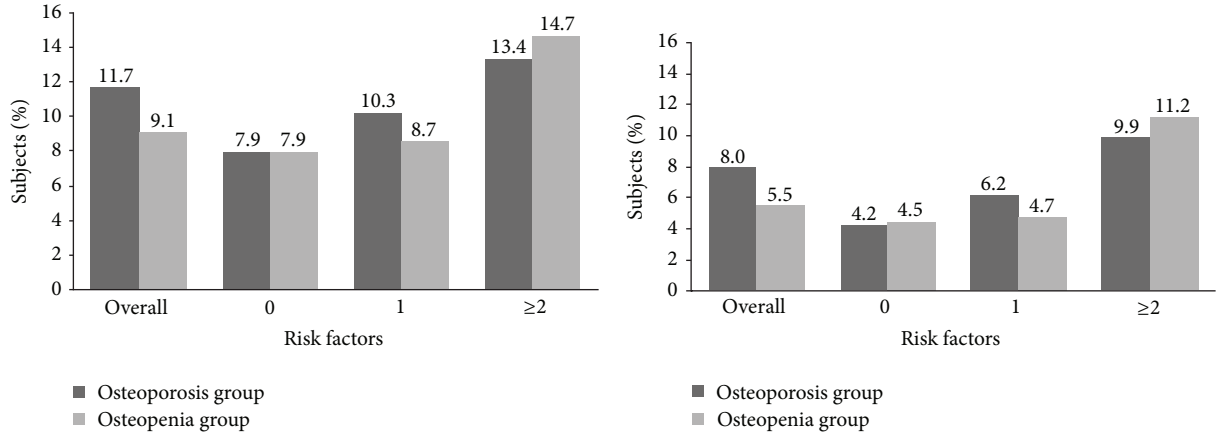


Figure 2: Prevalence of fracture risk factors among subjects with osteoporosis or osteopenia. *Number of subjects with minimum T-scores: 1,703 (88.9%) in osteoporosis subjects; 2,322 (92.4%) in osteopenia subjects. BMI, body mass index.



(a) Percentage of subjects with any on-study fracture

(b) Percentage of subjects with osteoporosis-related on-study fracture

Figure 3: Percentage of subjects reporting on-study fracture stratified by number of fracture risk factors.

Table 2: Subject-reported on-study osteoporosis-related fracture stratified by number of risk factors.

	Subjects with 0 risk factors	Subjects with 1 risk factor	Subjects with ≥2 risk factors
	n (%)	*n* (%)	*n* (%)
Osteoporotic subjects	214	663	1,039
Any osteoporosis-related fracture	9 (4.2)	41 (6.2)	103 (9.9)
Hip fracture	0 (0)	4 (0.6)	31 (3.0)
Spine fracture	2 (0.9)	7 (1.1)	26 (2.5)
Nonhip/nonspine fracture	7 (3.3)	33 (5.0)	64 (6.2)
Multiple osteoporosis-related fractures	1 (0.5)	10 (1.5)	35 (3.4)
Osteopenic subjects	1,322	843	348
Any osteoporosis-related fracture	59 (4.5)	40 (4.8)	39 (11.2)
Hip fracture	8 (0.6)	6 (0.7)	8 (2.3)
Spine fracture	11 (0.8)	7 (0.8)	11 (3.2)
Nonhip/nonspine fracture	47 (3.6)	28 (3.3)	24 (6.9)
Multiple osteoporosis-related fractures	14 (1.1)	10 (1.2)	13 (3.7)

Risk factors: age > 70 years, history of fracture since age 50, minimum hip or spine T-score ≤ −2.5 at diagnosis, body mass index < 18.5 kg/m^2, rheumatoid arthritis, parental history of hip fracture, current cigarette smoking, and glucocorticoid use in 6 months prior to study entry.

5.3% of the 1,093 whose FRAX scores were below the threshold ($P = 0.32$). There were also no statistically significant differences in the incidence of osteoporosis-related on-study fractures relative to the NOF risk thresholds.

In the 2,996 pharmacologically treated subjects with FRAX scores (Table 4), the incidence of any on-study fracture was significantly higher among subjects whose risk scores met or exceeded the NOF thresholds (16.7% versus 10.1%, $P < 0.0001$). The same pattern was observed for osteoporosis-related fractures, which occurred in 12.0% of pharmacologically treated subjects who met or exceeded the treatment threshold compared with 5.8% of pharmacologically treated subjects whose FRAX scores were below the NOF threshold ($P < 0.0001$). In combined analysis of pharmacologically treated and nonpharmacologically treated subjects with FRAX scores ($n = 4{,}295$), 13.6% of the subjects with predicted 10-year hip fracture risk of ≥3% experienced an on-study fracture, and 8.7% of the subjects with predicted hip fracture risk were below this threshold ($P < 0.0001$). Similarly, 16.2% of the subjects with predicted 10-year major osteoporotic fracture risk of ≥20% experienced an on-study fracture. This compares with 5.0% of the subjects who had a predicted risk of major osteoporosis fracture below this threshold ($P < 0.0001$).

4. Discussion

The prevalence of key risk factors for fracture has been evaluated in untreated populations in the course of developing and validating FRAX [2], and FRAX was recently validated in a Canadian cohort that included treated individuals [8]; however, this is the first study to our knowledge to examine the prevalence of these risk factors in a cohort of postmenopausal women who have been identified with, and are undergoing treatment for, osteoporosis or low bone mass in the primary care setting in the USA. In this treatment cohort, 1 in 2 osteoporotic women and nearly 1 in 7 osteopenic women had multiple risk factors for fracture. With a median followup

Table 3: Self-reported on-study fracture among nonpharmacologically treated[a] POSSIBLE US subjects stratified by the National Osteoporosis Foundation treatment thresholds.

	Number of subjects with FRAX score	FRAX predicted 10-year risk of ≥3% for hip fracture			FRAX predicted 10-year risk of ≥20% for major osteoporotic fracture			FRAX score ≥ intervention threshold for either hip or major osteoporotic fractures		
	(*N* = 1,299) *n* (%)	Yes (*N* = 407) *n* (%)	No (*N* = 892) *n* (%)	*P* value	Yes (*N* = 206) *n* (%)	No (*N* = 1,093) *n* (%)	*P* value	Yes (*N* = 411) *n* (%)	No (*N* = 888) *n* (%)	*P* value
No on-study fracture	1,227 (94.5)	384 (94.4)	843 (94.5)	0.91	192 (93.2)	1,035 (94.7)	0.39	388 (94.4)	839 (94.5)	0.95
Any on-study fracture	72 (5.5)	23 (5.7)	49 (5.5)		14 (6.8)	58 (5.3)		23 (5.6)	49 (5.5)	
Any osteoporosis-related fracture	47 (3.6)	18 (4.4)	29 (3.3)	0.29	12 (5.8)	35 (3.2)	0.06	18 (4.4)	29 (3.3)	0.33
Hip fracture	9 (0.7)	3 (0.7)	6 (0.7)	0.90	1 (0.5)	8 (0.7)	0.70	3 (0.7)	6 (0.7)	0.91
Spine fracture	9 (0.7)	4 (1.0)	5 (0.6)	0.40	4 (1.9)	5 (0.5)	0.02	4 (1.0)	5 (0.6)	0.41
Nonhip/non-spine fracture	31 (2.4)	11 (2.7)	20 (2.2)	0.61	7 (3.4)	24 (2.2)	0.30	11 (2.7)	20 (2.3)	0.64
Multiple osteoporosis-related fractures	11 (0.9)	6 (1.5)	5 (0.6)	0.10	4 (1.9)	7 (0.6)	0.06	6 (1.5)	5 (0.6)	0.10

[a]Subjects who reported no pharmacological therapy or using only calcium/vitamin D within 2 months of study entry.
P value is for pairwise (yes/no) comparisons with percentage of specified fracture risk outcome for each risk category.

Table 4: Self-reported on-study fracture among pharmacologically treated[a] POSSIBLE US subjects stratified by the National Osteoporosis Foundation treatment thresholds.

	Number of subjects with FRAX score	FRAX predicted 10-year risk of ≥3% for hip fracture			FRAX predicted 10-year risk of ≥20% for major osteoporotic fracture			FRAX score ≥ intervention threshold for either hip or major osteoporotic fractures		
	N = 2,996 *n* (%)	Yes (*N* = 1,039) *n* (%)	No (*N* = 1,957) *n* (%)	*P*-value	Yes (*N* = 610) *n* (%)	No (*N* = 2,386) *n* (%)	*P*-value	Yes (*N* = 1,062) *n* (%)	No (*N* = 1,934) *n* (%)	*P*-value
No on-study fracture	2,623 (87.6)	865 (83.3)	1,758 (89.8)	<0.0001	492 (80.7)	2,131 (89.3)	<0.0001	885 (83.3)	1,738 (89.9)	<0.0001
Any on-study fracture	373 (12.5)	174 (16.8)	199 (10.2)		118 (19.3)	255 (10.7)		177 (16.7)	196 (10.1)	
Any osteoporosis-related fracture	240 (8.0)	126 (12.1)	114 (5.8)	<0.0001	93 (15.3)	147 (6.2)	<0.0001	127 (12.0)	113 (5.8)	<0.0001
Hip fracture	48 (1.6)	34 (3.3)	14 (0.7)	<0.0001	30 (4.9)	18 (0.8)	<0.0001	35 (3.3)	13 (0.7)	<0.0001
Spine fracture	54 (1.8)	32 (3.1)	22 (1.1)	0.0001	29 (4.8)	25 (1.1)	<0.0001	32 (3.0)	22 (1.1)	0.0002
Nonhip/non-spine fracture	169 (5.6)	79 (7.6)	90 (4.6)	0.0007	52 (8.5)	117 (4.9)	0.0005	80 (7.5)	89 (4.6)	0.0009
Multiple osteoporosis-related fractures	72 (2.4)	41 (4.0)	31 (1.6)	0.0001	34 (5.6)	38 (1.6)	<0.0001	42 (4.0)	30 (1.6)	<0.0001

[a]Subjects who reported the use of pharmacological monotherapy or combination therapy at study entry or initiated pharmacological therapy within 2 months of entry.
P value is for pairwise (yes/no) comparisons with percentage of specified fracture risk outcome for each risk category.

period of approximately 2.6 years, 8.0% of women in the osteoporosis group and 5.5% of women in the osteopenia group experienced an osteoporosis-related on-study fracture with the majority (68.0% and 71.7%, resp.,) of these fractures occurring at sites other than the hip or spine in both groups. The on-study fracture incidence was even higher among subjects who met the NOF FRAX treatment thresholds.

Osteoporosis-related fractures were also significantly more common in women with multiple risk factors compared with women with a single risk factor. Interestingly, women

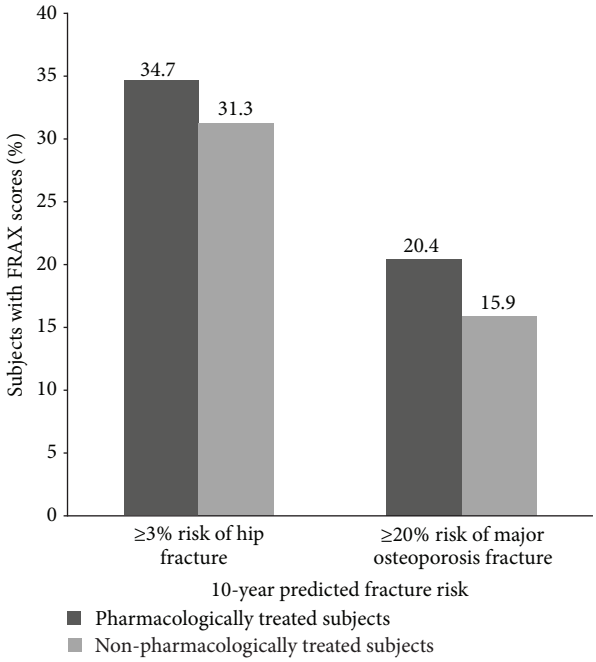


Figure 4: Percentage of subjects in the osteoporosis and osteopenia groups whose FRAX scores met or exceeded the National Osteoporosis Foundation treatment threshold.

with multiple risk factors had a similar fracture incidence regardless of whether they were in the osteoporosis group or osteopenia group.

For osteoporosis, effective treatment involves identifying at-risk individuals, determining the likely causes and factors contributing to low bone mass, and tailoring medical treatments and other interventions (e.g., fall prevention) to the individual patient's needs [9]. The results of our study may help inform primary care physicians' approaches to the first component of this treatment paradigm—patient identification. In particular, women of age > 70 years, with history of fracture, and/or current smoking (the most common non-BMD risk factors in this cohort of postmenopausal women) may merit further assessment of their bone health and potential fracture risk. Our results also suggest that women with multiple risk factors may be more likely to experience an osteoporosis-related fracture even after having bone-specific medications prescribed. The greater incidence of fractures among women with multiple risk factors during the relative short observation period (from 2 to 3 years) underscores the importance of determining how many fracture risk factors each patient has and suggests that primary care physicians may want to closely monitor patients with multiple risk factors, even after they initiate therapy.

Published guidelines recommend using risk factor profiles to identify candidates for osteoporosis therapy. As recently as 2003, osteoporosis guidelines advocated active patient identification even in the absence of a consensus about the best approach [10]. The World Health Organization has defined osteoporosis using a T-score cut-off, and BMD assessments were initially the primary tool for patient identification. More recently, other independent clinical risk factors (in addition to BMD) have been shown to enhance the efficiency of identifying candidates for therapy [11], and patient identification approaches have been broadened to include these other factors.

The FRAX tool, for example, was designed to help physicians in clinical practice to identify patients who are at high risk of fracture by estimating the 10-year fracture risk for an individual compared with a population of the same age and sex [12]. The recent incorporation of FRAX into the US osteoporosis screening guidelines acknowledges the importance of assessing risk factors beyond BMD and defines a routine role for FRAX in the primary care setting to identify women (and men) who would be most likely to benefit from osteoporosis therapy [13, 14]. FRAX is also included in osteoporosis guidelines for a number of countries outside of the USA [15, 16]. For example, Canada has adopted FRAX to identify candidates for osteoporosis therapy and also has created a customized version using the Canadian national hip fracture and mortality data [17, 18]. The use of FRAX in clinical practice has also been shown to improve prescribing practices for osteoporosis therapies [19]. Although, we found no association between on-study fracture experience and NOF FRAX treatment thresholds among subjects on non-pharmacologic therapy, we did observe a greater incidence of on-study fractures among pharmacologically treated subjects who met or exceeded the treatment thresholds compared with subjects whose scores were below the treatment thresholds.

This shift beyond using only BMD assessment to identify individuals at increased risk of fracture may, in part, also reflect concerns about inadequate access to dual energy X-ray absorptiometry (DXA) in Europe and the USA [11, 20], as well as increased understanding of the role of other risk factors. Recent estimates suggest that most European countries lack the DXA resources required for case finding and treatment monitoring, and in the USA, there is concern that reductions in Medicare reimbursements for DXA will result in the underuse of BMD assessments in Medicare populations [11, 20].

By 2025, an estimated 3 million osteoporotic fractures are projected to occur in the USA, and these fractures are associated with an increased risk of subsequent fractures, significant treatment costs, quality of life decrements, and increased mortality risk [21–25]. In the USA and elsewhere, a variety of effective therapies with different modes of administration and mechanisms of action are available for both the prevention and treatment of osteoporosis [26]. The combination of the large public health burden associated with osteoporosis-related fracture and the availability of effective osteoporosis therapies suggests that there may be substantial benefit from identifying individuals at greatest risk for fracture and providing those individuals with appropriate therapeutic interventions [26]. The results of our study support the current published recommendations to evaluate a variety of fracture risk factors, and suggest that, in addition to BMD, age, history of fracture, and smoking status may be the most common risk factors in postmenopausal women identified by primary care physicians for osteoporosis therapy.

Various limitations must be considered in interpreting the results of this study. On-study fracture was identified using subject-reported data. Therefore, the accuracy of these data may be affected by the use of a 6-month recall window and by other factors limiting subject recall. The physician-reported diagnoses used to characterize and group subjects may have been based on clinical judgment, in addition to explicit risk factors. This may explain why some women who were reported to have no risk factors in our analysis were actually receiving treatment. Although the women in this cohort are demographically similar to women treated for osteoporosis in the USA [5], they may not be representative of women with postmenopausal bone loss overall or those treated for bone loss in countries outside of the USA. In addition, in the time since the POSSIBLE US data were collected, evidence highlighting risks and limitations of bisphosphonate therapies has been published [27, 28]. In light of these findings, primary care physicians may have become less likely to recommend osteoporosis treatment for low risk patients. The utility of FRAX scores computed for the women in POSSIBLE US cohort is still being assessed because FRAX was initially developed to predict fracture risk only in untreated individuals. In this context, the "untreated" population includes individuals who have no pharmacological treatment history, as well as individuals who used oral bisphosphonates for <2 months in the previous 2 years and individuals with no estrogen, raloxifene, calcitonin, or denosumab use in the past year but who may have had prior use of these agents [29]. However, a recent validation study using 5 years of follow up data from a large Canadian population-based cohort concluded that FRAX predicted the risk of major osteoporotic and hip fractures equally well in untreated, currently treated, and previously treated women [8]. The authors note that this finding should not be interpreted as meaning that osteoporosis treatment is ineffective, and although their study was not powered to detect an antifracture benefit of therapy, the actual fracture experience in the most adherent patient group was lower than the one predicted by FRAX. Finally, there are a few data limitations which may impact the FRAX scores. BMD assessments were not required by the POSSIBLE US study protocol; 11.1% of subjects in the osteoporosis group and 7.6% of subjects in the osteopenia group did not have T-scores for use in the FRAX calculation. Also, by defaulting the alcohol use and secondary osteoporosis diagnosis risk factors to "no" in the FRAX calculator, we may have underestimated the 10-year predicted fracture risks for some subjects.

In summary, our study highlighted that multiple fracture risk factors were present in a significant proportion of the postmenopausal women with osteoporosis receiving treatment. Multiple risk factors were associated with a greater incidence of on-study fracture (overall and osteoporosis-related fractures), even though the women in this study had been identified by their primary care physicians to receive osteoporosis therapy during the followup period. By demonstrating the association between the presence of multiple risk factors and fracture incidence, this study underscores the importance of considering independent fracture risk factors (beyond BMD) to identify postmenopausal women who could benefit from osteoporosis treatments. These results also suggest that women with multiple risk factors may remain at elevated risk of fracture even after initiating therapy.

Conflict of Interests

This study was funded by Amgen Inc. Amgen employees assisted in the study design, analysis and interpretation of data, and paper development. N. Yurgin and D. Macarios are employees of Amgen Inc. and have stock and/or stock options in Amgen Inc. S. Wade and S. Satram-Hoang have received consulting fees from Amgen Inc. M. Hochberg has received remuneration from Amgen Inc. for serving on the POSSIBLE US Steering Committee.

Acknowledgments

The authors would like to acknowledge the other members of the POSSIBLE US Steering Committee: Elizabeth Barrett-Connor, Robert Downs, Ted Ganiats, Barbara Lukert, Robert Recker, Robert Rubin, and Anna Tosteson. They would also like to thank Patrick Ventura and Aalok Nadkar for data analysis assistance; Nathaniel McKinney, Chloe Schaefer, and Gordon Wade for FRAX score calculation; and Mandy Suggitt, holder of a B.A. degree and Erica Rockabrand holder of a Ph.D. degree, who provided editorial assistance on behalf of Amgen Inc.

References

[1] WHO Collaborating Centre for Metabolic Bone Diseases, "FRAX: WHO risk assessment tool," http://www.sheffield.ac.uk/FRAX.

[2] E. McCloskey, "FRAX: identifying people at high risk of fracture," http://www.osteoporosis.org.za/downloads/FRAX-report-09.pdf.

[3] U.S. Preventive Services Task Force, "Screening for osteoporosis: U.S. Preventive Services Task Force recommendation statement," *Annals of Internal Medicine*, vol. 154, no. 5, pp. 356–364, 2011.

[4] National Osteoporosis Foundation, *Clinician's Guide To Prevention and Treatment of Osteoporosis*, National Osteoporosis Foundation, Washington, DC, USA, 2010.

[5] E. Barrett-Connor, K. Ensrud, A. N. A. Tosteson et al., "Design of the POSSIBLE US Study: postmenopausal women's compliance and persistence with osteoporosis medications," *Osteoporosis International*, vol. 20, no. 3, pp. 463–472, 2009.

[6] A. H. Warriner, N. M. Patkar, J. R. Curtis et al., "Which fractures are most attributable to osteoporosis?" *Journal of Clinical Epidemiology*, vol. 64, no. 1, pp. 46–53, 2011.

[7] A. N. A. Tosteson, T. P. Do, S. W. Wade, M. S. Anthony, and R. W. Downs, "Persistence and switching patterns among women with varied osteoporosis medication histories: 12-month results from POSSIBLE US," *Osteoporosis International*, vol. 21, no. 10, pp. 1769–1780, 2010.

[8] W. D. Leslie, L. M. Lix, H. Johansson, A. Oden, E. McCloskey, and J. A. Kanis, "Does osteoporosis therapy invalidate FRAX for fracture prediction?" *Journal of Bone and Mineral Research*, vol. 27, no. 6, pp. 1243–1251, 2012.

[9] P. Geusens, "Strategies for treatment to prevent fragility fractures in postmenopausal women," *Best Practice and Research*, vol. 23, no. 6, pp. 727–740, 2009.

[10] P. P. Geusens, "Review of guidelines for testing and treatment of osteoporosis," *Current Osteoporosis Reports*, vol. 1, no. 2, pp. 59–65, 2003.

[11] C. De Laet, A. Odén, H. Johansson, O. Johnell, B. Jönsson, and J. A. Kanis, "The impact of the use of multiple risk indicators for fracture on case-finding strategies: a mathematical approach," *Osteoporosis International*, vol. 16, no. 3, pp. 313–318, 2005.

[12] E. S. Siris, S. Baim, and A. Nattiv, "Primary care use of FRAX: absolute fracture risk assessment in postmenopausal women and older men," *Postgraduate Medicine*, vol. 122, no. 1, pp. 82–90, 2010.

[13] J. A. Kanis, E. V. McCloskey, H. Johansson, and A. Oden, "Approaches to the targeting of treatment for osteoporosis," *Nature Reviews*, vol. 5, no. 8, pp. 425–431, 2009.

[14] J. A. Kanis, E. V. McCloskey, H. Johansson, A. Oden, O. Ström, and F. Borgström, "Development and use of FRAX in osteoporosis," *Osteoporosis international*, vol. 21, supplement 2, pp. S407–S413, 2010.

[15] J. A. Kanis, N. Burlet, C. Cooper et al., "European guidance for the diagnosis and management of osteoporosis in postmenopausal women," *Osteoporosis International*, vol. 19, no. 4, pp. 399–428, 2008.

[16] National Osteoporosis Guideline Group, http://www.shef.ac.uk/NOGG/.

[17] W. D. Leslie, L. M. Lix, L. Langsetmo et al., "Construction of a FRAX model for the assessment of fracture probability in Canada and implications for treatment," *Osteoporosis International*, vol. 22, no. 3, pp. 817–827, 2011.

[18] A. Papaioannou, S. Morin, A. M. Cheung et al., "2010 clinical practice guidelines for the diagnosis and management of osteoporosis in Canada: Summary," *Canadian Medical Association Journal*, vol. 182, no. 17, pp. 1864–1873, 2010.

[19] W. D. Leslie, S. Morin, and L. M. Lix, "A before-and-after study of fracture risk reporting and osteoporosis treatment initiation," *Annals of Internal Medicine*, vol. 153, no. 9, pp. 580–586, 2010.

[20] A. B. King and D. M. Fiorentino, "Medicare payment cuts for osteoporosis testing reduced use despite tests' benefit in reducing fractures," *Health Affairs*, vol. 30, no. 12, pp. 2362–2370, 2011.

[21] R. Burge, B. Dawson-Hughes, D. H. Solomon, J. B. Wong, A. King, and A. Tosteson, "Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025," *Journal of Bone and Mineral Research*, vol. 22, no. 3, pp. 465–475, 2007.

[22] J. R. Center, D. Bliuc, T. V. Nguyen, and J. A. Eisman, "Risk of subsequent fracture after low-trauma fracture in men and women," *Journal of the American Medical Association*, vol. 297, no. 4, pp. 387–394, 2007.

[23] D. W. Dempster, "Osteoporosis and the burden of osteoporosis-related fractures," *The American Journal of Managed Care*, vol. 17, supplement 6, pp. S164–S169, 2011.

[24] G. Ioannidis, A. Papaioannou, W. M. Hopman et al., "Relation between fractures and mortality: results from the Canadian Multicentre Osteoporosis Study," *Canadian Medical Association Journal*, vol. 181, no. 5, pp. 265–271, 2009.

[25] O. Strom, F. Borgstrom, J. A. Kanis et al., "Osteoporosis: burden, health care provision and opportunities in the EU: a report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA)," *Archives of Osteoporosis*, vol. 6, no. 1-2, pp. 59–155, 2011.

[26] T. D. Rachner, S. Khosla, and L. C. Hofbauer, "Osteoporosis: now and the future," *The Lancet*, vol. 377, no. 9773, pp. 1276–1287, 2011.

[27] D. M. Black, D. C. Bauer, A. V. Schwartz, S. R. Cummings, and C. J. Rosen, "Continuing bisphosphonate treatment for osteoporosis: for whom and for how long?" *The New England Journal of Medicine*, vol. 366, pp. 2051–2053, 2012.

[28] M. Whitaker, J. Guo, T. Kehoe, and G. Benson, "Bisphosphonates for osteoporosis: where do we go from here?" *The New England Journal of Medicine*, vol. 366, pp. 2048–2051, 2012.

[29] National Osteoporosis Foundation (NOF)/International Society for Clinical Densitometry (ISCD), National Osteoporosis Foundation (NOF)/International Society for Clinical Densitometry (ISCD) FRAX Implementation Guide, http://www.iscd.org/resources/fracture-risk-models/.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 368970, 6 pages
http://dx.doi.org/10.1155/2013/368970

Research Article

Omentin-1 Stimulates Human Osteoblast Proliferation through PI3K/Akt Signal Pathway

Shan-Shan Wu, Qiu-Hua Liang, Yuan Liu, Rong-Rong Cui, Ling-Qing Yuan, and Er-Yuan Liao

Institute of Metabolism and Endocrinology, Second Xiang-Ya Hospital, Central South University, Changsha 410011, Hunan, China

Correspondence should be addressed to Ling-Qing Yuan; allenylq@hotmail.com and Er-Yuan Liao; eyliao007@yahoo.com.cn

Received 3 January 2013; Accepted 8 March 2013

Academic Editor: Guang-Da Xiang

It has been presumed that adipokines deriving from adipose tissue may play important roles in bone metabolism. Omentin-1, a novel adipokine, which is selectively expressed in visceral adipose tissue, has been reported to stimulate proliferation and inhibit differentiation of mouse osteoblast. However, little information refers to the effect of omentin-1 on human osteoblast (hOB) proliferation. The current study examined the potential effects of omentin-1 on proliferation in hOB and the signal pathway involved. Omentin-1 promoted hOB proliferation in a dose-dependent manner as determined by [^{3}H]thymidine incorporation. Western blot analysis revealed that omentin-1 induced activation of Akt (phosphatidylinositol-3 kinase downstream effector) and such effect was impeded by transfection of hOB with Akt-siRNA. Furthermore, LY294002 (a selective PI3K inhibitor) and HIMO (a selective Akt inhibitor) abolished the omentin-1-induced hOB proliferation. These findings indicate that omentin-1 induces hOB proliferation via the PI3K/Akt signaling pathway and suggest that osteoblast is a direct target of omentin-1.

1. Introduction

Adipokines, secreted by adipose tissue, have been demonstrated to play critical roles in regulating metabolic homeostasis, insulin sensitivity, systemic inflammatory processes, cardiovascular function, and bone metabolism [1, 2]. Recently, adipokines have emerged as elements in the regulation of bone metabolism [3, 4]. Previous studies proved that the adipokines such as leptin and adiponectin could modulate bone metabolism both *in vitro* and *in vivo* [5–9]. Our previous work showed that apelin and vaspin inhibited the apoptosis of human osteoblast (hOB) [10, 11], and adiponectin stimulated proliferation and differentiation of hOB [12]. However, the function and mechanism involved await to be elucidated.

Omentin-1, also named intelectin-1, is a newly discovered 34 kDa adipokine selectively expressed in omental adipose tissue and abundantly present in plasma [13, 14]. Omentin-1 participates in multiple physiological processes including insulin action, cardiovascular function, and inflammatory response. It was reported that omentin-1 could modulate insulin sensitivity [14], inhibit TNF-induced vascular inflammation in human endothelial cells [15], and induce vasodilation [16]. Recent study demonstrated that omentin-1 played a protective role against vascular calcification [17]. Clinical studies showed that omentin-1 levels inversely correlated with obesity and insulin resistance [18]. Regarding its effects on bone, recent study reported that circulating omentin-1 levels had an inverse correlation with bone mineral density (BMD) at lumber spine in Iranian postmenopausal women [19]. Xie et al.'s study demonstrated that omentin-1 could alleviate the bone loss in osteoprotegerin-deficient or ovariectomized mice by regulating the proliferation and differentiation of the mouse osteoblast [20, 21]. However, research concerning the potential effects of omentin-1 on hOB proliferation remains relatively poor. Our present work focuses on the role of omentin-1 in controlling hOB proliferation and the signaling pathway involved.

2. Materials and Methods

2.1. Reagents. Recombinant omentin-1 was the product of Cell Science, Inc. (Canton, MA, USA). Anti-Akt and p-Akt

antibodies, anti-mouse, and rabbit IgG peroxidase conjugate antibodies were purchased from Santa Cruz Biotechnology Inc. (Waltham, MA, USA). LY294002 and HIMO were purchased from Calbiochem Corp. (San Diego, CA, USA).

2.2. Cell Cultures. Primary hOB was isolated from human trabecular bone obtained during surgery following traffic accident victims as previously described [22, 23], and after being approved by the Ethics Committee of the Second Xiangya Hospital of Central South University, China. None of the donors suffered from clinical symptoms or history of bone metabolic disorders. Briefly, samples were washed extensively with phosphate buffered saline (PBS) to remove blood cells and debris and finally washed in culture medium. Then, the sample was digested with type IV collagenase (Sigma) and cultured in phenol red-free α-MEM containing 10% fetal bovine serum (FBS, Gibco-BRL Corp. Grand Island, NY, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL ascorbic acid (Sigma) at 37°C in a humidified incubator with 5% CO_2. Medium was changed every 2 days and after approximately 4 weeks in culture, cells were harvested using trypsin EDTA and subcultured in α-MEM containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL ascorbic acid. Osteoblast was cultured to facilitate mineralization in differentiation medium containing 10% FBS, 50 μg/mL ascorbic acid, 10 nM dexamethasone, and 10 mM β-glycerophosphate. The phenotype of cells was characterized based on the ALP activity, osteocalcin (OC) secretion, and the formation of mineralization nodules as previously described [22, 23]. Briefly, ALP activity was assayed by spectrophotometric measurement of p-nitrophenol release at 37°C. ALP activity was normalized to total protein content of the cell layer. Osteocalcin released into the culture media was measured using a specific radioimmunoassay kit (DiaSorin, Stillwater, MN, USA). To normalize protein expression to total cellular protein, a fraction of the lysate solution was used in a Bradford protein assay. The formation of mineralization nodules was determined by Alizarin Red S staining.

2.3. Assessment of Cell Proliferation. HOB proliferation was assessed using [^{3}H]thymidine (2 mCi/mL) incorporation into trichloroacetic acid (TCA) insoluble material followed by scintillation counting. Briefly, cells were plated at a density of 2×10^4 cells/well in 24-well plates and treated with 25–200 ng/mL omentin-1 for 48 h, in the presence of [^{3}H]thymidine. 24 hours later, the plates were washed with PBS, and 10% TCA solution was added to the wells. Incorporated [^{3}H]thymidine was released by washing with 0.2 N of NaOH, and radioactivity was measured using a β-scintillation counter. Results are expressed as counts per minute.

To study the effects of inhibitors, cells were pretreated with PI3K inhibitor LY294002 (10 μM), or Akt inhibitor HIMO (10 μM) for 3 h prior to treatment with 200 ng/mL omentin-1.

2.4. Detection of Akt Activation. Briefly, hOBs were first treated with 200 ng/mL omentin-1 for 5–60 min. Then, cell monolayers were washed quickly with cold PBS containing 5 mM of EDTA and 0.1 mM of Na_3VO_4 and lysed with a lysis buffer consisting of 20 mM of Tris-HCl (pH 7.5), 150 mM of NaCl, 1% Triton X-100, 10 mM of NaH_2PO_4, 10% glycerol, 2 mM of Na_3VO_4, 10 mM of NaF, 1 mM of ABSF, 10 mg/mL leupeptin, and 10 mg/mL aprotinin. Protein concentrations were determined by Bradford assay. 10 μg of protein was loaded onto a 10% polyacrylamide gel. After electrophoresis, the SDS-PAGE separated proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked with 2.5% nonfat milk in PBS and incubated with anti-Akt and -phospho-Akt primary antibodies (Santa Cruz, Biotechnology, CA, USA) at 1 : 500 in PBS for 2 h. Then, the membrane was incubated with goat anti-mouse or rabbit IgG conjugated with horseradish peroxidase (Santa Cruz) at 1 : 1000 in PBS for 1 h. Blots were processed using an ECL (Santa Cruz) kit and exposed to X-ray film.

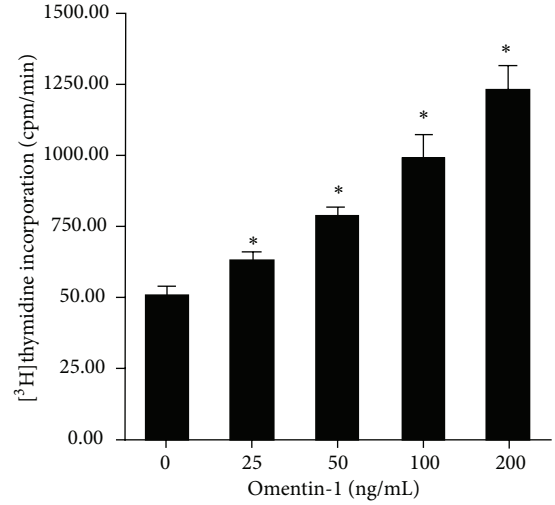


Figure 1: Omentin-1 stimulated the proliferation of hOB. Cells were exposed to 25–200 ng/mL omentin-1 for 48 h. Cell proliferation was determined by measuring [^{3}H]thymidine incorporation. Results are expressed as counts per minute. $^*P < 0.05$ versus control.

2.5. Genetic Suppression of Akt by siRNA. For gene knockdown experiments, hOBs were plated in 60 mm diameter dish and cultured for 24 h in medium without antibiotics. To suppress Akt, the hOBs were transfected with either Akt small interfering RNA (siRNA) or control siRNA (Santa Cruz Biotechnology Inc.) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The levels of Akt expression were analyzed by western blotting as described above.

2.6. Statistical Analyses. Data are presented as the mean ± SD. Comparisons were made using a one-way ANOVA. All experiments were repeated at least three times, and representative experiments are shown.

3. Results

3.1. Characterization of hOB. Cells were identified as osteoblast using several criteria, including high intrinsic ALP

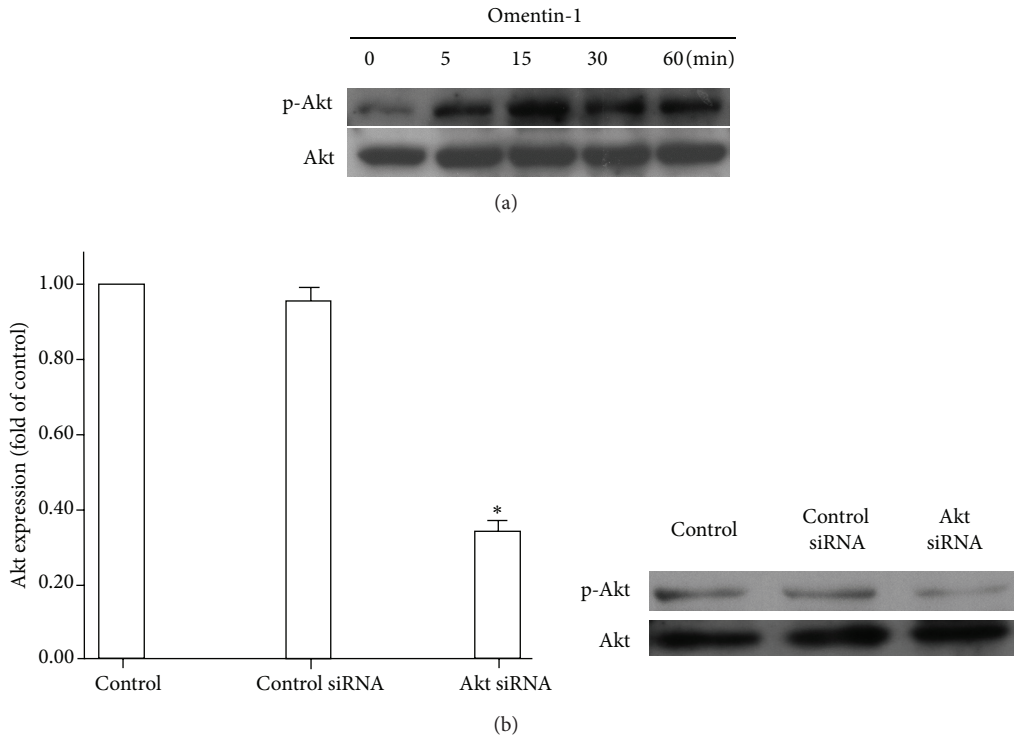


Figure 2: Omentin-1 activated Akt signal pathway in hOB. (a) Western blot analysis of Akt activation. The hOBs were cultured in serum-free a-MEM for 6 h and then treated with omentin-1 (200 ng/mL) for 5–60 min. The cell lysates were analyzed by western blotting and incubated with antibodies against p-Akt and Akt. Representative results are shown. (b) Either control siRNA or Akt siRNA was transfected into osteoblast. Akt siRNA significantly suppressed the expression of phosphor-Akt (left). Expression of Akt was determined by western blot analysis using an Akt antibody. Representative results are shown (right).

activity, secretion of OC, and mineralized nodule formation as previously described [24]. The ALP activities in normal hOB were 71.6 ± 6.3 nmol/min/mg protein. The OC levels in the culture supernatants from unstimulated human bone cells were 4.65 ± 0.36 ng/mg protein. After 21 days of culture with differentiation medium, the mineralized nodule formation was detected using Alizarin Red S staining in cultured hOB. Fulfilling the above criteria for osteoblast, our results demonstrated that the cells isolated were primary hOB from collagenase-digested human trabecular bone.

3.2. Omentin-1 Stimulated hOB Proliferation. Using [^{3}H]thymidine incorporation by cells to determine the proliferation of hOB, we confirmed that omentin-1 stimulated hOB proliferation in a dose-dependent manner. Compared to the control group, cells treated with omentin-1 at concentrations of 25, 50, 100, and 200 ng/mL increased the [^{3}H]thymidine incorporation of hOB by 23.66%, 53.29%, 93.93%, and 140.36%, respectively, with statistical significance (all $P < 0.05$) (Figure 1).

3.3. Omentin-1 Activated Akt Signaling Pathway in hOB. To investigate the signal pathway involving omentin-1, we determined if the Akt signaling pathway was inducible by omentin-1. As shown in Figure 2(a), omentin-1 stimulated the activity of Akt in hOB after 5 min incubation with omentin-1 as demonstrated by an increased phosphorylated Akt levels.

To determine the effect of Akt in the proliferation of omentin-1 on hOB, we used siRNA to knockdown the expression of Akt. As shown in Figure 2(b), transfection of hOB with Akt siRNA inhibited Akt protein expression.

3.4. Omentin-1 Regulated Proliferation of hOB through the PI3K/Akt Signaling Pathway. Because the results above demonstrated that omentin-1 activated Akt signaling pathway in hOB, we examined whether the omentin-1-induced proliferation is mediated via the activation of PI3K/Akt signaling pathway. Pretreatment of cells with the PI3K inhibitor LY294002 or Akt inhibitor HIMO abolished the omentin-1-induced cell proliferation (Figure 3). The observation from Akt siRNA treatment cohered with the current observation when cells are treated with LY294002 and HIMO. In conclusion, treatment of hOB with Akt siRNA suppressed the effects of omentin-1 on proliferation in hOB (Figure 3).

4. Discussion

The present study shows that treatment with omentin-1 stimulates proliferation of hOB, indicating a growth promotion

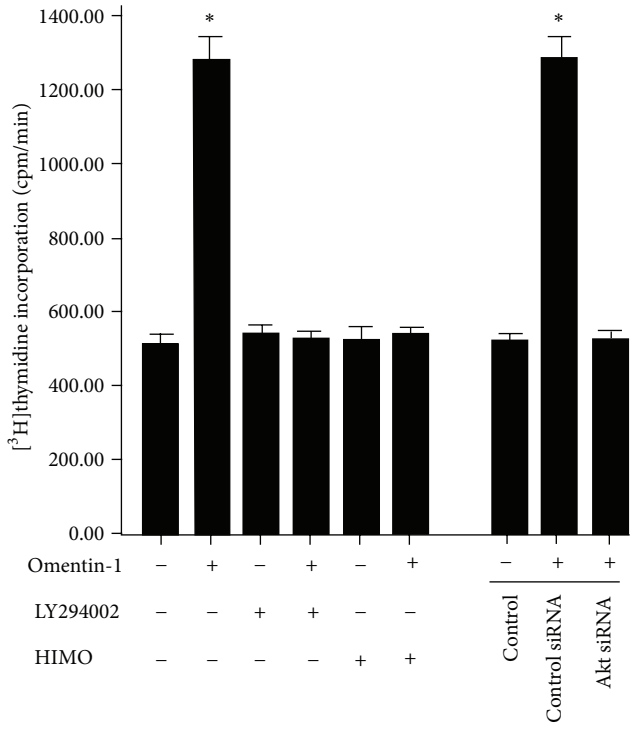


Figure 3: Omentin-1 regulated proliferation of hOB through the PI3K/Akt signaling pathway. The hOBs were pretreated with vehicle, PI3K inhibitor LY294002 (10 μM), or Akt inhibitor HIMO (10 μM) for 3 h prior to treatment with omentin-1 (200 ng/mL) for 48 h. The cells were also transfected with control siRNA or Akt siRNA before treatment with omentin-1 (200 ng/mL) for 48 h. Cell proliferation was determined by measuring [^{3}H]thymidine incorporation. Results are expressed as counts per minute. $^*P < 0.05$ versus control.

effect of omentin-1 on hOB. It is also shown that PI3K/Akt signal pathway is a key mediator of such effect.

Adipose tissue has been recognized as a highly active endocrine organ. In addition to the uptake, storage, and synthesis of lipids, adipose tissue secrets a variety of adipokines (e.g., adiponectin, leptin, resistin, vaspin, and visfatin). These adipokines control insulin sensitivity, neuroendocrine activity, food and water intake, breeding, inflammatory response, cardiovascular function, and bone metabolism [1, 21]. Omentin-1, a new adipokine, is primarily expressed in visceral adipose tissue and abundant in plasma [13, 14]. Previous studies have demonstrated that both omentin-1 mRNA and plasma levels inversely correlated to obesity, BMI, and insulin resistance [18, 25], and serum omentin-1 was found decreased in diabetic patients [26, 27]. Furthermore, omentin-1 plays critical roles in cardiovascular protecting, such as inducing vasodilation, inhibiting osteoblastic differentiation of vascular smooth muscle cells (VSMCs) [16, 17]. It has been noted that those effects of omentin-1 is similar to cardiovascular protecting adipokine adiponectin, an insulin sensitizer [28–30]. Our previous study has shown that adiponectin stimulated the proliferation and differentiation of hOB [12]. Several other adipokines such as leptin, resistin, and visfatin had been identified to induce the proliferation of osteoblast [31–33], suggesting a close relationship between adipokine and osteoblast. Clinical investigations have demonstrated that serum omentin-1 negatively correlated with BMD in the anorexia nervosa girls [34] and Iranian postmenopausal women [19]. Xie et al. have also found that omentin-1 ameliorated bone loss of ovary ectomized mice and OPG$^{-/-}$ mice, and that omentin-1 stimulated proliferation and inhibited differentiation of mouse primary osteoblast [20, 21].

In the present experiment, 25–200 ng/mL omentin-1 has been chosen to treat the hOB based on the previous research [21]. To determine the effect of omentin-1 on hOB proliferation, [^{3}H]thymidine incorporation assay was adopted. Our data have demonstrated that omentin-1 stimulated the proliferation of hOB in a dose-dependent manner. The proliferative effect of omentin-1 on hOB is therefore consistent with the report by Xie et al. [21].

To gain further insight into the underlying mechanism about how omentin-1 stimulated hOB proliferation, we evaluated signaling pathway that was potentially involved. Multiple signaling pathways, such as MAPK, Wnt, AMP, and PI3K-Akt, were found to participate in the modulation of osteoblast proliferation [35–38]. Among this, PI3K-Akt, which existed in all mammalian cells and exerted profound effects on diverse processes including cell proliferation, survival, differentiation, migration, and metabolism, was the most important one. Our study showed that omentin-1 induced the activation of Akt in hOB, whereas knocking down the expression of Akt with siRNA impeded the stimulatory effect of omentin-1 in hOB proliferation. In addition, pretreatment of hOB with LY294002 or HIMO could also block the effect of omentin-1. Collectively, these results suggest that omentin-1 promotes the hOB proliferation via the PI3K/Akt signaling pathway. Several studies [39–41] have also uncovered that PI3K/Akt activation plays an essential role in osteoblast proliferation and recent data has demonstrated that PI3K/Akt plays a crucial role in osteoblastic differentiation of CVSMCs (calcifying vascular smooth muscle cells) induced by omentin-1 [17], findings that are in line with our present results.

In summary, our present study identified omentin-1 as an important regulator in human bone remodeling by promoting the hOB proliferation through the PI3K/Akt signaling pathway. These findings revealed the relationship between adipokines and bone metabolism and provided us a better understanding of the involved signaling mechanisms. Further investigation of omentin-1 and osteoblast may offer us a new target for osteoporotic prevention and treatment.

Conflict of Interests

The authors have declared that no competing interests exist.

Authors' Contribution

Shan-Shan Wu and Qiu-Hua Liang contributed equally to this work.

Acknowledgments

This work was supported by funding from The National Natural Science Foundation of China (Grants nos. 81270962, 81070246, and 30801174) and the National Science Foundation for Postdoctoral Scientists of China (Grant no. 2012M521568).

References

[1] S. P. Poulos, D. B. Hausman, and G. J. Hausman, "The development and endocrine functions of adipose tissue," *Molecular and Cellular Endocrinology*, vol. 323, no. 1, pp. 20–34, 2010.

[2] B. Gustafson, "Adipose tissue, inflammation and atherosclerosis," *Journal of Atherosclerosis and Thrombosis*, vol. 17, no. 4, pp. 332–341, 2010.

[3] M. Holecki and A. Więcek, "Relationship between body fat mass and bone metabolism," *Polskie Archiwum Medycyny Wewnetrznej*, vol. 120, no. 9, pp. 361–367, 2010.

[4] Y. Liu, C. Y. Song, S. S. Wu, Q. H. Liang, L. Q. Yuan, and E. Y. Liao, "Novel adipokines and bone metabolism," *International Journal of Endocrinology*, vol. 2013, Article ID 895045, 2013.

[5] G. A. Williams, Y. Wang, K. E. Callon et al., "*In vitro* and *in vivo* effects of adiponectin on bone," *Endocrinology*, vol. 150, no. 8, pp. 3603–3610, 2009.

[6] K. N. Ealey, J. Kaludjerovic, M. C. Archer, and W. E. Ward, "Adiponectin is a negative regulator of bone mineral and bone strength in growing mice," *Experimental Biology and Medicine*, vol. 233, no. 12, pp. 1546–1553, 2008.

[7] S. M. Bartell, S. Rayalam, S. Ambati et al., "Central (ICV) leptin injection increases bone formation, bone mineral density, muscle mass, serum IGF-1, and the expression of osteogenic genes in leptin-deficient ob/ob mice," *Journal of Bone and Mineral Research*, vol. 26, no. 8, pp. 1710–1720, 2011.

[8] M. W. Hamrick, M. A. Della-Fera, Y. H. Choi, C. Pennington, D. Hartzell, and C. A. Baile, "Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice," *Journal of Bone and Mineral Research*, vol. 20, no. 6, pp. 994–1001, 2005.

[9] H. S. Berner, S. P. Lyngstadaas, A. Spahr et al., "Adiponectin and its receptors are expressed in bone-forming cells," *Bone*, vol. 35, no. 4, pp. 842–849, 2004.

[10] H. Xie, L. Q. Yuan, X. H. Luo et al., "Apelin suppresses apoptosis of human osteoblasts," *Apoptosis*, vol. 12, no. 1, pp. 247–254, 2007.

[11] X. Zhu, Y. Jiang, P. F. Shan et al., "Vaspin attenuates the apoptosis of human osteoblasts through ERK signaling pathway," *Amino Acids*, vol. 44, no. 3, pp. 961–968, 2013.

[12] X. H. Luo, L. J. Guo, L. Q. Yuan et al., "Adiponectin stimulates human osteoblasts proliferation and differentiation via the MAPK signaling pathway," *Experimental Cell Research*, vol. 309, no. 1, pp. 99–109, 2005.

[13] A. Schäffler, M. Neumeier, H. Herfarth, A. Fürst, J. Schölmerich, and C. Büchler, "Genomic structure of human omentin, a new adipocytokine expressed in omental adipose tissue," *Biochimica et Biophysica Acta*, vol. 1732, no. 1–3, pp. 96–102, 2005.

[14] R. Z. Yang, M. J. Lee, H. Hu et al., "Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action," *American Journal of Physiology*, vol. 290, no. 6, pp. E1253–E1261, 2006.

[15] H. Yamawaki, J. Kuramoto, S. Kameshima, T. Usui, M. Okada, and Y. Hara, "Omentin, a novel adipocytokine inhibits TNF-induced vascular inflammation in human endothelial cells," *Biochemical and Biophysical Research Communications*, vol. 408, no. 2, pp. 339–343, 2011.

[16] H. Yamawaki, N. Tsubaki, M. Mukohda, M. Okada, and Y. Hara, "Omentin, a novel adipokine, induces vasodilation in rat isolated blood vessels," *Biochemical and Biophysical Research Communications*, vol. 393, no. 4, pp. 668–672, 2010.

[17] X. Y. Duan, P. L. Xie, Y. L. Ma, and S. Y. Tang, "Omentin inhibits osteoblastic differentiation of calcifying vascular smooth muscle cells through the PI3K/Akt pathway," *Amino Acids*, vol. 41, no. 5, pp. 1223–1231, 2011.

[18] C. M. de Souza Batista, R. Z. Yang, M. J. Lee et al., "Omentin plasma levels and gene expression are decreased in obesity," *Diabetes*, vol. 56, no. 6, pp. 1655–1661, 2007.

[19] M. Tohidi, S. Akbarzadeh, B. Larijani et al., "Omentin-1, visfatin and adiponectin levels in relation to bone mineral density in Iranian postmenopausal women," *Bone*, vol. 51, no. 5, pp. 876–881, 2012.

[20] H. Xie, P. L. Xie, X. H. Luo et al., "Omentin-1 exerts bone-sparing effect in ovariectomized mice," *Osteoporosis International*, vol. 23, no. 4, pp. 1425–1436, 2012.

[21] H. Xie, P. L. Xie, X. P. Wu et al., "Omentin-1 attenuates arterial calcification and bone loss in osteoprotegerin-deficient mice by inhibition of RANKL expression," *Cardiovascular Research*, vol. 92, no. 2, pp. 296–306, 2011.

[22] Y. S. Liu, Y. Lu, W. Liu et al., "Connective tissue growth factor is a downstream mediator for preptin-induced proliferation and differentiation in human osteoblasts," *Amino Acids*, vol. 38, no. 3, pp. 763–769, 2010.

[23] L. Q. Yuan, H. Xie, X. H. Luo et al., "Taurine transporter is expressed in osteoblasts," *Amino Acids*, vol. 31, no. 2, pp. 157–163, 2006.

[24] L. J. Guo, H. Xie, H. D. Zhou, X. H. Luo, Y. Q. Peng, and E. Y. Liao, "Stimulation of RANKL and inhibition of membrane-type matrix metalloproteinase-1 expression by parathyroid hormone in normal human osteoblasts," *Endocrine Research*, vol. 30, no. 3, pp. 369–377, 2004.

[25] J. M. Moreno-Navarrete, V. Cataln, F. Ortega et al., "Circulating omentin concentration increases after weight loss," *Nutrition and Metabolism*, vol. 7, article 27, 2010.

[26] B. K. Tan, S. Pua, F. Syed, K. C. Lewandowski, J. P. O'Hare, and H. S. Randeva, "Decreased plasma omentin-1 levels in Type 1 diabetes mellitus," *Diabetic Medicine*, vol. 25, no. 10, pp. 1254–1255, 2008.

[27] H. Y. Pan, L. Guo, and Q. Li, "Changes of serum omentin-1 levels in normal subjects and in patients with impaired glucose regulation and with newly diagnosed and untreated type 2 diabetes," *Diabetes Research and Clinical Practice*, vol. 88, no. 1, pp. 29–33, 2010.

[28] J. J. Díez and P. Iglesias, "The role of the novel adipocyte-derived protein adiponectin in human disease: an update," *Mini-Reviews in Medicinal Chemistry*, vol. 10, no. 9, pp. 856–869, 2010.

[29] Y. Matsuzawa, "Adiponectin: a key player in obesity related disorders," *Current Pharmaceutical Design*, vol. 16, no. 17, pp. 1896–1901, 2010.

[30] P. Chiarugi and T. Fiaschi, "Adiponectin in health and diseases: from metabolic syndrome to tissue regeneration," *Expert Opinion on Therapeutic Targets*, vol. 14, no. 2, pp. 193–206, 2010.

[31] J. Cornish, K. E. Callon, U. Bava et al., "Leptin directly regulates bone cell function *in vitro* and reduces bone fragility *in vivo*," *Journal of Endocrinology*, vol. 175, no. 2, pp. 405–415, 2002.

[32] L. Thommesen, A. K. Stunes, M. Monjo et al., "Expression and regulation of resistin in osteoblasts and osteoclasts indicate a role in bone metabolism," *Journal of Cellular Biochemistry*, vol. 99, no. 3, pp. 824–834, 2006.

[33] H. Xie, S. Y. Tang, X. H. Luo et al., "Insulin-like effects of visfatin on human osteoblasts," *Calcified Tissue International*, vol. 80, no. 3, pp. 201–210, 2007.

[34] L. J. Guo, T. J. Jiang, L. Liao, H. Liu, and H. B. He, "Relationship between serum omentin-1 level and bone mineral density in girls with anorexia nervosa," *Journal of Endocrinological Investigation*, vol. 2012, 2012.

[35] A. Y. Au, R. Y. Au, J. L. Demko, R. M. McLaughlin, B. E. Eves, and C. G. Frondoza, "Consil bioactive glass particles enhance osteoblast proliferation and selectively modulate cell signaling pathways *in vitro*," *Journal of Biomedical Materials Research A*, vol. 94, no. 2, pp. 380–388, 2010.

[36] I. Kanazawa, T. Yamaguchi, S. Yano, M. Yamauchi, M. Yamamoto, and T. Sugimoto, "Adiponectin and AMP kinase activator stimulate proliferation, differentiation, and mineralization of osteoblastic MC3T3-E1 cells," *BMC Cell Biology*, vol. 8, article 51, 2007.

[37] W. Zhang, X. Shen, C. Wan et al., "Effects of insulin and insulin-like growth factor 1 on osteoblast proliferation and differentiation: differential signalling via Akt and ERK," *Cell Biochemistry and Function*, vol. 30, no. 4, pp. 297–302, 2012.

[38] E. Haÿ, A. Nouraud, and P. J. Marie, "N-cadherin negatively regulates osteoblast proliferation and survival by antagonizing Wnt, ERK and PI3K/Akt signalling," *PloS ONE*, vol. 4, no. 12, p. e8284, 2009.

[39] Y. X. Gu, J. Du, M. S. Si, J. J. Mo, S. C. Qiao, and H. C. Lai, "The roles of PI3K/Akt signaling pathway in regulating MC3T3-E1 preosteoblast proliferation and differentiation on SLA and SLActive titanium surfaces," *Journal of Biomedical Materials Research A*, vol. 101, no. 3, pp. 748–754, 2013.

[40] S. Katz, V. Ayala, G. Santillan, and R. Boland, "Activation of the PI3K/Akt signaling pathway through $P2Y_2$ receptors by extracellular ATP is involved in osteoblastic cell proliferation," *Archives of Biochemistry and Biophysics*, vol. 513, no. 2, pp. 144–152, 2011.

[41] P. Ma, B. Gu, J. Ma et al., "Glimepiride induces proliferation and differentiation of rat osteoblasts via the PI3-kinase/Akt pathway," *Metabolism*, vol. 59, no. 3, pp. 359–366, 2010.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 895474, 5 pages
http://dx.doi.org/10.1155/2013/895474

Clinical Study

Comparison of QCT and DXA: Osteoporosis Detection Rates in Postmenopausal Women

Na Li,[1] Xin-min Li,[1] Li Xu,[1] Wei-jie Sun,[1] Xiao-guang Cheng,[1] and Wei Tian[2]

[1] *Department of Radiology, Beijing Jishuitan Hospital, No. 31 Xinjiekou Street, Xicheng District, Beijing 100035, China*
[2] *Department of Spine Surgery, Beijing Jishuitan Hospital, No. 31 Xinjiekou Street, Xicheng District, Beijing 100035, China*

Correspondence should be addressed to Wei Tian; tianweia@163bj.com

Received 5 December 2012; Revised 1 March 2013; Accepted 4 March 2013

Academic Editor: Peng-Fei Shan

Objective. To compare the osteoporosis detection rates in postmenopausal women when measuring bone mineral density (BMD) with quantitative computed tomography (QCT) in the spine versus dual X-ray absorptiometry (DXA) in the spine and hip and to investigate the reasons for the discrepancy between the two techniques. *Methods*. Spinal volumetric BMD was measured with QCT, and areal spinal and hip BMDs were measured with DXA in 140 postmenopausal women. We calculated the osteoporosis detection rate for the two methods. Lumbar CT images of patients who had a discrepancy between QCT and DXA findings were reviewed to evaluate vertebral fractures, spinal degeneration, and abdominal aortic calcification. *Results*. For the entire 140 patients, the detection rate was 17.1% for DXA and 46.4% for QCT, a significant difference ($P < 0.01$). Of the 41 patients with conflicting diagnoses, 7 whose diagnosis by QCT was osteoporosis had vertebral fractures even though their DXA findings did not indicate osteoporosis. Varying degrees of spinal degeneration were seen in all of the 41 patients. *Conclusion*. QCT may avoid the overestimation of BMD by DXA associated with spinal degeneration, abdominal aortic calcification, and other sclerotic lesions. It may be more sensitive than DXA for detecting osteoporosis in postmenopausal women.

1. Introduction

Osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to increased bone fragility and a consequent increase in fracture risk. Fractures may lead to a decreased quality of life and increased medical costs. Thus, osteoporosis is widely considered a major health concern.

Consequently, noninvasive techniques for measuring bone mineral density (BMD) play an important role in the clinical diagnosis of osteoporosis and in monitoring its progression. Dual X-ray absorptiometry (DXA) and quantitative computed tomography (QCT) are the most common tools for measuring BMD. DXA determines BMD in two dimensions, including both trabecular and cortical bone, with the results expressed as areal density (grams per square centimeter). QCT allows measurement of volumetric trabecular bone density without superimposition of cortical bone and other tissues, with the results expressed in milligrams per cubic centimeter of calcium hydroxyapatite.

DXA and QCT findings cannot be compared directly, and sometimes the diagnosis indicated by BMD findings differs between the two modalities. Therefore, we compared the detection rate of osteoporosis for posteroanterior DXAs (PA-DXA) with the rate for QCT and analyzed the reasons for the differences between the two.

2. Materials and Methods

Between February 2010 and February 2013, we reviewed data, for our study, for 194 postmenopausal women who underwent QCT and areal spinal and hip DXA in our department with an interval between QCT and DXA scans of two months. Study exclusion criteria included a history of multiple myeloma, rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus (SLE), connective tissue disease, metabolic and endocrine diseases, or bone tumors. Fifty-four patients were excluded, leaving 140 postmenopausal women as study participants.

Table 1: Diagnostic results of DXA versus QCT for 140 participants.

	Normal (%)	Osteopenia (%)	Osteoporosis (%)
Lumbar PA-DXA	69 (49.3)	47 (33.6)	24 (17.1)
Any femoral site by DXA	52 (37.1)	70 (50.0)	18 (12.9)
Any spinal or femoral site by DXA	40 (28.6)	72 (51.4)	28 (20.0)
Lumbar QCT	19 (13.6)	56 (40.0)	65 (46.4)

DXA: dual X-ray absorptiometry; PA-DXA: posteroanterior DXA; QCT: quantitative computed tomography.

DXA measurements were obtained using a Prodigy DXA scanner (GE, Lunar, Madison, WI, USA) and were analyzed using the manufacturer's software. The DXA T-score was calculated on the basis of the Chinese reference database [1]. Vertebrae from L1 to L4 and the left hip were scanned in the supine position using posteroanterior projections. The T-score for L1–L4 and for the femoral neck plus the total hip measurement by DXA were used to diagnose osteoporosis. We used the diagnostic criteria established by the World Health Organization (WHO) in 1994 [2].

QCT measurements were obtained with an Aquilion 64-slice CT scanner (Toshiba, Tokyo, Japan) with a solid Mindways QCT phantom (Mindways Software Inc., Austin, TX, USA). Vertebrae from L1 to L4 were scanned in the supine position. Images were analyzed using the Mindways software. Elliptical regions of interest were put in the midplane of three vertebral bodies (L2–L4) in the trabecular bone automatically, avoiding the cortical bone of the vertebrae. Fractured vertebrae were excluded from measurement. Average BMD is expressed in milligrams per cubic centimeter of calcium hydroxyapatite. For the BMD of spinal trabecular bone, thresholds of 120 mg/cm^3 for osteopenia (equivalent to a DXA T-score of −1.0 SD) and 80 mg/cm^3 for osteoporosis (equivalent to a DXA T-score of −2.5 SD) were suggested by the International Society for Clinical Densitometry in 2007 [3] and by the American College of Radiology in 2008 [4].

To estimate the degree of spinal degeneration and abdominal aortic calcification (AAC), two radiologists who were blinded to the BMD results independently reviewed lumbar CT images. The diagnosis in questionable cases was determined by consensus.

The difference between the osteoporosis detection rates for DXA versus QCT was analyzed using the chi-square test. Results were specified with a 95% confidence interval.

3. Results

The 140 study participants ranged in age from 47.1 to 85.9 years (mean: 63.2 ± 8.1 years). The interval time between DXA and QCT scans ranged from 0 to 43 days (mean: 4.3 ± 9.7 days). The BMD of L1–L4 as measured by DXA ranged from 0.575 to 1.621 g/cm^2 (mean: 0.973 ± 0.169 g/cm^2). The trabecular BMD of the lumbar spine as measured by QCT ranged from −5.0 to 199.4 mg/cm^3 (mean: 81.7 ± 34.8 mg/cm^3).

The osteoporosis detection rates for lumbar PA-DXA, lowest BMD of the femoral neck and total hip, lowest BMD for spinal and hip DXA, and lumbar QCT were 17.1%, 12.9%, 20.0%, and 46.4%, respectively (Table 1). The intragroup detection rates for QCT versus DXA were significantly different ($P < 0.01$), with QCT detecting osteoporosis more frequently than spinal and hip DXA, did.

Of the 140 participants, 41 (29.3%) were found by QCT but not by DXA to have osteoporosis. Of these, 7 (17.1%) had single or multiple vertebral fractures (Figure 1). One of them underwent percutaneous vertebroplasty later. All 41 (100%) had vertebral osteophytes or end plate sclerosis, 33 (80.5%) had facet joint osteoarthritis, 14 (34.1%) had spinous process osteophytes, and 25 (61.0%) had AAC. In addition, 3 participants had bone islands or focal sclerosis (Figure 2). One participant had multiple calcifications in the abdomen.

4. Discussion

The diagnostic criteria for DXA established by WHO in 1994 have long been used as the gold standard in the clinical diagnosis of osteoporosis. The sites most commonly measured are the lumbar spine and hip. PA-DXA determines BMD in two dimensions. Spinal degeneration and AAC may lead to a false finding of increased BMD. By contrast, the site most commonly measured in QCT is the lumbar spine. Osteoporotic bone loss occurs mainly in trabecular bone. The turnover rate of trabecular bone is higher than that of cortical bone. Our study showed a significant difference in osteoporosis detection rates between DXA and QCT, providing clinical evidence that QCT has a greater diagnostic sensitivity than DXA.

Osteoporosis, spinal degeneration, and AAC are most commonly seen in the elderly, and the consequences of these conditions are more serious with increased age. This is why it can be problematic to use DXA rather than QCT; even though clinical findings may indicate osteoporosis, DXA may still indicate that BMD is normal. A possible explanation for the superior performance of QCT may be spinal degeneration and calcinations in the soft tissue around the spine. Yu et al. reported that BMD measured by PA-DXA was significantly higher in patients with spinal degenerative joint disease changes than in those without such changes, particularly when osteophytes were present at the vertebral bodies and facet joints [5]. Ito et al. indicated that the presence of osteophytes is associated with higher BMD when measured with DXA [6]. Our results show that spinal degeneration and AAC may be associated with the overestimation of BMD and the underestimation of osteoporosis by DXA. This may diminish the sensitivity of DXA for assessing osteoporosis.

Several clinical techniques for BMD measurement are available, including DXA, QCT, and ultrasound, each with

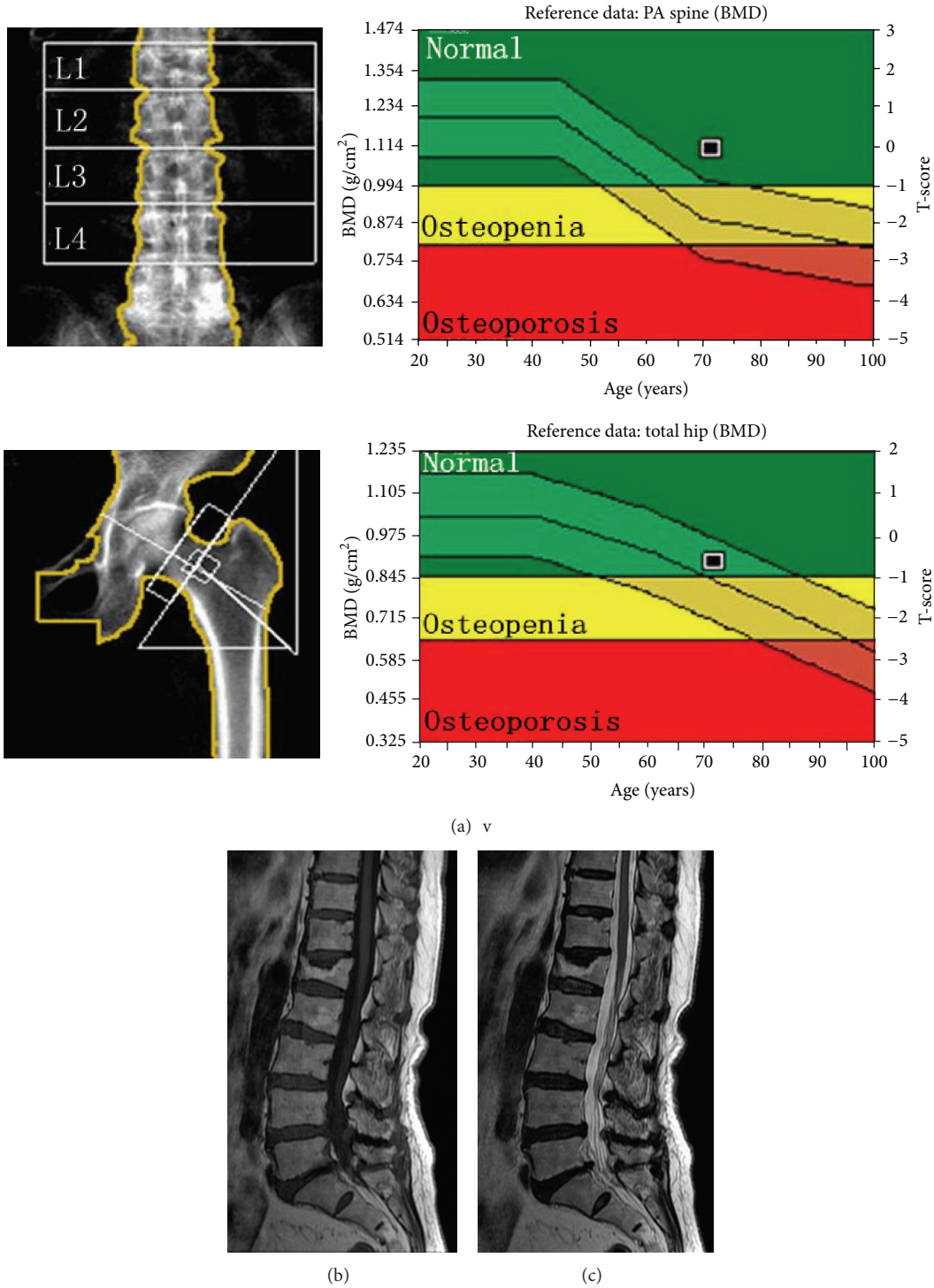


Figure 1: Images obtained from a 71.7-year-old woman whose bone mineral density was found to be normal on dual X-ray absorptiometry. (a) The T-scores for lumbar posteroanterior dual X-ray absorptiometry, the femoral neck, and the total hip were −0.1, −0.8, and −0.7, respectively. The trabecular bone mineral density of L2–L4 was 36.1 mg/cm^3, and the diagnosis via lumbar quantitative computed tomography was osteoporosis. Sagittal lumbar spine images show two adjacent vertebral fractures with wedging, deformation of the end plates, and degenerative disc disease: (b) T_1 weighted and (c) T_2 weighted.

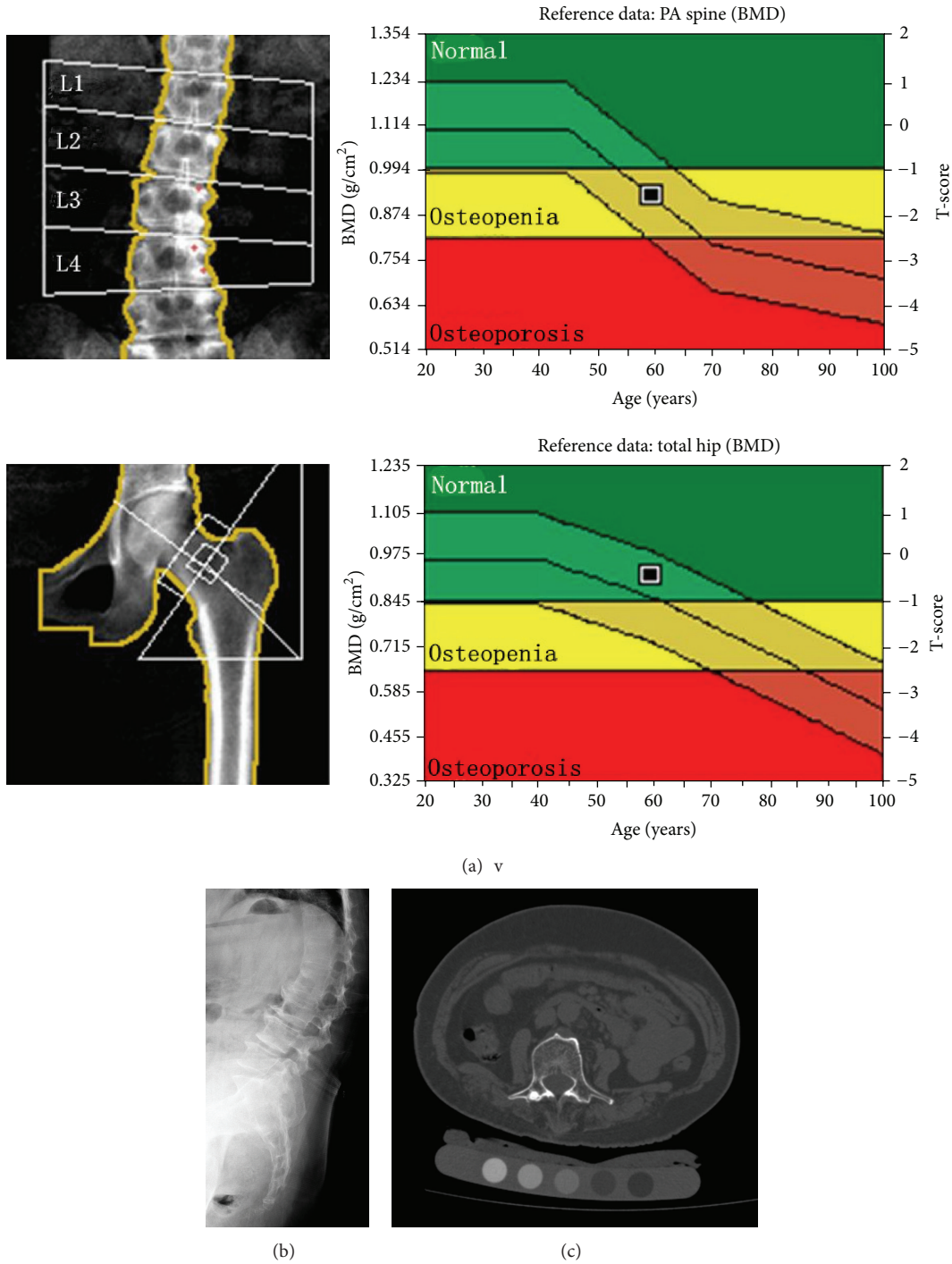


Figure 2: Images obtained from a 59.5-year-old woman who was found by dual X-ray absorptiometry to have osteopenia. (a) The T-scores for lumbar posteroanterior dual X-ray absorptiometry, the femoral neck, and total hip were −1.6, −0.5, and −0.4, respectively. The trabecular bone mineral density of L2–L4 was 71.4 mg/cm^3, indicating a potential diagnosis of osteoporosis according to the American College of Radiology guidelines. A lateral lumbar radiograph (b) and an axial computed tomography image (c) showed severe osteophytes and end plate sclerosis of the lumbar vertebrae. Focal sclerosis is apparent on the right accessory of L3.

its own advantages and shortcomings. Appropriate choice of technique and measurement site is important for the accurate diagnosis of osteoporosis. Schneider et al. found that, under the WHO classification, women with spinal osteoarthritis were more likely to be given a diagnosis of osteoporosis of the femoral neck and hip than those without spinal osteoarthritis but less likely to receive such a diagnosis when BMD was based on the PA spine (14.4% versus 24.5%). Schneider et al.

recommended that, in women aged 65 years and older who are likely to have spinal osteoarthritis, DXA of the hip be used for identification of osteoporosis [7]. However, DXA of the hip still includes cortical bone, so, findings can be influenced by degenerative changes, leading to a decrease in the ability to detect osteoporosis. In our study, the detection rate of osteoporosis at any spinal or femoral site by DXA was significantly lower than the rate for QCT, and no femoral site was superior to the PA spinal site. QCT is truly a three-dimensional technique for quantifying BMD. It may measure BMD more accurately and reproducibly, especially in patients with spinal deformity, severe degenerative changes, extreme obesity, or low body mass index. QCT may be particularly useful in China, where DXA scanners are not available in most areas.

Greenspan et al. found that vertebral fractures were present in 18.3% of asymptomatic postmenopausal women and that 11.0% to 18.7% of individuals with clinical osteoporosis would have been classified as having normal bone by BMD criteria alone [8]. Ling et al. reported that vertebral fractures were present in 15% of women aged 50 years or older in Beijing [9]. In our study, we found vertebral fractures in 17.1% of women in whom QCT but not DXA showed osteoporosis. The extent of osteoporosis in these women may have been underestimated by DXA.

5. Conclusions

As our study demonstrated, QCT may avoid the overestimation of BMD by DXA associated with spinal degeneration, AAC, and other sclerosis lesions, such as bone islands. QCT may be more sensitive for detecting osteoporosis, but this must be validated in a larger population.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

Part of this work was supported by Beijing Bureau of Health 215 program. Medical editor Katharine O'Moore-Klopf, ELS (East Setauket, NY, USA), provided professional English language editing of this paper.

References

[1] X. G. Cheng, D. Z. Yang, Q. Zhou et al., "Age-related bone mineral density, bone loss rate, prevalence of osteoporosis, and reference database of women at multiple centers in China," *Journal of Clinical Densitometry*, vol. 10, no. 3, pp. 276–284, 2007.

[2] J. A. Kanis, L. J. Melton III, C. Christiansen, C. C. Johnston, and N. Khaltaev, "The diagnosis of osteoporosis," *Journal of Bone and Mineral Research*, vol. 9, no. 8, pp. 1137–1141, 1994.

[3] K. Engelke, J. E. Adams, G. Armbrecht et al., "Clinical use of quantitative computed tomography and peripheral quantitative computed tomography in the management of osteoporosis in adults: the 2007 ISCD Official Positions," *Journal of Clinical Densitometry*, vol. 11, no. 1, pp. 123–162, 2008.

[4] American College of Radiology, "ACR Practice Guideline for the Performance of Quantitative Computed Tomography (QCT) Bone Densitometry (Resolution 33)," Reston, Va, USA, 2008, http://www.acr.org/~/media/ACR/Documents/PGTS/guidelines/QCT.pdf.

[5] W. Yu, C. C. Glüer, T. Fuerst et al., "Influence of degenerative joint disease on spinal bone mineral measurements in postmenopausal women," *Calcified Tissue International*, vol. 57, no. 3, pp. 169–174, 1995.

[6] M. Ito, K. Hayashi, M. Yamada, M. Uetani, and T. Nakamura, "Relationship of osteophytes to bone mineral density and spinal fracture in men," *Radiology*, vol. 189, no. 2, pp. 497–502, 1993.

[7] D. L. Schneider, R. Bettencourt, and E. Barrett-Connor, "The clinical utility of spine bone density in elderly women," *Journal of Clinical Densitometry*, vol. 9, no. 3, pp. 255–260, 2006.

[8] S. L. Greenspan, E. Von Stetten, S. K. Emond, L. Jones, and R. A. Parker, "Instant vertebral assessment: a noninvasive dual X-ray absorptiometry technique to avoid misclassification and clinical mismanagement of osteoporosis," *Journal of Clinical Densitometry*, vol. 4, no. 4, pp. 373–380, 2001.

[9] X. Ling, S. R. Cummings, Q. Mingwei et al., "Vertebral fractures in Beijing, China: the Beijing Osteoporosis Project," *Journal of Bone and Mineral Research*, vol. 15, no. 10, pp. 2019–2025, 2000.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 125932, 7 pages
http://dx.doi.org/10.1155/2013/125932

Research Article

Establishment of OPG Transgenic Mice and the Effect of OPG on Bone Microarchitecture

Ying Wu,[1,2] Jianghua Liu,[1,2] Hui Guo,[1] Qiong Luo,[1] Ziying Yu,[1] Eryuan Liao,[3] and Xuyu Zu[1,2]

[1] *Department of Metabolism and Endocrinology, The First Affiliated Hospital of University of South China, Hengyang, Hunan 421000, China*
[2] *Institute of Clinical Medicine, The First Affiliated Hospital of University of South China, Hengyang, Hunan 421000, China*
[3] *Institute of Metabolism and Endocrinology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China*

Correspondence should be addressed to Jianghua Liu; jianghua990@126.com and Xuyu Zu; zuxuyu0108@hotmail.com

Received 3 January 2013; Accepted 5 February 2013

Academic Editor: Guang-Da Xiang

Osteoprotegerin (OPG) plays a determinant role in regulating bone metabolism, but the effect of OPG on bone microarchitecture needs to be further elucidated. We attempted to construct pCI-hOPGp-mOPG vector containing human OPG promoter and FLAG tag and to microinject vector into fertilized zygotes from C57BL/6J × CBA mice to prepare transgenic mice. The OPG transgenic positive mice were identified by PCR and western blotting. Twelve-week-old OPG transgenic mice (OPG-Tg mice) and wild-type mice (WT mice) were utilized in the study of bone microarchitecture. Microcomputed tomography (micro-CT) data showed that compared with WT mice, the tibia of OPG-Tg mice showed an increased volumetric BMD (vBMD), tissue BMD (tBMD), trabecular thickness (Tb.Th), and trabecular number (Tb.N), and a decreased trabecular separation (Th.Sp) ($P < 0.05$) . The cortical bone microarchitecture parameters, such as cortical area (Ct.Ar), cortical thickness (Ct.Th), cortical BMD (Ct.BMD), cortical BMC (Ct.BMC), BMD, and BMC of femur, were increased, and the inner perimeter (In.Pm) was decreased, in OPG-Tg mice, compared to those in WT mice ($P < 0.05$). The established OPG transgenic mouse model could be valuable for further studying the biological significance and gene regulation of OPG in vivo.

1. Introduction

The skeleton is in a dynamic state, being continually degraded and renewed in a tightly regulated remodeling process that involves a complex network of systemic hormones and local factors. Among the local signaling factors implicated in this process is OPG/RANK/RANKL system. OPG, a secreted member of the tumor necrosis factor receptor superfamily, has been identified as an osteoblast-derived regulator of bone resorption and bone mass, and it is implicated in the pathogens of postmenopausal osteoporosis and other metabolic bone diseases. OPG acts by neutralizing RANKL, an essential cytokine required for osteoclast formation and activation [1]. Prior studies have showed that OPG is not only an important regulatory factor of bone growth, bone modeling, and bone remodeling [2, 3], but also an intermediary factor of a wide variety of hormones, cytokine, and growth factors which are involved in regulating bone metabolism [4]. In vitro, osteoclast differentiation which is induced by 1,25-$(OH)_2D_3$, PTH, PGE_2, and IL-4 is blocked by OPG in a dose-dependent manner [5, 6]; in vivo, recombinant OPG increases significantly bone mass and bone mineral density of lumbar spine and femoral of ovariectomized rat [7]. In clinical trials, recombinant OPG can be used for postmenopausal women for the treatment of osteoporosis [8]. These data suggested that OPG could act as a key factor in the regulation of bone mass and implied a utility for OPG in the treatment of metabolic bone disease. In addition to the effect on bone tissue, OPG was also reported to influence cardiovascular [9] and immune system [10]. Therefore, a suitable animal model with OPG overexpression is needed for the further insight into the implication of OPG in metabolic bone disease.

Transgenesis is a process where foreign genes are inserted into an animal's DNA, and it is popular in the biomedical science. Scientists create, so-called transgenic animals to investigate disease treatments, produce natural material, and expand scientific knowledge. Transgenic mice are the most widely used as experimental models to unravel gene phenotype and they are valuable tools for biomedical research [11]. In our previous study, OPG knockout mice showed increased bone remodeling, deceased bone density, and reduction in bone strength [12]. In the present work, we firstly created OPG transgenic mice containing human OPG promoter and FLAG tag. Furthermore, we obtained the quantitative data of bone microarchitecture in OPG transgenic mice for the first time.

2. Materials and Methods

2.1. Animals. C57BL/6J mice (WT mice) were purchased from the Shanghai Research Center for Biomodel Organisms. All studies were performed with the approval of the Experimental Animal Committee at the University of South China. All the animals were housed under specific pathogen-free conditions (22°C, 12 h/12 h light/dark, 50%–55% humidity) with free access to food pellets and tap water in the experimental animal center of the University of South China.

2.2. Construction of Gene Targeting Plasmid. The liver RNA extracted from C57BL/6J mice was used to perform two PCR amplification using Pfu PCR enzyme to obtain mOPG cDNA sequence. Then mOPG cDNA sequence and pCI-NEO-OPG-LacZ plasmid which contained human OPG promoter were double digested by using Not I and Sal I. The digested fragments were connected by using T4 ligase, and then the connected fragments were transferred into competent cells from Escherichia coli and tiled in LB plate. Monoclonal bacteria were selected and the pCI-hOPGp-mOPG plasmid was confirmed by enzyme digestion and sequence reaction. The vector was digested by using BglII/KpnI to obtain linear DNA fragment for further transgenic mice establishment.

2.3. Generation of Transgenic Mice. Six–eight-week C57BL/6J × CBA female mice were selected to perform ovarian stimulation and subsequently mated with healthy male mice overnight. The female mice which have vaginal pessary were selected as receptor for embryo transplantation, and then fertilized eggs were obtained from the receptors by surgery. Linear DNA was injected into fertilized eggs (pronuclear stage) by using an inverted microscope, micromanipulation equipment, and injection/holding devices. To generate OPG-Tg mice, 15~30 recombinant cells were injected into C57BL/6J blastocysts and then transferred into uterus of pseudopregnant mice. After young mice were born, progeny was screened for the presence of the OPG by PCR and western Blot.

2.4. Genotype Identification. Genomic DNA was extracted and purified from the tail tissue of each mouse. To genotype the OPG-Tg mice, PCR was performed using the following primers: OPG-JD-1 : 5-TCA AAG GCA GGC GAT ACT-3 and OPG-JD-2 : 5-CAA TGT CTT CCT CCT CAC TGT-3.

2.5. OPG Protein Expression Analysis. The mice were sacrificed after 12 weeks, and proteins were extracted from the livers. The OPG protein levels in the mice were detected by performing a western blot assay using rabbit anti-FlAG polyclonal antibodies [13, 14].

2.6. Micro-CT. The right tibia was fixed with 4% paraformaldehyde for 24 h and subsequently washed with 10% saccharose solution for 12 h. Micro-CT scanning was performed using the GE explore Locus SP system. The proximal tibia was selected as the region for scanning by the fluoro method. The center of rotation and the CT value were artificially modified and the entire results obtained for the samples scanned with an isotropic resolution of $8.0 \times 8.0 \times 8.0\,\mu$m voxels were reconstructed after completion of the scanning. For the cancellous bone analysis, bone tissue of 0.8 mm thickness at a distance of 0.16 mm from the distal end of the growth plate was selected from the image as the region of interest (ROI) in order to perform a three-dimensional reconstruction of $8.0 \times 8.0 \times 8.0\,\mu$m voxels; image information was obtained based on the automatic domain value provided by the computer in order to accomplish binary conversion of the image. Selected areas of cancellous bone within the ROI were demonstrated three dimensionally. For the cortical bone analysis, bone tissue, 1.0 mm in length and 0.16 mm in thickness, from the middle diaphysis of the tibia was selected as the ROI from the reconstructed image to perform three-dimensional reconstruction of $8.0 \times 8.0 \times 8.0\,\mu$m voxels, and image information was obtained based on the automatic domain value yielded by the computer. The 2.0^{+}ABA Microview software of the micro-CT system was applied to perform quantitative analysis on the reconstructed images.

2.7. DXA Scanning and Image Analysis. The left femur was fixed onto the scanning table along the longitudinal axis and subsequently scanned by dual-energy X-ray absorptiometry (DXA) using a PIXImus densitometer (GE Lunar, Madison, WI) to determine the BMD and BMC. Scanning parameters are as follows: the scan spacing was 1.0×1.0 mm, scanning speed was 60.0 mm/s, and the accuracy was 1.0%.

2.8. Statistics. The SPSS 15.0 statistical software was used for statistical analysis. Data are reported as mean ± SD. Data were analyzed by analysis of variance followed by the Student-Newman-Keuls Multiple Comparisons test, and statistical significance was considered when P was less than 0.05.

3. Results

3.1. Construction of Gene Targeting Plasmid. The liver RNA extracted from C57BL/6J mice was utilized to perform RT-PCR to obtain OPG cDNA sequence. OPG cDNA sequence and pCI-NEO-OPG-LacZ plasmid (Figure 1(a)) containing human OPG promoter were double digested by using Not I and Sal I, and a 1.2 kb fragment from mOPG cDNA and a 10.4 kb fragment from pCI-NEO-OPG-LacZ were observed

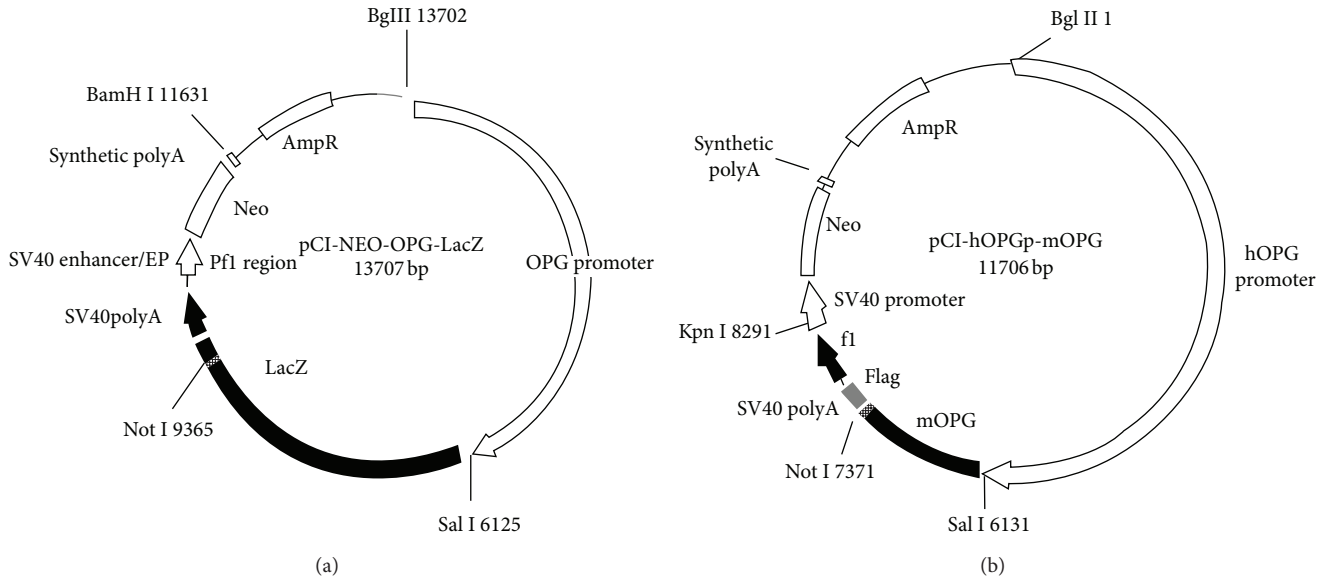


Figure 1: Structure scheme of plasmids. (a) PCI-NEO-OPG-LacZ plasmid profile. (b) PCI-hOPGp-mOPG plasmid profile.

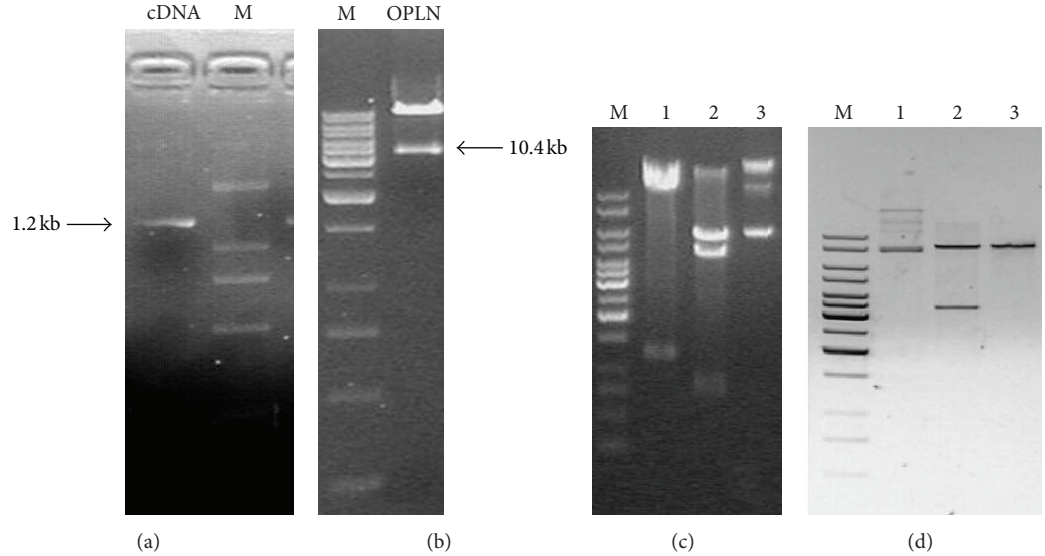


Figure 2: Recombinant PCI-hOPGp-mOPG plasmid construction and plasmid linearization. (a) mCPG DNA was digested by Not I and Sal I. (b) PCI-NEO-OPG-LacZ was digested by restriction enzymes Not I and Sal. (c) Identification of the digested pCI-hOPG-mOPG.M:Marker1Kb Ladder. 1: pCI-hOPG-mOPG was digested by NotI and SalI; 2: pCI-hOPG-mOPG was digested by EcoRI; 3: pCI-hOPG-mOPG plasmid. (d) linear pCI-hOPGp-mOPG plasmid DNA.M:1KbLadder; 1: pCI-hOPG-mOPG palsmid; 2: pCI-hOPG-mOPG digested by BglII/KpnI; 3: pCI-hOPG-mOPG digested by BglII/KpnI was recovered.

(Figures 2(a) and 2(b)). The recombinant pCI-hOPGp-mOPG plasmid (Figure 1(b)) showed a 1.2 kb fragment and a 10.4 kb fragment with the digestion of Not I and Sal I. 766 bp, and 5354 bp and 5586 bp fragments were observed after the digestion EcoR (Figure 2(c)). The recombinant pCI-hOPGp-mOPG plasmid was linealized by the digestion with BglII and KpnI to obtain two DNA fragments of 8291 bp and 3411 bp, and the 8291 bp linearized pCI-hOPGp-mOPG fragment was microinjected into mouse fertilized egg cells (Figure 2(d)).

3.2. Positive Transgenic Mice Identification. During the course of transgenic mice preparation, total 426 injection eggs were transplanted into 17 pseudopregnant female mice tuba, and 12 female mice of them were shown to be pregnant. Total 69 mice were born and 7 positive transgenic mice were confirmed by PCR screening. Furthermore, genomic DNA extracted from positive mice and WT mice tails was used for double-blinded PCR to further confirm the seven positive transgenic mice (Figure 3(a)). The transgenic founder female mice were fed together with WT male mice in the cage

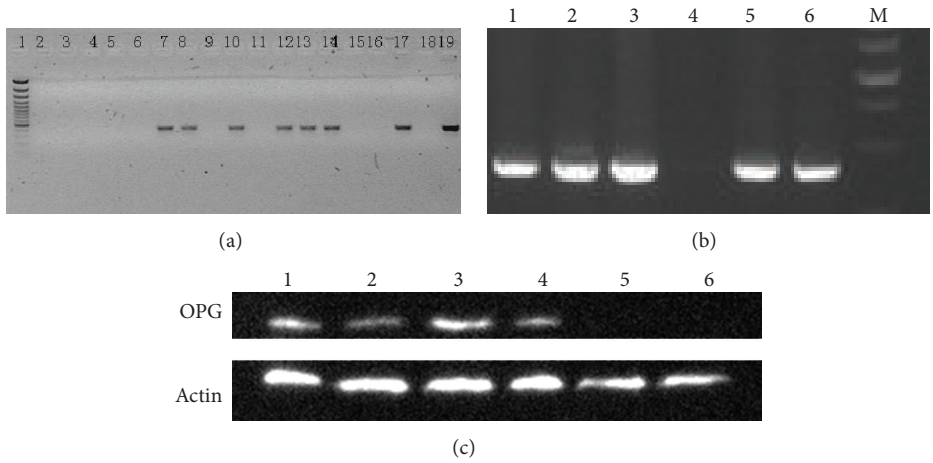


Figure 3: Identification of genotypes of OPG-Tg mice. (a) Identification of OPG genotypes in OPG-Tg founder mice; 1: GenenRuler 100 bp DNA Ladder; 2–17: mice samples; 18: ddH2O; 19: positive control. (b) Identification of young mice genotypes. M: GenenRuler100 bp DNA Ladder; 1–3: young pup samples; 4: ddH_2O; 5-6: positive control. (c) OPG-Tg mice liver's western blot examination. 1–4: OPG-Tg mouse; 5: WT mouse; 6: H_2O.

Table 1: Femur overall bone mineral density and bone mineral content of two types of mice.

	BMD (mg/cm^2)	BMC (mg)
OPG-Tg	32.3 ± 1.2[a]	7.3 ± 1.1[a]
WT	22.7 ± 0.9	2.9 ± 0.7

Values are means ± SD of 4 mice. [a] Compared with WT group, $P < 0.05$.

Table 2: Comparison of trabecular bone structural parameter.

	WT	OPG-Tg
vBMD (mg/mm^3)	207.9 ± 27.6	292.3 ± 30.1[a]
tBMD (mg/mm^3)	621.7 ± 26.9	694.5 ± 33.1[a]
Tb.Th (mm)	0.015 ± 0.001	0.024 ± 0.002[b]
Tb.N (mm^{-1})	3.11 ± 0.63	5.04 ± 0.82[a]
Tb.SP (mm)	0.175 ± 0.021	0.092 ± 0.010[b]

Values are means ± SD of 6 mice. [a] Compared with WT group, $P < 0.05$. [b] Compared with WT group, $P < 0.01$.

rearing at the ratio of 1 : 1, and transgenic founder male mice were fed with WT female mice in cage at the ratio of 1 : 3. To identify the genotype of the offspring, special primers across the hOPG promoter and mOPG were used for the PCR screening, and as shown in Figure 3(b), no band was detected in WT mice and one 385 bp band was observed in OPG-Tg mice. To further confirm the genotype of the transgenic mice, the proteins were extracted from the mice livers and western blot was performed using rabbit anti-FLAG polyclonal antibodies. As shown in Figure 3(c), the FLAG tag could not be detected in the WT mice, whereas a 60-kD OPG protein containing FLAG tag could be detected in the OPG-Tg mice.

3.3. BMD and BMC Measurement. The whole left femur was scanned by DXA to determine the BMD and BMC. It was found that OPG-Tg mice showed increases of 42.3% and 151.7% of overall femoral BMD and BMC, respectively, compared to those of WT mice (Table 1).

3.4. Micro-CT. To observe the effect of OPG on bone microarchitecture, micro-CT was used. As shown in Figure 4, compared with WT mice, cancellous bone trabecular number of OPG-Tg mice increased significantly; distribution of bone trabecula in OPG-Tg mice became denser and more continuous and trabecular separation of OPG-Tg mice became smaller. Micro-CT scanning also showed that the OPG-Tg mice had an increase of 40.6%, 11.7%, 60%, and 62.1% for vBMD, tBMD, Tb.Th, and Tb.N, respectively, and a decrease of 47.4% for Th.Sp compared to those of WT mice (Table 2). The cortical bone structural parameter also showed difference between OPG-Tg mice and WT mice. OPG-Tg mice showed obviously thicker cortical bone and narrower lumen (Figure 4). It was also confirmed that the OPG-Tg mice mice showed an increase of 81.0%, 132.9%, 15.7%, and 118.2% for Ct.Ar, Ct.Th, Ct.BMD, and Ct.BMC respectively, and a decrease of 15.4% for In.Pm compared to those of WT mice. No significant outer perimeter (Ot.Pm) difference was observed between OPG-Tg mice and WT mice (Table 3).

4. Discussion

OPG, a novel secreted member of the TNFR superfamily, acts as a soluble receptor antagonist of RANKL by preventing it from binding to RANK, and the interaction of RANKL and RANK has been shown to be required for osteoclast differentiation. Any dysregulation of their respective expression leads to bone tumor-associated osteolysis, immune disease, and cardiovascular pathology. Many researches have proved that OPG can inhibit osteoclast maturation and protect bone

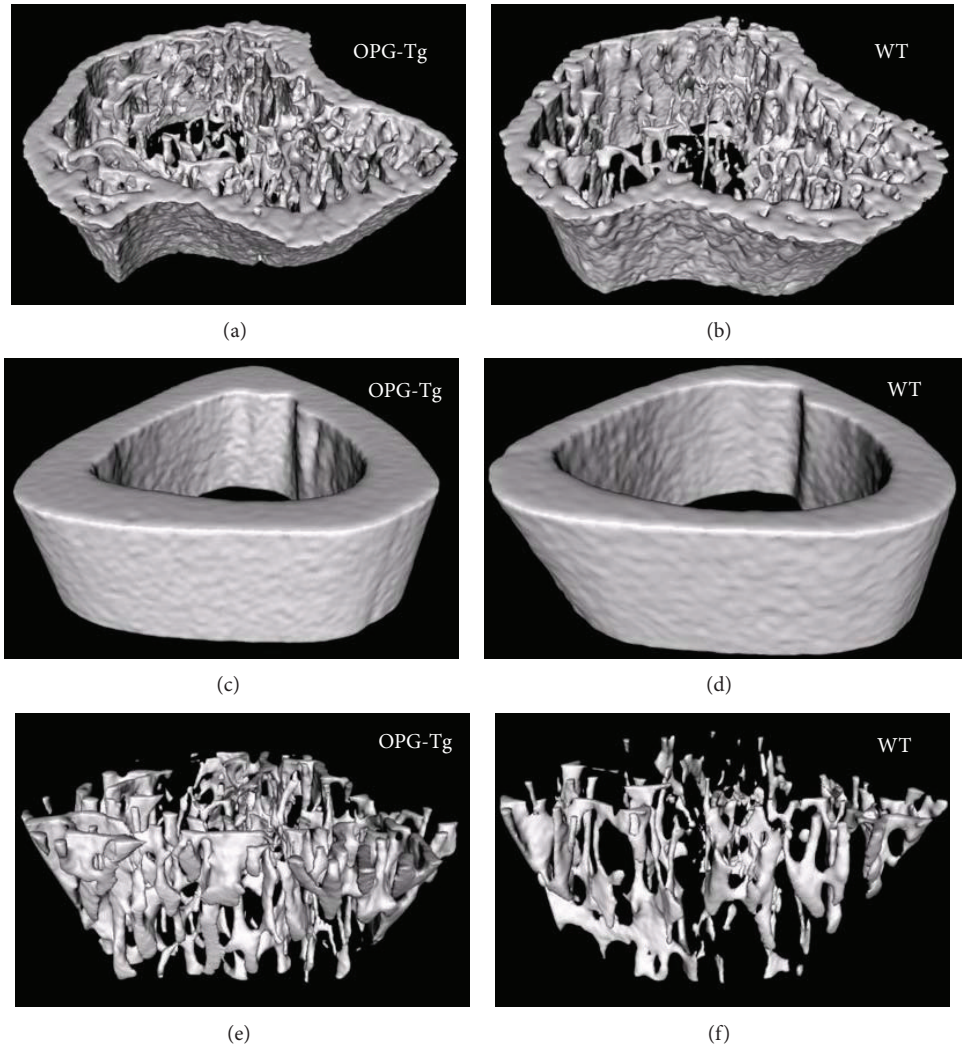


Figure 4: Three-dimensional micro-CT images of the right tibia from two types of mice. (a) Image of the right tibia of OPG-Tg mice; (b) image of the right tibia of WT mice; (c) image of the cortex of OPG-Tg mice; (d) image of the cortex of WT mice; (e) image of the trabecular of OPG-Tg mice; (f) image of the trabecular of WT mice.

Table 3: Comparison of cortical bone structural parameter.

	WT	OPG-Tg
In.Pm (mm)	2.317 ± 0.098	1.961 ± 0.082[a]
Ot.Pm (mm)	3.677 ± 0.271	3.881 ± 0.541
Ct.Ar (mm^2)	0.263 ± 0.014	0.476 ± 0.032[b]
Ct.Th (mm)	0.082 ± 0.001	0.191 ± 0.004[b]
Ct.BMD (mg/mm^3)	1017.3 ± 9.3	1176.8 ± 10.9[b]
Ct.BMC (g)	0.0022 ± 0.0001	0.0048 ± 0.0006[b]

Values are means ± SD of 6 mice. [a] Compared with WT group, $P < 0.05$. [b] Compared with WT group, $P < 0.01$.

from both normal osteoclast remodeling and ovariectomy-associated bone loss, indicating that OPG might be a key determinant in regulating bone metabolism [15]. Firstly, RANKL is a membrane-bound protein of the tumor necrosis factor ligand family that is expressed on the osteoblast cell surface and has been shown to play a major role in osteoclast differentiation along with macrophage colony stimulating factor. RANKL binds to its receptor RANK on hematopoietic cells and initiates a cascade of signaling events that leads to osteoclast differentiation. As a decoy receptor for RANKL, OPG can prevent its interaction with the cognate receptor RANK. OPG has been shown to be a potent inhibitor of bone resorption through interfering osteoclast survival, differentiation, and biological activity in vitro. Secondly, OPG can prevent TAF6 activation with RANK/RANKL, thus inhibiting osteoclast activation and maturation, and this could ultimately result in the reduction of functional osteoclast. Thirdly, OPG can also prevent stroma cell from the interaction with osteoclast, and this inhibitory ability can be protected in the presence of caspase-3 inhibitor, indicating that OPG can prevent bone loss through inducing osteoclast apoptosis [16]. Finally, OPG can also affect the function of osteoclast directly by increasing the protease and trypsin inhibitor expression. In summary, the mechanism

of OPG in regulating bone metabolism is complicated and deserves more investigations [17]. OPG was also shown to influence cardiovascular and immune system except for the bone tissue. OPG-deficient mice have shown early onset arterial calcification [18, 19], suggesting an important role of OPG in the protection of blood vessels, whereas recent studies showed that OPG might increase adhesion function of endothelial cell and serum OPG level is associated with atherosclerosis severity. In the immunity system, OPG can regulate dendritic cell differentiation and maturation, affecting lymphocyte development and function [20]. Due to the diverse function of OPG, a suitable animal model is needed for the comprehensive understanding of OPG function.

Recently, the advances in transgenic technologies have made it possible for the developments of different transgenic animal models. Compared with other animal model, mouse shows higher genome homology with human, so it is widely used as transgenic animal model for biomedical research. Hence, the mouse is unique in offering the possibility to understand genotype-phenotype relationships that are relevant for unraveling the biologic role of the genes in human [11]. OPG-deficient mice exhibited bone loss with an increase in both bone resorption and formation, which just like high bone turnover postmenopausal osteoporosis [21]. In this work, we ligated full-length mouse OPG cDNA sequence with pCI-NEO-LacZ plasmid which contains human OPG promoter to obtain pCI-hOPGp-mOPG vector. Because variety of FLAG-tagged protein can retain their biochemical activity, FLAG tag is widely utilized as screening marker in the development of transgenic cells and animals models [22]. So FLAG tag was added to pCI-hOPGp-mOPG vector to improve the screen efficiency of OPG-Tg mice. Because human OPG promoter has a transcriptional activity without tissue specificity, it could be anticipated that the established OPG transgenic mice could have a widely exogenous expression of OPG in different tissues. In order to avoid possible noise from endogenous OPG in the screening process, specific primers that span from the human OPG promoter to mOPG were designed to improve the correct rate in the OPG-Tg mice screening; moreover, FLAG protein expression in the OPG-Tg mice was also detected to further devoid those transgenic mice without OPG expression due to different genomic context. Since different founder mice had different insertion sites, we got at least two transgenic mice lines to observe their phenotype, and the same phenotypes from different transgenic mice lines were considered as phenotypes of *OPG* gene.

Micro-CT is an evolving technique that has the ability to measure three-dimensional (3D) bone microstructure in arbitrary orientations in a highly automated, objective, nondestructive manner, allowing great number of samples for unbiased comparisons between controls and the disordered or treated. Compared to two-dimensional image (2D) data of DXA, micro-CT can not only directly observe the 3D images of cortical bone and trabecular bone microstructure, but also make a quantitative analysis of the 3D structure such as trabecular volume, trabecular thickness, number, separation, structure model index, degree of anisotropy, and connectivity, in a model-independent manner. Quantitative analysis of 3D bone microstructure characteristics may improve our ability to understand the pathophysiology of osteoporosis, to test the efficacy of pharmaceutical intervention, and to estimate bone biomechanical properties [23]. So it is very meaningful to measure 3D bone microstructure of OPG transgenic mice to observe the direct effect of OPG on bone mass. Previous research has reported the quantitative analysis of 3D bone microstructure in the OPG transgenic rats [24], and in the present study, we for the first time quantified 3D bone microstructure in OPG transgenic mice. Micro-CT data showed that compared with wild-type mice, the OPG-Tg mice showed increased tibial cancellous bone microstructural parameters such as vBMD, tBMD, Tb.Th, and Tb.N than those in wild-type mice. The cortical bone microarchitecture parameters of tibia such as Ct.Ar, Ct.Th, Ct.BMD, and Ct.BMC in OPG-Tg mice were significantly higher than those in WT mice. The 3D structure diagram of tibia showed that cortical bone appeared thick and luminal appeared narrow in OPG-Tg mice. The 2D image data of DXA also showed that BMD and BMC were significantly improved in OPG-Tg mice.

5. Conclusion

In summary, we for the first time developed OPG transgenic mice model containing human OPG promoter and FLAG tag, and a quantitative analysis of the 3D bone microarchitecture in OPG transgenic mice was also carried out. Our results show that the bone mass of cancellous and cortical bone in OPG transgenic mice is significantly increased. The established OPG transgenic mouse model would offer a suitable animal model for further insight into the regulating mechanism of bone metabolism.

Conflict of Interests

All authors disclose that there is no financial or ethical conflict of interests.

Authors' Contribution

Y. Wu and J. Liu contributed equally to this work.

Acknowledgments

The investigators acknowledge the assistance of Shanghai Research Center for Biomodel Organisms and the suggestions from Dr. Jing Zhong, Dr. Huifang, Tang and Dr. Xinhua Xiao for the revision of the paper. This work was supported by National Natural Science Foundation (no. 81170807 to J. Liu) and a Grant from Scientific and Technological Department of Hunan province (no. 2010FJ3153 to J. Liu).

References

[1] D. L. Lacey, W. J. Boyle, W. S. Simonet et al., "Bench to bedside: elucidation of the OPG-RANK-RANKL pathway and the development of denosumab," *Nature Reviews Drug Discovery*, vol. 11, no. 5, pp. 401–419, 2012.

[2] S. Honsawek, T. Chaiwatanarat, P. Vejchapipat, V. Chongsrisawat, N. Thawornsuk, and Y. Poovorawan, "Relationships between OPG, RANKL, bone metabolism, and bone mineral density in biliary atresia," *Pediatric Surgery International*, vol. 25, no. 3, pp. 261–267, 2009.

[3] K. H. Baek, K. W. Oh, W. Y. Lee et al., "Changes in the serum sex steroids, IL-7 and RANKL-OPG system after bone marrow transplantation: influences on bone and mineral metabolism," *Bone*, vol. 39, no. 6, pp. 1352–1360, 2006.

[4] S. Jurado, N. Garcia-Giralt, A. Diez-Perez et al., "Effect of IL-1β, PGE2, and TGF-β1 on the expression of OPG and RANKL in normal and osteoporotic primary human osteoblasts," *Journal of Cellular Biochemistry*, vol. 110, no. 2, pp. 304–310, 2010.

[5] S. Kwan Tat, J. P. Pelletier, D. Lajeunesse, H. Fahmi, M. Lavigne, and J. Martel-Pelletier, "The differential expression of osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) in human osteoarthritic subchondral bone osteoblasts is an indicator of the metabolic state of these disease cells," *Clinical and Experimental Rheumatology*, vol. 26, no. 2, pp. 295–304, 2008.

[6] S. K. Tat, J. P. Pelletier, D. Lajeunesse, H. Fahmi, N. Duval, and J. Martel-Pelletier, "Differential modulation of RANKL isoforms by human osteoarthritic subchondral bone osteoblasts: influence of osteotropic factors," *Bone*, vol. 43, no. 2, pp. 284–291, 2008.

[7] Y. Wang, P. Huang, P. F. Tang, K. M. Chan, and G. Li, "Alendronate (ALN) combined with Osteoprotegerin (OPG) significantly improves mechanical properties of long bone than the single use of ALN or OPG in the ovariectomized rats," *Journal of Orthopaedic Surgery and Research*, vol. 6, no. 1, article 34, 2011.

[8] J. Zupan, S. Mencej-Bedrač, S. Jurković-Mlakar, J. Preželj, and J. Marc, "Gene-gene interactions in RANK/RANKL/OPG system influence bone mineral density in postmenopausal women," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 118, no. 1-2, pp. 102–106, 2010.

[9] D. E. Malliga, D. Wagner, and A. Fahrleitner-Pammer, "The role of osteoprotegerin (OPG) receptor activator for nuclear factor kappaB ligand (RANKL) in cardiovascular pathology—a review," *Wiener Medizinische Wochenschrift*, vol. 161, no. 23-24, pp. 565–570, 2011.

[10] F. Montecucco, S. Steffens, and F. Mach, "The immune response is involved in atherosclerotic plaque calcification: could the RANKL/RANK/OPG system be a marker of plaque instability?" *Clinical & Developmental Immunology*, vol. 2007, p. 75805, 2007.

[11] X. Miao, "Recent advances in the development of new transgenic animal technology," *Cellular and Molecular Life Sciences*, vol. 70, no. 5, pp. 815–828, 2012.

[12] J. Liu, K. Xu, G. Wen et al., "Comparison of the effects of genistein and zoledronic acid on the bone loss in OPG-deficient mice," *Bone*, vol. 42, no. 5, pp. 950–959, 2008.

[13] R. R. Cui, S. J. Li, L. J. Liu et al., "MicroRNA-204 regulates vascular smooth muscle cell calcification in vitro and in vivo," *Cardiovascular Research*, vol. 96, pp. 320–329, 2012.

[14] Q. H. Liang, Y. Jiang, X. Zhu et al., "Ghrelin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the ERK pathway," *PLoS ONE*, vol. 7, no. 4, p. 33126, 2012.

[15] A. P. Trouvin and V. Goëb, "Receptor activator of nuclear factor-κB ligand and osteoprotegerin: maintaining the balance to prevent bone loss," *Clinical Interventions in Aging*, vol. 5, pp. 345–354, 2010.

[16] M. Montagnana, G. Lippi, E. Danese, and G. C. Guidi, "The role of osteoprotegerin in cardiovascular disease," *Annals of Internal Medicine*, 2012.

[17] L. L. E. Hou, J. F. A. Hou, J. Yao, and Z. L. Zhou, "Effects of osteoprotegerin from transfection of pcDNA3.1(+)/chOPG on bioactivity of chicken osteoclasts," *Acta Veterinaria Scandinavica*, vol. 53, article 21, 2011.

[18] N. Bucay, I. Sarosi, C. R. Dunstan et al., "Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification," *Genes and Development*, vol. 12, no. 9, pp. 1260–1268, 1998.

[19] T. Omland, T. Ueland, A. M. Jansson et al., "Circulating osteoprotegerin levels and long-term prognosis in patients with acute coronary syndromes," *Journal of the American College of Cardiology*, vol. 51, no. 6, pp. 627–633, 2008.

[20] C. G. Mueller and E. Hess, "Emerging functions of RANKL in lymphoid tissues," *Frontiers in Immunology*, vol. 3, p. 261, 2012.

[21] M. Tabuchi, K. Miyazawa, M. Kimura et al., "Enhancement of crude bone morphogenetic protein-induced new bone formation and normalization of endochondral ossification by bisphosphonate treatment in osteoprotegerin-deficient mice," *Calcified Tissue International*, vol. 77, no. 4, pp. 239–249, 2005.

[22] F. Sasaki, T. Okuno, K. Saeki et al., "A high-affinity monoclonal antibody against the FLAG tag useful for G-protein-coupled receptor study," *Analytical Biochemistry*, vol. 425, no. 2, pp. 157–165, 2012.

[23] S. X. Vasquez, N. Shah, and A. M. Hoberman, "Small animal imaging and examination by micro-CT," *Methods in Molecular Biology*, vol. 947, pp. 223–231, 2013.

[24] M. S. Ominsky, M. Stolina, X. Li et al., "One year of transgenic overexpression of osteoprotegerin in rats suppressed bone resorption and increased vertebral bone volume, density, and strength," *Journal of Bone and Mineral Research*, vol. 24, no. 7, pp. 1234–1246, 2009.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 897193, 9 pages
http://dx.doi.org/10.1155/2013/897193

Clinical Study

Selective Determinants of Low Bone Mineral Mass in Adult Women with Anorexia Nervosa

Andrea Trombetti,[1] Laura Richert,[1] François R. Herrmann,[1] Thierry Chevalley,[1] Jean-Daniel Graf,[2] and René Rizzoli[1]

[1] *Bone Diseases Service, Department of Internal Medicine Specialties, Geneva University Hospitals and Faculty of Medicine, Rue Gabrielle-Perret-Gentil 4, 1211 Geneva 14, Switzerland*
[2] *Central Laboratory of Clinical Chemistry, Geneva University Hospitals, 1221 Geneva, Switzerland*

Correspondence should be addressed to Andrea Trombetti; andrea.trombetti@hcuge.ch

Received 7 November 2012; Revised 2 February 2013; Accepted 14 February 2013

Academic Editor: Ling-Qing Yuan

We investigated the relative effect of amenorrhea and insulin-like growth factor-I (sIGF-I) levels on cancellous and cortical bone density and size. We investigated 66 adult women with anorexia nervosa. Lumbar spine and proximal femur bone mineral density was measured by DXA. We calculated bone mineral apparent density. Structural geometry of the spine and the hip was determined from DXA images. Weight and BMI, but not height, as well as bone mineral content and density, but not area and geometry parameters, were lower in patients with anorexia nervosa as compared with the control group. Amenorrhea, disease duration, and sIGF-I were significantly associated with lumbar spine and proximal femur BMD. In a multiple regression model, we found that sIGF-I was the only significant independent predictor of proximal femur BMD, while duration of amenorrhea was the only factor associated with lumbar spine BMD. Finally, femoral neck bone mineral apparent density, but not hip geometry variables, was correlated with sIGF-I. In anorexia nervosa, spine BMD was related to hypogonadism, whereas sIGF-I predicted proximal femur BMD. The site-specific effect of sIGF-I could be related to reduced volumetric BMD rather than to modified hip geometry.

1. Introduction

Significant bone loss is a major feature of anorexia nervosa [1, 2]. Bone mineral density (BMD) has been reported to be reduced by more than 2.5 SD at either the hip or spine in 38% of women with anorexia nervosa and by more than 1.0 SD in 92% [3]. Bone loss predisposes to an increased risk of fractures [2, 4, 5]. The possible mechanisms of bone loss include nutritionally mediated changes in gonadal steroid concentrations [6] as well as alterations in the GH-IGF-I system, with markedly low serum insulin-like growth factor-I (sIGF-I) concentrations [7–11]. IGF-I stimulates bone formation through its interaction with the type I IGF-I receptor on the osteoblast [12–16]. A number of studies have shown an imbalanced state of bone turnover in such patients, with decreased markers of bone formation and increased or normal markers of bone resorption. The latter observation appears to be primarily driven by estrogen deficiency, while decreased bone formation has been attributed to low sIGF-I levels [7, 17]. Indeed, several studies of anorexia patients found lower levels of sIGF-I and a direct correlation of this hormone with weight or body mass index (BMI). However, data demonstrating that reduced serum concentrations of sIGF-I contribute to low bone mineral density and whether different skeletal sites are affected in a similar way are scarce. The relative impact of estrogen deficiency versus low sIGF-I on volumetric density or size is not well established. In a cohort of patients with anorexia nervosa, we studied skeletal site specificity of the association between bone density or size, and sIGF-I level or sex hormone deficiency.

2. Subjects and Methods

2.1. Patients and Controls. The study is a retrospective analysis in a selected group of patients with anorexia nervosa which were consecutively referred to our consultation with data prospectively collected. The participants with anorexia

nervosa met at a given time DSM-IV criteria [18], including weight less than 85% of expected, intense fear of being fat, disturbed body image, and amenorrhea for three or more consecutive menstrual cycles. Women who had regular uterine withdrawal bleeding while receiving estrogen therapy and women with patterns of concomitant binge eating and purging type were also included. We also included 11 patients who had resumed menses, but still with an active disease. All except one patients had secondary amenorrhea. One patient had primary amenorrhea.

Height was determined by a Holtain stadiometer (Holtain, Crosswell, Wales). Weight was determined on a calibrated scale to the nearest 0.1 kg. Percentage of ideal body weight [18] and body mass index (with weight and height measured in kilograms/height in meters^2) were calculated. Age at menarche, time since last menstrual period for patients with amenorrhea at time of evaluation, and total duration of amenorrhea were determined, as well as previous and current estrogen use and fracture history during the course of anorexia nervosa. Calcium and protein intakes were recorded by a validated food frequency questionnaire [19] in a subset of 56 patients. Fifty-two subjects completed a validated questionnaire for a detailed assessment of their specific weekly sports activities, including physical education classes, organized sports, recreational activities, walking, and cycling [20]. Their physical activities were computed in hours per week.

Sixty-six normal women of the same age were used as a control population. Controls were recruited mainly among the hospital staff and their families.

2.2. Areal Bone Mineral Density (BMD). Areal BMD of the lumbar spine (LS) in anteroposterior view, femoral neck (FN) and proximal femur (PF) were measured by dual-energy X-ray absorptiometry (DXA) using Hologic QDR 2000 or 4500 instruments (Hologic, Inc., Waltham, MA).

The coefficient of variation of repeated BMD measurements was 1%-2% [21, 22]. The BMD (in g/cm^2) of the LS (L2–L4), FN, and PF was also expressed as a T-Score which compares individual BMD values to those of a young normal population of the same gender (number of standard deviations from mean) [23]. According to criteria defined by the World Health Organization, T-Scores between –1 and –2.49 indicate osteopenia, whereas T-Scores below −2.5 standard deviations (≤−2.5) indicate osteoporosis [24]. The T-Score was applied in patients older than 18. BMD (g/cm^2) of the LS, and those of FN were also expressed in terms of standard deviations from mean gender- and age-matched controls (Z-Scores).

Skeletal size was estimated from the scanned bone projected area of LS and PF. At the spine level, the height and the width at three levels (high, middle, and lower level) of vertebral body L2 were measured. Structural geometry parameters of the hip were measured from conventional DXA outputs of the femoral neck region of interest. We measured the internal and external cortical thickness, the diameter of the femoral shaft at a fixed level of the femoral diaphysis (10 lines from the lower edge of the trochanter minor), neck width (area divided by the mean diameter of the femoral neck), and hip length (measuring the central line from the femoral head to the trochanteric region). The number of measured pixels was multiplied by a conversion factor of 1 pixel = 0.43 mm for the Hologic 2000 device and 1 pixel = 0.45 mm for the Hologic 4500 device. We determined the reproducibility of the bone geometry parameters measurements. All measures were done by the same trained technician. Sample size for the studied group was estimated based on Walter et al.'s approximation method [25]. Assuming a minimal intraclass correlation coefficient (ICC) of 0.5 (p0) against a desired of 0.8 (p1), based on $\alpha = 0.05$ and $\beta = 0.20$, at least 14 participants were required, if measured 3 times. CV (%) was computed as the 100 times the standard deviation divided by the average of 3 measures repeated in 14 patients. The mean CV of the morphological DXA parameters ranged from 0.12% ± 0.06% to 1.09% ± 0.74%. Bone mineral apparent density (BMAD) was calculated as previously described for LS and FN [26, 27]. At the lumbar spine and femoral neck level, BMAD was computed by using the equation BMAD = BMC/projected area$^{1.5}$ and BMAD = BMC/projected area^2, respectively.

2.3. Serum and Urinary Measurements. Prolactin, FSH, and TSH were determined using standard methods, for patients with amenorrhea and not receiving any form of estrogen. PTH was determined by immunoradiometric assay with an intrassay coefficient of variation of 1.8%–3.4% (Nichols Institute diagnostics). Calcifediol (25-OH-D_3) was determined by chemiluminescence assay (Nichols Advantage, San Clemente, CA). The first 19 sIGF-I determinations, that is, before March 2002, were performed by RIA after acid-ethanol extraction (Nichols Institute diagnostics). Thereafter, a fully automated chemiluminescence assay (Nichols Institute diagnostics) was used. Intra- and interassay coefficients of variation were 6.4% and 10% for RIA measurement, and 4.8% and 6.7% for chemiluminescence assay. The two assays were highly correlated ($r = 0.99$), with a regression analysis giving the following results in our laboratory: Nichols Advantage IGF-I assay (y) versus Nichols IGF-I by extraction (RIA) assay (x); $y = 0.84 * x - 2.15$. RIA values were transformed accordingly.

IGFBP-3 was measured by an enzyme-labeled chemiluminescent immunometric assay (Immulite, DPC). Plasma and urinary calcium, phosphate, and creatinine were measured using an automatic analyser. Plasma calcium was adjusted for protein levels (adjusted Ca = Ca/[(protein/160) + 0.55]). The following parameters were calculated: (a) fasting urinary Ca-to-creatinine ratio was taken as a reflection of net bone resorption (Bone Resorption Index, BRI); (b) renal tubular reabsorption of phosphate (TmPi/GFR) and of calcium (TRCaI) was calculated as described elsewhere [28, 29].

The marker of bone formation osteocalcin was determined with an immunoradiometric assay (CIS-Bio, Gif-sur-Yvette, France). The specific urine marker of bone resorption deoxypyridinoline was measured by fluorescence emission after acid hydrolysis and high-performance liquid chromatography separation (Bio-Rad system, Munich, Germany) and expressed as a creatinine ratio.

Table 1: Patients' characteristics: demographic, anthropometric, dietary, and bone data[#].

Demographic and dietary data	Patients	Controls	*P*-value
Number	66	66	
Age (yrs)	20.0 (16–33)	21 (16–22)	ns
Restricting type (%)	68%		
Age at menarche (yrs)	13.0 (11–16)	13 (11–15)	ns
Age of onset of anorexia (yrs)	16.0 (13–27)		
Duration of illness (yrs)	3.0 (0.7–12)		
History of low traumatic fractures, % (*n*)	12% (8)		
Time since last menstrual period (mo)[°]	9.0 (3–33)		
Total duration of amenorrhea (mo)	12.0 (2–84)		
Estrogen use, % (*n*)[*]	39% (26)		
Estrogen use (mo)	38.8 ± 40.3		
Current estrogen use	30% (20)		
Weight (kg)	42.1 ± 6.9	58.9 ± 8.1	<0.007
Weight (kg) before the onset of illness[**]	54.7 ± 7.5		
Height (cm)	165.0 ± 7.1	164.3 ± 6.0	ns
Body Mass Index (kg/m^2)	15.4 ± 2.1	21.8 ± 3.0	<0.007
% of ideal body weight	71.1 ± 10.0		
Calcium intake (mg/d)	835 (281–2000)	835 (510–1074)	ns
Protein intake (g/d)	45 (12–132)	42 (25–57)	ns
Protein intake (g/kg of body weight)	1.11 (0.29–2.92)	0.7 (0.4–1.1)	ns
Physical activity (h/week)	5.6 ± 4.0		
Anteroposterior spine BMD (g/cm^2)	0.887 ± 0.12	1.030 ± 0.12	<0.0001
Femoral neck BMD (g/cm^2)	0.753 ± 0.11	0.862 ± 0.11	<0.0001
Total hip BMD (g/cm^2)	0.788 ± 0.11	0.873 ± 0.15	<0.0009

[#]For skew data, median and interquartile (IQR) range are reported. Otherwise, mean ± SD.
[°]For amenorrheic women at time of examination.
[*]At any time since the onset of anorexia.
[**]Applicable if the onset of the disease occurred after the time of menarche.

2.4. Statistics. Summary results are presented as the mean ± standard deviation. When the data are not normally distributed, the summary descriptive statistics are the median and interquartile range. All non-Gaussian variables were successfully normalized using simple mathematical transformations. Normality was verified by using Shapiro-Francia tests. Comparisons between patients and age-matched controls for continuous variables were performed with a Wilcoxon matched-pairs signed-ranks test to take into account the matched design. Comparisons between two or more groups for continuous variables were performed with Student's *t*-test or ANOVA for unmatched groups. When appropriate, a nonparametric Kruskal-Wallis test was applied. Chi-squared tests were used for comparing proportions. BMI was calculated in kilograms/meters^2.

Correlations between bone mineral density and recorded variables were determined by simple or multiple (backward stepwise) linear regression analyses. Standard multiple regression models were constructed for each skeletal site by using duration of anorexia nervosa, total duration of amenorrhea, age at the time of evaluation, BMI, and sIGF-I as covariates. Adjusted regression coefficients and confidence intervals were determined for each covariate. The significance level for two-sided *P* values was 0.05 in all tests. The data were analyzed using the STATA statistical software package (version 9.2; Stata Corporation, College Station, TX).

3. Results

3.1. Patients' Characteristics. The subjects were in the early twenties (the age range and median age and interquartile range were 15 to 46 years, median: 20, and interquartile range: 16–33), had a low weight (weight range: 26 to 60 kg, median: 43, and interquartile range: 30–55), and a low BMD (Table 1). The median height was 165, with a range of 146 to 180 cm. Their mean T-Scores (in patients aged more than 18 years) were −1.52 ± 1.23 SD for the anterior-posterior view of LS, −0.93 ± 0.92 SD for the FN, and −1.34 ± 0.92 for the PF, significantly lower as compared with the normal population (Table 1). Osteopenia was found in at least one skeletal site in 63% of patients and osteoporosis in 17% of patients. Five patients had a family history of osteoporosis. No differences in BMD expressed as T-Scores were observed between patients with anorexia nervosa alone and those with the binge-purge subtype of anorexia nervosa at any site measured (data not shown). Eight patients reported a history of low trauma fracture (elbow, wrist or hand ($n = 4$), foot or ankle ($n = 2$), rib ($n = 1$), vertebrae ($n = 1$)). Median time

Table 2: Serum and urinary measurements (mean ± SD, or median values). Patient's characteristics by estrogen use and menstrual history.

Laboratory test	Normal range	N	All patients	Amenorrheic women[1]	Current estrogen use	Eugonadal women
			$N = 66$	$N = 38$	$N = 17$	$N = 11$
Serum corrected calcium	[2.25–2.60 mmol/L]	64	2.35 ± 0 .09	2.34 ± 0.1	2.35 ± 0.1	2.36 ± 0.1
Serum phosphate	[0.80–1.4 mmol/L]	63	1.3 (0.3)	1.3 (0.2)	1.3 (0.2)	1.5 (0.4)
Serum creatinine	[35–88 μmol/L]	65	71.85 ± 11.32	71.9 ± 12.9	74.1 ± 8.4	67.8 ± 8.6
Serum protein	[61–79 g/L]	45	66.0 ± 6.4	65.9 ± 6.6	65.8 ± 6.4	66.4 ± 6.8
Serum albumin	[35–48 g/L]	64	38.26 ± 4.51	40.5 ± 4.5°	37.3 ± 3.6	38.1 ± 4.7
Serum osteocalcin	[8.8–29.7 μg/L]	44	20.49 ± 11.30	19.2 ± 11.2	19.3 ± 9.0	28.4 ± 14.1
Serum total alkaline phosphatase	[30–125 IU/L]	61	46 (18)	48 (21)	41 (10)*	51 (14)
Serum IGF-I	[116–447 ng/mL]	66	118 (86)	103 (81)	129 (81)	144 (133)
Serum IGFBP-3	[3.3–6.7 μg/mL]	34	4.63 ± 1.21	4.4 ± 1.3	4.9 ± 1.0	4.9 ± 1.2
Serum parathyroid hormone	[1.1–6.8 pmol/L]	58	3.1 (1.5)	3.0 (1.4)	3.0 (1.8)	3.7 (1.3)
25-hydroxyvitamin D	[25–120 nmol/L]	57	74 (43)	69 (36)	76 (66)	73 (65)
Urinary calcium/creatinine	[0.10–0.50 mmol/mmol]	59	0.37 (0.44)	0.6 (0.6)°	0.3 (0.3)	0.2 (0.3)
Urinary D-pyr/cr[2]	[4.2–18.2 nmol/mmol]	52	17.7 (10.1)	18.1 (7.0)	13.4 (11.0)	16.0 (10.1)
TRCaI/GFR[3]	[2.4–2.9 mmol/L GFR]	55	2.59 ± 0 .21	2.54 ± 0.2	2.64 ± 0.2	2.67 ± 0.2
TmPi/GFR[4]	[0.8–1.4 mmol/L GFR]	55	1.4 (0.3)	1.4 (0.3)	1.3 (0.3)	1.5 (0.5)

Age-adjusted normal values were taken into account.
[1]At the time of evaluation, and including 3 patients taking oral contraceptives, but continuing to be amenorrheic.
[2]Deoxypyridinoline/creatinine.
[3]Renal tubular reabsorption of calcium index.
[4]Renal tubular reabsorption of phosphate.
°$P < 0.05$ versus current estrogen use and eugonadal women.
*$P < 0.05$ current estrogen versus eugonadal women.

to the first fracture was 6 years (IQR: 5) after the onset of the disease. Their BMD tended to be lower, but not significantly, than that observed in patients ($n = 54$) without a history of low traumatic fracture (data not shown). The age of the fractured and the non-fractured was 24.5±7.7 versus 22.1±6.4 years (ns), duration of amenorrhea was 50.4 ± 60.5 versus 21.4 ± 31.0 months ($P < 0.14$), and disease evolution was 7.3±4.5 versus 4.6±4.5 years (ns). Fifty-five patients reported at least one hospitalization during the course of the disease.

Tanner stages at the time of anorexia onset were not recorded. Girls usually complete puberty by ages 15–17, and menarche occurs generally at Tanner stage 4, at the age of 13. In order to clarify this aspect, we determined the number of patients with a disease onset before menarche. Six patients had a disease onset before the first periods. For 3 of them, the interval was lower than 1 year. Three others had a delayed menarche (after 16), 4 to 5 year after disease onset, and thus possibly before pubertal skeletal growth.

Twenty patients (30%) were current estrogen users (median duration: 27 months, IQR: 61). Six patients (9%) were previous estrogen users (median duration: 8 months, IQR: 29). Age, age at menarche, age at the onset of anorexia nervosa, total duration of amenorrhea, protein and calcium intake, sIGF-I, and physical activity did not differ according to estrogen use and menstrual history at the time of examination (Table 2). Amenorrheic women had a significantly lower BMI (15.3 ± 2.2 in untreated women versus 15.6 ± 2.0 in current estrogen users, $P < 0.05$). Age- and sex-adjusted BMD values (Z-Score) were similar (data not shown).

Serum levels of albumin and protein were lower than the normal range in 17% and 29% of patients, respectively (Table 2), while sIGF-I was below the lower limit of the normal level in 58% of patients. The sIGF-I levels correlated with BMI ($r = 0.43$, $P < 0.001$). This was not the case for serum albumin or protein. Eleven patients displayed vitamin D insufficiency (below 50 nmol/L) and one displayed a deficiency (below 25 nmol/L). Among them, six patients had secondary hyperparathyroidism. Mean serum LH and FSH were at the lowest limit of the normal range.

Markers of bone turnover (total alkaline phosphatase, but not osteocalcin, and fasting urinary calcium-to-creatinine ratio) were lower in estrogen users or estrogen replete patients (Table 2).

3.2. Bone Geometry in Patients and Controls (Table 3). Bone mineral content was lower in patients as compared with controls. Bone area was in contrast similar or tended to be higher in anorexia nervosa patients. Thus, compatible with a reduced volumetric bone density, rather than altered bone geometry, BMAD was lower, as was also cortical thickness at the level of the femoral shaft.

Bone geometry parameters were compared to the control population according to the disease onset, that is, in those below or above the median age of disease onset, and this did not affect the results (data not shown).

3.3. Relationship between BMD, Bone Geometry and Clinical and Biological Variables (Table 4). LS BMD correlated to

Table 3: Bone geometry as measured by DXA in patients with anorexia nervosa and in controls.

	Patients	Controls	P
Lumbar spine			
BMC (g)	40.1 ± 7.2	43.6 ± 7.1	0.01
Area (cm^2)	44.9 ± 4.8	42.3 ± 4.0	0.002
BMAD (g/cm^3)	0.13 ± 0.01	0.16 ± 0.02	0.0001
L2 vertebral height (mm)	29.1 ± 1.8	28.0 ± 2.3	0.0029
L2 width: higher part (mm)	40.2 ± 2.9	38.9 ± 3.3	0.02
L2 width: middle part (mm)	34.8 ± 2.7	33.8 ± 2.4	ns
L2 width: lower part (mm)	38.5 ± 3.2	35.3 ± 2.9	0.001
Hip			
BMC (g)/neck	4.0 ± 0.7	4.4 ± 0.7	0.003
Area (cm^2)/neck	5.3 ± 0.4	5.1 ± 0.5	0.01
BMC (g)/total hip	24.1 ± 4.2	29.3 ± 3.9	0.0001
Area (cm^2)/total hip	31.2 ± 3.1	30.9 ± 2.0	ns
Hip midline (mm)	83.7 ± 4.6	81.0 ± 5.0	0.002
Neck diameter (mm)	31.1 ± 2.2	30.3 ± 2.1	ns
BMAD (g/cm^3)	0.14 ± 0.02	0.17 ± 0.03	0.0001
BMC (g)/trochanter	5.4 ± 1.1	6.3 ± 1.3	0.0007
Area (cm^2)/trochanter	9.0 ± 1.0	8.9 ± 0.9	ns
Femoral shaft (mm)	27.2 ± 2.2	26.5 ± 2.5	ns
Internal cortical thickness (mm)	5.8 ± 1.0	6.3 ± 0.9	0.008
External cortical thickness (mm)	5.0 ± 0.8	5.0 ± 0.8	ns

Mean ± SD.

the duration of anorexia nervosa, total duration of amenorrhea, sIGF-I, and BMI. FN BMD was associated ($P < 0.05$ for all comparisons, Table 4) with age at the time of evaluation, duration of anorexia nervosa, total duration of amenorrhea, sIGF-I, and IGFBP-3. BMD at any site was not correlated with the degree of physical activity.

To assess the relative contribution of the variables in determining BMD, multiple regression models were constructed using a backward stepwise process. The following parameters were included: duration of anorexia nervosa, total duration of amenorrhea, age at the time of evaluation, BMI, and sIGF-I. Physical activity was not included in the model, as severely affected patients were unable to exert a high level of such activity. Furthermore, many patients were advised to avoid sports activities in order to conserve their energy. Given the high variability of the recorded calcium and protein intakes and the absence of any correlation in the univariate model, these two variables were not included. LS BMD was significantly correlated with total duration of amenorrhea ($P < 0.01$). The serum level of IGF-I was the only significant independent predictor of FN BMD and remained significant even after adjusting for height. We performed the same analysis excluding the 20 patients receiving a hormone replacement therapy at the time of evaluation and found similar results (data not shown).

We examined whether the influence of the sIGF-I level on BMD could be explained by an effect on true volumetric density or on bone size. The sIGF-I levels correlated with FN BMAD (multiple regression model: $r = 0.001$, $P < 0.04$), but not with hip geometry variables (neck width, hip axis length, cortical thickness, and femoral shaft diameter).

3.4. Relationship between Bone Markers and Demographic, Nutritional, or Hormonal Parameters (Table 5). There was a correlation between osteocalcin (a bone-specific formation marker) and nutritional variables such as BMI, 25-hydroxy vitamin D, sIGF-I, and IGF-BP3. In a multiple stepwise backward regression model, BMI and 25-hydroxy vitamin D were independent predictors of serum osteocalcin levels.

Urinary deoxypyridinoline and calcium-to-creatinine ratios were significantly higher in patients with amenorrhea at the time of evaluation as compared with those spontaneously menstruating or being treated with estrogen (Table 2).

Deoxypyridinoline positively correlated with age at the time of evaluation, amenorrhea at the time of evaluation, 25-hydroxy vitamin D, and PTH levels. In a stepwise multiple regression, the serum PTH level and amenorrhea at the time of evaluation were the most significant predictors of deoxypyridinoline levels, explaining 36% of its variance.

4. Discussion

Our study showed a high prevalence and profound degree of low bone mineral mass in women with anorexia nervosa. Indeed, osteoporosis was present in 17% of patients and osteopenia in 63%. Altogether, 12% reported a history of low trauma fracture. Similar figures have been found in other studies [1–3]. Our results suggest that low bone mass is the consequence of a reduced bone density rather than a reduced bone size.

The pathogenesis of bone loss in anorexia nervosa is still not completely understood. It may result from a number of mechanisms, including estrogen deficiency and inadequate nutritional intakes. We found, as other groups, that age-adjusted bone mineral density correlates with the duration of amenorrhea [6, 9, 30]. In our population, estrogen deficiency seemed to equally affect the spine and the proximal femur. Decreased calcium intake and excessive physical activity may also impact on the degree of bone loss, but we did not find any correlation between physical activity and LS BMD. A beneficial effect of physical activity had been suggested in an observational study published by Seeman et al. [31]. Conversely, another recent study showed that excessive moderate loading exercise may put patients at higher risk of low bone mass, but high bone loading activities may provoke bone accrual during recovery [32]. In our study, data about nutritional intakes and exercise were obtained through a questionnaire completed by the patients, with its associated limitations. Since denial is common in anorexia nervosa, these data should be considered with caution. These young women typically over reported food intakes and under reported exercise [33]. We therefore excluded these variables from the multivariate models. Undernutrition and nutritionally dependent factors are likely to play a major role in the bone loss associated with anorexia nervosa. There

Table 4: Relationship of BMD and variables measured (univariate regression analyses and multiple regression models constructed by backward stepwise).

Variables	AP spine BMD						Femoral neck BMD					
	Univariate			Multivariate			Univariate			Multivariate		
	Regres. coeff. (95% CI)	P	R^2	Regres.coeff. (95% CI)	P	R^2	Regres. coeff. (95% CI)	P	R^2	Regres. coeff. (95% CI)	P	R^2
Age (yrs)[#]	0.29 (−0.81; 1.39)	ns					1.24 (0.28; 2.19)	0.01	9%			
Disease duration (yrs)[°]	−0.01 (−0.02; −0.00)	0.03	7%				−0.01 (−0.02; −0.00)	0.01	10%			
Duration of amenorrhea (months)[*]	−0.03 (−0.06; −0.01)	0.01	10%	−0.03 (−0.05; −0.01)	0.01		−0.03 (−0.05; −0.00)	0.02	8%			
BMI (Kg/m^2)	0.02 (0.00; 0.03)	0.02	9%				0.01 (−0.01; 0.02)	ns				
IGF-I (μg/mL)[°]	0.01 (0.00; 0.02)	0.01	11%				0.02 (0.01; 0.02)	0.001	22%	0.01 (0.01; 0.02)	0.001	
IGFBP-3 (mg/mL)	0.01 (−0.01; 0.04)	ns					0.03 (0.00; 0.05)	0.02	16%			
Parathyroid hormone[#]	0.11 (−0.14; 0.35)	ns					0.19 (−0.05; 0.44)	ns				
25 OH-Vitamin D (nmol/l)[°]	0.00 (−0.01; 0.02)	ns					−0.001 (−0.01; 0.01)	ns				
Physical activity (h/week)[°]	0.03 (−0.00; 0.06)	ns					0.01 (−0.02; 0.04)	ns				
Protein intake (g/kg of body weight × d)[°]	−0.07 (−0.16; 0.01)	ns					−0.04 (−0.11; 0.04)	ns				
Calcium intake (mg/d)[*]	−0.04 (−0.10; 0.02)	ns					−0.02 (−0.07; 0.03)	ns				
						21%						29%

[#]Reciprocal root variable (1/square root).
[°]Square root transformed variable.
[*]Log transformed variable.

Table 5: Relationship between bone markers and demographic, nutritional, or hormonal parameters (univariate regression analyses).

Variables	Serum osteocalcin	(n = 44)		Urinary deoxypyridinoline	(n = 52)	
	Regres. coef. (95% CI)	P univar.	R^2	Regres. coef. (95% CI)	P univar.	R^2
Age (yrs)	−0.114 (−0.600; 0.373)	ns		−0.349 (−0.716; 0.017)	0.06	7%
Disease duration (yrs)	−0.205 (−0.989; 0.580)	ns		−0.520 (−1.049; −0.008)	0.053	5%
Total duration of amenorrhea (months)	0.024 (−0.077; 0.125)	ns		0.000 (−0.078; 0.08)	ns	
BMI (Kg/m^2)	3.157 (1.416; 4.898)	0.001	24%	−0.450 (−1.561; 0.660)	ns	
IGF-I (μg/mL):	0.040 (−0.001; 0.08)	0.058	8%	−0.002 (−0.031; 0.027)	ns	
IGFBP-3 (mg/mL)	3.76 (0.630; 6.889)	0.021	24%	1.0 (−1.319; 3.314)	ns	
Estrogen use	−2.342 (−10.121; 5.436)	ns		−4.702 (−9.34; −0.059)	0.05	8%
Parathyroid hormone (pmol/L)	0.831 (−3.268; 1.604)	ns		−1.743 (−3.014; −0. 472)	0.01	14%
25-Hydroxyvitamin D (nmol/L)	−0.103 (−0.198; −0.009)	0.03	12%	−0.039 (−0.082; 0.002)	0.063	8%
Protein intake (g/kg of body weight × d)	−1.996 (−5.116; 1.124)	ns		0.027 (−3.080; 3.134)	ns	
Calcium intake (mg/d)	−0.000 (−0.006; 0.006)	ns		0.000 (−0.006; 0.004)	ns	

were marked effects of nutritional status on bone formation markers which correlated with BMI, 25-hydroxy vitamin D, serum IGF-I, and IGFBP-3, in our study as in others [7, 8, 34]. Caloric and protein deprivation in anorexia nervosa leads to increased GH and decreased IGF-I. This could be explained by a downregulation of the GH receptor or its postreceptor mechanisms, inducing a fall in the GH-dependent serum proteins IGF-I and IGFBP-3 levels [35]. The rise in GH is presumably due to decreased feedback inhibition by sIGF-I. Fibroblast growth factor-21 may be a putative factor of GH resistance in anorexia nervosa [36].

Our study confirms the low sIGF-I levels previously reported in low-weight patients with anorexia nervosa [9, 10]. Sixty percent of the patients had sIGF-I values below the age-adjusted reference range; yet, only 17% of the patients showed lower than normal serum albumin concentration, a usual indicator of nutritional status. The reason of these discrepancies in the results is not clear. sIGF-I is closely related to nutritional status as suggested by the highly significant correlation with BMI ($P < 0.001$). Several studies showed that sIGF-I concentration is a more reliable index of nutritional status as compared with others markers [37–39].

The most intriguing finding was the high and independent association of sIGF-I and IGFBP-3 levels with age-adjusted FN BMD. We examined whether differences in bone size may explain the association. In a first step, we incorporated body height in the multiple regression model. This adjustment did not affect the relation between FN BMD and sIGF-I. In a second step, parameters of structural geometry of the hip were measured using conventional DXA outputs obtained from the FN region of interest. We measured medial and lateral cortical thickness at a fixed level of the femoral diaphysis, neck width, hip lengths, and femoral shaft diameter. None of them showed any relation with sIGF-I. In a third step, we examined the impact of another analytic strategy, that of BMAD, a volumetric bone density estimate. We found a positive correlation which suggests that the relation between sIGF-I and FN BMD might not be related to differences in bone size. Note that part of the femoral neck region of interest is intracapsular and thus devoid of periosteum. Data on structural geometry of the hip are scarce. A recent study (Hip Structural Analysis) found an increase in the outer and inner diameter of the hip and a decrease in the cortical thickness at the femoral shaft. These structural modifications were not correlated with sIGF-I [40].

Anorexia nervosa affects patients at a time of acquisition of peak bone mass, and one possible consequence is growth retardation and an effect on bone modelling. Short stature may be present in those starting in the infancy or early childhood [41]. For those beginning their eating disorders later, that is, during or after puberty, a study found that majority of patients with anorexia reached their expected height [42]. In our population, the median age of onset of anorexia was 16 years. A small number of patient had a disease onset before menarche, and had probably reached their adult stature and peak bone mass. This may explain the absence of decrease in bone size.

This study has several limitations, particularly the relative heterogeneity of the study population, with three groups of patients at different stages of severity (active disease, and spontaneously menstruated) and taking or not estrogens. It may be advocated that the inclusion of patients who were taking estrogen confounds the interpretation of the results that spine bone mineral density was related to hypogonadism. Although the benefits of estrogen in the bone density issue of patient with anorexia nervosa were refuted in the last decade, a recent publication suggested that physiologic estrogen replacement given transdermally may be beneficial to bone [43]. Excluding those patients taking estrogen, we found similar results.

Male patients were not included in the study even if they display similar hormonal issues, given the aim of study in analyzing bone size.

In conclusion, in a large cohort of patients with anorexia nervosa, spine and hip BMD values were related to hypogonadism, whereas sIGF-I was the most significant predictor of bone mass at the FN level. Our morphometric analysis suggests that the site-specific effect of sIGF-I could be related to reduced volumetric BMD rather than to modified hip geometry.

Acknowledgments

The authors thank Rosemary Sudan and Katy Giroux for invaluable editorial assistance. They thank also Giulio Conicella and Anne Sigaud for their assistance.

References

[1] N. A. Rigotti, S. R. Nussbaum, D. B. Herzog, and R. M. Neer, "Osteoporosis in women with anorexia nervosa," *The New England Journal of Medicine*, vol. 311, no. 25, pp. 1601–1606, 1984.

[2] N. A. Rigotti, R. M. Neer, S. J. Skates, D. B. Herzog, and S. R. Nussbaum, "The clinical course of osteoporosis in anorexia nervosa. A longitudinal study of cortical bone mass," *JAMA*, vol. 265, no. 9, pp. 1133–1138, 1991.

[3] S. Grinspoon, E. Thomas, S. Pitts et al., "Prevalence and predictive factors for regional osteopenia in women with anorexia nervosa," *Annals of Internal Medicine*, vol. 133, no. 10, pp. 790–I42, 2000.

[4] P. Vestergaard, C. Emborg, R. K. Støving, C. Hagen, L. Mosekilde, and K. Brixen, "Fractures in patients with anorexia nervosa, bulimia nervosa, and other eating disorders—a nationwide register study," *International Journal of Eating Disorders*, vol. 32, no. 3, pp. 301–308, 2002.

[5] Y. M. Maugars, J. M. M. Berthelot, R. Forestier et al., "Follow-up of bone mineral density in 27 cases of anorexia nervosa," *European Journal of Endocrinology*, vol. 135, no. 5, pp. 591–597, 1996.

[6] B. M. K. Biller, V. Saxe, D. B. Herzog, D. I. Rosenthal, S. Holzman, and A. Klibanski, "Mechanisms of osteoporosis in adult and adolescent women with anorexia nervosa," *Journal of Clinical Endocrinology and Metabolism*, vol. 68, no. 3, pp. 548–554, 1989.

[7] S. Grinspoon, H. Baum, K. Lee, E. Anderson, D. Herzog, and A. Klibanski, "Effects of short-term recombinant human insulin-like growth factor I administration on bone turnover in osteopenic women with anorexia nervosa," *Journal of Clinical*

Endocrinology and Metabolism, vol. 81, no. 11, pp. 3864–3870, 1996.

[8] D. R. Counts, H. Gwirtsman, L. M. S. Carlsson, M. Lesem, and G. B. Cutler, "The effect of anorexia nervosa and refeeding on growth hormone-binding protein, the insulin-like growth factors (IGFs), and the IGF-binding proteins," *Journal of Clinical Endocrinology and Metabolism*, vol. 75, no. 3, pp. 762–767, 1992.

[9] I. Legroux-Gérot, J. Vignau, M. D'Herbomez et al., "Evaluation of bone loss and its mechanisms in anorexia nervosa," *Calcified Tissue International*, vol. 81, no. 3, pp. 174–182, 2007.

[10] L. Audí, D. M. Vargas, M. Gussinyé, D. Yeste, G. Martí, and A. Carrascosa, "Clinical and biochemical determinants of bone metabolism and bone mass in adolescent female patients with anorexia nervosa," *Pediatric Research*, vol. 51, no. 4, pp. 497–504, 2002.

[11] E. A. Lawson, K. K. Miller, M. A. Bredella et al., "Hormone predictors of abnormal bone microarchitecture in women with anorexia nervosa," *Bone*, vol. 46, no. 2, pp. 458–463, 2010.

[12] A. Bennett, T. Chen, and D. Feldman, "Characterization of insulin-like growth factor I receptors on cultured rat bone cells: regulation of receptor concentration by glucocorticoids," *Endocrinology*, vol. 115, no. 4, pp. 1577–1583, 1984.

[13] E. Canalis, "Effect of insulinlike growth factor I on DNA and protein synthesis in cultured rat calvaria," *The Journal of Clinical Investigation*, vol. 66, no. 4, pp. 709–719, 1980.

[14] J. M. Hock, M. Centrella, and E. Canalis, "Insulin-like growth factor I has independent effects on bone matrix formation and cell replication," *Endocrinology*, vol. 122, no. 1, pp. 254–260, 1988.

[15] O. G. Isaksson, A. Lindahl, A. Nilsson, and J. Isgaard, "Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth," *Endocrine Reviews*, vol. 8, no. 4, pp. 426–438, 1987.

[16] T. L. McCarthy, M. Centrella, and E. Canalis, "Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures," *Endocrinology*, vol. 124, no. 1, pp. 301–309, 1989.

[17] L. A. Soyka, S. Grinspoon, L. L. Levitsky, D. B. Herzog, and A. Klibanski, "The effects of anorexia nervosa on bone metabolism in female adolescents," *Journal of Clinical Endocrinology and Metabolism*, vol. 84, no. 12, pp. 4489–4496, 1999.

[18] "New weight standards for men and women," *Statistical Bulletin-Metropolitan Life Insurance Company*, vol. 40, pp. 1–40, 1959.

[19] P. Morin, F. Herrmann, P. Ammann, B. Uebelhart, and R. Rizzoli, "A rapid self-administered food frequency questionnaire for the evaluation of dietary protein intake," *Clinical Nutrition*, vol. 24, no. 5, pp. 768–774, 2005.

[20] A. M. Boot, M. A. J. De Ridder, H. A. P. Pols, E. P. Krenning, and S. M. P. F. De Muinck Keizer-Schrama, "Bone mineral density in children and adolescents: relation to puberty, calcium intake, and physical activity," *Journal of Clinical Endocrinology and Metabolism*, vol. 82, no. 1, pp. 57–62, 1997.

[21] D. O. Slosman, R. Rizzoli, C. Pichard, A. Donath, and J. P. Bonjour, "Longitudinal measurement of regional and whole body bone mass in young healthy adults," *Osteoporosis International*, vol. 4, no. 4, pp. 185–190, 1994.

[22] D. O. Slosman, J. P. Casez, C. Pichard et al., "Assessment of whole-body composition with dual-energy X-ray absorptiometry," *Radiology*, vol. 185, no. 2, pp. 593–598, 1992.

[23] A. C. Looker, H. W. Wahner, W. L. Dunn et al., "Updated data on proximal femur bone mineral levels of US adults," *Osteoporosis International*, vol. 8, no. 5, pp. 468–489, 1998.

[24] J. A. Kanis, L. J. Melton, C. Christiansen, C. C. Johnston, and N. Khaltaev, "Perpective: the diagnosis of osteoporosis," *Journal of Bone and Mineral Research*, vol. 9, article 1137, 1994.

[25] S. D. Walter, M. Eliasziw, and A. Donner, "Sample size and optimal designs for reliability studies," *Statistics in Medicine*, vol. 17, pp. 101–110, 1998.

[26] D. K. Katzman, L. K. Bachrach, D. R. Carter, and R. Marcus, "Clinical and anthropometric correlates of bone mineral acquisition in healthy adolescent girls," *Journal of Clinical Endocrinology and Metabolism*, vol. 73, no. 6, pp. 1332–1339, 1991.

[27] D. R. Carter, M. L. Bouxsein, and R. Marcus, "New approaches for interpreting projected bone densitometry data," *Journal of Bone and Mineral Research*, vol. 7, no. 2, pp. 137–145, 1992.

[28] O. L. M. Bijvoet, D. B. Morgan, and P. Fourman, "The assessment of phosphate reabsorption," *Clinica Chimica Acta*, vol. 26, no. 1, pp. 15–24, 1969.

[29] B. Buchs, R. Rizzoli, and J. P. Bonjour, "Evaluation of bone resorption and renal tubular reabsorption of calcium and phosphate in malignant and nonmalignant hypercalcemia," *Bone*, vol. 12, no. 1, pp. 47–56, 1991.

[30] L. K. Bachrach, D. Guido, D. Katzman, I. F. Litt, and R. Marcus, "Decreased bone density in adolescent girls with anorexia nervosa," *Pediatrics*, vol. 86, no. 3, pp. 440–447, 1990.

[31] E. Seeman, G. I. Szmukler, C. Formica, C. Tsalamandris, and R. Mestrovic, "Osteoporosis in anorexia nervosa: the influence of peak bone density, bone loss, oral contraceptive use, and exercise," *Journal of Bone and Mineral Research*, vol. 7, no. 12, pp. 1467–1474, 1992.

[32] E. J. Waugh, D. B. Woodside, D. E. Beaton, P. CotI, and G. A. Hawker, "Effects of exercise on bone mass in young women with anorexia nervosa," *Medicine and Science in Sports and Exercise*, vol. 43, no. 5, pp. 755–763, 2011.

[33] J. E. Schebendach, K. J. Porter, C. Wolper, B. T. Walsh, and L. E. S. Mayer, "Accuracy of self-reported energy intake in weight-restored patients with anorexia nervosa compared with obese and normal weight individuals," *International Journal of Eating Disorders*, vol. 45, no. 4, pp. 570–574, 2012.

[34] M. Hotta, I. Fukuda, K. Sato, N. Hizuka, T. Shibasaki, and K. Takano, "The relationship between bone turnover and body weight, serum insulin-like growth factor (IGF) I, and serum IGF-binding protein levels in patients with anorexia nervosa," *Journal of Clinical Endocrinology and Metabolism*, vol. 85, no. 1, pp. 200–206, 2000.

[35] J. P. Thissen, S. Triest, L. E. Underwood, M. Maes, and J. M. Ketelslegers, "Divergent responses of serum insulin-like growth factor-I and liver growth hormone (GH) receptors to exogenous GH in protein-restricted rats," *Endocrinology*, vol. 126, no. 2, pp. 908–913, 1990.

[36] P. K. Fazeli, M. Misra, M. Goldstein, K. K. Miller, and A. Klibanski, "Fibroblast growth factor-21 may mediate growth hormone resistance in anorexia nervosa," *Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 1, pp. 369–374, 2010.

[37] F. Minuto, A. Barreca, G. F. Adami et al., "Insulin-like growth factor-I in human malnutrition: relationship with some body composition and nutritional parameters," *Journal of Parenteral and Enteral Nutrition*, vol. 13, no. 4, pp. 392–396, 1989.

[38] J. Niebauer, C. D. Pflaum, A. L. Clark et al., "Deficient insulin-like growth factor I in chronic heart failure predicts altered body composition, anabolic deficiency, cytokine and neurohormonal activation," *Journal of the American College of Cardiology*, vol. 32, no. 2, pp. 393–397, 1998.

[39] A. R. Qureshi, A. Alvestrand, A. Danielsson et al., "Factors predicting malnutrition in hemodialysis patients: a cross- sectional study," *Kidney International*, vol. 53, no. 3, pp. 773–782, 1998.

[40] A. D. DiVasta, T. J. Beck, M. A. Petit, H. A. Feldman, M. S. LeBoff, and C. M. Gordon, "Bone cross-sectional geometry in adolescents and young women with anorexia nervosa: a hip structural analysis study," *Osteoporosis International*, vol. 18, no. 6, pp. 797–804, 2007.

[41] J. S. Garrow, "Underfeeding and overfeeding and their clinical consequences," *Proceedings of the Nutrition Society*, vol. 35, no. 3, pp. 363–368, 1976.

[42] R. J. Pfeiffer, A. R. Lucas, and D. M. Ilstrup, "Effect of anorexia nervosa on linear growth," *Clinical Pediatrics*, vol. 25, no. 1, pp. 7–12, 1986.

[43] M. Misra, D. Katzman, K. K. Miller et al., "Physiologic estrogen replacement increases bone density in adolescent girls with anorexia nervosa," *Journal of Bone and Mineral Research*, vol. 26, no. 10, pp. 2430–2438, 2011.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 679763, 9 pages
http://dx.doi.org/10.1155/2013/679763

Research Article

Dysfunction of Collagen Synthesis and Secretion in Chondrocytes Induced by *Wisp3* Mutation

Min Wang,[1,2] Xiao-Fei Man,[1] Ya-Qing Liu,[1] Er-Yuan Liao,[1] Zhi-Feng Shen,[1] Xiang-Hang Luo,[1] Li-Juan Guo,[1,2] Xian-Ping Wu,[1] and Hou-De Zhou[1]

[1] *Institute of Endocrinology and Metabolism, The Second Xiang-Ya Hospital of Central South University, Changsha, Hunan 410011, China*
[2] *Department of Endocrinology and Metabolism, Xiang-Ya Hospital of Central South University, Changsha, Hunan 410008, China*

Correspondence should be addressed to Hou-De Zhou; houdezhou@xysm.net

Received 23 December 2012; Revised 21 January 2013; Accepted 28 January 2013

Academic Editor: Peng-Fei Shan

Wisp3 gene mutation was shown to cause spondyloepiphyseal dysplasia tarda with progressive arthropathy (SRDT-PA), but the underlying mechanism is not clear. To clarify this mechanism, we constructed the wild and mutated *Wisp3* expression vectors and transfected into human chondrocytes lines C-20/A4; *Wisp3* proteins subcellular localization, cell proliferation, cell apoptosis, and *Wisp3*-mediated gene expression were determined, and dynamic secretion of collagen in transfected chondrocytes was analyzed by ^{14}C-proline incorporation experiment. Mutated *Wisp3* protein increased proliferation activity, decreased apoptosis of C-20/A4 cells, and aggregated abnormally in cytoplasm. Expression of collagen II was also downregulated in C-20/A4 cells transfected with mutated *Wisp3*. Wild type *Wisp3* transfection increased intracellular collagen content and extracellular collagen secretion, but the mutated *Wisp3* lost this function, and the peak phase of collagen secretion was delayed in mutated *Wisp3* transfected cells. Thus abnormal protein distribution, cell proliferation, collagen synthesis, and secretion in *Wisp3* mutated chondrocytes might contribute to the pathogenesis of SEDT-PA.

1. Introduction

Wnt-1-induced secreted protein 3 (*Wisp3*/CCN6) is a cysteine-rich protein that belongs to the cysteine-rich 61-connective tissue growth factor, nephroblastoma overexpressed CCN family members, maps to chromosome 6q21-22, and encodes a 354 amino acid secreted protein [1]. *Wisp3* proteins are characterized by an N-terminal secretory signal followed by four structural domains with partial sequence identity to insulin-like growth factor binding protein (IGFBP) (GCGCCXXC); Von Willebrand factor type C like motif, thrombospondin type 1 module, and a C-terminal cysteine knot-like domain (CK) putatively involved in dimerization [1, 2], and IGFBP can be upregulated by implementation of exercise [3]. The members of CCN family are multifunctional in which they are involved in regulation of cell adhesion, migration, proliferation, growth arrest, survival, apoptosis, differentiation, endochondral ossification, and extracellular matrix production [4–6].

Wisp3 mutations have been demonstrated in most patients of an autosomal recessive hereditary cartilage metabolic disorder, spondyloepiphyseal dysplasia tarda with progressive arthropathy (SEDT-PA), or progressive pseudorheumatoid dysplasia (PPD), which characterized by deformation and limitation of most large and small joints clinically, and continuous degeneration and loss of articular cartilage pathologically [7–11]. In our previous work, we found a novel compound mutation (840delT/T1000C) of *Wisp3* in Chinese PPD kindred [12, 13]; the two probands carried a substitution mutation (1000T → C, Ser334Pro) in paternal allele, and a deletion (840delT) mutation in maternal allele that caused a truncated *Wisp3* protein to miss 43 residues in C-terminus [14], and we also discovered the biological behavior changes of the articular chondrocytes (ACs) separated from the patients [15]. *Wisp3* also had growth-, invasion-, and angiogenesis-inhibitory functions in inflammatory breast cancer (IBC) in vitro and in vivo [16] and was a key genetic determinant of the IBC phenotype [17]. However,

the precise action of *Wisp3* in cartilage maintenance and metabolism and the mechanisms of SEDT-PA/PPD caused by *Wisp3* mutations have not been elucidated.

The present study was undertaken to investigate the subcellular localization and function of mutant *Wisp3* in chondrocytes. The results suggest that mutated *Wisp3* protein aggregated abnormally in cytoplasm, and mutated *Wisp3* failed to inhibit cell proliferation and modulate the expression of type II collagen in chondrocytes, which may be an important molecule mechanism involved in the pathogenesis of SEDT-PA/PPD.

2. Materials and Methods

2.1. Cell Cultures. The immortalized human chondrocytes cell lines C-20/A4 were derived from human juvenile costal cartilage and generated by infection with a replication defective retroviral vector expressing SV40 large T antigen. Cultures of C-20/A4 cells were maintained in DMEM/Ham's F-12(1 : 1,v/v) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, USA) in a 5%CO_2 incubator at 37°C and passaged at subconfluence every 5-6 days.

2.2. Wild Type and Mutant Wisp3 Expression Construct. Human *Wisp3* was cloned from ACs cDNA by PCR amplification with *Wisp3*-specific primers, bearing Hind III and BamH I restriction enzyme sites at their flanking ends, for the purpose of subcloning into the expression vector pcDNA3.1(+) and pEGFP-C2 (Invitrogen). The primers used for cloning *Wisp3* into pcDNA3.1 were 5′-GTAAGCTTAGCGACATGCAGGGGCTCCTCTT-3′ (forward) and 5′-GCGGATCCTTACAGAATCTTGAGCTCAG-3′ (reverse), and pEGFP-C2 were 5′-TCAAGCTTCGACGTACAGGGGCTCCTCTT-3′ (forward, exclude start coden) and 5′-GCGGATCCTTACAGAATCTTGAGCTCAG-3′ (reverse). The amplified *Wisp3* gene products (~1.1 kb) were ligated to pGEM-T easy (Promega, Madison, WI, USA), and the products were used as templates in the PCR reaction of the site-directed mutagenesis (SDM).

The mutants *Wisp3* ($MUT^{1000T/C}$ and $MUT^{840delT}$) were constructed using SDM separately. The mutant primers for $MUT^{1000T/C}$ had the sequences 5′-CCTTGTGTGTGTCAGAGAAA-3′ (forward) and 5′-CCTTCTACGACACCTAATGT-3′ (reverse), and for $MUT^{840delT}$ had the sequences 5′-AATTGTCTTTTCTGGATGCTCA-3′ (forward) and 5′-AAGGTTGAGAGGTTTCGACTTT-3′ (reverse). After confirmed by restriction endonuclease analysis and sequencing, the target fragments (WT-*Wisp3*, $MUT^{1000T/C}$, and $MUT^{840delT}$) were subcloned to Hind III and BamH I sites of expression vector pcDNA3.1(+) and pEGFP-C2. The recombined expression plasmids with pcDNA3.1(+) were used for all functional studies of wild and mutant *Wisp3*, and those with pEGFP-C2 were used for subcellular localization of wild and mutant *Wisp3* proteins.

2.3. Cell Transfection. Lipofectamine was used for transfecting C-20/A4 chondrocytes cell lines with the recombined plasmids and empty vector constructs. Briefly, cells (2.5~5 × 10^5/mL) were plated 1 day before transfection in 6-well tissue culture plates (2 mL/well) and incubated at 37°C in 5% CO_2. A complex of the plasmid DNA (<1 μg) with 6 μL PLUS reagent in 100 μL of serum-free, antibiotic-free medium was prepared in a sterile microfuge tube for 15 minutes; dilute 4 μL of Lipofectamine into 100 μL of serum-free medium and added to each reaction mixture, and incubated at room temperature for additional 15–30 min. A similar complex was prepared for each well of a 6-well plate. The cells in each well of the plate were washed with sterile PBS and then added 800 μL serum-free medium and the transfection mixture drop wise to each plate, and incubated for 4 h, after which 1 mL of culture medium with 5% FBS was added to each well. After 24 hours, the transfection mixture was replaced with fresh culture medium containing 10% FBS. The incubation was continued for an additional 24–26 hours, and the cells were used for observation by laser scanning confocal microscopy (LSCM) and harvested for either RNA or protein extraction. After 24 hours of transfection, the cells were placed in 25 cm^2 flasks for stable transfection with selection by G418 (400 μL /mL).

2.4. Wild and Mutant Wisp3 Proteins Subcellular localization. After 48 h of transiently transfection with WT-*Wisp3*/pEGFP-C2, $MUT^{1000T/C}$/pEGFP-C2, $MUT^{840delT}$/pEGFP-C2, or empty vector, cells were rinsed once with PBS after removal of culture medium, and then fixed for 15 min with freshly prepared 4% paraformaldehyde, and then incubated with 0.25% Triton X100 at 37°C for 20 min. 4′-6-Diamino-2-phenylindole-2HCl (DAPI; Sigma) was used at a final concentration of 100 ng/mL to stain cell nuclei. After washing three times with PBS at room temperature for 10 min, the fluorescence was observed under LSCM.

2.5. Cell Viability Assay. Cell viability was determined using a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, cells stably transfected with $MUT^{1000T/C}$/pcDNA3.1(+), $MUT^{840delT}$/pcDNA3.1(+), or empty vector were seeded 10^4 cells/well into 96-well plates. Following 24 h in culture, 5 μL MTT was added into each well and cells were incubated for an additional 3 h. The medium was discarded and the cells were solubilized in 100 μL dimethyl sulphoxide (DMSO), and then shaken for 1 min, and incubated for 5 min at room temperature, and the absorbance at 570 nm was read on Micro ELISA reader (Molecular Devices, CA, USA).

2.6. Cell Cycle and Apoptosis Analysis. Cells stably transfected with $MUT^{1000T/C}$/pcDNA3.1(+), $MUT^{840delT}$/pcDNA3.1(+), or empty vector were seeded into in 25 cm^2 flasks at a density of 2 × 10^5 cells/mL and cultured for 24 h. After cultured in serum-free medium for 24 h, cells were digested with 0.05% trypsin-EDTA, rinsed with PBS, fixed with 75% ethanol over night at 4°C, and stained with propidium iodide. Cell cycle and apoptosis were evaluated using FAC flow cytometry (BD Biosciences). Cell proliferation index (PI) was calculated using the equation (PI) = (G2+S)/(G1+S+G2).

Table 1: Primer pairs and experimental conditions.

Gene product	Forward and reverse primers (5′-3′)	Expected product size, bp	Annealing temperature (°C)
COL2A1	CCTAATGGAGATGCTGGTCG	187 bp	57
	CCAGGGAATCCAATGTTGC		
COL1A1	ATCCAGCTGACCTTCCTGCG	322 bp	60
	TCGAAGCCGAATTCCTGGTCT		
Wisp3	GTAAGCTTAGCGACATGCAGGGGCTCCTCTT	1065 bp	62
	GCGGATCCTTACAGAATCTTGAGCTCAG		
Fibronectin	GTGTGACCCTCATGAGGCAAC	299 bp	60
	TACTCTCGGGAATCTTCTCTGT		
SOX9	CACACTACAGCCCCTCCTAC	258 bp	60
	CCTCCTCAAGGTCGAGTGAG		
MMP-1	ATGCTGAAACCCTGAAGGTG	305 bp	60
	CAAGATTTCCTCCAGGTCCA		
β-actin	TCCTGTGCATCCACGAAACT	310 bp	58
	GAAGCATTTGCGGTGGACGAT		

Apoptosis was also studied morphologically using fluorescent dyes that intercalate DNA. Acridine orange stains DNA bright green, allowing visualization of the nuclear chromatin pattern. Apoptotic cells have condensed chromatin that is uniformly stained. Ethidium bromide stains DNA orange, but is excluded by viable cells. Cells stably transfected with $MUT^{1000T/C}$/pcDNA3.1(+), $MUT^{840delT}$/pcDNA3.1(+), or empty vector were stained by acridine orange and ethidium bromide, respectively, and observed using LSCM.

2.7. RNA Extraction and RT-PCR. The C-20/A4 chondrocytes were transfected with WT-*Wisp3*, $MUT^{1000T/C}$, $MUT^{840delT}$, or empty vector, and the total RNA was extracted using TRizol Reagent (Invitrogen). RNA was treated with RNase-free DNase (Promega). Reverse transcriptase-PCR (RT-PCR) was performed using a reverse transcription kit according to the instructions of the manufacturer (Invitrogen). Primers specific for type II collagen, type I collagen, *SOX9*, fibronectin, *MMP-1*, and β-actin were used for estimating the levels of expression of the corresponding mRNA. During cDNA synthesis, 2 μg of RNA was used for each specimen, and 30 cycles of PCR were carried out. The β-actin gene was used as an internal control. Table 1 summarizes the primer pairs and experimental conditions used for RT-PCR analysis.

2.8. Preparation of Whole Cell Protein Lysates and Western Blot. To prepare whole cell lysates, cells transfected with WT-*Wisp3*, $MUT^{1000T/C}$, $MUT^{840delT}$, or empty vector were rinsed once with precooling PBS after removal of culture medium and treated with precooling Triton lyses buffer (50 mmol/L Tris-HCL, PH8.0 containing 150 mmol/L NaCL, 1%Triton X100, 10 mmol/L EDTA, 0.2%NaN_3, 10 μg/mL Aprotinin, and 1 μg/mL phenylmethylsulfonyl fluoride) on ice for 20 minutes and the protein concentrations were determined using Bradford protein assay. Approximately 50 μg of each cell lysate was mixed with 2×SDS gel-loading buffer (100 mmol/L Tris-HCL, PH6.8, and 200 mmol/L DDT, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) and then heated to 95°C for 5 min. The samples were loaded onto a polyacrylamide gel (7.5 for type II collagen, 12% for *Wisp3* and β-actin), and prestained molecular weight standard (Bio-Rad, CA, USA) was also loaded onto the gels. After electrophoresis, the SDS-PAGE separated proteins were transferred to a nitrocellulose (GE, Pittsburgh, USA). The membrane was blocked with 5% nonfat milk in PBS for 60 min and then incubated with 2 μg/mL goat monoclonal antihuman type II collagen, *Wisp3*, or β-actin (Santa Cruz, CA, USA) for 60 min. After extensive washing with PBS, the membrane was reprobed with mouse anti-goat IgG conjugated with horseradish peroxidase (GE) at 1 : 1000 in PBS for 1 h at room temperature. Blots were visualized with chemiluminescence as described previously [18].

2.9. ^{14}C-Proline Incorporation Analysis. Chondrocytes were seeded into 24-well plates (5×10^4 cells/cm^2) in DMEM with 10% FBS for 24 hours, then cultured in 500 μL of serum-free DMEM for 4 hours. For ^{14}C-proline incorporation, each well was added with 10 μCi of ^{14}C-proline (100 μCi/mL) (GE) and 100 μg/mL aminopropionitrile, then incubated for 2 h; cells were rinsed five times with PBS, and complete medium was added and incubated at 37°C for 0, 30, 60, 120, 180, 240, and 300 minutes; supernatant and cell lysates were collected at these time points. For the collection of cell lysates, cells were rinsed two times with PBS after the supernatant collection, two times with 5% cold trichloroacetic acid, two times with 80% ethanol and finally lysed at 37°C for 2 h in 0.5 mL of 10 mM EDTA; 0.2 mL aliquots of the lysates and supernatant were dissolved in 10 mL Ecoscint H (Prolabo, Briare, France) separately and counted by scintillation; the quantification of intracellular collagen content and extracellular secretion was determined by the radioactivity in the cell lysate and supernatant, and the ratio of extracellular collagen secretion to intracellular collagen content was counted.

2.10. Statistical Analysis. SPSS 11.0 software was used for statistical analysis and data are presented as the mean ± SD, with the exception of gene analysis data. Data were compared using one-way ANOVA or the student's *t*-test. All experiments were repeated at least 3 times; the representative experiments are shown.

3. Results

3.1. Abnormal Protein Localization of Cells Transfected with Wisp3 Mutants. The recombined plasmids WT-*Wisp3*/pEGFP-C2, $MUT^{1000T/C}$/pEGFP-C2, and $MUT^{840delT}$/pEGFP-C2 were transfected transiently into human chondrocytes cell line C-20/A4, and pEGFP-C2 vector was used as a control. The expression and localization of green fluorescence protein (GFP) fusion proteins were observed using LSCM after 48 hours of transfection. GFP signal was distributed throughout the cells transfected with pEGFP-C2 vector (Figure 1(a)) and uniformly in cytoplasm and cell membrane transfected with WT-*Wisp3*/pEGFP-C2 (Figure 1(b)); however, the fluorescence signal aggregated to speckles or agglomerates in cytoplasm transfected with $MUT^{1000T/C}$/pEGFP-C2 and $MUT^{840delT}$/pEGFP-C2 (Figures 1(c) and 1(d)).

3.2. Increased Cell Proliferation and Decreased Cell Apoptosis Ratio of Cells Transfected with Wisp3 Mutants. MTT assays showed cell viability in C-20/A4 cells stably transfected with $MUT^{1000T/C}$/pcDNA3.1(+) and $MUT^{840delT}$/pcDNA3.1(+) was obviously higher (0.38 ± 0.03 and 0.42 ± 0.04, $P < 0.01$) than that in cells stably transfected with pcDNA3.1(+) (0.24 ± 0.02) (Figure 2(a)).

Overproliferation of cells stably transfected with mutant *wisp3* was further demonstrated by flow cytometry analysis, which indicated that the cell numbers in the G2-M plus S phases were significantly higher than that of control cells (33.6±4.0%, $P < 0.05$, Figure 2(b)), with a proliferation index of 49.8 ± 5.0% and 53.2 ± 4.5%, respectively (Figures 2(c) and 2(d)).

The apoptosis rate of control cells was 26.1 ± 4.0% after cultured in serum-free medium for 24 h (Figure 3(a)), while that of cells stably transfected with $MUT^{1000T/C}$/pcDNA3.1(+) and $MUT^{840delT}$/pcDNA3.1(+) was decreased (8.5 ± 2.6% and 6.9 ± 2.4%) (Figures 3(b) and 3(c)). Acridine orange and ethidium bromide staining also suggested that the apoptosis rate was decreased dramatically in cells stably transfected with mutant *Wisp3*; there were no apparent apoptotic cells in them (Figures 3(d)–3(f)).

3.3. Dysfunction of Collagen Production in Cells Transfected with Mutants. The function of *Wisp3* gene in chondrocytes and the mechanism of disorders in cartilage tissue caused by *Wisp3* mutation were still unclear. The C-20/A4 chondrocytes lines express very low levels of *Wisp3* (Figures 4(a) and 5(a)) and cartilage specific collagens. To investigate the function of wild and mutant *Wisp3* gene in chondrocytes, the mutant and control plasmids were transfected stably into chondrocyte cell line C-20/A4. The WT-*Wisp3*/pcDNA3.1(+) transfected C-20/A4 cells expressed 3.3-fold higher levels of COL2A1 mRNA than the cells transfected with the control vector (Figure 4(b)). Figure 5(b) demonstrated that stable transfection of C-20/A4 cells with WT-*Wisp3*/pcDNA3.1(+) upregulates type II collagen protein expression ($P < 0.05$). However, the COL2A1 expression did not change in C-20/A4 cells transfected with $MUT^{1000T/C}$ /pcDNA3.1(+) and $MUT^{840delT}$/pcDNA3.1(+) both in mRNA and protein level, compared with the cells transfected with the empty vector.

In contrast, minimal changes were observed in the levels of mRNA of type I collagen, *SOX9*, and fibronectin in response to either wild or mutant *Wisp3* (Figures 4(c), 4(d), and 4(e)). The mRNA expression of *MMP-1*, which had been found dramatically decreased in articular chondrocytes separated from SEDT-PA/PPD patient [15] (Figure 4(f)), wasn't changed in the mutant *Wisp3* transfected chondrocytes.

3.4. Abnormal Intracellular Collagen Content and Secretion in Mutant Chondrocyte. By ^{14}C-proline incorporation analysis, very low radioactivity was detected in the supernatant of and cell lysate of C-20/A4 cells transfected with control vectors, which indicated that very low collagen synthesis and secretion in this cell line (Figures 6(a) and 6(b)), and the ratio of extracellular collagen secretion to intracellular content is approximately 1. However, in wild type *Wisp3* stably transfected C-20/A4 cells, high radioactivity, were detected in the culture supernatant (3000 CPM to 7000 CPM) and cell lysate (700 CPM to 1000 CPM); the peak collagen secretion and intracellular content were appeared at 120 min and 60 min separately after refreshment of the complete medium, compared to C-20/A4 cells transfected with control vector; *Wisp3* increased the intracellular collagen content to about 5–10 times ($P < 0.01$), and especially increased the extracellular collagen secretion to 10–20 times ($P < 0.01$), and the ratio of extracellular collagen secretion to intracellular content is 3.5–10 (Figure 6(c)). In mutant *Wisp3* ($MUT^{840delT}$ and $MUT^{1000T/C}$) transfected cells, the peak collagen secretion and intracellular content were backward to 120 min and 180 min separately, although the radioactivity of collagen secretion was slightly higher than that of intracellular collagen content, the extracellular collagen secretion was decreased obviously compared to the wild type *Wisp3* transfected cells, and the ratio of extracellular collagen secretion to intracellular content is about 1.5.

4. Discussion

PPD was attributed to mutations of *Wisp3* gene; we previously identified a novel compound heterozygous mutation (840delT/T1000C) of *Wisp3* in a SEDT-PA/PPD family, and this mutation results in a dramatic decrease in the tensile strength of articular cartilage; however, the detail mechanism is not clear.

By bioinformatics analysis, we predicted that the compound heterozygous mutation formed a truncated Wisp protein and a Ser334Pro mutated proteins [14]. The 3D-conformational change of the 840delT truncated mutant

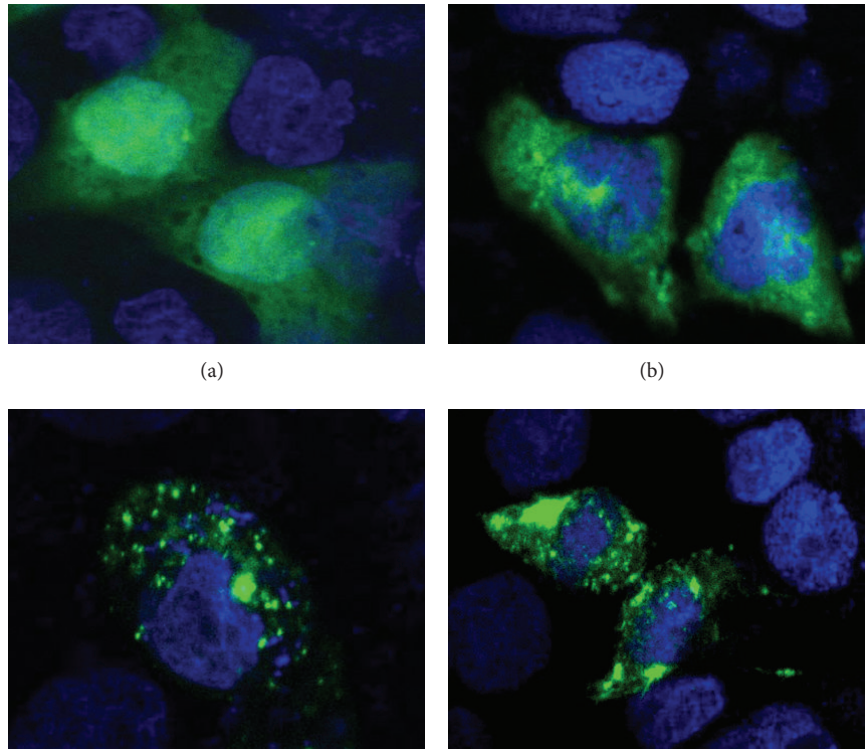


Figure 1: Localization of wild and mutated *Wisp3* protein in C20/A4 cells by confocal microscope. Recombined plasmids WT-*Wisp3*/pEGFP-C2, MUT$^{1000T/C}$/pEGFP-C2, and MUT^{840delT}/pEGFP-C2 were transfected transiently into human chondrocyte cell line C20/A4, and pEGFP-C2 vector was used as a control. The cells were observed using a confocal laser scanning microscope after 48 hours of transfection at magnification 1000x. (a) EGFP; (b) WT-*Wisp3*; (c) MUT$^{1000T/C}$; (d) MUT^{840delT}; Green fluorescence indicate the *Wisp3* EGFP fusion protein. Blue fluorescence shows cell nuclei dye by DAPI. Note the distribution of WT-*Wisp3* in cytoplasm and cell membrane uniformly. In contrast, the majority of MUT$^{1000T/C}$ and MUT^{840delT} were aggregated to speckles or agglomerates in cytoplasm.

Wisp3 protein is the single long peptide loop in the region from signal peptide to the beginning 24 amino acid residues in the first domain (IGFBP) which was subjected to folding into two smaller cross-loops accompanied with a much shorter C-terminus. It has been noted that the function of the first (IGFBP) domain of *Wisp3* is involved in inhibiting the function of IGF-1 to the chondrocytes and the fourth (CK) domain is involved in disulfide-linked dimerisation and is necessary for dimer formation in the endoplasmic reticulum, an important function for the establishment and maintenance of normal 3D-conformation of *Wisp3* protein [18–20]. Through GFP labeled protein localization analysis, we found that wild type *Wisp3* protein did localize in cytoplasm and cell membrane of C-20/A4 cells, but the two mutated *Wisp3* proteins aggregated abnormally in cytoplasm of C-20/A4 cells transfected with MUT$^{1000T/C}$ and MUT^{840delT}. It needs further research to validate the hypothesis that 3D-conformational change causes localization change of mutated *Wisp3* protein.

Wisp3 belongs to the CCN family of proteins, which play important roles in development during chondrogenesis and enchondromatosis and encode cysteine-rich secreted proteins with roles in cell growth and differentiation [21]. To investigate the effects of the T1000C and 840delT mutations on *Wisp3* function in chondrocytes, we compared biological behaviors in C-20/A4 cells transfected separately with WT-*Wisp3*, MUT$^{1000T/C}$, and MUT^{840delT}. MUT$^{1000T/C}$ *Wisp3* and MUT^{840delT} *Wisp3* increased proliferation activity as well as decreased apoptosis of C-20/A4 cells obviously, which shared the phenotype of articular chondrocytes (ACs), separated from SEDT-PA patients we described before [15]. Therefore, inhibition of cell proliferation and promotion of precursor cell differentiation are major effects of *Wisp3* on chondrocytes, through which *Wisp3* modulates the balance of cartilage metabolism.

We previously found that PPD cartilage had lost its flexibility, and the main matrix component of cartilage is collagen, so we detected the effect of *Wisp3* gene mutation on the collagen expression in chondrocytes. The results demonstrated that both mutant *Wisp3* lose the function to modulate the expression of cartilage specific matrix type II collagen when compared with wild *Wisp3*, which consisted of results in C-28/I2 and T/C-28a2 cells transfected with another SEDT-PA related *Wisp3* mutation (Cis78-Arg) [22], but the modulation effect of *Wisp3* may not be via activation of *SOX9* in our study since no change of SOX expression was

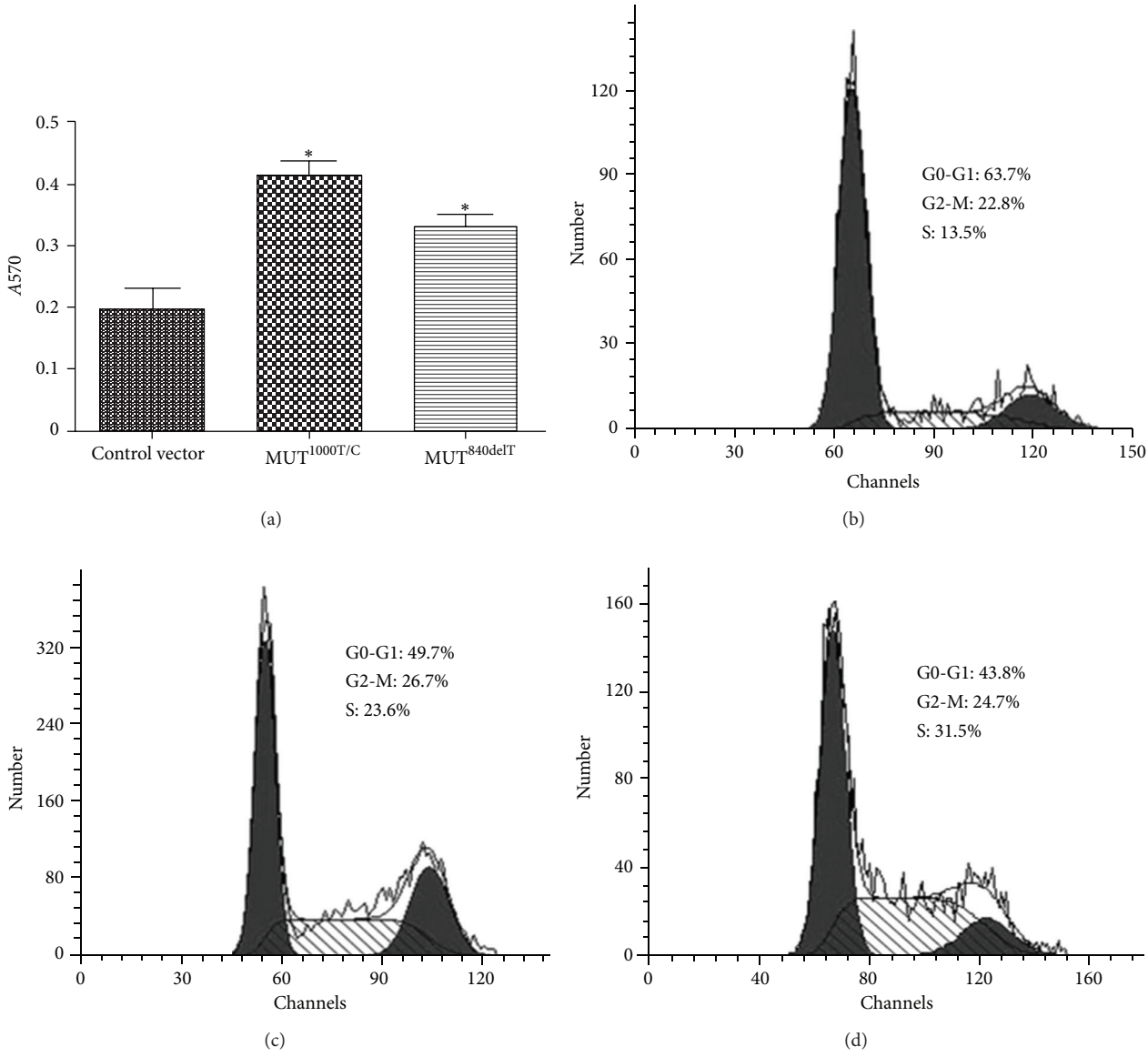


Figure 2: Cell viability and cycle analysis in C20/A4 cells transfected with *Wisp3*. Cells stably transfected with $MUT^{1000T/C}$/pcDNA3.1(+), $MUT^{840delT}$/pcDNA3.1(+) or empty vector, and cell viability (a) were determined by MTT and cell cycle was evaluated using flow cytometry. (b) Empty vector; (c) $MUT^{1000T/C}$; (d) $MUT^{840delT}$. $^{*}P < 0.01$ compared with C20/A4 cells transfected with empty vector.

found. After the collagen synthesis, it need to be secreted into the extracellular matrix, if the collagen secretion was changed by gene mutation or 3D-conformational alteration, the function of cartilage will be abnormal, and to further study the dynamic collagen synthesis and secretion, we use ^{14}C-proline, which is the major material of collagen synthesis and a major determinant of collagen tertiary structure, to label the new synthesized collagen, through detection of the radioactivity in cell lysate and supernatant to quantify the intracellular collagen content and extracellular secretion at different time points. Compared to the wild type *Wisp3*, $MUT^{1000T/C}$ or $MUT^{840delT}$ *Wisp3* lost the function of increasing the extracellular collagen secretion, delaying the intracellular collagen synthesis, which is one of the important mechanisms for the collage size and density decrease in PPD cartilage described previously.

However, we could not find the difference of *MMP-1* mRNA levels between the C-20/A4 cells transfected with wild and mutant *Wisp3*, which was dramatically decreased in ACs separated from SEDT-PA/PPD patients compared with normal ACs. The paradoxical phenomenon may be related to the following causes: (1) C-20/A4 is an immortalized cell line derived from human juvenile costal cartilage and is highly proliferative and not contact-inhibited compared with primary cells, which may have influence on the expressions of matrix and other genes at reasonable levels [23]. (2) $MUT^{1000T/C}$ or $MUT^{840delT}$ results in obviously changed biological behaviors of chondrocytes; however, that

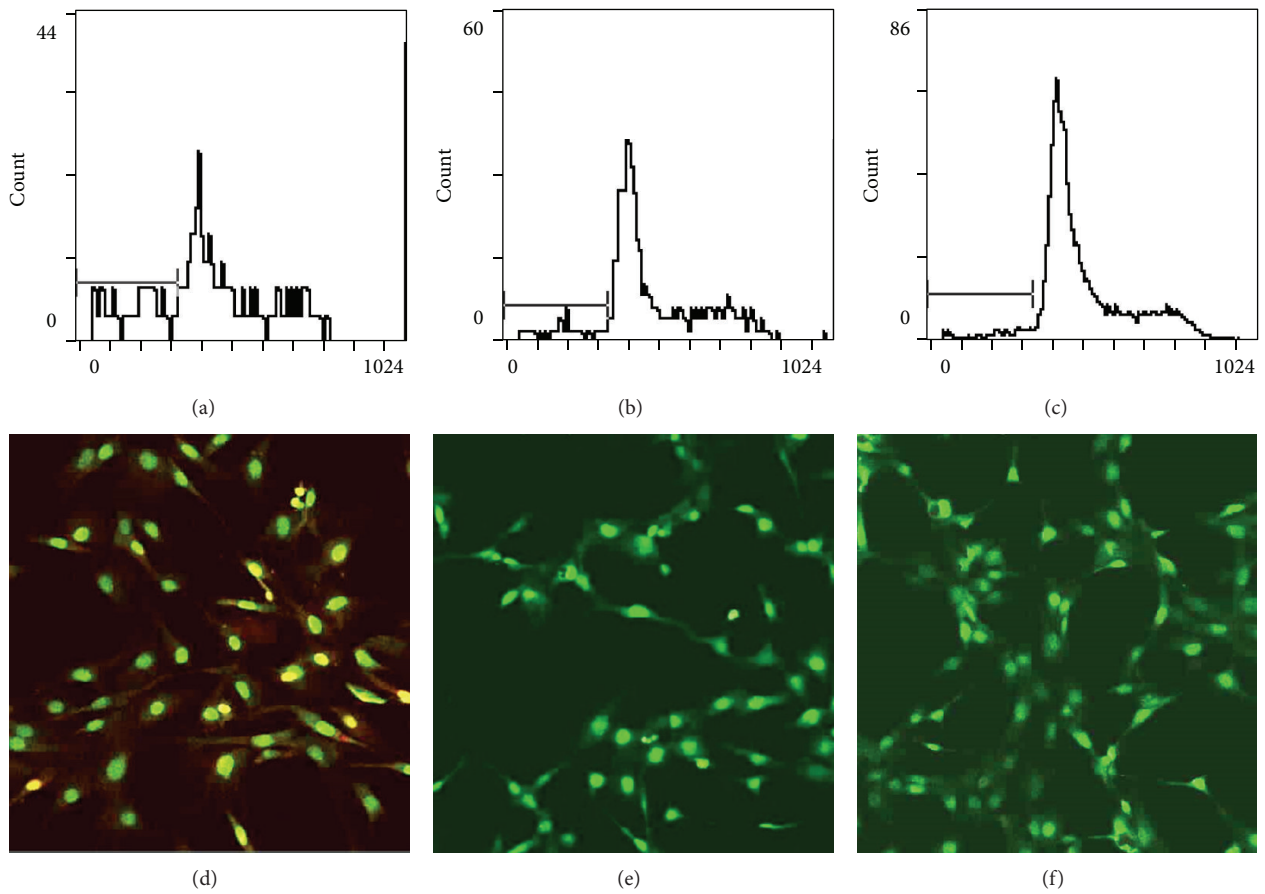


Figure 3: Cell apoptosis analysis in C20/A4 cells transfected with mutant *Wisp3* Cells stably transfected with $MUT^{1000T/C}$/pcDNA3.1(+), $MUT^{840delT}$/pcDNA3.1(+), or empty vector; cell apoptosis was evaluated by using FAC flow cytometry (a–c) and acridine orange/ethidium bromide staining (d–f) (apoptotic cells stained with yellow, condensed, or fragmented nuclei) analysis. (a) and (d) empty vector apoptosis rate is 27.1%; (b and e) $MUT^{1000T/C}$ apoptosis rate is 9.2%; (c and f) $MUT^{840delT}$ apoptosis rate is 7.8%. Magnification is 200 x.

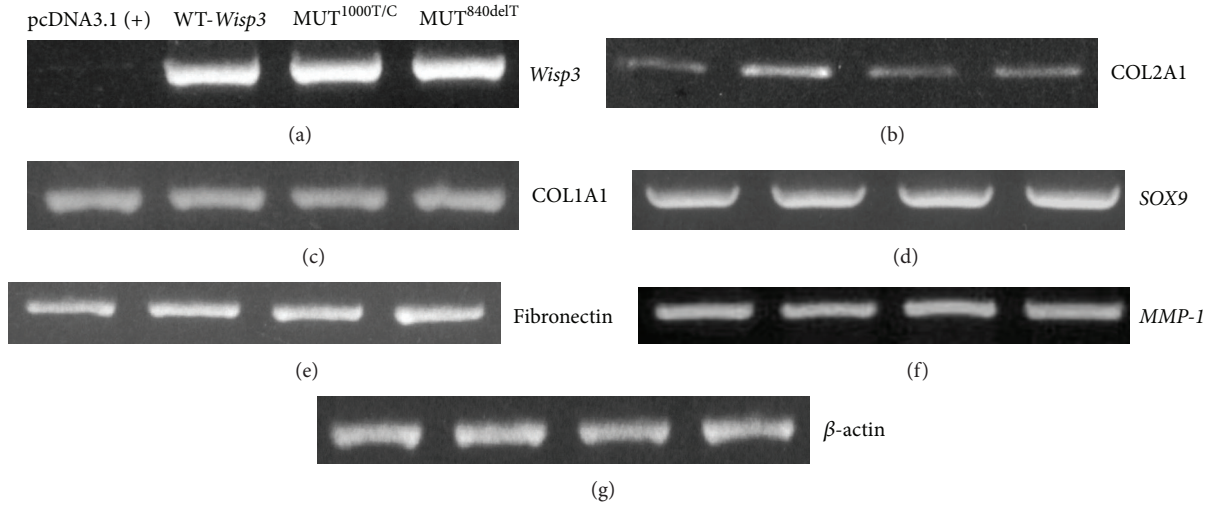


Figure 4: mRNA expression of cartilage-specific genes in C20/A4 cells transfected with wild and mutant *Wisp3*. Cells stably transfected with WT-*Wisp3*/pcDNA3.1(+), $MUT^{1000T/C}$/pcDNA3.1(+), $MUT^{840delT}$/pcDNA3.1(+), or empty vector. mRNA expression of cartilage-specific genes in C20/A4 cells was determined by RT-PCR. (a)–(g) represent *Wisp3*, COL2A1, COL1A1, *SOX9*, fibronectin, *MMP-1*, and *β*-actin separately.

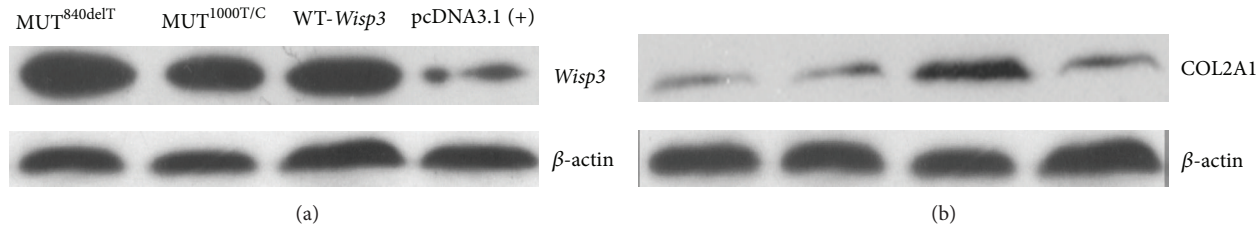


Figure 5: *Wisp3* and COL2A1 protein expression in C20/A4 cells transfected with wild and mutant *Wisp3* analyzed by western blot.

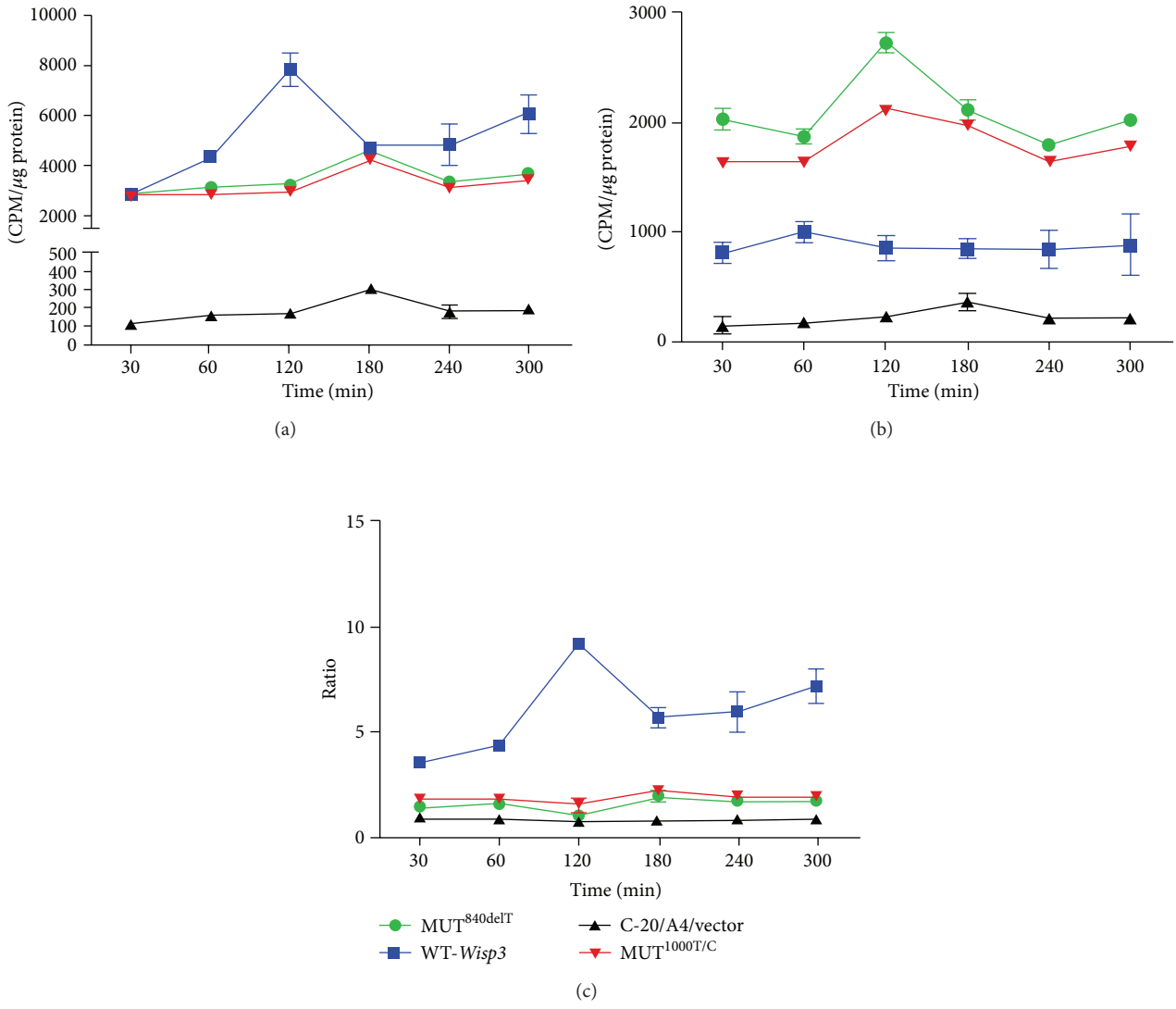


Figure 6: Change of intracellular collagen content and extracellular collagen secretion in mutant chondrocytes analyzed by ^{14}C-proline incorporation assay. (a) Time course of ^{14}C-proline labeled collagen (detected by radioactivity) secreted to the supernatant of the cultured chondrocytes. (b) Time course of ^{14}C-proline labeled collagen content in cultured chondrocytes. (c) Ratio of secreted collagen to intracellular collagen.

cannot fully represent the effect of compound mutation (840delT/T1000C) on the cartilage metabolism. (3) The low expression level of *Wisp3* in C-20/A4 cell line may have interacted influence on the expressions of other genes.

5. Conclusions

Wisp3 mutations resulted in abnormal protein distribution and dysfunction of cell proliferation, collagen production, and dynamic secretion in chondrocytes, which may be involved in the pathogenesis of SEDT-PA.

Acknowledgments

This work was supported by Grant nos. 81070278, 30872617 from China National Natural Scientific Foundation, Natural Science Foundation from Hunan Provincial (100JJ1007), and Central South University (2010QZZD025).

References

[1] J. R. Hurvitz, W. M. Suwairi, W. Van Hul et al., "Mutations in the CCN gene family member *WISP3* cause progressive pseudorheumatoid dysplasia," *Nature Genetics*, vol. 23, no. 1, pp. 94–98, 1999.

[2] B. Perbal, C. Martinerie, R. Sainson, M. Werner, B. He, and B. Roizman, "The C-terminal domain of the regulatory protein NOVH is sufficient to promote interaction with fibulin 1C: a clue for a role of NOVH in cell-adhesion signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 3, pp. 869–874, 1999.

[3] Y. Nishida, T. Matsubara, T. Tobina et al., "Effect of low-intensity aerobic exercise on insulin-like growth factor-I and insulin-like growth factor-binding proteins in healthy men," *International Journal of Endocrinology*, vol. 2010, Article ID 452820, 8 pages, 2010.

[4] B. Perbal, D. R. Brigstock, and L. F. Lau, "Report on the second international workshop on the CCN family of genes," *Journal of Clinical Pathology*, vol. 56, no. 2, pp. 80–85, 2003.

[5] G. W. Zuo, C. D. Kohls, B. C. He et al., "The CCN proteins: important signaling mediators in stem cell differentiation and tumorigenesis," *Histology and Histopathology*, vol. 25, no. 6, pp. 795–806, 2010.

[6] A. Leask, "CCN6 (*WISP3*): a new anti-cancer therapy?" *Journal of Cell Communication and Signaling*, vol. 4, no. 4, pp. 199–200, 2010.

[7] S. A. Al-Awadi, T. I. Farag, and K. Naguib, "Spondyloepiphyseal dysplasia tarda with progressive arthropathy," *Journal of Medical Genetics*, vol. 21, no. 3, pp. 193–196, 1984.

[8] A. Dalal, G. Bhavani SL, P. P. Togarrati et al., "Analysis of the *WISP3* gene in Indian families with progressive pseudorheumatoid dysplasia," *American Journal of Medical Genetics A*, vol. 158, pp. 2820–2828, 2012.

[9] S. Ehl, M. Uhl, R. Berner, L. Bonafé, A. Superti-Furga, and A. Kirchhoff, "Clinical, radiographic, and genetic diagnosis of progressive pseudorheumatoid dysplasia in a patient with severe polyarthropathy," *Rheumatology International*, vol. 24, no. 1, pp. 53–56, 2004.

[10] J. Ye, H. W. Zhang, W. J. Qiu et al., "Patients with progressive pseudorheumatoid dysplasia: from clinical diagnosis to molecular studies," *Molecular Medicine Reports*, vol. 5, pp. 190–195, 2012.

[11] M. F. Kahn, "Chondrodysplasic rheumatism," *British Journal of Rheumatology*, vol. 37, no. 8, article 917, 1998.

[12] E. Y. Liao, Y. Q. Peng, H. D. Zhou et al., "Gene symbol: *WISP3*. Disease: spondyloepiphyseal dysplasia tarda with progressive arthropathy," *Human Genetics*, vol. 115, no. 2, article 169, 2004.

[13] E. Y. Liao, Y. Q. Peng, H. D. Zhou et al., "Gene symbol: *WISP3*. Disease: spondyloepiphyseal dysplasia tarda with progressive arthropathy," *Human Genetics*, vol. 115, no. 2, article 174, 2004.

[14] Y. Q. Peng, E. Y. Liao, H. M. Gu et al., "Pathology and molecular pathogenesis of spondyloepiphyseal dysplasia tarda with progressive arthropathy caused by compound CCN6 heterogeneous gene mutations," *Zhonghua Yi Xue Za Zhi*, vol. 84, no. 21, pp. 1796–1803, 2004.

[15] H. D. Zhou, Y. H. Bu, Y. Q. Peng et al., "Cellular and molecular responses in progressive pseudorheumatoid dysplasia articular cartilage associated with compound heterozygous *WISP3* gene mutation," *Journal of Molecular Medicine*, vol. 85, no. 9, pp. 985–996, 2007.

[16] A. Pal, W. Huang, X. Li, K. A. Toy, Z. Nikolovska-Coleska, and C. G. Kleer, "CCN6 modulates BMP signaling via the Smad-independent TAK1/p38 pathway, acting to suppress metastasis of breast cancer," *Cancer Research*, vol. 72, pp. 4818–4828, 2012.

[17] W. Huang, M. E. Gonzalez, K. A. Toy, M. Banerjee, and C. G. Kleer, "Blockade of CCN6 (*WISP3*) activates growth factor-independent survival and resistance to anoikis in human mammary epithelial cells," *Cancer Research*, vol. 70, no. 8, pp. 3340–3350, 2010.

[18] Q. H. Liang, Y. Jiang, X. Zhu et al., "Ghrelin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the ERK pathway," *PLoS ONE*, vol. 7, no. 4, Article ID e33126, 2012.

[19] C. G. Kleer, Y. Zhang, and S. D. Merajver, "CCN6 (*WISP3*) as a new regulator of the epithelial phenotype in breast cancer," *Cells Tissues Organs*, vol. 185, no. 1–3, pp. 95–99, 2007.

[20] A. Katsumi, E. A. Tuley, I. Bodo, and J. E. Sadler, "Localization of disulfide bonds in the cystine knot domain of human von Willebrand factor," *Journal of Biological Chemistry*, vol. 275, no. 33, pp. 25585–25594, 2000.

[21] W. E. Kutz, Y. Gong, and M. L. Warman, "*WISP3*, the gene responsible for the human skeletal disease progressive pseudorheumatoid dysplasia, is not essential for skeletal function in mice," *Molecular and Cellular Biology*, vol. 25, no. 1, pp. 414–421, 2005.

[22] M. B. Goldring, J. R. Birkhead, L. F. Suen et al., "Interleukin-1β-modulated gene expression in immortalized human chondrocytes," *Journal of Clinical Investigation*, vol. 94, no. 6, pp. 2307–2316, 1994.

[23] B. Perbal, "NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues," *Journal of Clinical Pathology*, vol. 54, no. 2, pp. 57–79, 2001.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 213234, 9 pages
http://dx.doi.org/10.1155/2013/213234

Review Article

Age-Related Changes in Trabecular and Cortical Bone Microstructure

Huayue Chen,[1] Xiangrong Zhou,[2] Hiroshi Fujita,[2] Minoru Onozuka,[3] and Kin-Ya Kubo[4]

[1] *Department of Anatomy, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan*
[2] *Department of Intelligent Image Information, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan*
[3] *Nittai Jusei Medical College for Judo Therapeutics, 2-2-7 Yoga, Setagaya-ku, Tokyo 158-0097, Japan*
[4] *Seijoh University Graduate School of Health Care Studies, 2-172 Fukinodai, Tokai, Aichi 476-8588, Japan*

Correspondence should be addressed to Huayue Chen; huayue@gifu-u.ac.jp

Received 24 October 2012; Accepted 14 February 2013

Academic Editor: Ling-Qing Yuan

The elderly population has substantially increased worldwide. Aging is a complex process, and the effects of aging are myriad and insidious, leading to progressive deterioration of various organs, including the skeleton. Age-related bone loss and resultant osteoporosis in the elderly population increase the risk for fractures and morbidity. Osteoporosis is one of the most common conditions associated with aging, and age is an independent risk factor for osteoporotic fractures. With the development of non-invasive imaging techniques such as computed tomography (CT), micro-CT, and high resolution peripheral quantitative CT (HR-pQCT), imaging of the bone architecture provides important information about age-related changes in bone microstructure and estimates of bone strength. In the past two decades, studies of human specimens using imaging techniques have revealed decreased bone strength in older adults compared with younger adults. The present paper addresses recently studied age-related changes in trabecular and cortical bone microstructure based primarily on HR-pQCT and micro-CT. We specifically focus on the three-dimensional microstructure of the vertebrae, femoral neck, and distal radius, which are common osteoporotic fracture sites.

1. Introduction

The proportion of elderly persons in the world population has increased substantially and will continue to do so in the coming years. Aging has multiple complex effects that result in the progressive deterioration of various organs, including the skeleton. Age-related bone loss and osteoporosis in the elderly increase the risk of fractures and morbidity in this population [1]. Osteoporosis is a common disease of the elderly [2–4], characterized by low bone mass and microstructural deterioration of bone tissue, with an increased fracture risk. Osteoporosis is defined by the World Health Organization as a bone mineral density (BMD) at least 2.5 standard deviations below the mean peak bone mass of young, healthy adults as measured by dual-emission X-ray absorptiometry [5]. With the aging population, osteoporosis and its related fractures have become an increasingly important health and socioeconomic issue. It is important to identify the possible pathologic mechanisms underlying bone fragility in old age. During life, mammalian bone undergoes a process of continuous remodeling in which old bone is resorbed and replaced with newly formed bone. In young adults, the overall amounts of resorbed and formed bone are balanced. With aging, however, this remodeling balance becomes negative, resulting in a decreased bone mass. The decline in bone mass is associated with reduced bone strength, resulting in osteoporosis [6, 7].

Osteoporosis is generally asymptomatic. The main consequence of osteoporosis is the increased risk of bone fractures. The vertebrae, femoral neck, and distal radius are highly susceptible to fracture in patients with osteoporosis [1–3]. Although BMD is an important predictor of subsequent fracture risk, age itself is also a major determinant factor of the fracture risk, independent of BMD [6, 7]. The effects of age on fracture risk could be due to a number of factors, including bone microstructural deterioration and possible changes in bone material properties, such as the composition and degree of collagen cross-linking. Although techniques to

noninvasively measure bone material properties may be validated and available in the future, high-resolution computed-tomography (CT) currently allows for three-dimensional (3D) assessment of the bone microstructure and evaluation of the trabecular and cortical microstructure separately [8]. The present paper discusses recently studied age-related changes in trabecular and cortical bone microstructure, based primarily on high resolution peripheral quantitative CT (HR-pQCT) and micro-CT. As these changes take a somewhat different course in females and males and in different skeletal sites, these variations are also reviewed.

2. Vertebrae

Fractures caused by osteoporosis most often occur in the vertebrae. The vertebrae from the mid-thoracic region down are more likely to sustain osteoporosis-related fractures [9]. Osteoporosis-related vertebral fractures may occur spontaneously or during everyday activities, such as opening a window, an insignificant fall, or twisting while lifting. The vertebrae bear the weight of the upper body and withstand substantial physical pressure. The vertebral body, a box-shaped block of bone, comprises an elliptical block of trabecular bone covered by a thin shell of cortical bone. Vertebral bodies are connected to each other by intervertebral discs at the upper and lower ends and ligaments at the anterior/posterior lateral sides, forming a flexible column. This design provides a lightweight structure that involves a minimum of material in its construction. The quality of the trabecular bone in vertebral bodies plays an important role in the performance of the entire spine [10, 11].

2.1. Vertebral Trabecular BMD. The trabecular BMD in the vertebrae is metabolically more active and may therefore serve as an early indicator of vertebral osteoporosis [8]. Vertebral trabecular BMD is significantly correlated with vertebral fracture [10]. Worldwide, the number of subjects undergoing thoracic and abdominal CT examinations has increased dramatically over the last two decades [8, 12]. In an age- and sex-stratified population sample of 541 women and 490 men aged 17 to 88 years, we examined the relationship between vertebral trabecular volumetric BMD (vBMD) and age [13]. The CT images included all thoracic and lumbar vertebrae and were scanned using standard setting (120 kV, auto mAs, 1.25 mm thick slice, pitch = 0.49–0.88 mm). Slice intervals were modified to the same value as the pitch using sinc interpolation to keep each voxel in an isotropic size in three dimensions. A standard phan-tom (B-MAS 200; Kyoto Kagaku, Kyoto, Japan) was used to calibrate the CT Hounsfield units to equivalent bone mineral concentration [13]. Trabecular vBMD of both women and men tended to decrease gradually from the first thoracic vertebra (Th1) to the third lumbar vertebra (L3) in all age categories. With regard to the vertebral level, L3 had the lowest vBMD among the thoracic and lumbar vertebrae. Compared with Th1, trabecular vBMD of L3 was lower than that of Th1 by about 30% [13]. Trabecular vBMD of L3 tended to decrease with aging for both women and men. The vBMD in subjects over 70 years of age was lower than that of adults under 40 years of age by about 70%. In women 50 years of age and older, the vBMD was considerably lower than that in women under 50 years of age. In men, trabecular vBMD declined at an almost constant rate with aging (Figure 1).

2.2. Vertebral Trabecular Microstructure. Vertebral trabecular bone has a complex inhomogeneous 3D microstructure [14, 15]. The central and anterosuperior regions of the vertebral body have a lower bone volume fraction (BV/TV) than the corresponding posterior region [14, 16]. A thorough understanding of the regional variations in microstructural properties is crucial for evaluating age- and sex-related bone loss of the vertebrae and may provide more insight into the mechanisms of vertebral osteoporosis and the related fracture risks. We studied 56 fourth lumbar vertebrae (L4) from Japanese cadaver donors aged 57 to 98 years [14]. An 8 mm thick sagittal section close to the mid-line of the L4 vertebral body was harvested using a water-cooled low-speed diamond saw (Buehler IsoMet, Illinois, USA). The central trabecular bone of the vertebral body was examined by micro-CT and scanning electron microscopy. The trabecular bone volume fraction (BV/TV) and trabecular number (Tb.N) significantly decreased with aging. Between the ages of 60 and 90 years, BV/TV declined by 22% and 24% in both women and men. Decreases in BV/TV with aging were similar in women and men. Tb.N also declined with aging, by 16% in women and 19% in men. Consistently, trabecular separation (Tb.Sp) increased with aging. The age-related decrease in trabecular thickness (Tb.Th) was not statistically significant [14, 15]. The reduction of BV/TB with aging is associated primarily with reduced Tb.N and increased Tb.Sp [14, 17].

Age-related changes of Tb.Th are quite controversial. Some studies report a greater relative loss and thinning with age for all trabeculae [18], or for horizontal trabeculae only [19]. With the loss of horizontal trabeculae, the remaining vertical trabeculae tend to maintain their thickness and might even increase in thickness with aging [14, 19]. Some studies indicate that there are no significant changes in Tb.Th with aging [14]. The reduced BV/TV due to decreases in Tb.N and increases in Tb.Sp, with or without thinning of Tb.Th, has formed the basis of the plausible hypothesis for age-related trabecular bone loss [20].

Some microstructural parameters differ significantly between women and men [14, 21]. Compared with women, men have higher BV/TV and Tb.N. Scanning electron microscopic images revealed increased resorbing surfaces, perforated or disconnected trabeculae, and microcallus formations in elderly subjects [14, 22, 23]. Therefore, it is conceivable that age-related vertebral trabecular bone loss is caused by increased bone resorption activity [9, 10, 14]. These findings illustrate some potential mechanisms underlying vertebral fractures.

2.3. Vertebral Cortical Bone. The cortical shell of the vertebral body is thin and porous. Thus, one difficulty in sorting out the role of the vertical cortex, particularly in aged individuals, is that the extreme thinness of the cortex makes

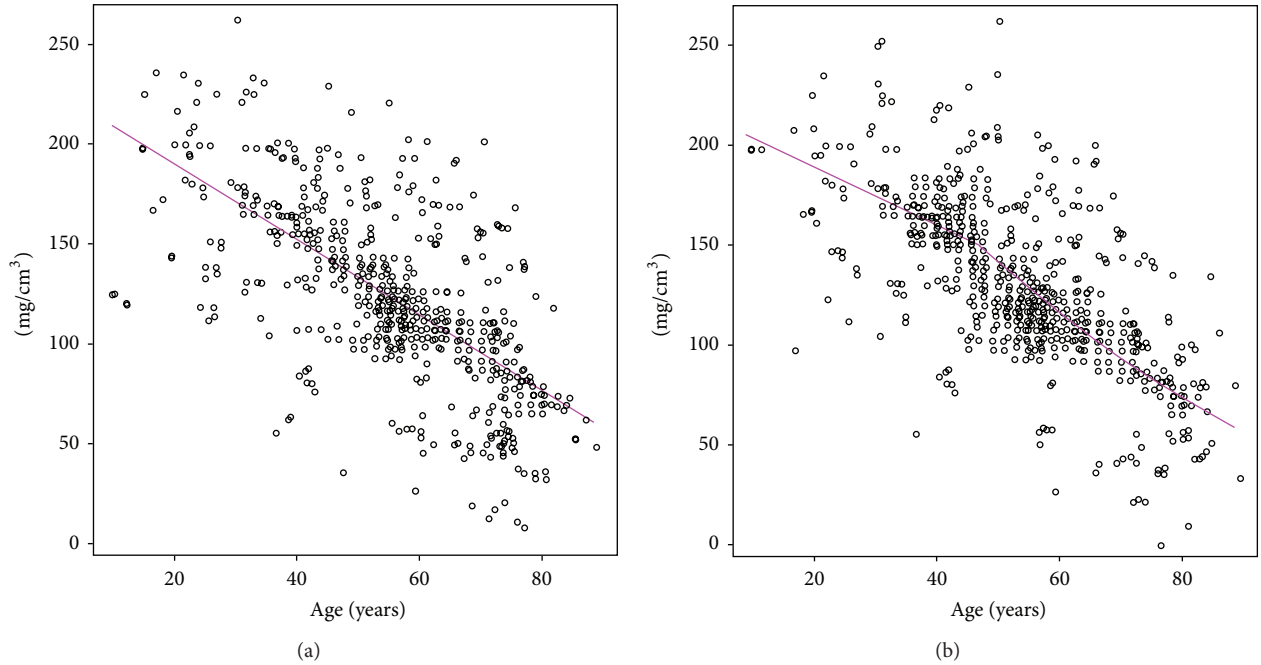


Figure 1: Relationship between age and trabecular vBMD at the third lumbar vertebra for men (a) and women (b) [13].

it difficult to measure with most nondestructive techniques. The relative contribution of the cortical shell to whole bone strength remains poorly understood. The vertebral cortical thickness ranges from 180 to 600 μm, with a mean thickness of 380 μm [24–27]. The cortical thickness of the thoracic vertebrae is thinner than that of the cervical and lumbar vertebrae. The mean thickness of the ventral shell is in general greater than that of the dorsal shell. Cortical thickness is not sex specific. Cortical thickness slightly decreases with aging. Although most studies emphasize the important role of trabecular bone in age-related vertebral fragility, both old and new studies point to an important role for the cortical shell, particularly when trabecular bone volume is low, in elderly subjects [24–27].

3. Femoral Neck

Femoral neck fractures are the most common injury observed in elderly subjects. This type of fracture is a major cause of morbidity in the elderly as it leaves many patients immobile and confined to their bed [28]. The risk of femoral neck fracture increases 10-fold with every 20 years of age. Femoral neck fractures usually occur due to falls, which are common among the elderly. Femoral neck fractures are attributed to both cortical and trabecular bone loss. The anatomic distribution of cortical and trabecular bone in the femoral neck might be critical in determining resistance to fracture.

3.1. Microstructure of Femoral Neck Trabecular Bone. An approximately 15 mm segment of femoral neck was harvested by cutting at the base of femoral head and at the base of femoral neck. Trabecular specimen of $8 \times 8 \times 8$ mm cube was obtained in the middle of femoral neck for micro-CT analysis. Age-related changes in trabecular bone of the femoral neck include a decrease in BV/TV, Tb.N, and an increase in Tb.Sp [29, 30]. BV/TV declines by 22% and 18% between ages 60 and 90 years. Tb.N and Tb.Th decrease, and Tb.Sp increases in both women and men. The reduction of BV/TV with aging is associated with a decline in Tb.N and Tb.Th and increase in Tb.Sp [21, 29–32]. Trabecular bone in the femoral neck has a complex 3D structure that consists of interconnecting plates and rods. Plate or rod characteristics of trabeculae can be estimated by measuring the structure model index (SMI). This is an important structural feature that strongly impacts the mechanical properties of trabeculae [33]. The SMI in the femoral neck and vertebrae significantly increases with aging [29, 32]. A more rod-like structure of trabecular bone is observed in the femoral neck with aging, and, hence, the femoral neck is likely to be more susceptible to bending and buckling failure modes. Trabecular connectivity is a fundamental property of 3D networks. Connectivity density (Conn.D) is crucial in the maintenance of bone strength. Conn.D in femoral neck and vertebra decreases significantly with aging [14, 29]. As the trabecular bone volume decreases, there is a corresponding decrease in Conn.D, possibly due to the loss of small interconnecting trabeculae with a small initial diameter [14]. The degree of anisotropy (DA) defines the direction and magnitude of the preferred orientation of trabeculae and uses the ratio between the maximum and minimum radii of the mean intercept length ellipsoid [34]. DA is a measure of how highly oriented substructures are within a volume, which is an important trabecular bone microstructural parameter. In the trabecular bone of femoral neck and vertebra, we did not find any significant differences between DA and age.

3.2. Microstructure of Femoral Neck Cortical Bone. The morphology of the femoral neck shows marked regional heterogeneity [29, 35–37]. As the body weight rests vertically and unidirectionally on the hip joint, cortical bone in the superior region is thinner than that in the inferior region of the femoral neck. The cortices in the elderly exhibit marked thinning in the superior region, but the inferior cortices are thicker compared with those in younger adults [35–37]. Relative to the mean value at age 60 years, cortical thickness in the superoposterior octant, which is compressed most in a sideways fall, declines in women by 6.4% per decade. Similar but significantly smaller effects are evident in men. This thinning compromises the capacity of the femoral neck to absorb energy independently of osteoporosis [29, 36].

The cortical porosity (Ct.Po) of the femoral neck ranges from 5% to 13% [29, 36–38]. With aging, pores within the cortex adjacent to the marrow cavity coalesce, leaving cortical remnants that look similar to trabeculae. The remaining thinned cortex beneath the periosteum retains a compact appearance and contains enlarged though not confluent pores. In elderly subjects, excavation of the remaining compact cortex leaves further cortical remnants. Figure 2 shows the age-related changes in cortical porosity of the femoral neck. Between ages 60 and 90 years, cortical thickness (Ct.Th) decreases by 3% to 5% per decade, and Ct.Po increases by 31% to 33% per decade [29]. The pore diameter increases, with no significant changes in the pore number [29, 36–38]. Consequently, we consider that cortical porosity with aging is mainly due to enlarged intracortical pores. Relative to men, women have a higher Ct.Po and pore diameter. Therefore, while age is the most important factor, sex also has a role in Ct.Po and pore size. With aging, cortical pores fuse together to form giant pores with diameters exceeding 385 μm [29, 36, 39]. Thus, the formation of giant pores could be considered a pivotal process in the focal loss of cortical thickness and strength.

The most obvious age-related change in femoral neck is the increase in Ct.Po. The decrease of BV/TV with aging is more noticeable than that of Ct.Th. There is a significant inverse correlation between Ct.Po and BV/TV for both women and men. As compared with women, men have higher Ct.Th and BV/TV and lower Ct.Po. These findings may serve as reference for ethnic comparison with aging and sex and may provide more insight into femoral neck fracture risk [29, 36, 39].

4. Radius

Fractures of the distal radius are one of the most common injury types, especially in pediatric and elderly populations, which are at greatest risk for this injury [40]. As the population continues to age, the incidence of osteoporotic distal radius fractures will also increase. In the elderly population, radial fractures frequently result from falls from a standing height and other low-energy traumas [41].

Population studies using HR-pQCT imaging revealed that in the trabecular bone of the distal radius, between the ages of 20 and 90 years, BV/TV decreases by 27% in women and 26% in men. BV/TV remains relatively constant at the distal radius until midlife and declines thereafter. Tb.N declines and Tb.Sp increases with aging in women. Trabecular bone mass is higher in men than in women of the same age; age-related declines of the trabecular BMD and BV/TV are similar in women and men between the ages of 20 and 90 years [42–45]. The microstructural basis for the decrease in trabecular volume differs between women and men [44, 45]. Women appear to lose trabeculae, primarily with reductions in Tb.N and increases in Tb.Sp, while in men the main mechanism for the decrease in BV/TV is trabecular thinning, resulting in a marked decrease of Tb.Th and unchanged Tb.N [44, 45].

Cortical bone was examined morphologically with HR-pQCT. Cortical bone strength (failure load) and the load distribution were estimated using finite-element analysis as reported previously [46]. Cortical vBMD is significantly lower in older women than in younger women. There is no significant change in the cortical vBMD with age in men [44]. Ct.Po is very low in the radius, ranging from 0.2% to 2.4% [44, 45]. Older women and men have an increased Ct.Po and cortical pore diameter, compared with younger subjects. Cortical bone strength correlates negatively with Ct.Po, which is an important component of bone quality that deteriorates with aging, independent of BMD [45, 47, 48]. The age-related increase in Ct.Po is more than 2-fold greater in women than in men. Cortical thickness also tends to decline more with aging in women than in men. Bone strength is greater in men than in women at the distal radius. The sex difference is probably caused by greater cortical porosity in women.

5. Tibia

Although the tibia is commonly measured with pQCT, there are currently no recommendations for using the tibia for bone health assessment or hip fracture risk prediction. Given that the tibiae are exposed to multiple modes, frequencies, durations, amplitudes, and rates of mechanical loading from physical activities, tibial characteristics may be more closely related to those of the hip than the forearm [49, 50].

Trabecular bone specimens from the medial compartment of the proximal tibial metaphyses have been examined with micro-CT and scanning electron microscopy [51–53]. Trabecular BMD and BV/TV of the proximal tibia show age-related decreases in women and men [51–53]. Figure 3 shows the age-related changes of trabecular bone at the proximal tibia. From 57 to 98 years of age, BV/TV decreases by 7% and 6% per decade for women and men, respectively, while BMD declines by around 4% per decade. The rate of decline with aging is similar for women and men. Women, however, have a consistently lower BMD and BV/TV than men of the same age. Possible explanations for the sex differences are that women reach a lower peak bone mass before they start losing bone, or that any accelerated loss occurs earlier, perhaps, as has been suggested, around menopause [51, 54]. It is likely that much of the female preponderance for fractures is related to the lower bone mass of women compared with men.

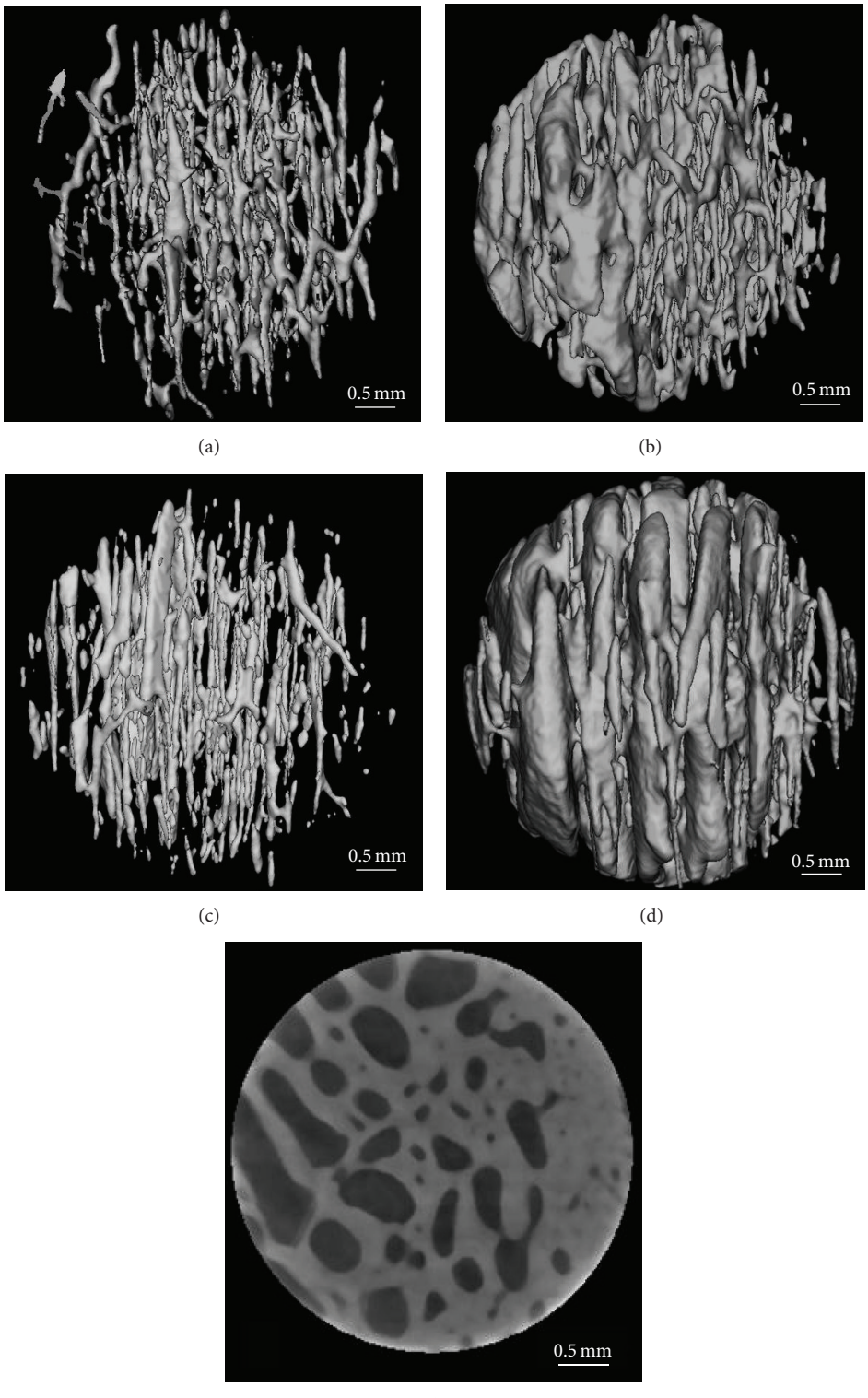


Figure 2: 3D reconstructed images of cortical porosity at the inferior femoral neck from a man aged 62 years (a), a man aged 92 years (b), a woman aged 62 years (c), and a woman aged 92 years (d). There are more enlarged pores in the 92-year-old group than those of the 62-year-old group. Representative 2D micro-CT image of the femoral neck cortex from a woman aged 92 years (e) is shown. The periosteal surface faces right for all specimens [29].

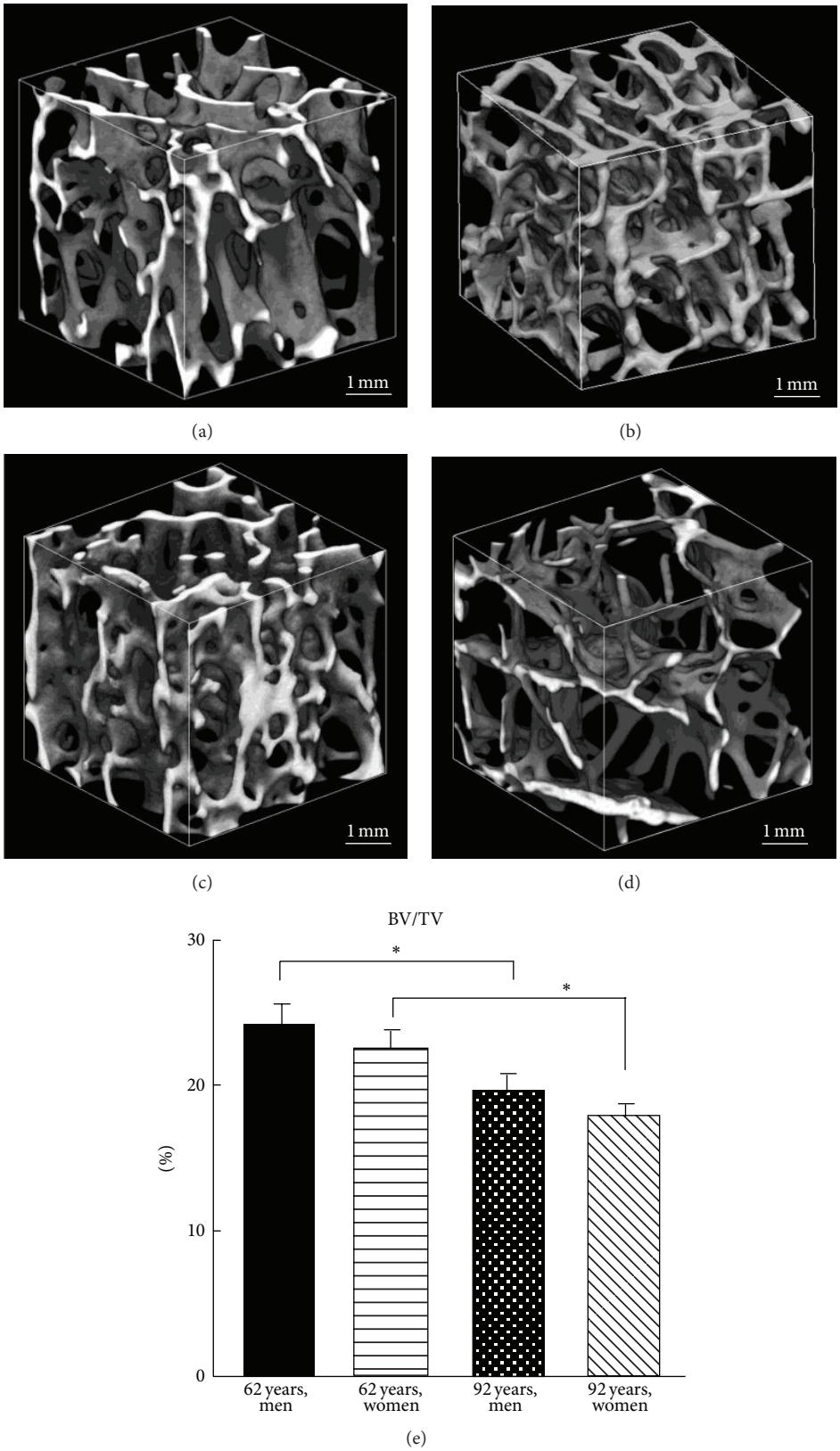


Figure 3: 3D reconstructed images of trabecular microstructure at the proximal tibia from a man aged 62 years (a), a man aged 92 years (b), a woman aged 62 years (c), a woman aged 92 years (d), and the corresponding values for BV/TV (e). The trabecular bone volume fraction is highest in man aged 62 years and lowest in woman aged 92 years ($^*P < 0.05$) [51].

Age-related trabecular microstructural changes at the proximal tibia include a decrease in BV/TV, Tb.Th, and Conn.D, as well as an increase in Tb.Sp and SMI [51–53]. The decline in BV/TV and Tb.Th with aging is similar for women and men. The age-related decrease in Tb.N for women is nearly twice that in men. Age-related bone loss at the proximal tibia in women is considered to be due to decreases in both Tb.N and Tb.Th, whereas in men, the primary mechanism for the decrease in BV/TV is trabecular thinning. Based on finite element modeling, reductions in Tb.N have a 2- to 5-fold greater impact on bone strength compared with reductions in Tb.Th that result in similar decreases in bone volume [55]. The SMI increases with aging. A shift toward a more rod-like structure with aging is observed in the proximal tibia [51, 53].

Scanning electron microscopy revealed that the percentage area of trabecular resorbing surface increases significantly with aging. Some trabeculae are completely perforated or disconnected. Age-related trabecular bone loss at the proximal tibia is caused by trabecular perforation and thinning. Several trabecular microcallus formations on the thin trabeculae are observed in elderly subjects. A microcallus is a small mass of woven bone often observed at the vertebra, mainly on the vertical trabeculae [14, 23, 51]. A microcallus can be seen as an attempt to preserve or repair a trabecula [14, 56, 57]. What triggers the microcallus formation, however, remains a subject of debate.

Cortical thickness of tibia tends to decline more with aging in women than in men. Ct.Po in the tibia ranges from 0.3% to 7.1%, which is lower than that of the femur and higher than that of the radius [44, 45, 48, 58]. Men have a larger cortical area and thicker cortices than women. Older women and men have an increased Ct.Po and cortical pore diameter compared with younger subjects. The age-related increase in Ct.Po is more prominent in women. The sex difference in tibial strength is due to greater trabecular bone mass and lower cortical porosity.

6. Conclusion

Age-related bone loss and resultant osteoporosis in a substantial proportion of the elderly population is multifaceted and multifactorial, involving a progressive loss of both bone quantity and quality. A number of genetic, hormonal, and biochemical players are implicated in this process. The role of aging pathways, such as increased oxidative stress and telomere shortening, in bone loss is also becoming apparent. The role of nutrition and lifestyle choices such as exercise is better appreciated with newer studies linking decreased bone density and increased fracture risk in nutritionally compromised and sedentary elderly individuals.

Age-related microstructural changes in bone are complex. There are three major age-related processes that lead to bone loss. The first and most important is trabecular bone loss. The decrease in trabecular bone is caused by thinning of the trabeculae and, especially in early postmenopausal women, by disruption of the trabecular microstructure and loss of trabecular elements. Trabecular bone loss over life is one-half at the vertebra and one-quarter at the femur, radius, and tibia. The second process contributing to bone loss is a decrease in cortical bone, mainly caused by increased porosity from both an increase in resorption cavities and an accumulation of incompletely closed osteons with aging. The third process is continued net resorption at the endocortical surface. Bone loss over life from this process is approximately 25% to 40% at the femoral neck and distal radius, but less at the tibia.

Vertebral strength, a key etiologic factor of osteoporotic fractures, is maintained mainly by trabecular bone. Vertebral trabecular bone mass is lower than peripheral bone mass. Vertebral trabeculae are microstructurally heterogeneous, with lower bone mass at the central and anterosuperior regions of the vertebral body. Decreased trabecular bone mass and especially increased cortical porosity might be the most important causes of femoral neck and tibial fragility in the elderly subjects. Cortical porosity is an important component of bone quality at the distal radius that deteriorates with aging. Although our understanding of the pathogenesis of aging bone is appreciable, it is not yet exhaustive. Further studies are needed to define the extent to which deterioration of the cortical and trabecular microstructure contributes to the effect of age on bone fragility at common sites of osteoporotic fractures.

Abbreviations

BMD:	Bone mineral density
BV/TV:	Bone volume fraction
Tb.Th:	Trabecular thickness
Tb.N:	Trabecular number
Tb.Sp:	Trabecular separation
Ct.Po:	Cortical porosity
SMI:	Structural model index
Conn.D:	Connectivity density
DA:	Degree of anisotropy
vBMD:	Volumetric bone mineral density
CT:	Computed tomography
HR-pQCT:	High resolution peripheral quantitative CT.

Acknowledgment

This research work was funded in part by a Grant-in-Aid for Scientific Research on Innovative Areas, MEXT, Japan.

References

[1] T. D. Rachner, S. Khosla, and L. C. Hofbauer, "Osteoporosis: now and the future," *The Lancet*, vol. 377, no. 9773, pp. 1276–1287, 2011.

[2] G. Duque and B. R. Troen, "Understanding the mechanisms of senile osteoporosis: new facts for a major geriatric syndrome," *Journal of the American Geriatrics Society*, vol. 56, no. 5, pp. 935–941, 2008.

[3] P. Pietschmann, M. Rauner, W. Sipos, and K. Kerschan-Schindl, "Osteoporosis: an age-related and gender-specific disease—a mini-review," *Gerontology*, vol. 55, no. 1, pp. 3–12, 2009.

[4] J. Jasien, C. M. Daimon, S. Maudsley, B. K. Shapiro, and B. Martin, "Aging and bone health in individuals with developmental disabilities," *International Journal of Endocrinology*, vol. 2012, Article ID 469235, 10 pages, 2012.

[5] World Health Ooganization, "Assessment of fracture risk and its application to screening for postmenopausal osteoporosis," in *Report of a WHO Study Group*, vol. 843 of *World Health Organization Technical Report Series*, pp. 1–129, 1994.

[6] F. A. Syed and A. C. Ng, "The pathophysiology of the aging skeleton," *Current Osteoporosis Reports*, vol. 8, no. 4, pp. 235–240, 2010.

[7] Y. Gabet and I. Bab, "Microstructural changes in the aging skeleton," *Current Osteoporosis Reports*, vol. 9, no. 4, pp. 177–183, 2011.

[8] H. Chen, T. Hayashi, X. Zhou, H. Fujita, M. Onozuka, and K. Kubo, "Sophisticated imaging technology in the assessment of osteoporosis risk," in *Osteoporosis*, Y. Dionyssiotis, Ed., pp. 181–194, InTech, Rijeka, Croatia, 2012.

[9] R. D. Wasnich, "Vertebral fracture epidemiology," *Bone*, vol. 18, no. 3, supplement, pp. 179S–183S, 1996.

[10] P. McDonnell, P. E. McHugh, and D. O'Mahoney, "Vertebral osteoporosis and trabecular bone quality," *Annals of Biomedical Engineering*, vol. 35, no. 2, pp. 170–189, 2007.

[11] A. Ruyssen-Witrand, L. Gossec, S. Kolta, M. Dougados, and C. Roux, "Vertebral dimensions as risk factor of vertebral fracture in osteoporotic patients: a systematic literature review," *Osteoporosis International*, vol. 18, no. 9, pp. 1271–1278, 2007.

[12] C. H. McCollough, L. Guimarães, and J. G. Fletcher, "In defense of body CT," *American Journal of Roentgenology*, vol. 193, no. 1, pp. 28–39, 2009.

[13] T. Hayashi, H. Chen, K. Miyamoto et al., "Analysis of bone mineral density distribution at trabecular bones in thoracic and lumbar vertebrae using X-ray CT images," *Journal of Bone and Mineral Metabolism*, vol. 29, no. 2, pp. 174–185, 2011.

[14] H. Chen, S. Shoumura, S. Emura, and Y. Bunai, "Regional variations of vertebral trabecular bone microstructure with age and gender," *Osteoporosis International*, vol. 19, no. 10, pp. 1473–1483, 2008.

[15] P. A. Hulme, S. K. Boyd, and S. J. Ferguson, "Regional variation in vertebral bone morphology and its contribution to vertebral fracture strength," *Bone*, vol. 41, no. 6, pp. 946–957, 2007.

[16] H. Gong, M. Zhang, H. Y. Yeung, and L. Qin, "Regional variations in microstructural properties of vertebral trabeculae with aging," *Journal of Bone and Mineral Metabolism*, vol. 23, no. 2, pp. 174–180, 2005.

[17] F. Eckstein, M. Fischbeck, V. Kuhn, T. M. Link, M. Priemel, and E. M. Lochmüller, "Determinants and heterogeneity of mechanical competence throughout the thoracolumbar spine of elderly women and men," *Bone*, vol. 35, no. 2, pp. 364–374, 2004.

[18] R. W. McCalden, J. A. McGeough, and C. M. Court-Brown, "Age-related changes in the compressive strength of cancellous bone. The relative importance of changes in density and trabecular architecture," *Journal of Bone and Joint Surgery (American Volume)*, vol. 79, no. 3, pp. 421–427, 1997.

[19] J. S. Thomsen, E. N. Ebbesen, and L. I. Mosekilde, "Age-related differences between thinning of horizontal and vertical trabeculae in human lumbar bone as assessed by a new computerized method," *Bone*, vol. 31, no. 1, pp. 136–142, 2002.

[20] B. L. Riggs and A. M. Parfitt, "Drugs used to treat osteoporosis: the critical need for a uniform nomenclature based on their action on bone remodeling," *Journal of Bone and Mineral Research*, vol. 20, no. 2, pp. 177–184, 2005.

[21] E. M. Lochmüller, M. Matsuura, J. Bauer et al., "Site-specific deterioration of trabecular bone architecture in men and women with advancing age," *Journal of Bone and Mineral Research*, vol. 23, no. 12, pp. 1964–1973, 2008.

[22] J. A. P. Jayasinghe, S. J. Jones, and A. Boyde, "Scanning electron microscopy of human lumbar vertebral trabecular bone surfaces," *Virchows Archiv*, vol. 422, no. 1, pp. 25–34, 1993.

[23] M. Hahn, M. Vogel, M. Amling, H. Ritzel, and G. Delling, "Microcallus formations of the cancellous bone: a quantitative analysis of the human spine," *Journal of Bone and Mineral Research*, vol. 10, no. 9, pp. 1410–1416, 1995.

[24] H. Ritzel, M. Amling, M. Pösl, M. Hahn, and G. Delling, "The thickness of human vertebral cortical bone and its changes in aging and osteoporosis: a histomorphometric analysis of the complete spinal column from thirty-seven autopsy specimens," *Journal of Bone and Mineral Research*, vol. 12, no. 1, pp. 89–95, 1997.

[25] J. P. Roux, J. Wegrzyn, M. E. Arlot et al., "Contribution of trabecular and cortical components to biomechanical behavior of human vertebrae: an ex vivo study," *Journal of Bone and Mineral Research*, vol. 25, no. 2, pp. 356–361, 2010.

[26] S. K. Eswaran, A. Gupta, M. F. Adams, and T. M. Keaveny, "Cortical and trabecular load sharing in the human vertebral body," *Journal of Bone and Mineral Research*, vol. 21, no. 2, pp. 307–314, 2006.

[27] B. A. Christiansen, D. L. Kopperdahl, D. P. Kiel, T. M. Keaveny, and M. L. Bouxsein, "Mechanical contributions of the cortical and trabecular compartments contribute to differences in age-related changes in vertebral body strength in men and women assessed by QCT-based finite element analysis," *Journal of Bone and Mineral Research*, vol. 26, no. 5, pp. 974–983, 2011.

[28] O. Johnell and J. A. Kanis, "An estimate of the worldwide prevalence, mortality and disability associated with hip fracture," *Osteoporosis International*, vol. 15, no. 11, pp. 897–902, 2004.

[29] H. Chen, X. Zhou, S. Shoumura, S. Emura, and Y. Bunai, "Age- And gender-dependent changes in three-dimensional microstructure of cortical and trabecular bone at the human femoral neck," *Osteoporosis International*, vol. 21, no. 4, pp. 627–636, 2010.

[30] W. Q. Cui, Y. Y. Won, M. H. Baek et al., "Age- and region-dependent changes in three-dimensional microstructural properties of proximal femoral trabeculae," *Osteoporosis International*, vol. 19, no. 11, pp. 1579–1587, 2008.

[31] F. Eckstein, M. Matsuura, V. Kuhn et al., "Sex differences of human trabecular bone microstructure in aging are site-dependent," *Journal of Bone and Mineral Research*, vol. 22, no. 6, pp. 817–824, 2007.

[32] M. Stauber and M. Müller, "Age-related changes in trabecular bone microstructures: global and local morphometry," *Osteoporosis International*, vol. 17, no. 4, pp. 616–626, 2006.

[33] T. Hildebrand and P. Rüegsegger, "Quantification of bone microarchitecture with the structure model index," *Computer Methods in Biomechanics and Biomedical Engineering*, vol. 1, no. 1, pp. 15–23, 1997.

[34] T. P. Harrigan and R. W. Mann, "Characterization of microstructural anisotropy in orthotropic materials using a second rank tensor," *Journal of Materials Science*, vol. 19, no. 3, pp. 761–767, 1984.

[35] K. E. S. Poole, P. M. Mayhew, C. M. Rose et al., "Changing structure of the femoral neck across the adult female lifespan," *Journal of Bone and Mineral Research*, vol. 25, no. 3, pp. 482–491, 2010.

[36] P. M. Mayhew, C. D. Thomas, J. G. Clement et al., "Relation between age, femoral neck cortical stability, and hip fracture risk," *The Lancet*, vol. 366, no. 9480, pp. 129–135, 2005.

[37] V. Bousson, F. Peyrin, C. Bergot, M. Hausard, A. Sautet, and J. D. Laredo, "Cortical bone in the human femoral neck: three-dimensional appearance and porosity using synchrotron radiation," *Journal of Bone and Mineral Research*, vol. 19, no. 5, pp. 794–801, 2004.

[38] C. Chappard, S. Bensalah, C. Olivier et al., "3D characterization of pores in the cortical bone of human femur in the elderly at different locations as determined by synchrotron micro-computed tomography images," *Osteoporosis International*, vol. 24, no. 3, pp. 1023–1033, 2013.

[39] G. R. Jordan, N. Loveridge, K. L. Bell, J. Power, N. Rushton, and J. Reeve, "Spatial clustering of remodeling osteons in the femoral neck cortex: a cause of weakness in hip fracture?" *Bone*, vol. 26, no. 3, pp. 305–313, 2000.

[40] K. W. Nellans, E. Kowalski, and K. C. Chung, "The epidemiology of distal radius fractures," *Hand Clinics*, vol. 28, no. 2, pp. 113–125, 2012.

[41] A. P. Diamantopoulos, G. Rohde, I. Johnsrud, I. M. Skoie, M. Hochberg, and G. Haugeberg, "The epidemiology of low- and high-energy distal radius fracture in middle-aged and elderly men and women in Southern Norway," *PLoS One*, vol. 7, no. 8, Article ID e43367, 2012.

[42] B. L. Riggs, L. J. Melton, R. A. Robb et al., "Population-based study of age and sex differences in bone volumetric density, size, geometry, and structure at different skeletal sites," *Journal of Bone and Mineral Research*, vol. 19, no. 12, pp. 1945–1954, 2004.

[43] S. Khosla, B. L. Riggs, E. J. Atkinson et al., "Effects of sex and age on bone microstructure at the ultradistal radius: a population-based noninvasive in vivo assessment," *Journal of Bone and Mineral Research*, vol. 21, no. 1, pp. 124–131, 2006.

[44] K. M. Nicks, S. Amin, E. J. Atkinson, B. L. Riggs, L. J. Melton III, and S. Khosla, "Relationship of age to bone microstructure independent of areal bone mineral density," *Journal of Bone and Mineral Research*, vol. 27, no. 3, pp. 637–644, 2012.

[45] H. M. MacDonald, K. K. Nishiyama, J. Kang, D. A. Hanley, and S. K. Boyd, "Age-related patterns of trabecular and cortical bone loss differ between sexes and skeletal sites: a population-based HR-pQCT study," *Journal of Bone and Mineral Research*, vol. 26, no. 1, pp. 50–62, 2011.

[46] J. A. MacNeil and S. K. Boyd, "Bone strength at the distal radius can be estimated from high-resolution peripheral quantitative computed tomography and the finite element method," *Bone*, vol. 42, no. 6, pp. 1203–1213, 2008.

[47] R. M. Zebaze, A. Ghasem-Zadeh, A. Bohte et al., "Intracortical remodelling and porosity in the distal radius and post-mortem femurs of women: a cross-sectional study," *The Lancet*, vol. 375, no. 9727, pp. 1729–1736, 2010.

[48] A. J. Burghardt, G. J. Kazakia, S. Ramachandran, T. M. Link, and S. Majumdar, "Age- and gender-related differences in the geometric properties and biomechanical significance of intracortical porosity in the distal radius and tibia," *Journal of Bone and Mineral Research*, vol. 25, no. 5, pp. 983–993, 2010.

[49] V. D. Sherk, D. A. Bemben, M. G. Bemben, and M. A. Anderson, "Age and sex differences in tibia morphology in healthy adult Caucasians," *Bone*, vol. 50, no. 6, pp. 1324–1331, 2012.

[50] D. Liu, S. L. Manske, S. A. Kontulainen et al., "Tibial geometry is associated with failure load ex vivo: a MRI, pQCT and DXA study," *Osteoporosis International*, vol. 18, no. 7, pp. 991–997, 2007.

[51] H. Chen, Y. Washimi, K. Y. Kubo, and M. Onozuka, "Gender-related changes in three-dimensional microstructure of trabecular bone at the human proximal tibia with aging," *Histology and Histopathology*, vol. 26, no. 5, pp. 563–570, 2011.

[52] M. Ding and I. Hvid, "Quantification of age-related changes in the structure model type and trabecular thickness of human tibial cancellous bone," *Bone*, vol. 26, no. 3, pp. 291–295, 2000.

[53] M. Ding, A. Odgaard, F. Linde, and I. Hvid, "Age-related variations in the microstructure of human tibial cancellous bone," *Journal of Orthopaedic Research*, vol. 20, no. 3, pp. 615–621, 2002.

[54] E. Seeman, "Pathogenesis of bone fragility in women and men," *The Lancet*, vol. 359, no. 9320, pp. 1841–1850, 2002.

[55] M. J. Silva and L. J. Gibson, "Modeling the mechanical behavior of vertebral trabecular bone: effects of age-related changes in microstructure," *Bone*, vol. 21, no. 2, pp. 191–199, 1997.

[56] X. G. Cheng, P. H. F. Nicholson, G. Lowet et al., "Prevalence of trabecular microcallus formation in the vertebral body and the femoral neck," *Calcified Tissue International*, vol. 60, no. 5, pp. 479–484, 1997.

[57] X. Banse, J. P. Devogelaer, D. Holmyard, and M. Grynpas, "Vertebral cancellous bone turn-over: microcallus and bridges in backscatter electron microscopy," *Micron*, vol. 36, no. 7-8, pp. 710–714, 2005.

[58] N. Dalzell, S. Kaptoge, N. Morris et al., "Bone micro-architecture and determinants of strength in the radius and tibia: age-related changes in a population-based study of normal adults measured with high-resolution pQCT," *Osteoporosis International*, vol. 20, no. 10, pp. 1683–1694, 2009.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 148673, 10 pages
http://dx.doi.org/10.1155/2013/148673

Review Article

The Role of Vitamin D Deficiency in the Incidence, Progression, and Complications of Type 1 Diabetes Mellitus

Marlene Chakhtoura and Sami T. Azar

Department of Internal Medicine, Division of Endocrinology and Metabolism, American University of Beirut-Medical Center, 3 Dag Hammarskjold Plaza, 8th floor, New York, NY 10017, USA

Correspondence should be addressed to Sami T. Azar; sazar@aub.edu.lb

Received 19 November 2012; Revised 23 January 2013; Accepted 29 January 2013

Academic Editor: Guang-Da Xiang

The "nonclassic" role of 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) has been recently widely recognized. In type 1 diabetes mellitus (T1D), it plays an immunomodulatory role through the vitamin D receptor (VDR) present on pancreatic and immune cells. Specific VDR allelic variants have been associated with T1D in many countries. Furthermore, vitamin D deficiency has been prevalent in T1D, and the seasonal and latitude variability in the incidence of T1D can be partly explained by the related variability in vitamin D level. In fact, retrospective studies of vitamin D supplementation during pregnancy or infancy showed a lower incidence of T1D. We will review the different mechanisms of the vitamin D protective effect against insulitis and present the available data on the role of vitamin D deficiency in the control, progression, and complications of T1D.

1. Introduction

Type 1 diabetes (T1DM) is an autoimmune disease occurring in the pancreatic islets [1]. It accounts for 90% of diabetes in children and adolescents [2]. Its incidence varies considerably worldwide, being highest in Finland and Sardinia [3], probably related to genetic, dietary, and environmental factors that might interfere with its pathogenesis [4]. The annual incidence has been increasing worldwide, possibly related to higher socioeconomic status and degree of urbanization [5]. Recently, there has been appealing evidence on the "nonclassic" role of vitamin D in many autoimmune diseases including rheumatoid arthritis, scleroderma, psoriasis, multiple sclerosis, and also T1DM [6, 7]. In fact, in addition to its skeletal effects and control of calcium hemostasis, 1,25-DihydroxyvitaminD3 ($1,25(OH)_2D_3$) showed potent antiproliferative and immunomodulatory properties [8].

In this paper, we will review the available data on the relationship between vitamin D and T1DM trying to elucidate the immunomodulatory mechanisms of vitamin D on pancreatic insulitis, seasonal and latitude effects, protective effects of supplements on T1DM incidence, complications and progression.

2. Immunomodulatory Effect of Vitamin D

$1,25(OH)_2D_3$ plays an immunomodulatory role in the prevention of T1DM, through the vitamin D receptor (VDR) expressed in antigen presenting cells, activated T cells [9], and pancreatic islet β-cells [10]; this has been demonstrated in many trials done on nonobese diabetic mice (NOD)—a murine model of human IDDM, spontaneously developing diabetes mellitus (DM)—using $1,25(OH)_2D_3$ or its analogue ($1,25(OH)_2D_3$, MC1288 (20-epi-$1,25(OH)_2D_3$), or KH1060 ($1,25(OH)_2$-20-epi-22-oxa-24,26,27,-trishomovitamin D) [9]. Conversely, $1,25(OH)_2D_3$-deficient mice were at higher risk of developing DM, with a more aggressive course when deficiency is present early in life [11, 12]. $1,25(OH)_2D_3$, administered early on, protects against or reduces the severity of pancreatic insulitis via a dual action, on the pancreatic beta cells and on the immune cells [13]. Furthermore, administration of $1,25(OH)_2D_3$ in combination with cyclosporine A, after the onset of the autoimmune attack, which is known as a prediabetic state, can prevent clinical diabetes [14].

At the level of the pancreatic islets, $1,25(OH)_2D_3$ decreased in vivo and in vitro proinflammatory chemokine and cytokine expression (e.g., IL6), which are implicated in

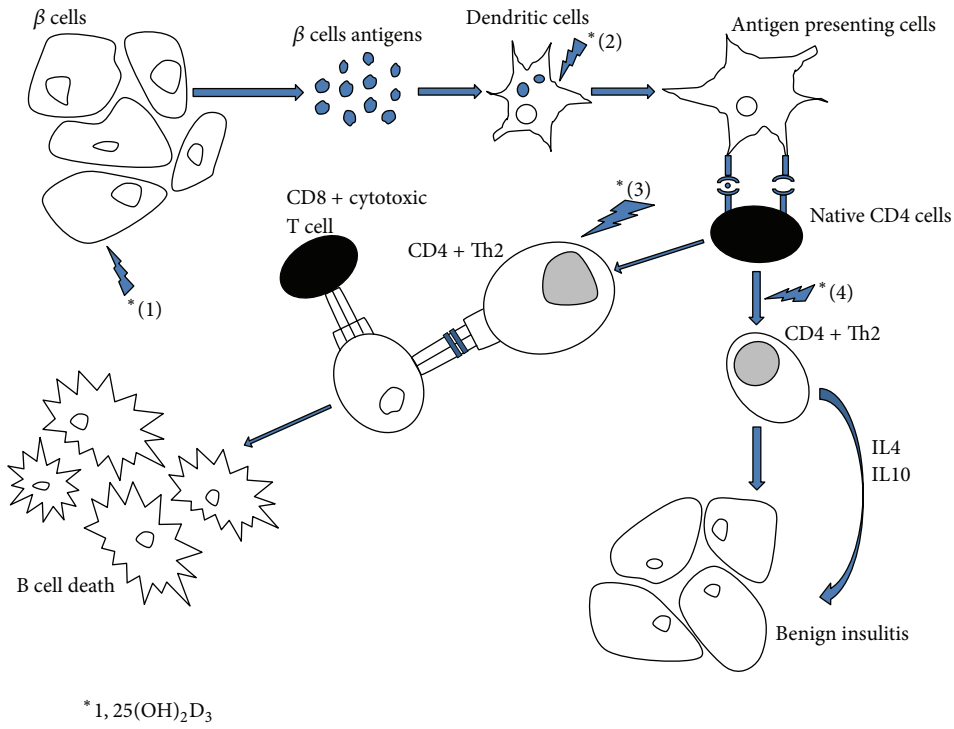


Figure 1

the pathogenesis of T1DM making β-cells less chemoattractive and less prone to inflammation; this results in decreased T cell recruitment and infiltration, increased regulatory cells, and arrest of the autoimmune process [15–17]. Furthermore, $1,25(OH)_2D_3$ decreases MHC class I expression leading to reduced vulnerability of islet β-cells to cytotoxic T lymphocytes [18].

At the level of the immune system, $1,25(OH)_2D_3$ inhibits the differentiation and maturation of dendritic cells and promotes their apoptosis [19], preventing their transformation into antigen presenting cells which is the first step in the initiation of an immune response [20]. It has been also demonstrated that $1,25(OH)_2D_3$ restores the suppressor cells, decreases Th1 cytokine production—responsible for β-cell death—and shifts the immune response toward Th2 pathway, leading to benign insulitis [21–24]. The addition of $1,25(OH)_2D_3$ inhibits the production of Il-6, a direct stimulator of Th17 cells [19], implicated in many autoimmune diseases, including T1D [20]. On the other hand, $1,25(OH)_2D_3$ exerts antiapoptotic effects on the cytokine-induced pancreatic β-cells apoptosis. It induces and maintains high levels of A20 gene protein, which leads to decreased nitric oxide (NO) levels. In fact, NO induces directly beta cell dysfunction and death, and, indirectly, through the induction of Fas expression [25]; Fas is a transmembrane cell surface receptor and a member of the tumor necrosis factor (TNF) receptor family. It is stimulated by inflammatory cytokines secreted by islet-infiltrating mononuclear cells. It renders the β-cells in T1DM susceptible to Fas-Ligand-induced apoptosis mediated by tissue-infiltrating Fas-Ligand-positive T lymphocytes [26]. Decreasing NO levels leads to down regulation of all the aforementioned mechanisms and allows cytoprotective effects on islet cells. In addition, $1,25(OH)_2D_3$ has been found to be able to counteract the cytokine-induced Fas expression in human pancreatic islets, both at the mRNA and protein levels, modulating the cascade of death signals and preventing cell apoptosis [27] (Figure 1).

3. Vitamin D Polymorphism

Vitamin D and its analogues exert their actions through the nuclear VDR which is responsible for transducing the action of the active form of vitamin D, $1,25(OH)_2D_3$ [28]. The VDR gene is located on chromosome 12q12-q14 in humans [29]. Polymorphisms within the VDR gene may be associated with altered gene expression or gene function [29], and many reports revealed their association with many physiologic and pathologic phenotypes, though inconsistently [30]. Five single nucleotide polymorphisms (SNP) in exon 2 (*Fok*I), intron 8 (*Bsm*I, *Tru9*I, *Apa*I), and exon 9 (*Taq*I) have been defined historically in VDR gene, by the associated restriction enzyme [31]. Association studies of VDR allelic variations and T1D done in many countries, including different populations (southern [32] and northern [33] India, Iran [34], Spain [35], Romania [36, 37], Turkey [38, 39], Hungary [40], Portugal [41], UK, US, Norway [42], Japan [42, 43], Finland [42, 44], Poland [45], Croatia [46, 47], Brazil [48], Uruguay [49], Germany [50–52], Greece [53], Bangladesh [54], Taiwan [55],

Table 1: VDR gene polymorphism and type 1 DM.

Population	VDR polymorphism associated with IDDM
Bangladesh	*Fok*I, *Bsm*I, *Apa*I, *Taq*I
Brazil	No association
Chile	*Bsm*I, *Apa*I, *Taq*I[1]
Croatia	*Tru*9I, *Fok*I[2]
Finland	No association
Germany	*Tak*I, *Apa*I, *Bsm*I[1], *Tru*I[3]
Greece	*Fok*I, *Bsm*I, *Apa*I, *Taq*I
Hungary	*Bsm*I, *Apa*I, *Tru*9I[4]
India Northern	*Fok*I, *Taq*I
India Southern	*Bsm*I
Italy	No association
Iran	*Taq*I
Japan	*Bsm*I, *Fok*I
Norway	No association
Polish	No association
Portugal	No association
Romania	No association
Spain	*Fok*I
Taiwan	*Bsm*I, *Apa*I
Turkey	*Fok*I
United States	No association
United Kingdom	No association

[1]Combined.
[2]Dalmatian population.
[3]In one study, in combination with *Tru*I, VDR polymorphisms were protective against DM type I.
[4]Combined, only in girls.

Chile [56], and Italy [57]) yielded conflicting results; some showed significant association while others failed to reach statistical significance, as shown in Table 1. These different results may be related to differences in ethnic background of the populations studied, interactions with other genetic or environmental factors involved in the pathogenesis of T1DM [34], and possibly differences in ultraviolet radiation exposure [58]. In fact, VDR polymorphisms, with the potential exception of the *Fok*I allele variant which has a differential effect on the immune system [59], may not have any functional effect, so, the *VDR* itself may not be the disease affecting locus but rather a marker locus in linkage disequilibrium with the real disease locus, and the discrepant findings may reflect variable strength of linkage disequilibrium in different populations [41].

The largest meta-analysis to date investigating the association between polymorphisms in VDR gene and T1DM risk found that *Bsm*I polymorphism is associated with a significantly increased risk of T1DM, whereas the *Fok*I, *Apa*I, and *Taq*I polymorphisms do not appear to have a significant association with overall T1DM risk. The *Bsm*I variant B allele (BB or Bb) carriers might have a 30% increased risk of T1DM when compared with the bb homozygote carriers [60].

Table 2: Mean 25OH D level in T1DM in different countries.

Country	Mean 25OH D level (nmol/L)
Australia	78.7
Egypt	46.75
Florida	53
Qatar	39.8
Sweden	82.5
Switzerland	45.7
USA (North Eastern)	67

4. Prevalence of Low Vitamin D Level in Type I DM

Given the association between vitamin D and T1DM and the possible role that vitamin D deficiency might play in its pathogenesis, many observational studies have assessed the 25-hydroxyvitamin D (25-OH D) level in T1DM patients (Table 2) and found a significant higher prevalence of 25-OH D deficiency in T1DM patients compared to controls. In Switzerland, in a cross-sectional study, 60–84% of T1DM were 25-OH D deficient [61]. In Qatar, in a case control study, 90.6% of T1DM children versus 85.3% of nondiabetic children had vitamin D deficiency [62]. Similarly, in North India in a case-control study, 58% of T1DM and only 32% of controls had 25-OH D deficiency [63]. In Northeastern US, in a cross-sectional study, it has been found that 15% of T1D patients were 25-OH D deficient and 61% were insufficient, findings inversely associated with age [64]. All these studies showed significantly lower mean 25-OH D level in T1DM compared to controls [62, 63, 65–68]. In addition, in the Diabetes Incidence Study in Sweden (DISS), 25-OH D level was lower in diabetics compared to controls, not only at the onset of diabetes but also at 8-year followup [69].

However, only one study, in Florida, a solar rich region in the United States, found no difference in 25-OH D levels in diabetics (recently or more than 5 months diagnosed) compared to their first degree relatives and controls [70].

A pilot study, comparing 25-OH D level in T1DM and type 2 diabetes mellitus (T2DM) showed a higher prevalence of deficiency in T2DM compared to T1DM, and more severe deficiency, independent of age, sex, BMI, and insulin treatment (mean adjusted 25-OH D level 18.1 ± 1.4 ng/mL in T2DM versus 22.9 ± 1.6 ng/mL in T1DM) [71].

5. Effect of Latitude on Vitamin D Level and T1DM Incidence

Dermal vitamin D synthesis is a major source of circulating 25-OH D and its metabolites [72]. Sun exposure, strongly related to latitude, predicts 25-OH D level. Many observational studies showed increased T1DM prevalence at northern latitudes where sun exposure is reduced [6].

In Australia, an ecologic analysis of immune-related disorders showed a positive association of T1DM prevalence

with both increasing southern latitude of residence and decreasing regional annual ambient ultraviolet radiation (UVR), with an evident threefold increase in prevalence from the northernmost region to the southernmost region [73]. Similar results were found with increasing latitude in Sweden [74] and China [75]. In Norway, a nationwide prospective study showed higher rate of T1DM in southern county and lowest in northern county [76].

The EURODIAB collaborative, a large multicenter case-control study including 7 centers, Austria, Bucharest, Bulgaria, Latvia, Lithuania, Luxembourg, North Ireland representing most European countries and Israel, in a report based on 16 362 cases registered during the period 1989–1994 by 44 centers and covering a population of about 28 million children, found a high incidence rate in northern and north western Europe and low in central, southern, and eastern Europe with the exception of Sardinia which presented higher rates than neighboring countries [77], with reverse prevalence, being higher in southern areas [78]. In a worldwide study assessing the pattern of incidence of diabetes in 51 different countries, according to latitude and solar UVR, the incidence rates were higher at higher latitudes and lower ultraviolet B irradiance, adjusted for cloud cover, as inversely associated with incidence rates [79].

Note that interpretation of international correlations is particularly difficult because there are many confounding factors such as affluence and genetic variation. Within country analysis provides probably more precise information [80].

6. Seasonal Variability in the Incidence of T1DM

Variability in sun exposure during pregnancy or early developmental stages in infancy has been also suggested as an important environmental factor influencing T1DM onset, possibly related to changes in 25-OH D levels, with highest birth dates of diabetic patients in spring–summer months with an opposite pattern of disease onset peaking in autumn and winter [80]. Consistent results were found in Ukraine (highest variability in western Europe) [81], Sweden [82], Greece [83], Ireland (significant in boys only) [84], Slovenia [85], Germany [86], The Netherlands [87], Britain [88], New Zealand [89], and Sardinia [90]. However, a multicenter cohort study in Europe found no seasonal variations [91]. Similarly, no significant differences in parameters studied in diabetics and controls were detected in Denmark [92]. In a Lebanese T1D population, El Baba et al. showed seasonal variation in glucose control but failed to establish a significant correlation between seasonal changes in 25-OH D levels and HbA1c [93, 94]. In fact, ethnicity may be a confounding factor [95]. Furthermore, none of these studies have shown data about 25-OH D levels, given that they were retrospective. Also, given that viral infections—proven to be involved in the pathogenesis of T1DM—may have also seasonal variations, the evidence of vitamin D involvement in seasonal variations of T1DM needs to be demonstrated with more accurate data.

7. Vitamin D Supplementation and Risk of Developing T1DM

Many studies have assessed the effect of vitamin D supplementation during pregnancy, infancy, or early adulthood and the risk of developing T1DM later on in life (Table 3).

The EURODIAB focused on early exposures and risk of T1DM. Vitamin D intake during infancy was assessed by questionnaire or interview (recalled). It showed that vitamin D supplements (given for the prevention of rickets) have a protective effect, even after adjustment for various confounders [96].

Hyppönen et al., in his finish birth cohort study found that vitamin D supplementation of 200 IU daily (as cod liver oil), given to children, was associated with a lower incidence of T1DM during a follow-up period of 31 years [97].

In the Norway pilot study, Stene et al. demonstrated a protective effect of vitamin D supplements, only when given as cod liver oil to pregnant women and not when given in other forms of supplementation or when given to children, suggesting a protective effect in utero [98]. However, in his larger case control study, he found a protective effect of cod liver oil when given during the first year of life only and when given ≥5 times per week. No protective effect was detected if vitamin D was given during pregnancy, conflicting results with what have been shown previously by the same group for unknown reasons [99].

Similarly, in the DAISY (Diabetes Autoimmunity Study in the Young) study in Colorado, that recruited at birth and followed children at increased risk for T1DM, as determined by HLA-DR genotype or by family history of T1DM, there was a protective effect of vitamin D taken through food only and not as supplements [100]. More recently, Tenconi et al. demonstrated a protective effect of vitamin D given during lactation [101].

The ABIS (All Babies in Southeast Sweden) study is a large, prospective, population-based cohort study in Sweden that found vitamin D supplementation, given as drops 10 mcg daily, decreased significantly the incidence of glutamic acid decarboxylase autoantibodies or IA-2A in the offspring at 1 year, but not at 2.5 years [102].

Furthermore, the Diabetes Prediction and Prevention study (DIPP), which is a population-based birth cohort of infants at genetic risk of T1DM, showed no significant protective effects of vitamin D whether given with food or as supplements [103].

A meta-analysis of the results of observational studies suggests that the risk of T1DM is 29% reduced in those who were supplemented in childhood with vitamin D compared to those who were not [104]. There was some evidence of dose-response effect—higher supplementation resulting in better protection—and the timing of supplementation predicted a favorable response when given between 7 and 12 months, critical period for immunity to become competent [105].

To note that all these studies have several limitations including recall bias, the absence of 25-OH D level, and the absence of quantitative assessment of vitamin D intake; the dose of vitamin D given was not always mentioned. Randomized controlled trials with long periods of followup

Table 3: Vitamin D supplementation and risk of T1DM development.

Author Study design (year)	Country	Population	Vitamin D supplements	Duration	Results (relative risk of T1D with vitamin D supplements)
EURODIAB (no authors listed) Case control study (1999) [96]	7 countries in Europe	746 T1DM and 2188 controls	Vitamin D supplementation during infancy	31 years	0.67 (0.53, 0.86)
Stene et al. Case control study (2000) [98]	Norway	78 T1DM and 980 controls	Cod liver oil to pregnant women	16 years	0.36 (0.14–0.9)
Hyppönen et al. Birth control, prospective study (2001) [97]	Finland	81 T1DM and 10366 controls	Cod liver oil to children during the first year of life (2000IU daily)	31 years	0.22 (0.05–0.89) for regular or irregular vitamin D intake versus no supplements 0.12 (0.03–0.51) for regular vitamin D supplements versus no supplements
Fronczak et al. Cohort study (2003) [100]	Colorado	16 T1DM and 206 controls	Vitamin D supplementation in food, during the third trimester of pregnancy (250IU daily)	4 years	0.37 (0.17–0.78)
Stene and Joner Case control study (2003) [99]	Norway	545 T1DM and 1668 controls	Cod liver oil in the first year of life, at 7–12 months of age (10 mcg daily for at least 5 times per week)	15 years	0.74 (0.56–0.99)
Tenconi et al. Case control study (2007) [101]	North Italy	159 T1DM and 318 controls	Vitamin D supplementation during lactation	29 years	0.33 (0.14–0.81)
Brekke et al. Cohort retrospective and prospective study (2007) [102]	Sweden	8.7% at 1 year and 8.9% at 2.5 years had positive antibodies	Vitamin D supplementation during pregnancy (10 mcg daily)	2.5 years	0.71 (0.17–0.78)
Marjamäki et al. Birth cohort study (2010) [103]	Finland	165 patients with positive antibodies and 4297 control	Vitamin D supplements during pregnancy (mean supplements 5.1 mcg and 1.3 mcg in food, daily)	4 years	No significant protective effect

are needed to establish causality and to suggest the best formulation, dose, duration, and period of supplementation with vitamin D that would allow appropriate protection against T1DM [104].

8. Possible Explanation of Vitamin D Deficiency in Diabetes

One of the plausible mechanisms of vitamin D deficiency in diabetics is decreased binding proteins; this has been initially demonstrated in diabetic rats [106]. Later on, in humans, it has been found that the urinary loss of vitamin D binding protein (VDBP) is secondary to diminished function or availability of megalin or low-density lipoprotein-related protein 2 (LRP2), correlated with proteinuria. In fact, Megalin is a receptor to many ligands, including albumin, vitamin-binding protein, lipoproteins, hormones, enzymes, and drugs responsible for their reabsorption in the proximal tubule. It facilitates the generation of $1,25(OH)_2D_3$ following the reabsorption of the VDBP—25OHD complex by via megalin endocytic receptor [107]. Furthermore, a study on pubertal T1DM patients showed altered vitamin D regulatory mechanisms with relative decrease in $1,25(OH)_2D_3$ plasma concentration and increased 24,25-dihydroxyvitamin D levels in diabetics compared to their healthy counterparts [68]. Note that 25-OH D level upon presentation with diabetic ketoacidosis can be falsely lowered by acidosis and improves with its resolution without any supplementation [108].

9. Vitamin D Deficiency and Risk of Diabetic Complications

Vitamin D deficiency is associated with increased inflammatory markers in diabetics including CRP, monocyte toll-like receptor (TLR) 2, TLR4, and nuclear factor-κB (NFκB) expression; this might predict increased microvascular complications. However, no statistically significant difference was found in 25-OH D levels in diabetics with microvascular complications compared to those without [109]. On the other hand, another study showed that persistent microalbuminuria is associated with lower 25-OH D levels in T1DM compared to controls [110]. Cardiovascular diseases increased with low 25-OH D levels in the general population [111] but these results have not been specifically studied in diabetics.

25-OH D deficiency has been prevalent upon the initial presentation of T1DM patients who presented with DKA, making it a contributing factor. However, given that levels improved spontaneously after correction of acidosis, the direct contribution of 25-OH D deficiency in the acute presentation of DKA remains controversial [108].

10. Vitamin D Supplementation Effect on Progression and Control of Diabetes

Given that vitamin D deficiency increases the risk of diabetes development and supplementation showed protective effects, many studies looked at the protective effect of vitamin D on diabetes progression and control. One randomized controlled study aimed to assess the effect calcitriol (given as 0.25 mcg every other day) compared to nicotinamide, within 4 weeks of diabetes diagnosis, on the preservation of β-cell function; it showed no improvement in C-peptide and HbA1c levels but significantly lower insulin doses in the calcitriol-treated group [112]. Even when the dose of calcitriol was increased to 0.25 mcg daily and after a followup of 2 years, there was no protective effect of such supplementation on C-peptide levels [113]. Conversely, in LADA patients, when calcitriol (0.5 mcg daily) was added to insulin, it showed stabilization or improvement in fasting and 2 h after 75-g glucose load C-peptide level at 1 year, especially in those whose diabetes duration was less than 1 year [114]. Similarly, in a study in Saudi Arabia, vitamin D_3 supplementation to T1DM patients who were deficient showed improvement in glucose control (with significantly lower HbA1c) when 25OH D level reached >75 nmol/L at 12 weeks [115].

11. Guidelines of Vitamin D Supplementation in Children

The American Academy of Pediatrics and the Canadian Pediatric Association recommended vitamin D supplementation of 400 IU daily, starting the first few days of life [116]. The Institute of Medicine (IOM) recommended that the adequate intake and RDA for children below 1 year of age is 400 IU/d and for all individuals of 1 year to 70 years should be 600 IU/d [117]. It seems prudent to ensure that all infants in the United States and other areas with comparable sunlight exposure receive enough vitamin D, especially in winter [118]. Whether these recommended doses are enough to allow extraskeletal benefits of vitamin D is still unknown.

Until now, no specific recommendations regarding vitamin D supplementation in patients with T1DM or at risk of developing autoimmune diabetes [119] but intakes between 5 mcg daily and the 25 mcg daily, tolerable upper intake level, may be desirable [118].

12. Conclusion

$1,25(OH)_2D_3$ immunomodulatory effects have shown significant protection against pancreatic insulitis in animal studies [13–18, 21, 22, 120, 121]. In humans, retrospective analysis and observational studies demonstrated high prevalence of 25-OH D deficiency in patients with T1DM [61–68] and suggested a contributory role in the pathogenesis of T1DM, specially with certain allelic variations of the VDR [32–57]. Conversely, vitamin D supplementation during pregnancy and early childhood decreased the risk of autoimmune diabetes [96–104] and perhaps, even after the onset of diabetes, it may improve glycemic control [114, 115]. Despite all these data, the best dose to be used and the target population in order to decrease the incidence of T1DM have not been yet defined. Abiding by the IOM and the American Academy of Pediatrics recommendations on vitamin D supplementations, at least, improves the 25OH D level.

References

[1] L. C. Harrison, M. C. Honeyman, G. Morahan et al., "Type 1 diabetes: lessons for other autoimmune diseases?" *Journal of Autoimmunity*, vol. 31, no. 3, pp. 306–310, 2008.

[2] M. E. Craig, A. Hattersley, and K. Donaghue, "Definition, epidemiology and classification of diabetes in children and adolescents," *Pediatric Diabetes*, vol. 7, no. 10, supplement 12, pp. 3–12, 2009.

[3] N. Lammi, O. Taskinen, E. Moltchanova et al., "A high incidence of type 1 diabetes and an alarming increase in the incidence of type 2 diabetes among young adults in Finland between 1992 and 1996," *Diabetologia*, vol. 50, no. 7, pp. 1393–1400, 2007.

[4] G. P. Forlenza and M. Rewers, "The epidemic of type 1 diabetes: what is it telling us?" *Current Opinion in Endocrinology, Diabetes and Obesity*, vol. 18, no. 4, pp. 248–251, 2011.

[5] L. Moroni, I. Bianchi, and A. Lleo, "Geoepidemiology, gender and autoimmune disease," *Autoimmunity Reviews*, vol. 11, pp. A386–A392, 2012.

[6] M. A. Kriegel, J. E. Manson, and K. H. Costenbader, "Does vitamin D affect risk of developing autoimmune disease?: a systematic review," *Seminars in Arthritis and Rheumatism*, vol. 40, no. 6, pp. 512–531, 2011.

[7] M. Cutolo, C. Pizzorni, and A. Sulli, "Vitamin D endocrine system involvement in autoimmune rheumatic diseases," *Autoimmunity Reviews*, vol. 11, pp. 84–87, 2011.

[8] S. Nagpal, S. Na, and R. Rathnachalam, "Noncalcemic actions of vitamin D receptor ligands," *Endocrine Reviews*, vol. 26, no. 5, pp. 662–687, 2005.

[9] E. van Etten and C. Mathieu, "Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 97, no. 1-2, pp. 93–101, 2005.

[10] S. Lee, S. A. Clark, R. K. Gill, and S. Christakos, "1,25-Dihydroxyvitamin D3 and pancreatic β-cell function: vitamin D receptors, gene expression, and insulin secretion," *Endocrinology*, vol. 134, no. 4, pp. 1602–1610, 1994.

[11] J. B. Zella, L. C. McCary, and H. F. DeLuca, "Oral administration of 1,25-dihydroxyvitamin D3 completely protects NOD mice from insulin-dependent diabetes mellitus," *Archives of Biochemistry and Biophysics*, vol. 417, no. 1, pp. 77–80, 2003.

[12] A. Giulietti, C. Gysemans, K. Stoffels et al., "Vitamin D deficiency in early life accelerates Type 1 diabetes in non-obese diabetic mice," *Diabetologia*, vol. 47, no. 3, pp. 451–462, 2004.

[13] C. Mathieu, J. Laureys, H. Sobis, M. Vandeputte, M. Waer, and R. Bouillon, "1,25-Dihydroxyvitamin D3 prevents insulitis in NOD mice," *Diabetes*, vol. 41, no. 11, pp. 1491–1495, 1992.

[14] K. M. Casteels, C. Mathieu, M. Waer et al., "Prevention of type I diabetes in NOD mice by nonhypercalcemic doses of a new structural analog of 1,25-dihydroxyvitamin D3, KH1060," *Endocrinology*, vol. 136, no. 3, pp. 866–872, 1995.

[15] C. A. Gysemans, A. K. Cardozo, H. Callewaert et al., "1,25-Dihydroxyvitamin D3 modulates expression of chemokines and cytokines in pancreatic islets: implications for prevention of diabetes in nonobese diabetic mice," *Endocrinology*, vol. 146, no. 4, pp. 1956–1964, 2005.

[16] N. Giarratana, G. Penna, S. Amuchastegui, R. Mariani, K. C. Daniel, and L. Adorini, "A vitamin D analog down-regulates proinflammatory chemokine production by pancreatic islets inhibiting T cell recruitment and type 1 diabetes development," *Journal of Immunology*, vol. 173, no. 4, pp. 2280–2287, 2004.

[17] S. Gregori, N. Giarratana, S. Smiroldo, M. Uskokovic, and L. Adorini, "A 1α,25-dihydroxyvitamin D3 analog enhances regulatory T-cells and arrests autoimmune diabetes in NOD mice," *Diabetes*, vol. 51, no. 5, pp. 1367–1374, 2002.

[18] R. Riachy, B. Vandewalle, S. Belaich et al., "Beneficial effect of 1,25 dihydroxyvitamin D3 on cytokine-treated human pancreatic islets," *Journal of Endocrinology*, vol. 169, no. 1, pp. 161–168, 2001.

[19] J. A. Emamaullee, J. Davis, S. Merani et al., "Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice," *Diabetes*, vol. 58, no. 6, pp. 1302–1311, 2009.

[20] Y. Arnson, H. Amital, and Y. Shoenfeld, "Vitamin D and autoimmunity: new aetiological and therapeutic considerations," *Annals of the Rheumatic Diseases*, vol. 66, no. 9, pp. 1137–1142, 2007.

[21] C. Mathieu, M. Waer, J. Laureys, O. Rutgeerts, and R. Bouillon, "Prevention of autoimmune diabetes in NOD mice by 1,25 dihydroxyvitamin D3," *Diabetologia*, vol. 37, no. 6, pp. 552–558, 1994.

[22] C. Mathieu, M. Waer, K. Casteels, J. Laureys, and R. Bouillon, "Prevention of type I diabetes in NOD mice by non-hypercalcemic doses of a new structural analog of 1,25-dihydroxyvitamin D3, KH1060," *Endocrinology*, vol. 136, no. 3, pp. 866–872, 1995.

[23] L. Overbergh, B. Decallonne, M. Waer et al., "1α,25-dihydroxyvitamin D3 induces an autoantigen-specific T-helper 1/T-helper 2 immune shift in NOD mice immunized with GAD65 (p524–543)," *Diabetes*, vol. 49, no. 8, pp. 1301–1307, 2000.

[24] A. L. Khoo, I. Joosten, and M. Michels, "1, 25-Dihydroxyvitamin D3 inhibits proliferation but not the suppressive function of regulatory T cells in the absence of antigen-presenting cells," *Immunology*, vol. 134, no. 4, pp. 459–468, 2011.

[25] R. Riachy, B. Vandewalle, J. K. Conte et al., "1,25-dihydroxyvitamin D3 protects RINm5F and human islet cells against cytokine-induced apoptosis: implication of the antiapoptotic protein A20," *Endocrinology*, vol. 143, no. 12, pp. 4809–4819, 2002.

[26] A. Y. Savinov, A. Tcherepanov, and E. A. Green, "Contribution of Fas to diabetes development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, pp. 628–632, 2003.

[27] R. Riachy, B. Vandewalle, E. Moerman et al., "1,25-dihydroxyvitamin D3 protects human pancreatic islets against cytokine-induced apoptosis via down-regulation of the Fas receptor," *Apoptosis*, vol. 11, no. 2, pp. 151–159, 2006.

[28] S. Nagpal, S. Na, and R. Rathnachalam, "Noncalcemic actions of vitamin D receptor ligands," *Endocrine Reviews*, vol. 26, no. 5, pp. 662–687, 2005.

[29] A. G. Uitterlinden, Y. Fang, J. B. J. Van Meurs, H. A. P. Pols, and J. P. T. M. Van Leeuwen, "Genetics and biology of vitamin D receptor polymorphisms," *Gene*, vol. 338, no. 2, pp. 143–156, 2004.

[30] A. F. Reis, O. M. Hauache, and G. Velho, "Vitamin D endocrine system and the genetic susceptibility to diabetes, obesity and vascular disease. A review of evidence," *Diabetes and Metabolism*, vol. 31, no. 4, part 1, pp. 318–325, 2005.

[31] E. Ramos-Lopez, T. Jansen, V. Ivaskevicius et al., "Protection from type 1 diabetes by vitamin D receptor haplotypes," *Annals of the New York Academy of Sciences*, vol. 1079, pp. 327–334, 2006.

[32] M. F. McDermott, A. Ramachandran, B. W. Ogunkolade et al., "Allelic variation in the vitamin D receptor influences susceptibility to IDDM in Indian Asians," *Diabetologia*, vol. 40, no. 8, pp. 971–975, 1997.

[33] N. Israni, R. Goswami, A. Kumar, and R. Rani, "Interaction of Vitamin D receptor with HLA DRB1∗0301 in Type 1 diabetes patients from North India," *PLoS ONE*, vol. 4, no. 12, Article ID e8023, 2009.

[34] Z. Mohammadnejad, M. Ghanbari, R. Ganjali et al., "Association between vitamin D receptor gene polymorphisms and type 1 diabetes mellitus in Iranian population," *Molecular Biology Reports*, vol. 39, no. 2, pp. 831–837, 2012.

[35] G. Martí, L. Audí, C. Esteban et al., "Association of vitamin D receptor gene polymorphism with type 1 diabetes mellitus in two Spanish populations," *Medicina Clinica*, vol. 123, no. 8, pp. 286–290, 2004.

[36] S. Nejentsev, J. D. Cooper, L. Godfrey et al., "Analysis of the vitamin D receptor gene sequence variants in type 1 diabetes," *Diabetes*, vol. 53, no. 10, pp. 2709–2712, 2004.

[37] C. Guja, S. Marshall, K. Welsh et al., "The study of CTLA-4 and vitamin D receptor polymorphisms in the Romanian type 1 diabetes population," *Journal of Cellular and Molecular Medicine*, vol. 6, no. 1, pp. 75–81, 2002.

[38] S. B. Sahin, S. Cetinkalp, and M. Erdogan, "Fas, Fas ligand, and vitamin D receptor fokI gene polymorphisms in patients with Type 1 diabetes mellitus in the aegean region of Turkey," *Genet Test Mol Biomarkers*, vol. 16, no. 10, pp. 1179–1183, 2012.

[39] D. Gogas Yavuz, L. Keskin, S. Kıyıcı et al., "Vitamin D receptor gene BsmI, FokI, ApaI, TaqI polymorphisms and bone mineral density in a group of Turkish type 1 diabetic patients," *Acta Diabetologica*, vol. 48, no. 4, pp. 329–336, 2011.

[40] B. Györffy, B. Vásárhelyi, D. Krikovszky et al., "Gender-specific association of vitamin D receptor polymorphism combinations with type 1 diabetes mellitus," *European Journal of Endocrinology*, vol. 147, no. 6, pp. 803–808, 2002.

[41] M. Lemosa, A. Fagulhab, and E. Coutinhoc, "Lack of association of vitamin D receptor gene polymorphisms with susceptibility to type 1 diabetes mellitus in the Portuguese population," *Human Immunology*, vol. 69, pp. 134–138, 2008.

[42] A. Shimada, Y. Kanazawa, Y. Motohashi et al., "Evidence for association between vitamin D receptor BsmI polymorphism and type 1 diabetes in Japanese," *Journal of Autoimmunity*, vol. 30, no. 4, pp. 207–211, 2008.

[43] Y. Ban, M. Taniyama, T. Yanagawa et al., "Vitamin D receptor initiation codon polymorphism influences genetic susceptibility to type 1 diabetes mellitus in the Japanese population," *BMC Medical Genetics*, vol. 2, article 7, 2001.

[44] H. Turpeinen, R. Hermann, S. Vaara et al., "Vitamin D receptor polymorphisms: no association with type 1 diabetes in the Finnish population," *European Journal of Endocrinology*, vol. 149, no. 6, pp. 591–596, 2003.

[45] M. Fichna, M. Zurawek, D. Januszkiewicz-Lewandowska, P. Fichna, and J. Nowak, "PTPN22, PDCD1 and CYP27B1 polymorphisms and susceptibility to type 1 diabetes in Polish patients," *International Journal of Immunogenetics*, vol. 37, no. 5, pp. 367–372, 2010.

[46] V. Boraska, V. Škrabić, E. Zeggini et al., "Family-based analysis of vitamin D receptor gene polymorphisms and type 1 diabetes in the population of South Croatia," *Journal of Human Genetics*, vol. 53, no. 3, pp. 210–214, 2008.

[47] T. Zemunik, V. Škrabić, V. Boraska et al., "Fokl polymorphism, vitamin D receptor, and interleukin-1 receptor haplotypes are associated with type 1 diabetes in the Dalmatian population," *Journal of Molecular Diagnostics*, vol. 7, no. 5, pp. 600–604, 2005.

[48] D. B. Mory, E. R. Rocco, W. L. Miranda, T. Kasamatsu, F. Crispim, and S. A. Dib, "Prevalence of vitamin D receptor gene polymorphisms FokI and BsmI in Brazilian individuals with type 1 diabetes and their relation to β-cell autoimmunity and to remaining β-cell function," *Human Immunology*, vol. 70, no. 6, pp. 447–451, 2009.

[49] A. Mimbacas, J. Trujillo, C. Gascue, G. Javiel, and H. Cardoso, "Prevalence of vitamin D receptor gene polymorphism in a Uruguayan population and its relation to type 1 diabetes mellitus," *Genetics and Molecular Research*, vol. 6, no. 3, pp. 534–542, 2007.

[50] W. J. Fassbender, B. Goertz, K. Weismüller et al., "VDR gene polymorphisms are overrepresented in German patients with type 1 diabetes compared to healthy controls without effect on biochemical parameters of bone metabolism," *Hormone and Metabolic Research*, vol. 34, no. 6, pp. 330–337, 2002.

[51] M. A. Pani, M. Knapp, H. Donner et al., "Vitamin D receptor allele combinations influence genetic susceptibility to 1 diabetes in Germans," *Diabetes*, vol. 49, no. 3, pp. 504–507, 2000.

[52] E. Ramos-Lopez, T. Jansen, V. Ivaskevicius et al., "Protection from type 1 diabetes by vitamin D receptor haplotypes," *Annals of the New York Academy of Sciences*, vol. 1079, pp. 327–334, 2006.

[53] C. Panierakis, G. Goulielmos, D. Mamoulakis, E. Petraki, E. Papavasiliou, and E. Galanakis, "Vitamin D receptor gene polymorphisms and susceptibility to type 1 diabetes in Crete, Greece," *Clinical Immunology*, vol. 133, no. 2, pp. 276–281, 2009.

[54] B. W. Ogunkolade, B. J. Boucher, J. M. Prahl et al., "Vitamin D receptor (VDR) mRNA and VDR protein levels in relation to vitamin D status, insulin secretory capacity, and VDR genotype in Bangladeshi Asians," *Diabetes*, vol. 51, no. 7, pp. 2294–2300, 2002.

[55] T. J. Chang, H. H. Lei, J. I. Yeh et al., "Vitamin D receptor gene polymorphisms influence susceptibility to type 1 diabetes mellitus in the Taiwanese population," *Clinical Endocrinology*, vol. 52, no. 5, pp. 575–580, 2000.

[56] D. García, B. Angel, E. Carrasco, C. Albala, J. L. Santos, and F. Pérez-Bravo, "VDR polymorphisms influence the immune response in type 1 diabetic children from Santiago, Chile," *Diabetes Research and Clinical Practice*, vol. 77, no. 1, pp. 134–140, 2007.

[57] M. G. Bianco, L. Minicucci, M. G. Calevo, and R. Lorini, "Vitamin D receptor polymorphisms: are they really associated with type 1 diabetes?" *European Journal of Endocrinology*, vol. 151, no. 5, pp. 641–642, 2004.

[58] A. L. Ponsonby, A. Pezic, J. Ellis et al., "Variation in associations between allelic variants of the vitamin D receptor gene and onset of type 1 diabetes mellitus by ambient winter ultraviolet radiation levels: a meta-regression analysis," *American Journal of Epidemiology*, vol. 168, no. 4, pp. 358–365, 2008.

[59] E. Van Etten, L. Verlinden, A. Giulietti et al., "The vitamin D receptor gene FokI polymorphism: functional impact on the immune system," *European Journal of Immunology*, vol. 37, no. 2, pp. 395–405, 2007.

[60] J. Zhang, W. Li, and J. Liu, "Polymorphisms in the vitamin D receptor gene and type 1 diabetes mellitus risk: an update by meta-analysis," *Molecular and Cellular Endocrinology*, vol. 355, pp. 135–142, 2012.

[61] M. Janner, P. Ballinari, P. E. Mullis, and C. E. Flück, "High prevalence of vitamin D deficiency in children and adolescents

with type 1 diabetes," *Swiss Medical Weekly*, vol. 140, p. w13091, 2010.

[62] A. Bener, A. Alsaied, M. Al-Ali et al., "High prevalence of vitamin D deficiency in type 1 diabetes mellitus and healthy children," *Acta Diabetologica*, vol. 46, no. 3, pp. 183–189, 2009.

[63] V. V. Borkar, V. S. Devidayal, and A. K. Bhalla, "Low levels of vitamin D in North Indian children with newly diagnosed type 1 diabetes," *Pediatric Diabetes*, vol. 11, no. 5, pp. 345–350, 2010.

[64] B. M. Svoren, L. K. Volkening, J. R. Wood, and L. M. B. Laffel, "Significant vitamin D deficiency in youth with type 1 diabetes mellitus," *Journal of Pediatrics*, vol. 154, no. 1, pp. 132–134, 2009.

[65] P. Pozzilli, S. Manfrini, A. Crinò et al., "Low levels of 25-hydroxyvitamin D3 and 1, 25-dihydroxyvitamin D3 in patients with newly diagnosed type 1 diabetes," *Hormone and Metabolic Research*, vol. 37, no. 11, pp. 680–683, 2005.

[66] R. M. Greer, S. L. Portelli, and B. S. Hung, "Serum vitamin D levels are lower in Australian children and adolescents with type 1 diabetes than in children without diabetes," *Pediatric Diabetes*, vol. 14, no. 1, pp. 31–41, 2012.

[67] E. A. Hamed, N. H. Faddan, and H. A. Elhafeez, "Parathormone—25(OH)-vitamin D axis and bone status in children and adolescents with type 1 diabetes mellitus," *Pediatr Diabete*, vol. 12, no. 6, pp. 536–546, 2011.

[68] O. Rodland, T. Markestad, L. Aksnes, and D. Aarskog, "Plasma concentration of vitamin D metabolites during puberty of diabetic children," *Diabetologia*, vol. 28, no. 9, pp. 663–666, 1985.

[69] B. Littorin, P. Blom, A. Schölin et al., "Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS)," *Diabetologia*, vol. 49, no. 12, pp. 2847–2852, 2006.

[70] L. Bierschenk, J. Alexander, C. Wasserfall, M. Haller, D. Schatz, and M. Atkinson, "Vitamin D levels in subjects with and without type 1 diabetes residing in a solar rich environment," *Diabetes Care*, vol. 32, no. 11, pp. 1977–1979, 2009.

[71] D. J. Di Cesar, R. Ploutz-Snyder, R. S. Weinstock, and A. M. Moses, "Vitamin D deficiency is more common in type 2 than in type 1 diabetes [6]," *Diabetes Care*, vol. 29, no. 1, p. 174, 2006.

[72] H. F. DeLuca, "Overview of general physiologic features and functions of vitamin D," *The American Journal of Clinical Nutrition*, vol. 80, no. 6, supplement, pp. 1689S–1696S, 2004.

[73] J. A. Staples, A. L. Ponsonby, L. L. Y. Lim, and A. J. McMichael, "Ecologic analysis of some immune-related disorders, including type 1 diabetes, in Australia: iatitude, regional ultraviolet radiation, and disease prevalence," *Environmental Health Perspectives*, vol. 111, no. 4, pp. 518–523, 2003.

[74] L. Nystrom, G. Dahlquist, J. Ostman et al., "Risk of developing insulin-dependent diabetes mellitus (IDDM) before 35 years of age: indications of climatological determinants for age at onset," *International Journal of Epidemiology*, vol. 21, no. 2, pp. 352–358, 1992.

[75] Z. Yang, K. Wang, L. I. Tianlin et al., "Childhood diabetes in China: enormous variation by place and ethnic group," *Diabetes Care*, vol. 21, no. 4, pp. 525–529, 1998.

[76] G. Joner, L. C. Stene, and O. Søvik, "Nationwide, prospective registration of type 1 diabetes in children aged <15 years in Norway 1989–1998: no increase but significant regional variation in incidence," *Diabetes Care*, vol. 27, no. 7, pp. 1618–1622, 2004.

[77] "Variation and trends in incidence of childhood diabetes in Europe," *The Lancet*, vol. 355, no. 9207, pp. 873–876, 2000.

[78] A. Casu, C. Pascutto, L. Bernardinelli, and M. Songini, "Bayesian approach to study the temporal trend and the geographical variation in the risk of type 1 diabetes: the Sardinian Conscript type 1 diabetes registry," *Pediatric Diabetes*, vol. 5, no. 1, pp. 32–38, 2004.

[79] S. B. Mohr, C. F. Garland, E. D. Gorham, and F. C. Garland, "The association between ultraviolet B irradiance, vitamin D status and incidence rates of type 1 diabetes in 51 regions worldwide," *Diabetologia*, vol. 51, no. 8, pp. 1391–1398, 2008.

[80] T. R. Merriman, "Type 1 diabetes, the A1 milk hypothesis and vitamin D deficiency," *Diabetes Research and Clinical Practice*, vol. 83, no. 2, pp. 149–156, 2009.

[81] A. M. Vaiserman, B. Carstensen, V. P. Voitenko et al., "Seasonality of birth in children and young adults (0-29 years) with type 1 diabetes in Ukraine," *Diabetologia*, vol. 50, no. 1, pp. 32–35, 2007.

[82] U. Samuelsson, C. Johansson, and J. Ludvigsson, "Month of birth and risk of developing insulin dependent diabetes in south east Sweden," *Archives of Disease in Childhood*, vol. 81, no. 2, pp. 143–146, 1999.

[83] M. I. Kalliora, A. Vazeou, D. Delis, E. Bozas, I. Thymelli, and C. S. Bartsocas, "Seasonal variation of type 1 diabetes mellitus diagnosis in Greek children," *Hormones*, vol. 10, no. 1, pp. 67–71, 2011.

[84] E. F. Roche, H. Lewy, H. M. C. V. Hoey, and Z. Laron, "Differences between males and females in the seasonality of birth and month of clinical onset of disease in chilren with type 1 diabetes mellitus in Ireland," *Journal of Pediatric Endocrinology and Metabolism*, vol. 16, no. 5, pp. 779–782, 2003.

[85] N. Ursic-Bratina, T. Battelino, C. Kržišnik, T. Laron-Kenet, I. Ashkenazi, and Z. Laron, "Seasonality of birth in children (0–14 years) with type 1 diabetes mellitus in Slovenia," *Journal of Pediatric Endocrinology and Metabolism*, vol. 14, no. 1, pp. 47–52, 2001.

[86] O. Kordonouri, N. Shuga, H. Lewy, I. Ashkenazi, and Z. Laron, "Seasonality of month of birth of children and adolescents with type 1 diabetes mellitus in Berlin differs from the general population," *European Journal of Pediatrics*, vol. 161, no. 5, pp. 291–292, 2002.

[87] P. H. Jongbloet, H. M. Groenewoud, R. A. Hirasing, and S. Van Buuren, "Seasonality of birth in patients with childhood diabetes in The Netherlands," *Diabetes Care*, vol. 21, no. 1, pp. 190–191, 1998.

[88] P. M. Rothwell, A. Staines, P. Smail, E. Wadsworth, and P. McKinney, "Seasonality of birth of patients with childhood diabetes in Britain," *British Medical Journal*, vol. 312, no. 7044, pp. 1456–1457, 1996.

[89] J. A. Willis, R. S. Scott, B. A. Darlow, H. Lewy, I. Ashkenazi, and Z. Laron, "Seasonality of birth and onset of clinical disease in children and adolescents (0–19 years) with type 1 diabetes mellitus in Canterbury, New Zealand," *Journal of Pediatric Endocrinology and Metabolism*, vol. 15, no. 5, pp. 645–647, 2002.

[90] M. Songini, A. Casu, I. Ashkenazi, and Z. Laron, "Seasonality of birth in children (0–14 years) and young adults (0–29 years) with type 1 diabetes mellitus in Sardinia differs from that in the general population," *Journal of Pediatric Endocrinology and Metabolism*, vol. 14, no. 6, pp. 781–783, 2001.

[91] P. M. Rothwell, S. A. Gutnikov, P. A. McKinney, E. Schober, C. Ionescu-Tirgoviste, and A. Neu, "Seasonality of birth in children with diabetes in Europe: multicentre cohort study," *British Medical Journal*, vol. 319, no. 7214, pp. 887–888, 1999.

[92] T. Bock, C. R. Pedersen, A. Volund, C. S. Pallesen, and K. Buschard, "Perinatal determinants among children who later develop IDDM," *Diabetes Care*, vol. 17, no. 10, pp. 1154–1157, 1994.

[93] K. El Baba, M. S. Zantout, A. Arabi, and S. T. Azar, "Seasonal variations of glucose control in Lebanese patients with type 1 diabetes," *Biological Rhythm Research*, vol. 41, no. 2, pp. 91–97, 2010.

[94] K. El Baba, M. Zantout, R. Akel, and S. Azar, "Seasonal variation in vitamin D and HbA(1c) levels in patients with type 1 diabetes mellitus in the Middle East," *International Journal of General Medicine*, vol. 4, pp. 635–638, 2011.

[95] Z. Laron, H. Lewy, I. Wilderman et al., "Seasonality of month of birth of children and adolescents with type 1 diabetes mellitus in homogenous and heterogeneous populations," *Israel Medical Association Journal*, vol. 7, no. 6, pp. 381–384, 2005.

[96] "Vitamin D supplement in early childhood and risk for Type I (insulin- dependent) diabetes mellitus," *Diabetologia*, vol. 42, no. 1, pp. 51–54, 1999.

[97] E. Hyppönen, E. Läärä, A. Reunanen, M. R. Järvelin, and S. M. Virtanen, "Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study," *The Lancet*, vol. 358, no. 9292, pp. 1500–1503, 2001.

[98] L. C. Stene, J. Ulriksen, P. Magnus, and G. Joner, "Use of cod liver oil during pregnancy associated with lower risk of Type I diabetes in the offspring," *Diabetologia*, vol. 43, no. 9, pp. 1093–1098, 2000.

[99] L. C. Stene and G. Joner, "Use of cod liver oil during the first year of life is associated with lower risk of childhood-onset type 1 diabetes: a large, population-based, case-control study," *American Journal of Clinical Nutrition*, vol. 78, no. 6, pp. 1128–1134, 2003.

[100] C. M. Fronczak, A. E. Barón, H. P. Chase et al., "In utero dietary exposures and risk of islet autoimmunity in children," *Diabetes Care*, vol. 26, no. 12, pp. 3237–3242, 2003.

[101] M. T. Tenconi, G. Devoti, M. Comelli et al., "Major childhood infectious diseases and other determinants associated with type 1 diabetes: a case-control study," *Acta Diabetologica*, vol. 44, no. 1, pp. 14–19, 2007.

[102] H. K. Brekke and J. Ludvigsson, "Vitamin D supplementation and diabetes-related autoimmunity in the ABIS study," *Pediatric Diabetes*, vol. 8, no. 1, pp. 11–14, 2007.

[103] L. Marjamäki, S. Niinistö, M. G. Kenward et al., "Maternal intake of vitamin D during pregnancy and risk of advanced beta cell autoimmunity and type 1 diabetes in offspring," *Diabetologia*, vol. 53, no. 8, pp. 1599–1607, 2010.

[104] C. S. Zipitis and A. K. Akobeng, "Vitamin D supplementation in early childhood and risk of type 1 diabetes: a systematic review and meta-analysis," *Archives of Disease in Childhood*, vol. 93, no. 6, pp. 512–517, 2008.

[105] A. Antico, M. Tampoia, R. Tozzoli, and N. Bizzaro, "Can supplementation with Vitamin D reduce the risk or modify the course of autoimmune diseases? A systematic review of the literature," *Autoimmunity Reviews*, vol. 12, pp. 127–136, 2012.

[106] B. L. Nyomba, J. Verhaeghe, M. Thomasset, W. Lissens, and R. Bouillon, "Bone mineral homeostasis in spontaneously diabetic BB rats. I. Abnormal vitamin D metabolism and impaired active intestinal calcium absorption," *Endocrinology*, vol. 124, no. 2, pp. 565–572, 1989.

[107] K. M. Thrailkill, C. H. Jo, and G. E. Cockrell, "Enhanced excretion of vitamin D binding protein in type 1 diabetes: a role in vitamin D deficiency?" *The Journal of Clinical Endocrinology & Metabolism*, vol. 96, no. 1, pp. 142–149, 2011.

[108] T. Huynh, R. M. Greer, O. Nyunt et al., "The association between ketoacidosis and 25(OH)-vitamin D3 levels at presentation in children with type 1 diabetes mellitus," *Pediatric Diabetes*, vol. 10, no. 1, pp. 38–43, 2009.

[109] S. Devaraj, J. M. Yun, C. R. Duncan-Staley, and I. Jialal, "Low vitamin d levels correlate with the proinflammatory state in type 1 diabetic subjects with and without microvascular complications," *American Journal of Clinical Pathology*, vol. 135, no. 3, pp. 429–433, 2011.

[110] A. Verrotti, F. Basciani, F. Carle, G. Morgese, and F. Chiarelli, "Calcium metabolism in adolescents and young adults with type 1 diabetes mellitus without and with persistent microalbuminuria," *Journal of Endocrinological Investigation*, vol. 22, no. 3, pp. 198–202, 1999.

[111] S. Judd, V. Tangpricha, and D. Vitamin, "Deficiency and risk for cardiovascular disease," *Circulation*, vol. 117, no. 4, pp. 503–511, 2008.

[112] D. Pitocco, A. Crinò, E. Di Stasio et al., "The effects of calcitriol and nicotinamide on residual pancreatic β-cell function in patients with recent-onset Type 1 diabetes (IMDIAB XI)," *Diabetic Medicine*, vol. 23, no. 8, pp. 920–923, 2006.

[113] C. Bizzarri, D. Pitocco, N. Napoli et al., "No protective effect of calcitriol on β-cell function in recent-onset type 1 diabetes: the IMDIAB XIII trial," *Diabetes Care*, vol. 33, no. 9, pp. 1962–1963, 2010.

[114] X. Li, L. Liao, X. Yan et al., "Protective effects of 1-α-hydroxyvitamin D3 on residual β-cell function in patients with adult-onset latent autoimmune diabetes (LADA)," *Diabetes/Metabolism Research and Reviews*, vol. 25, no. 5, pp. 411–416, 2009.

[115] K. S. Aljabri, S. A. Bokhari, and M. J. Khan, "Glycemic changes after vitamin D supplementation in patients with type 1 diabetes mellitus and vitamin D deficiency," *Annals of Saudi Medicine*, vol. 30, no. 6, pp. 454–508, 2010.

[116] C. L. Wagner and F. R. Greer, "Prevention of rickets and vitamin D deficiency in infants, children, and adolescents," *Pediatrics*, vol. 122, no. 5, pp. 1142–1152, 2008.

[117] A. C. Ross, J. E. Manson, S. A. Abrams et al., "The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know," *Journal of Clinical Endocrinology and Metabolism*, vol. 96, no. 1, pp. 53–58, 2011.

[118] S. Harris, "Can vitamin D supplementation in infancy prevent type 1 diabetes?" *Nutrition Reviews*, vol. 60, no. 4, pp. 118–121, 2002.

[119] M. Holick, N. Binkley, H. Bischoff-Ferrari et al., "Endocrine Society. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline," *The Journal of Clinical Endocrinology & Metabolism*, vol. 96, no. 7, pp. 1911–1930, 2011.

[120] G. Penna and L. Adorini, "1α,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation," *Journal of Immunology*, vol. 164, no. 5, pp. 2405–2411, 2000.

[121] L. Piemonti, P. Monti, M. Sironi et al., "Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells," *Journal of Immunology*, vol. 164, no. 9, pp. 4443–4451, 2000.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 570413, 6 pages
http://dx.doi.org/10.1155/2013/570413

Clinical Study

Effects of Low-Dose Testosterone Undecanoate Treatment on Bone Mineral Density and Bone Turnover Markers in Elderly Male Osteoporosis with Low Serum Testosterone

Yan-Jiao Wang, Jun-Kun Zhan, Wu Huang, Yi Wang, Yuan Liu, Sha Wang, Pan Tan, Zhi-Yong Tang, and You-Shuo Liu

Geriatric Department of the Second Xiang-Ya Hospital, Institute of Aging and Geriatric Research, Central South University, No. 139 Middle Renmin Road, Changsha, Hunan 410011, China

Correspondence should be addressed to You-Shuo Liu; liuyoushuo@yeah.net

Received 24 December 2012; Revised 23 January 2013; Accepted 30 January 2013

Academic Editor: Guang-Da Xiang

This prospective 2-year, single-center, randomized, placebo-controlled, open-label clinical trial was performed to evaluate the efficacy of low-dose testosterone undecanoate (TU) treatment on bone mineral density (BMD) and biochemical markers of bone turnover in elderly male osteoporosis with low serum testosterone. A total of 186 elderly male osteoporosis patients with low serum testosterone were randomized into three groups: low-dose TU (20 mg, per day), standard-dose TU (40 mg, per day), and placebo group with a 24-month followup. Since the 6th month in standard-dose TU group or since the 12th month followup in low-dose TU group and throughout the study, lumbar spine and femoral neck BMD and serum levels of free testosterone, estradiol, and bone alkaline phosphatase significantly increased. There were no significant differences between groups of low-dose TU and standard dose TU in the percentage of changes of these data since the 18th month followup and throughout the study. No side effects on prostate glands including prostate specific antigen were found. In conclusion, low-dose TU (20 mg, per day) may be a cost effective and safe protocol for treating elderly male osteoporosis with low serum testosterone.

1. Introduction

With the aging of the population, osteoporosis in men is becoming an increasingly important public health problem. Aging men lose bone mineral density (BMD) at a rate of approximately 1% per year [1]. Johnell and Kanis [2] recently updated the worldwide prevalence of osteoporotic fractures using data from published sources. One in five men over the age of 50 will suffer an osteoporotic fracture during their lifetime. Of the annual incidence of 9 million fractures, 39% were in men. Thirty percent of hip fractures occurred in men. Hip fractures are the most main reason leading to death or disability in all kinds of fractures.

As is the case in women, the mainstays of therapy for osteoporosis in men are the bisphosphonates. For patients at high risk of fracture, use of teriparatide in men has also been approved by the FDA [3]. However, the levels of serum testosterone are lower in fifty percent of hip fractures occurred in elderly men. Endogenous testosterone and their metabolites play a role in maintaining bone health [4]. Regarding the issue of testosterone and/or the adrenal androgen dehydroepiandrosterone (DHEA), treatment of elderly men for preservation of bone and muscle mass remains an active area of debate and discussion. Based on the current state of uncertainty regarding testosterone treatment of aging men, the Institute of Medicine has recommended that a series of clinical trials be done to help determine the efficacy of testosterone for several important outcomes, including bone [5]. Oral testosterone undecanoate (TU) is the only oral form of testosterone replacement therapy and is available in many countries. Due to the most common adverse drug reaction (ADR) of testosterone supplementation for aging males is increase in serum prostate specific antigen (PSA), with a potential threat for developing prostate grand tumour cancer [6, 7], it is essential to find out a effective and safe testosterone supplementation protocol. However, there is so

little study reports on evaluating the effect and safety of low-dose testosterone treatment for elderly male osteoporosis up till now. So we perform this study to evaluate the effects of low-dose TU treatment on bone mineral density (BMD) and biochemical markers of bone turnover in elderly male osteoporosis with low serum testosterone and to observe the side effects of low-dose TU on PSA and prostate grand.

2. Material and Methods

2.1. Inclusion and Exclusion Criteria of Participants. With reference to the World Health Organization (WHO) definition [8], subjects with a BMD of 2.5 SD lower than the peak mean of the same gender ($T \leqslant -2.5$) were determined as osteoporotic. Men (aged >60 years) were screened if they had a T-score less than or equal to −2.5 (if no prevalent vertebral fracture) or less than or equal to −2.0 (if one prevalent vertebral fracture) at the femoral neck, total hip, trochanter, or lumbar spine and more than −4.0 at all sites. We enrolled 186 elderly male osteoporosis (average age 68.2 ± 5.2 years) with low serum testosterone (serum T < 300 ng/dL).

The exclusion criteria were clinical or laboratory evidence of systemic disease; presence or history of vertebral, hip, or wrist fractures; other metabolic bone diseases; prostate grand tumour; cancer; poorly controlled diabetes with HbA1c ⩾ 10%; uncontrolled hypertension with blood pressure ⩾ 180/100 mmHg; uncontrolled hypothyroidism; uncontrolled hyperthyroidism; hyperparathyroidism; abnormal liver function with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values >2-fold upper limits, or renal disease with serum creatinine >2 mg/dL; the use of HT, selective estrogen (or androgen) receptor modulators, or the use of calcitonin, chronic systemic corticosteroid, or any other treatment affecting BMD within the previous 6 months; or any use of bisphosphonate within the previous 12 months.

2.2. Study Design. This study was designed as a 2-year, single-center, randomized, placebo-controlled, open-label clinical trial. Patients were recruited from Changsha city and its surrounding area in Hunan province of China. All the participants were randomly divided into three group: standard-dose TU group (group A), low-dose TU group (group B), and placebo group (group C). The study protocol was approved by the second Xiangya hospital of central south university ethics committees in accordance with the Declaration of Helsinki and Good Clinical Practices Guidelines.

2.3. Treatment Assignments. Each subject in the standard-dose TU group (group A, $n = 62$) took Andriol Testocaps when having breakfast (Testosterone Undecanoate, Merck Sharp and Dohmo Ltd., China) 40 mg per day. Each subject in the low-dose TU group (group B, $n = 62$) took Andriol Testocaps 20 mg per day (each 40 mg capsule was divided into two average capsules). The remaining subjects (group C, $n = 62$) took one placebo capsules every day. All patients were also supplemented with calcium (600 mg) and vitamin D3 (125 IU) daily. Follow-up period lasted 24 months. Participants were requested to maintain their habitual diet and exercise patterns.

2.4. Assessment Methods

2.4.1. Areal Bone Mineral Density Assessment. The parameters including the projected areal bone mineral density (a BMD, g/cm^2) were measured by DXA using QDR 4500A fan beam bone densitometer (Hologic Inc., Bedford, MA, USA), according to the manufacturer's recommended standard analysis procedures for the PA lumbar spine (vertebrae L2–L4) and hip femoral neck. A long-term (exceeding 15 years) coefficient of variation (CV) for the BMC and BMD was not greater than 0.40%.

2.4.2. Vertebral Fractures Assessment. Lumbar spine X-rays for vertebral fractures were given to participants at baseline and 6, 12, 18, 24 months after randomization. Genant's vertebral fractures assessment method [9] was used.

2.4.3. Laboratory Tests. After an overnight fast, venous blood was sampled to determine plasma glucose (FPG), glycosylated hemoglobin A1c(HbA1c), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-CH), low-density lipoprotein cholesterol (LDL-CH), triglyceride (TG), creatinine (Cr), ALT, and AST at baseline and at 6, 12, 18, and 24 months (Automatic Analyzer 7600-020, Hitachi.) Serum bone-specific alkaline phosphatase (BAP, Beckman Access Ostase, Fullerton, CA, USA; interassay coefficient of variation CV = 9% and intra-assay CV = 4%) and urine collected for routine urinalysis and N-telopeptide of type 1 collagen (NTx, Vitros Immunodiagnostic Products, Ortho-Clinical Diagnostics, Buckinghamshire, UK; interassay CV = 10% and intra-assay CV = 5%) were examined at baseline and at 6, 12, 18, and 24 months. Serum concentrations of total testosterone (TT), free testosterone (fT), and estradiol (E2) were analyzed at baseline and at 6, 12, 18, and 24 months by chemical luminescence method.

2.4.4. Safety Assessment and Adverse Events. In addition to the aforementioned laboratory tests, the safety of the participants was further monitored by B-ultrasonography for prostate grand, by chemical luminescence method for serum prostate specific antigen (PSA). Adverse events were classified according to body system. Participants were asked about their symptoms at the clinics every 3 months.

2.5. Statistical Analysis. Descriptive data are given as the mean ± standard deviation (SD) for continuous variables. For continuous variables, differences in mean percentage changes from baseline between the two groups were evaluated by Student's t-test. A P value of 0.05 or less was considered statistically significant.

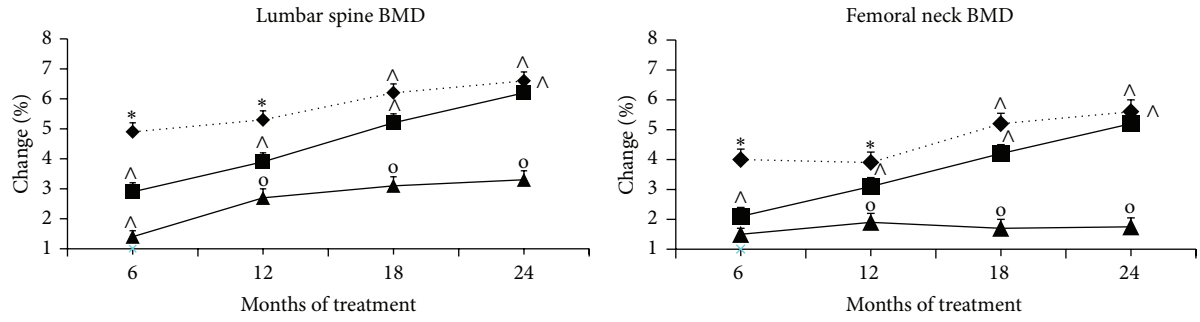
3. Results

No significant differences in age, body mass index (BMI), FPG, HbA1c, TC, TG, HDL-CH, LDL-CH, PSA, fPSA, lumbar spine and femoral neck BMD, TT, fT, E2, BAP, uNTX/Cr, and volume of prostate grand by B-ultrasonography were found at baseline between the three groups (Table 1).

Table 1: Characteristics of the patients at the entry (means ± SD).

Parameter	A	B	C
Regimen of treatment	TU 40 mg/d	TU 20 mg/d	Placebo 1 tablet/d
Patients (no)	62	62	62
Age (yr)	68.1 ± 5.4	68.4 ± 5.5	68.0 ± 4.8
Height (cm)	169.2 ± 2.7	168.3 ± 3.1	170.6 ± 2.5
Weight (kg)	82.0 ± 3.5	81.9 ± 4.8	84.1 ± 3.7
BMI (kg/m^2)	27.9 ± 3.2	28.2 ± 3.6	28.7 ± 2.9
SBP (mmHg)	136.2 ± 15.8	138.5 ± 9.9	142.8 ± 12.8
DBP (mmHg)	82.1 ± 4.5	86.2 ± 5.6	87.1 ± 6.2
FPG (mmol/L)	6.7 ± 0.6	6.3 ± 0.5	6.5 ± 0.7
HbA1c (%)	6.6 ± 0.7	6.8 ± 0.6	6.4 ± 0.8
TC (mmol/L)	4.9 ± 1.2	4.4 ± 1.0	5.1 ± 1.6
TG (mmol/L)	2.9 ± 1.2	2.6 ± 1.3	3.1 ± 1.4
HDL-CH (mmol/L)	0.9 ± 0.2	0.9 ± 0.3	0.8 ± 0.2
LDL-CH (mmol/L)	3.3 ± 0.9	3.1 ± 0.6	3.4 ± 1.2
PSA (ng/mL)	3.9 ± 0.8	3.6 ± 1.0	3.7 ± 0.7
fPSA (ng/mL)	0.7 ± 0.2	0.6 ± 0.3	0.6 ± 0.2
BMD (g/cm^2)			
Lumbar spine	0.783 ± 0.088	0.802 ± 0.085	0.797 ± 0.080
Femoral neck	0.598 ± 0.073	0.586 ± 0.076	0.592 ± 0.077
Sex hormones			
TT (ng/dL)	214.8 ± 22.4	218.3 ± 25.1	220.1 ± 20.7
fT (pg/mL)	4.2 ± 1.1	3.9 ± 0.9	3.8 ± 0.7
E2 (pg/mL)	10.8 ± 5.7	13.9 ± 7.6	15.3 ± 6.8
Bone turnover markers	33.6 ± 12.4	32.2 ± 10.9	30.9 ± 11.3
BAP (IU/L)	37.1 ± 3.4	36.0 ± 5.2	36.5 ± 5.1
uNTX/Cr (nmol/mmol)	6.9 ± 1.3	6.8 ± 1.0	6.6 ± 1.4

TU: testosterone undecanoate; BMI: body mass index; FPG: plasma glucose; HbA1c: glycosylated hemoglobin A1c; TC: total cholesterol; TG: triglyceride; HDL-CH: high-density lipoprotein cholesterol; LDL-CH: low-density lipoprotein cholesterol; PSA: prostate specific antigen; fPSA: free prostate specific antigen; BMD: bone mineral density; TT: total testosterone; fT: free testosterone; E2: estradiol; BAP: bone-specific alkaline phosphatase; uNTX/Cr: urine N-telopeptide of type 1 collagen/creatinine.



(a) (b)

Figure 1: Changes in lumbar spine and femoral neck BMD throughout 24 months of treatment. Note: data are means ± SD. BMD: bone mineral density; ∗: $P < 0.05$ versus baseline, groups B and C; ∧: $P < 0.05$ versus baseline and group C. ♦: group A, the standard-dose TU group; ■: group B, the low-dose TU group; ▲: group C, placebo group.

3.1. Percentage Changes in BMD and Vertebral Fractures throughout 24 Months of Treatment. At baseline, lumbar spine and femoral neck BMD was similar in the three groups (Table 1). Since the 6th month followup in group A or since the 12th month followup in group B and throughout the study, lumbar spine and femoral neck BMD significantly increased ($P < 0.05$) compared with baseline and group C (Figure 1). In addition, at the 12th month followup, percentage of changes of lumbar spine and femoral neck BMD was significantly ($P < 0.05$) higher in group A than in groups B and C (Figure 1).

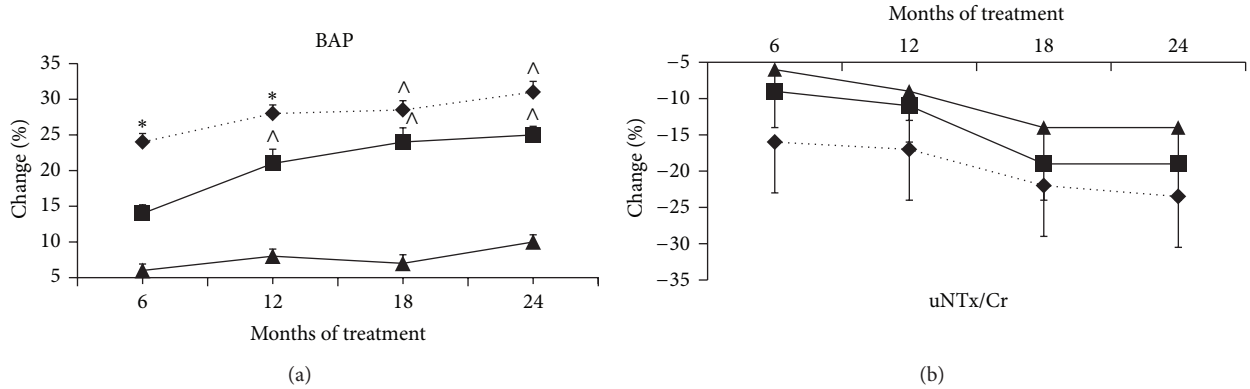


Figure 2: Changes in serum levels of BAP and uNTx/Cr throughout 24 months of treatment. Note: data are means ± SD. BAP: bone-specific alkaline phosphatase; uNTx/Cr: urine N-telopeptide of type 1 collagen/creatinine; ∗: $P < 0.05$ versus baseline, groups B and C; ∧: $P < 0.05$ versus baseline and group C. ⧫: group A, the standard-dose TU group; ■: group B, the low-dose TU group; ▲: group C, placebo group.

Since the 18th month followup and throughout the study, the percentage of changes of lumbar spine and femoral neck BMD was significantly higher ($P < 0.05$) in groups A and B than in group C (Figure 1), and no significant differences were found between groups A and B since that time (Figure 1). None of the patients had vertebral fractures during two years' TU treatment.

3.2. Bone Metabolism Markers. At baseline, the biochemical parameters of bone turnover were similar in the three groups (Table 1). Serum BAP levels significantly ($P < 0.05$) increased in group A since the 6th month followup and throughout the study and in group B since the 12th month followup and throughout the study (Figure 2). After 18 months of treatment, the percentage of increase in BAP levels was higher ($P < 0.05$) in groups A and B, without any difference between them, than in group C (Figure 2). Levels of uNTx/Cr were unchanged in all groups throughout the study period (Figure 2).

3.3. Sex Hormones. No significant differences in free T and E2 were found at baseline in all groups (Table 1). Serum-free T and E2 levels significantly ($P < 0.05$) increased in group A since the 6th month followup and throughout the study and in group B since the 12th month followup and throughout the study (Figure 3). After 18 months of treatment, the percentage of increase in free T and E2 levels was higher ($P < 0.05$) in groups A and B, without any difference between them, than in group C (Figure 3).

3.4. Side Effects and Dropouts. Throughout the 2-years observation, both doses of TU were equally well tolerated, and their safety profile was similar to that of placebo. There were no reports of nausea, vomiting, or diarrhea among study patients. All patients were monitored for serum levels of PSA, fPSA, and prostate grand by B-ultrasonography. There were no differences in these observed variables. No side effects on prostate glands were observed. There was also no significant difference in the incidence of all laboratory abnormalities between the three groups.

The numbers of withdrawals were similar in the three groups (five, four, and four men in groups A, B, and C). Dropouts were due to lack of compliance to the treatment in these patients.

4. Discussion

Although there have been considerable advances in our understanding and management options for male osteoporosis, there are a number of important gaps in knowledge [10]. The results of the present study show for the first time the effects of low-dose TU on BMD in aged male osteoporosis with low serum testosterone. Our results show that after the 24-month treatment, lumbar spine and femoral neck BMD and serum BAP levels increased significantly in both of low-dose TU (20 mg, per day) or standard-dose TU (40 mg, per day) treatment groups. There were no significant differences between groups of low-dose TU and standard-dose TU in the percentage of changes of lumbar spine and femoral neck BMD and serum levels of free testosterone, estradiol, and BAP since the 18th month followup and throughout the study. However, there were no differences for levels of uNTX in all groups. The elderly male patients tolerated the two doses of TU quite well. Adverse effects were similar and slight in the two groups. No side effects on prostate glands including PSA were found. None of the patients had vertebral fractures during the two years' TU treatment.

In a recent study, Nair and colleagues [11] performed a 2-year placebo-controlled, randomized, double-blind study involving 87 elderly men with low levels of DHEA sulfate and bioavailable testosterone (defined as below the 15th percentile for young normal men). Over 2-year of treatment, men who received testosterone transdermally or DHEA had a modest (~2%) increase in BMD at the femur neck but not at the spine, total hip, or radius. Neither treatments had major adverse effects, including prostate-specific antigen levels. These findings using transdermal testosterone at doses that had only modest effects on serum testosterone levels contrast with previous studies that used im testosterone administration and achieved higher circulating testosterone

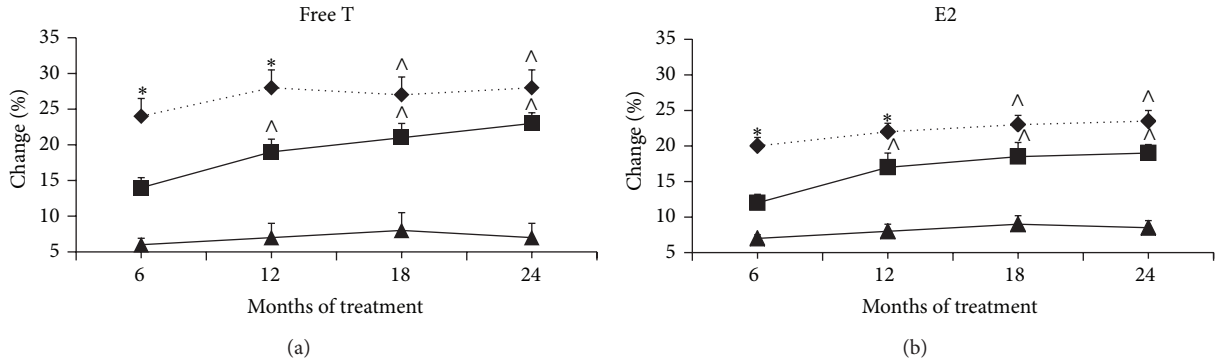


Figure 3: Changes in serum levels of free T and E2 throughout 24 months of treatment. Note: data are means ± SD. T: testosterone; E2: estradiol; ∗: $P < 0.05$ versus baseline, groups B and C; ∧: $P < 0.05$ versus baseline and group C. ♦: group A, the standard-dose TU group; ■: group B, the low-dose TU group; ▲: group C, placebo group.

levels [12]. Latter, testosterone therapy was associated with more clinically significant increases in bone mass.

The age-related decline in testosterone level was attributed to two factors, which were the degeneration of Leydig's cells and the increase of SHBG level with age [13]. In vitro studies demonstrated that androgen could increase the proliferation and decrease the apoptosis of osteoblast via regulation of protein kinase B [14]. It also played a vital role in the process of mineralization, which is the late differentiation stage of osteoblast [15, 16]. Androgen also prevented parathyroid-induced osteoclast formation [17] and decreased bone resorption activity of osteoclast via deactivation of lysosomal enzymes [18].

On the other hand, the traditional notion that estrogen is only important in maintenance of the female skeletal system while testosterone is vital for the male skeletal system is now challenged by several experiments of nature. Estrogen was found to be associated significantly with bone health status of elderly men in several large epidemiological studies. The Framingham study discovered that aged men with higher estradiol level had higher BMD, and the difference in BMD between the first quartile and the fourth quartile was equivalent to 10 years of aging on bone [19]. Positive and significant relationships between estradiol level and several hip strength parameters, especially cross-sectional area of bone, were also observed in the Boston Bone Health Study [20]. The estrogen hormone in men is produced via conversion of testosterone to estrogen via the aromatase enzyme (cytochrome 19) [21]. About 15% of the estrogen in men originates from the testes while the other 85% comes from peripheral tissue inclusive of bone. Furthermore, aromatase enzyme was found in osteoblasts, osteocytes, chondrocytes, and adipocytes but not in osteoclasts [22]. Therefore, it is reasonable to postulate that estrogen produced in the bone of men has paracrine or intracrine function [23].

However, there are reports of metastatic prostate cancer after testosterone administration in (elderly) men [24, 25]. This has raised concern that testosterone-replacement therapy should be given to aging men who do not have significantly high risk of developing prostate cancer. The current Endocrine Society Guidelines have been developed to render testosterone administration to elderly men acceptably safe therapy in men without a prior history of prostate carcinoma or without evidence of harboring a prostate carcinoma [26]. The members of the working group agreed that because the normative ranges for TT and FT in healthy young men vary among laboratories and assays (lower TT limits: 280–300 ng/dL; lower fT limits: 5–9 pg/mL) [27], clinicians should use the lower limit of normal range for healthy young men established in their laboratory. Members of the working group disagreed on T concentrations below which testosterone supplementation should be offered to older men with symptomatic hypogonadism. Some members of the working group recommended T supplementation in older men with TT level below 300 ng/dL, symptoms that might be attributable to low testosterone; others recommended T supplementation only in those with TT level below 200 ng/dL, because higher pretreatment T values are associated with lower beneficial effects of T therapy. A study in a worldwide sample of 1,438 men has found that the most common adverse drug reaction of injectable TU for the treatment of male hypogonadism is an increase in serum PSA with a potential threat for developing prostate grand tumour cancer [7], so to develop an effective and safe testosterone supplementation protocol is essential.

5. Conclusion

Treatment with low-dose TU (20 mg, per day) in elderly male osteoporosis with low serum testosterone effectively increases lumbar spine and femoral neck BMD and improves their bone turnover, similar to treatment with standard-dose TU (40 mg, per day). No side effects on prostate glands including prostate specific antigen were found. Low-dose TU may be a cost-effective and safe protocol for treating elderly male osteoporosis. Further clinical trials of large-sample, multi-center and longterm on the efficacy and safety of low-dose testosterone undecanoate treatment in elderly male osteoporosis with low serum testosterone is need.

Conflict of Interests

The authors declare that there is no conflict of interests associated with this paper.

Acknowledgments

This work was supported by the Foundation of the Ministry of Health of China (no. 201302008), the National Natural Science Foundation of China (no. 30871191), and the Hunan Provincial Natural Science Foundation (no. 09JJ3037).

References

[1] M. T. Hannan, D. T. Felson, B. Dawson-Hughes et al., "Risk factors for longitudinal bone loss in elderly men and women: the Framingham osteoporosis study," *Journal of Bone and Mineral Research*, vol. 15, no. 4, pp. 710–720, 2000.

[2] O. Johnell and J. A. Kanis, "An estimate of the worldwide prevalence and disability associated with osteoporotic fractures," *Osteoporosis International*, vol. 17, no. 12, pp. 1726–1733, 2006.

[3] S. Khosla, S. Amin, and E. Orwoll, "Osteoporosis in men," *Endocrine Reviews*, vol. 29, no. 4, pp. 441–464, 2008.

[4] A. C. Martin, "Osteoporosis in men: a review of endogenous sex hormones and testosterone replacement therapy," *Journal of Pharmacy Practice*, vol. 24, no. 3, pp. 307–315, 2011.

[5] Committee on Assessing the Need for Clinical Trials of Testosterone Replacement Therapy, "Executive summary," in *Testosterone and Aging: Clinical Research Directions*, C. T. Liverman and D. G. Blazer, Eds., pp. 1–10, The National Academic Press, Washington, DC, USA, 2004.

[6] A. Morales, B. Johnston, J. P. W. Heaton, and M. Lundie, "Testosterone supplementation for hypogonadal impotence: assessment of biochemical measures and therapeutic outcomes," *Journal of Urology*, vol. 157, no. 3, pp. 849–854, 1997.

[7] M. Zitzmann, A. Mattern, J. Hanisch, L. Gooren, H. Jones, and M. Maggi, "IPASS: a study on the tolerability and effectiveness of injectable testosterone undecanoate for the treatment of male hypogonadism in a worldwide sample of 1,438 men," *The Journal of Sexual Medicine*, vol. 10, no. 2, pp. 579–588, 2012.

[8] J. A. Kanis, L. J. Melton III, C. Christiansen, C. C. Johnston, and N. Khaltaev, "The diagnosis of osteoporosis," *Journal of Bone and Mineral Research*, vol. 9, no. 8, pp. 1137–1141, 1994.

[9] H. K. Genant, C. Y. Wu, C. Van Kuijk, and M. C. Nevitt, "Vertebral fracture assessment using a semiquantitative technique," *Journal of Bone and Mineral Research*, vol. 8, no. 9, pp. 1137–1148, 1993.

[10] S. Khosla, "Update in male osteoporosis," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 1, pp. 3–10, 2010.

[11] K. S. Nair, R. A. Rizza, P. O'Brien et al., "DHEA in elderly women and DHEA or testosterone in elderly men," *The New England Journal of Medicine*, vol. 355, no. 16, pp. 1647–1659, 2006.

[12] J. K. Amory, N. B. Watts, K. A. Easley et al., "Exogenous testosterone or testosterone with finasteride increases bone mineral density in older men with low serum testosterone," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 2, pp. 503–510, 2004.

[13] A. S. Midzak, H. Chen, V. Papadopoulos, and B. R. Zirkin, "Leydig cell aging and the mechanisms of reduced testosterone synthesis," *Molecular and Cellular Endocrinology*, vol. 299, no. 1, pp. 23–31, 2009.

[14] H. Y. Kang, C. L. Cho, K. L. Huang et al., "Nongenomic androgen activation of phosphatidylinositol 3-kinase/Akt signaling pathway in MC3T3-E1 osteoblasts," *Journal of Bone and Mineral Research*, vol. 19, no. 7, pp. 1181–1190, 2004.

[15] W. Balkan, K. L. Burnstein, P. C. Schiller et al., "Androgen-induced mineralization by MC3T3-E1 osteoblastic cells reveals a critical window of hormone responsiveness," *Biochemical and Biophysical Research Communications*, vol. 328, no. 3, pp. 783–789, 2005.

[16] K. M. Wiren, A. C. Evans, and X. W. Zhang, "Osteoblast differentiation influences androgen and estrogen receptor-α and -β expression," *Journal of Endocrinology*, vol. 175, no. 3, pp. 683–694, 2002.

[17] Q. Chen, H. Kaji, T. Sugimoto, and K. Chihara, "Testosterone inhibits osteoclast formation stimulated by parathyroid hormone through androgen receptor," *FEBS Letters*, vol. 491, no. 1-2, pp. 91–93, 2001.

[18] L. Pederson, M. Kremer, J. Judd et al., "Androgens regulate bone resorption activity of isolated osteoclasts *in vitro*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 2, pp. 505–510, 1999.

[19] S. Amin, Y. Zhang, and C. T. Sawin, "Association of hypogonadism and estradiol levels with bone mineral density in elderlymen from the Framingham study," *Annals of Internal Medicine*, vol. 133, no. 12, pp. 951–963, 2000.

[20] T. G. Travison, A. B. Araujo, T. J. Beck et al., "Relation between serum testosterone, serum estradiol, sex hormone-binding globulin, and geometrical measures of adult male proximal femur strength," *The Journal of Clinical Endocrinology & Metabolism*, vol. 94, no. 3, pp. 853–860, 2009.

[21] B. L. Riggs, S. Khosla, and L. J. Melton, "Sex steroids and the construction and conservation of the adult skeleton," *Endocrine Reviews*, vol. 23, no. 3, pp. 279–302, 2002.

[22] L. Gennari, R. Nuti, and J. P. Bilezikian, "Aromatase activity and bone homeostasis in men," *The Journal of Clinical Endocrinology & Metabolism*, vol. 89, no. 12, pp. 5898–5907, 2004.

[23] L. Vandenput and C. Ohlsson, "Sex steroid metabolism in the regulation of bone health in men," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 121, no. 3–5, pp. 582–588, 2010.

[24] E. L. Rhoden and A. Morgentaler, "Risks of testosterone-replacement therapy and recommendations for monitoring," *The New England Journal of Medicine*, vol. 350, no. 5, pp. 482–492, 2004.

[25] S. Sengupta, H. J. Duncan, R. J. Macgregor, and J. M. Russell, "The development of prostate cancer despite late onset androgen deficiency," *International Journal of Urology*, vol. 12, no. 9, pp. 847–848, 2005.

[26] S. Bhasin, G. R. Cunningham, F. J. Hayes et al., "Testosterone therapy in men with androgen deficiency syndromes: an endocrine society clinical practice guideline," *The Journal of Clinical Endocrinology & Metabolism*, vol. 95, no. 6, pp. 2536–2559, 2010.

[27] S. Bhasin, A. Zhang, A. Coviello et al., "The impact of assay quality and reference ranges on clinical decision making in the diagnosis of androgen disorders," *Steroids*, vol. 73, no. 13, pp. 1311–1317, 2008.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 842598, 7 pages
http://dx.doi.org/10.1155/2013/842598

Clinical Study

The Relationship between Serum Osteocalcin Concentration and Glucose Metabolism in Patients with Type 2 Diabetes Mellitus

Qingqing Wang,[1] Beibei Zhang,[1] Yulan Xu,[1] Hongdi Xu,[2] and Nan Zhang[1]

[1] *Department of Endocrinology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou 310016, China*
[2] *Department of Endocrinology, Zhejiang Qingchun Hospital, Hangzhou 310016, China*

Correspondence should be addressed to Nan Zhang; doczkm@yahoo.com.cn

Received 1 November 2012; Revised 9 January 2013; Accepted 12 February 2013

Academic Editor: Peng-Fei Shan

To study the correlations between serum osteocalcin and glucose metabolism in patients with type 2 diabetes, 66 cases were collected to determine total osteocalcin, undercarboxylated osteocalcin, fasting blood glucose, fasting insulin, and HbA1c. Osteocalcin concentrations were compared between groups of different levels of HbA1c, and parameters of glucose metabolism were compared between groups of different levels of total osteocalcin and undercarboxylated osteocalcin. The relationship between osteocalcin and parameters of glucose metabolism was also analyzed. We found that the total osteocalcin concentration of high-HbA1c group was significantly lower than that of low-HbA1c group. The fasting blood glucose of low-total-osteocalcin group was significantly higher than that of high-total-osteocalcin group in male participants, while the fasting blood glucose of low-undercarboxylated-osteocalcin group was significantly higher than that of high-undercarboxylated-osteocalcin group in all participants and in male participants. Total osteocalcin was inversely correlated with HbA1c, and undercarboxylated osteocalcin was inversely correlated with fasting blood glucose. However, no significant correlation was found between osteocalcin and HOMA-IR. Total osteocalcin was an independent related factor of HbA1c level. In summary, decreased serum total osteocalcin and undercarboxylated osteocalcin are closely related to the exacerbation of glucose metabolism disorder but have no relations with insulin resistance.

1. Introduction

Osteocalcin (OC) is a kind of noncollagenous protein which is synthesized and secreted by osteoblast. OC consists of undercarboxylated osteocalcin (ucOC) and fully carboxylated osteocalcin (cOC). cOC is formed after the glutamic acid residues of OC on the 17th, 21st, and 24th sites are carboxylated by vitamin K-dependent carboxylase. Osteocalcin with noncarboxylated glutamic acid residues is named as ucOC. Physiological functions of OC include maintaining normal bone mineralization, suppressing abnormal hydroxyapatite formation, and slowing down growth cartilage mineralization [1].

Previous human studies demonstrated that poorly controlled blood glucose could lead to reduced serum OC level in diabetic patients, while serum OC increased after blood glucose was well controlled [2]. These data indicated that changes of glucose metabolism could influence OC levels. Recent animal studies discovered that OC could reduce blood glucose, promote insulin secretion, and increase insulin sensitivity [3], indicating an important role of OC in the glucose metabolism regulation. Therefore, the theory that OC and glucose metabolism influenced each other was proposed.

To date, the evidences for OC influencing glucose metabolism were limited compared to those for glucose metabolism affecting OC. Whether OC affects energy metabolism still remains unclear in human. Moreover, ucOC was proved to play a leading role in promoting insulin synthesis and improving insulin resistance in animals [3], while several clinical studies suggested that cOC, rather than ucOC, played a more remarkable role in affecting insulin sensitivity in the human body [4, 5], which indicated that different components of OC might have different functions in human beings and animals. Therefore, accumulating data of ucOC and cOC, as well as total osteocalcin (tOC) is important to further discover the mechanisms of osteocalcin. However, data of current studies are mainly about tOC, while data of ucOC or cOC are rare. This study aims to further

investigate the influence of different OC components on glucose metabolism.

2. Materials and Methods

2.1. Objects and Grouping. We chose patients with type 2 diabetes mellitus (DM) who were admitted to Sir Run Run Shaw Hospital from March 2011 to March 2012 as research objects, referring to the 1999 WHO diagnostic criteria of diabetes mellitus. Patients with the following treatments or complications were excluded from the study: (i) acute diabetic complications, (ii) insulin treatment, (iii) using agents such as thiazolidinediones, statin, vitamin K, warfarin, vitamin D, calcium supplement, bisphosphonates, vitamin A, and hormones, (iv) bone diseases such as bone tumors, osteoporosis, and fracture, (v) nephropathy, liver dysfunction, kidney dysfunction, ovarian tumor, thyroid diseases, parathyroid diseases, and other endocrine diseases, and (vi) infection, trauma, major operation, and other stress. This study was approved by the medical ethic committee at Sir Run Run Shaw Hospital. All participants signed written informed consent.

First, the patients were divided into three groups, Group HbA1c-H (HbA1c $\geq$ 9%), Group HbA1c-M (7% $\leq$ HbA1c $<$ 9%), and Group HbA1c-L (HbA1c $<$ 7%) [6], as HbA1c $<$ 7% indicated well-controlled blood glucose and HbA1c $\geq$ 9% indicated marked hyperglycemia [7]. TOC, ucOC, and other indexes were compared among the three groups. Then, the patients were divided into two groups according to the average level of tOC, Group tOC-H and Group tOC-L. Indexes of glucose metabolism were compared between the two groups. At last, the patients were divided into two groups according to the average level of ucOC, Group ucOC-H and Group ucOC-L. Indexes of glucose metabolism were also compared between the two groups.

2.2. Specimen Collection. The age, gender, medication history, menstruation, course of diabetes mellitus, and complications of each patient were recorded. The liver and kidney functions, 24-hour urine microalbumin, creatinine clearance rate, bone mineral density, and thyroid function were also examined. Then, the research objects were chosen according to the inclusion and exclusion criteria. The venous blood of every research object was collected after 12-hour overnight fasting state, and levels of fasting blood glucose, fasting insulin, HbA1c, serum creatinine (sCr), blood calcium (Ca), bone-specific alkaline phosphatase (Ostase), and parathyroid hormone (PTH) were detected in the clinical laboratory of Sir Run Run Shaw hospital. Meanwhile, 4 mL of residual blood sample was collected and centrifuged, and the serum was kept in the fridge at the temperature of -20°C for tOC and ucOC detection. Homeostasis model assessment for beta-cell function (HOMA-β) was calculated to assess the basal insulin secretion of pancreatic beta cells. The formula was HOMA-β(%) = fasting insulin $\times$ 20/(fasting blood glucose $-$ 3.5). Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated to estimate insulin sensitivity. The formula was HOMA-IR = fasting blood glucose $\times$ fasting insulin/22.5. The unit of fasting blood glucose was mmol/L, and the unit of fasting insulin was μU/mL. The height and weight of the research objects were measured by a special-assigned person, and the body mass indexes (BMI) were calculated.

2.3. Assay of OC. TOC was detected by radioimmunoassay. The kit was provided by Beijing Atom HighTech Co., Ltd. The product batch number was 201203. UcOC was detected by enzyme-linked immunosorbent assay. The kit was provided by the United States R&D company. The product batch number was 10-25-931.

2.4. Statistical Method. Measurement data that conformed to the normal distribution were compared by Student's t-test. Data which did not conform to the normal distribution were converted to the normal distribution and compared. Data of sex composition ratio and postmenopausal women composition ratio were compared by chi-square test. The correlations of OC and other indexes were analyzed by Pearson correlation analysis. The correlations of HbA1c and other indexes were analyzed by Pearson correlation analysis and stepwise multiple regression analysis. $P < 0.05$ by two-tailed test was considered as significantly different between the two groups. Data were processed with the software package of SPSS 11.5.

3. Results

A number of 66 cases were finally included, consisting of 46 men and 20 women. Seventeen of the women were postmenopausal. The principal characteristics of the objects were displayed in Table 1. The average age of the female objects was older than that of the male objects, but there was no significant difference between the average ages of the male and all the objects. The average HOMA-β of the female objects was higher than that of the male objects.

3.1. Comparisons between Different Groups. Several parameters of bone and glucose metabolism were compared between different groups. Comparisons among Groups HbA1c-H, HbA1c-M, and HbA1c-L were displayed in Table 2. Comparisons between Groups tOC-H and tOC-L were displayed in Table 3. Comparisons between Groups ucOC-H and ucOC-L were displayed in Table 4.

3.2. Pearson Correlation Analysis. The Pearson correlation analysis showed that tOC was positively correlated with Ostase, negatively correlated with HbA1c (Figure 1), and not related with fasting blood glucose, fasting insulin, HOMA-β, HOMA-IR, or ucOC. ucOC was negatively correlated with fasting blood glucose (Figure 2), but not related with age, HbA1c, fasting insulin, HOMA-β, HOMA-IR, or tOC.

3.3. The Stepwise Multiple Regression Analysis of HbA1c and Other Indexes. We took HbA1c as a dependent variable. Age, BMI, course of DM, Ostase, Ca, sCr, fasting blood glucose, fasting insulin, HOMA-β, HOMA-IR, ucOC, and tOC were

Table 1: The principal characteristics of the research objects.

Parameters	All the objects ($n = 66$)	The male objects ($n = 46$)	The female objects ($n = 20$)
Age (year)	51.9 ± 13.3	49.8 ± 13.0	57.1 ± 12.0$^{\#}$
Height (cm)	164.76 ± 8.56	168.80 ± 6.45*	156.10 ± 5.49$^{**\#\#}$
Weight (kg)	65.92 ± 11.50	69.74 ± 9.97	57.45 ± 9.79$^{**\#\#}$
BMI (kg/m^2)	24.16 ± 3.09	24.43 ± 2.91	23.48 ± 3.29
Course of DM (month)	67.8 ± 53.7	56.2 ± 49.9	92.3 ± 54.6$^{\#}$
sCr (μmol/L)	72.4 ± 12.5	78.0 ± 11.0*	60.6 ± 9.2$^{**\#\#}$
Ca (mmol/L)	2.37 ± 0.13	2.37 ± 0.13	2.37 ± 0.11
Ostase (μg/L)	13.98 ± 4.09	13.34 ± 3.21	15.33 ± 5.35
PTH (ng/L)	36.41 ± 10.95	38.69 ± 15.53	35.89 ± 10.00
HbA1c (%)	9.6 ± 2.3	9.3 ± 2.3	9.9 ± 2.1
Fasting blood glucose (mmol/L)	7.92 ± 1.81	8.11 ± 1.64	7.90 ± 2.61
Fasting insulin (μIU/mL)	6.04 ± 3.06	5.74 ± 3.16	6.56 ± 2.60
HOMA-β (%)	33.56 ± 22.99	28.37 ± 15.82	43.74 ± 31.91$^{\#}$
HOMA-IR	2.13 ± 1.27	2.10 ± 1.36	2.23 ± 0.98
tOC (ng/mL)	4.08 ± 0.95	4.03 ± 0.91	4.31 ± 1.06
ucOC (pg/mL)	19.91 ± 9.76	21.04 ± 12.66	21.16 ± 10.53

$^{**}P < 0.01$ when compared with all the objects. $^{*}P < 0.05$ when compared with all the objects. $^{\#\#}P < 0.01$ when compared with the male objects. $^{\#}P < 0.05$ when compared with the male objects.

Table 2: Comparisons among Groups HbA1c-L, HbA1c-M, and HbA1c-H.

Parameters	HbA1c-L ($n = 11$)	HbA1c-M ($n = 16$)	HbA1c-H ($n = 36$)
Age (year)	46.5 ± 9.6	54.5 ± 11.9	52.5 ± 14.6
BMI (kg/m^2)	24.22 ± 2.54	24.25 ± 3.37	24.11 ± 3.20
sCr (μmol/L)	71.1 ± 9.9	73.8 ± 12.2	72.1 ± 13.6
Ca (mmol/L)	2.42 ± 0.14	2.39 ± 0.13	2.35 ± 0.12
Ostase (μg/L)	12.35 ± 3.63	13.82 ± 2.74	14.55 ± 4.63
PTH (ng/L)	40.71 ± 12.30	39.00 ± 10.77	33.94 ± 10.22
Postmenopausal ratio	10/10	16/16	33/36
Fasting blood glucose (mmol/L)	6.81 ± 1.13	8.37 ± 1.96*	8.06 ± 1.82*
Fasting insulin (μIU/mL)	5.62 ± 2.99	5.52 ± 2.05	6.40 ± 3.46
HOMA-β (%)	36.15 ± 16.79	27.95 ± 20.06	35.26 ± 25.76
HOMA-IR	1.75 ± 1.23	2.04 ± 0.86	2.29 ± 1.42
tOC (ng/mL)	4.54 ± 0.75	4.20 ± 1.25	3.89 ± 0.81*
ucOC (pg/mL)	20.24 ± 11.70	20.05 ± 8.90	19.75 ± 9.77

The objects were divided into three groups, Group HbA1c-H (HbA1c ≥ 9%), Group HbA1c-M (7% ≤ HbA1c < 9%) and Group HbA1c-L (HbA1c < 7%).
$^{*}P < 0.05$ when compared with Group HbA1c-L. $^{\#}P < 0.05$ when compared with Group HbA1c-M.

selected as independent variables. The stepwise multiple regression analysis showed that Ostase and tOC were both the independent relevant factors affecting HbA1c levels, whether the female objects were excluded or not (Table 5).

4. Discussion

4.1. tOC and Glucose Metabolism. The previous human studies demonstrated that poorly controlled blood glucose could lead to reduced serum OC level in diabetic patients, while serum OC would increase after blood glucose was controlled [2]. The animal model study conducted by Lee et al. found that OC gene knockout ($OC^{-/-}$) could lead to elevated blood glucose and insulin levels, as well as decreased insulin sensitivity [3], suggesting that OC could in turn regulate glucose metabolism. Does OC have the similar function in human body? The answer is uncertain, because the vast majority of the present human studies are cross-sectional studies.

Some studies on Caucasian and Asian populations [8–10] showed that tOC level was significantly negatively correlated with fasting blood glucose, fasting insulin, and HOMA-IR. However, several Chinese studies showed that tOC was not related to HOMA-IR [11, 12]. This study found that tOC level of Group HbA1c-H was significantly different from that of Group HbA1c-L, tOC was negatively correlated with HbA1c and had no relationship with fasting blood glucose, fasting insulin, or HOMA-IR, and tOC was an independent associated factor of HbA1c. After excluding all the female patients, we observed that the fasting blood glucose of Group

Table 3: Comparisons between Group tOC-H and Group tOC-L.

Parameters	All the objects		The male objects	
	tOC-H ($n = 35$)	tOC-L ($n = 31$)	tOC-H ($n = 23$)	tOC-L ($n = 23$)
Gender (female/male)	12/23	8/23	/	/
Age (year)	52.4 ± 11.5	51.2 ± 15.3	49.1 ± 11.9	50.2 ± 14.4
BMI (kg/m^2)	24.57 ± 2.86	23.77 ± 3.31	24.58 ± 2.87	24.25 ± 3.06
Course of DM (month)	61.7 ± 51.2	72.9 ± 58.5	53.8 ± 47.4	59.6 ± 54.2
sCr (μmol/L)	73.0 ± 11.5	72.3 ± 13.7	78.3 ± 9.8	76.4 ± 10.9
Ca (mmol/L)	2.40 ± 0.11	2.35 ± 0.14	2.40 ± 0.12	2.35 ± 0.15
Ostase (μg/L)	15.59 ± 3.99	16.05 ± 2.67	14.50 ± 3.52	14.89 ± 2.44
PTH (ng/L)	36.99 ± 11.35	36.86 ± 11.03	36.87 ± 11.78	37.43 ± 11.61
Postmenopausal ratio	34/35	29/31	/	/
Fasting blood glucose (mmol/L)	7.95 ± 2.10	8.28 ± 1.80	7.61 ± 1.47	8.60 ± 1.73*
Fasting insulin (μIU/mL)	6.34 ± 3.25	5.78 ± 2.75	6.21 ± 3.74	5.27 ± 2.50
HbA1c (%)	9.1 ± 2.3	10.0 ± 2.2	8.7 ± 2.2	10.0 ± 2.3
HOMA-β (%)	33.88 ± 20.44	30.30 ± 22.75	32.23 ± 16.81	23.83 ± 13.58
HOMA-IR	2.27 ± 1.40	2.08 ± 1.07	2.19 ± 1.63	2.01 ± 1.08

The objects were divided into two groups according to the average level of tOC, Group tOC-H and Group tOC-L.
* $P < 0.05$ when compared with Group tOC-H.

Table 4: Comparisons between Group ucOC-H and Group ucOC-L.

Parameters	All the objects		The male objects	
	ucOC-H ($n = 24$)	ucOC-L ($n = 42$)	ucOC-H ($n = 16$)	ucOC-L ($n = 30$)
Gender (female/male)	9/15	11/31	/	/
Age (year)	53.8 ± 12.8	50.8 ± 13.6	53.4 ± 12.1	47.8 ± 13.3
BMI (kg/m^2)	23.55 ± 2.84	24.55 ± 3.17	22.93 ± 2.62	25.16 ± 2.84*
Course of DM (month)	78.6 ± 66.0	60.6 ± 47.1	64.7 ± 71.5	52.6 ± 36.4
sCr (μmol/L)	70.6 ± 13.9	73.8 ± 11.6	78.9 ± 11.9	76.6 ± 9.4
Ca (mmol/L)	2.38 ± 0.14	2.38 ± 0.12	2.36 ± 0.15	2.38 ± 0.13
Ostase (μg/L)	14.82 ± 3.82	13.50 ± 3.84	14.27 ± 3.28	13.00 ± 3.13
PTH (ng/L)	38.78 ± 10.89	39.15 ± 10.71	34.75 ± 13.06	38.34 ± 10.78
Postmenopausal ratio	22/24	41/42	/	/
Fasting blood glucose (mmol/L)	7.60 ± 2.27	8.37 ± 1.74*	7.38 ± 1.23	8.45 ± 1.74*
Fasting insulin (μIU/mL)	5.49 ± 2.66	6.40 ± 3.19	4.67 ± 2.49	6.29 ± 3.40
HbA1c (%)	9.9 ± 2.5	9.3 ± 2.2	9.7 ± 2.8	9.2 ± 2.1
HOMA-β (%)	35.37 ± 27.83	30.55 ± 17.23	25.65 ± 13.29	29.36 ± 16.89
HOMA-IR	1.85 ± 1.04	2.36 ± 1.33	1.59 ± 1.10	2.35 ± 1.44*

The objects were divided into two groups according to the average level of ucOC, Group ucOC-H and Group ucOC-L. * $P < 0.05$ when compared with Group ucOC-H.

tOC-L was significantly different from that of Group tOC-H. These results suggested that decreased serum tOC was related with long-term hyperglycemia but had little impact on insulin resistance. The results of this study are similar to those of the other two Chinese studies, but different from those of the foreign studies. We think this may be caused by the differences in study populations (with different disease background and ages), races, genes, sunlight exposure, and diet habits.

Moreover, this study showed that HbA1c was positively correlated with Ostase while negatively correlated with blood calcium. The correlations disappeared after excluding the female objects. As we all know, the estrogen concentrations in postmenopausal women are lower. As a result, the lowered activity of vitamin D results in the decrease of blood calcium, the enhancement of bone turnover, and the increase of Ostase level. Therefore, we consider the phenomenon mentioned above may be related to the estrogen interference. However, the sample size became smaller after excluding the female objects, which limited the credibility of this study. Further researches are necessary to confirm the hypothesis above.

4.2. ucOC and Glucose Metabolism. Both *in vivo* and *in vitro* studies have indicated that it was probably the ucOC ingredient that played the leading role in lowering blood sugar, promoting insulin synthesis, and improving insulin resistance [3, 13]. Human studies in recent years have accumulated a small amount of ucOC data. These date, unlike the

Table 5: Stepwise multiple regression analysis showing variables independently associated with HbA1c.

	Independent variable	Regression coefficient	P value	95% C.I. of the regression coefficient
All the objects	Ostase	0.253	0.000^{**}	0.121~0.386
	tOC	−1.054	0.000^{**}	−1.624~ −0.483
The male objects	Ostase	0.215	0.044^{*}	0.007~0.424
	tOC	−1.108	0.005^{**}	−1.853~ −0.362

$^{**}P < 0.01$, $^{*}P < 0.05$. HbA1c was the dependent variable. C.I. represents confidence interval.

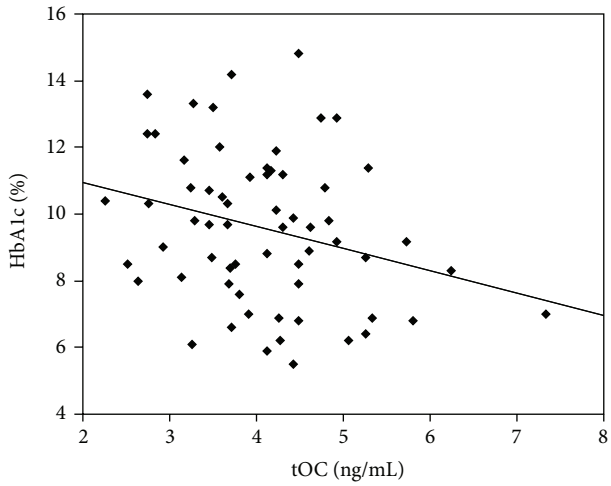


Figure 1: Negative correlation between tOC and HbA1c ($r = -0.277$, $P = 0.028$).

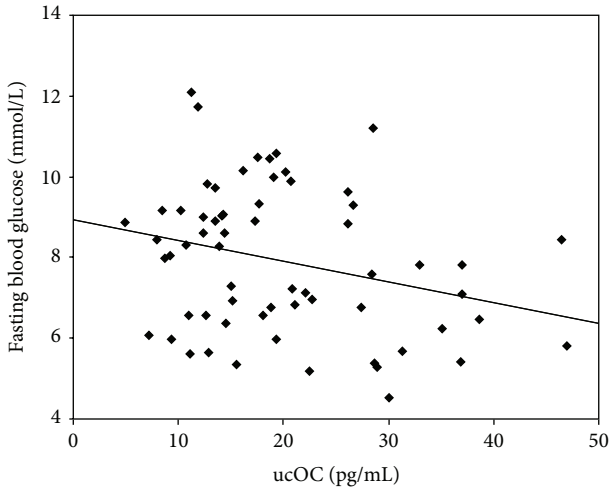


Figure 2: Negative correlation between ucOC and fasting blood glucose ($r = -0.278$, $P = 0.027$).

conclusions of animal studies, suggest that ucOC may have some association with insulin secretion and decreased blood glucose but have no relationship with insulin sensitivity [14–16]. This study found that the fasting blood glucose of Group ucOC-L was significantly higher than that of Group ucOC-H, and ucOC was negatively correlated with fasting blood glucose. But we failed to find any correlations between ucOC and HbA1c, fasting insulin, HOMA-β, HOMA-IR, and other indicators of glucose metabolism. These results suggested that ucOC decrease was related to the fasting blood glucose increase, but not related to the changes of insulin secretion and insulin resistance, partly in line with the conclusions of the aforementioned human studies.

Whether ucOC is able to improve insulin sensitivity is the main disagreement between animal studies and human studies. As a matter of fact, the human studies cited above and this study are all cross-sectional studies, which are unable to clarify the causality of serum osteocalcin variation and glucose metabolism changes. Two retrospective studies of a clinical trial [17] may be more persuasive. One study found that elevated cOC level and reduced ucOC% (the percentage of ucOC in OC) caused by vitamin K supplement might improve insulin resistance but had no effects on the basal secretion of insulin [4]. The other one also found that cOC had a greater impact on insulin sensitivity than ucOC [5]. These human studies inspire us to further investigate the mechanisms of different OC components on the regulation of glucose metabolism in humans. Perhaps it is the cOC rather than ucOC that plays a leading role in regulating insulin sensitivity in humans, which is distinctive from the mechanism in animals.

In addition, this study also found that HOMA-IR of Group ucOC-L was significantly higher than that of Group ucOC-H after excluding the female objects. But we were unable to draw the conclusion that reduced ucOC levels were related to the enhanced insulin resistance. Since the BMIs of Group ucOC-H and Group ucOC-L were significantly different after excluding the female patients, we considered the higher HOMA-IR level in Group ucOC-L was related to its higher mean BMI.

5. Deficiencies and Limitations

Most of the current studies about the relationship between osteocalcin and glucose metabolism only detected tOC levels. Data of ucOC are little. Our study investigates not only the serum tOC concentrations, but also the ucOC levels, accumulating more research data to help further explore the mechanism of osteocalcin in glucose metabolism. However, there are some deficiencies and limitations in this study. First, the sample size is small, and there are no healthy individuals served as normal controls. However, it is worth mentioning that specimens of healthy individuals are being collected as controls at present. Secondly, the correlation analysis showed that there was no linear relationship between tOC and ucOC, partly because of the different kits used for

determining tOC and ucOC. Gundberg et al. thought that serum ucOC was greatly impacted by serum tOC. Therefore they recommended ucOC% to reflect the serum ucOC level [18]. In this study, we could not calculate ucOC% because of the difference in kits, which is a disadvantage for the accuracy of analysis. Thirdly, we did not determine the serum cOC levels. Some human studies showed that cOC had a greater influence than ucOC on insulin sensitivity. Therefore, if tOC and cOC levels and ucOC% are simultaneously determined, the effects of different osteocalcin components on glucose metabolism may be further explored. Lastly, this research is a cross-sectional study, which cannot reveal the causal relationship of glucose metabolism and bone metabolism. Longitudinal studies are needed to further confirm whether the change of osteocalcin level, as an initiating factor, has some impact on insulin secretion and sensitivity.

6. Conclusions

By detecting and comparing the serum osteocalcin levels and glucose metabolism indicators of 66 patients with type 2 diabetes, we found that the tOC levels of patients with higher HbA1c were lower than those of the patients with well-controlled blood glucose levels, the fasting glucose levels were higher in male patients with lower tOC levels, tOC and HbA1c were significantly negatively correlated, and tOC was the independent associated factor affecting HbA1c level. These results indicated that there was a significant correlation between decreased tOC level and aggravating glucose metabolism disorder. In addition, we also collected data of ucOC, from which we found significantly higher fasting blood glucose levels in patients with lower ucOC levels. ucOC level was significantly inversely correlated with fasting blood glucose but was not associated with HOMA-IR. These results indicated that ucOC reduction may be associated with the aggravation of glucose metabolism disorders, but no influence of ucOC on insulin sensitivity could be proved.

Authors' Contribution

W. Qingqing and Z. Beibei contributed equally to this work.

Conflict of Interests

There is no conflict of interests among the authors.

Acknowledgment

This study was supported by Zhejiang province science and technology project no. 2010C33029.

References

[1] D. A. Pearson, "Bone health and osteoporosis: the role of vitamin K and potential antagonism by anticoagulants," *Nutrition in Clinical Practice*, vol. 22, no. 5, pp. 517–544, 2007.

[2] K. J. Motyl, L. R. McCabe, and A. V. Schwartz, "Bone and glucose metabolism: a two-way street," *Archives of Biochemistry and Biophysics*, vol. 503, no. 1, pp. 2–10, 2010.

[3] N. K. Lee, H. Sowa, E. Hinoi et al., "Endocrine regulation of energy metabolism by the skeleton," *Cell*, vol. 130, no. 3, pp. 456–469, 2007.

[4] M. Yoshida, P. F. Jacques, J. B. Meigs et al., "Effect of vitamin K supplementation on insulin resistance in older men and women," *Diabetes Care*, vol. 31, no. 11, pp. 2092–2096, 2008.

[5] M. K. Shea, C. M. Gundberg, J. B. Meigs et al., "γ-carboxylation of osteocalcin and insulin resistance in older men and women," *The American Journal of Clinical Nutrition*, vol. 90, no. 5, pp. 1230–1235, 2009.

[6] J. J. Wen, L. M. Chen, C. Y. Shan et al., "The correlation between glycated hemoglobin A1c and islet β cell function in poorly controlled type 2 diabetic patients treated with oral antidiabetic drugs," *Chinese Journal of Diabetes*, vol. 20, no. 2, pp. 129–132, 2012.

[7] S. Del Prato, A. M. Felton, N. Munro, R. Nesto, P. Zimmet, and B. Zinman, "Improving glucose management: ten steps to get more patients with type 2 diabetes to glycaemic goal," *International Journal of Clinical Practice*, vol. 59, no. 11, pp. 1345–1355, 2005.

[8] J. M. Kindblom, C. Ohlsson, O. Ljunggren et al., "Plasma osteocalcin is inversely related to fat mass and plasma glucose in elderly Swedish men," *Journal of Bone and Mineral Research*, vol. 24, no. 5, pp. 785–791, 2009.

[9] J. A. Im, B. P. Yu, J. Y. Jeon, and S. H. Kim, "Relationship between osteocalcin and glucose metabolism in postmenopausal women," *Clinica Chimica Acta*, vol. 396, no. 1-2, pp. 66–69, 2008.

[10] B. B. Yeap, S. A. P. Chubb, L. Flicker et al., "Reduced serum total osteocalcin is associated with metabolic syndrome in older men via waist circumference, hyperglycemia, and triglyceride levels," *European Journal of Endocrinology*, vol. 163, no. 2, pp. 265–272, 2010.

[11] M. Zhou, X. Ma, H. Li et al., "Serum osteocalcin concentrations in relation to glucose and lipid metabolism in Chinese individuals," *European Journal of Endocrinology*, vol. 161, no. 5, pp. 723–729, 2009.

[12] P. Du, G. Qin, and Z. Li, "Relationship between osteocalcin and type 2 diabetes mellitus," *Chinese Journal of Practical Meicine*, vol. 38, no. 7, pp. 48–49, 2011.

[13] M. Ferron, E. Hinoi, G. Karsenty, and P. Ducy, "Osteocalcin differentially regulates β cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 13, pp. 5266–5270, 2008.

[14] I. Kanazawa, T. Yamaguchi, M. Yamauchi et al., "Serum undercarboxylated osteocalcin was inversely associated with plasma glucose level and fat mass in type 2 diabetes mellitus," *Osteoporosis International*, vol. 22, no. 1, pp. 187–194, 2011.

[15] S. L. Booth, G. Dallal, M. K. Shea, C. Gundberg, J. W. Peterson, and B. Dawson-Hughes, "Effect of vitamin K supplementation on bone loss in elderly men and women," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 4, pp. 1217–1223, 2008.

[16] C. M. Gundberg, S. D. Nieman, S. Abrams, and H. Rosen, "Vitamin K status and bone health: an analysis of methods for determination of undercarboxylated osteocalcin," *Journal of Clinical Endocrinology and Metabolism*, vol. 83, no. 9, pp. 3258–3266, 1998.

[17] Y. C. Hwang, I. K. Jeong, K. J. Ahn, and H. Y. Chung, "The uncarboxylated form of osteocalcin is associated with improved

glucose tolerance and enhanced β-cell function in middle-aged male subjects," *Diabetes/Metabolism Research and Reviews*, vol. 25, no. 8, pp. 768–772, 2009.

[18] A. Prats-Puig, M. Mas-Parareda, E. Riera-Pérez et al., "Carboxylation of osteocalcin affects its association with metabolic parameters in healthy children," *Diabetes Care*, vol. 33, no. 3, pp. 661–663, 2010.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 513925, 7 pages
http://dx.doi.org/10.1155/2013/513925

Research Article

Establishing Reference Intervals for Bone Turnover Markers in the Healthy Shanghai Population and the Relationship with Bone Mineral Density in Postmenopausal Women

Wei-Wei Hu, Zeng Zhang, Jin-Wei He, Wen-Zhen Fu, Chun Wang, Hao Zhang, Hua Yue, Jie-Mei Gu, and Zhen-Lin Zhang

Metabolic Bone Disease and Genetic Research Unit, Department of Osteoporosis and Bone Disease, Shanghai Sixth People's Hospital Shanghai Jiao Tong University, 600 Yi-Shan Road, Shanghai 200233, China

Correspondence should be addressed to Zhen-Lin Zhang; zzl2002@medmail.com.cn

Received 12 November 2012; Revised 10 January 2013; Accepted 11 January 2013

Academic Editor: Ling-Qing Yuan

The reference ranges of bone turnover markers (BTMs) were important during the treatment of osteoporosis, and the associations with bone mineral density (BMD) were controversial. The aim of this study was to establish the reference ranges of N-terminal procollagen of type l collagen (P1NP), osteocalcin (OC), and beta C-terminal cross-linked telopeptides of type I collagen (β-CTX) in Shanghai area and to investigate the relationships between BTMs and BMD in postmenopausal women. 2,799 subjects recruited in Shanghai City were measured BTMs to establish the reference ranges. Additional 520 healthy postmenopausal women were also measured BTMs, these women measured BMD in addition. BTMs were measured using the Roche electrochemiluminescence system. We used the age range of 35 to 45-year-olds to calculate reference intervals. The reference range of OC was 4.91 to 13.90 ng/mL for women and 5.58 to 16.57 ng/mL for men, P1NP was 13.72 to 32.90 ng/mL for women and 16.89 to 42.43 ng/mL for men, and β-CTX was 0.112 to 0.210 ng/mL for women and 0.100 to 0.378 ng/mL for men. BTMs significantly negatively correlated with lumbar spine and femoral and total hip in postmenopausal women ($\text{Beta}_{\text{std}} = -0.157 \sim -0.217$, $P < 0.001$). We established the normal reference ranges of P1NP, OC, and β-CTX in the Shanghai area. This study also found that BTMs correlated with BMD and suggested that BTMs were the key determining factors of early BMD decreases.

1. Introduction

Bone mineral density (BMD) is used to diagnose osteoporosis and to predict the risk of osteoporotic fracture in postmenopausal women [1, 2]; however, the limitations of BMD measurements in assessing fracture risk have been documented, specifically that less than half of those who suffer a fracture have BMD values in the osteoporotic range [2, 3]. van Coeverden et al., as well as other researchers, confirmed that bone turnover markers (BTMs) had an important impact on bone mass, height, and other growth factors after the onset of puberty in boys and girls [4–6]. A recent study demonstrated that BTMs were useful in fracture prediction and in monitoring and evaluating drug intervention during clinical treatment and that they reflected the tested individual's bone turnover rate in clinical trials [1, 7]. In addition, many studies have found that BTMs can predict the risk of vertebral and hip fractures [8–10] and that they can predict the risk of hip fracture independent of hip BMD [9].

BTMs include several enzymes and their decomposition products that come from bone cells and bone matrix components. Among BTMs that reflect the activity of osteoblast cells are serum osteocalcin (OC) in form of N-terminal midmolecule fragment (N-MID) and undercarboxylated osteocalcin (UcOC), N-terminal procollagen of type l collagen (P1NP), and C-terminal procollagen of type l collagen (P1CP). The P1NP that is by the N-terminal enzymatic hydrolysis and is a specific marker that reflected in osteoblasts activity. Its conversion rate is also higher than the soft tissue sources, so the determination of the P1NP can reflect bone formation. BTMs that reflect the activity of osteoclast cells include cross-linked N-telopeptide of type l collagen (NTX),

cross-linked C-telopeptide of type l collagen (CTX), tartrate-resistant acid phosphatase (TRAP), and pyridinoline (Pyd). Because it is not being degraded in the blood and excreted by the kidneys, CTXs directly reflect bone matrix collagen degradation. During mature collagen degradation, the C-terminal peptide α aspartic acid is converted to β aspartic acid. So detection of β-CTX were reflected the state of bone resorption.

Most reference ranges have been provided by commercial laboratory kits using approximately 100–200 observations [11]. Therefore, it is essential to establish a valid reference range for clinical use. Once reference ranges for BTMs are established, comparisons of BTMs between healthy individuals and osteoporotic patients can be performed. Until now, there was no reference database of BTMs for the Chinese population. Whereas most studies have measured BTMs and BMD, age and other related factors have only been documented in small groups stratified by menopausal status, gender, or puberty stage, but no large-scale reference database has been compiled. One study indicated that there was no significant bone loss in 30- to 45-year-old women who were calcium balanced and of optimal bone health [12]. Therefore, it is important to establish reference intervals stratified by the known determinants of BTMs, such as age, sex, and body mass index (BMI). In this study, we established a reference database of β-CTX, P1NP, and OC levels in healthy Chinese men and women in Shanghai and investigated the relationships between BTMs and BMD in postmenopausal women.

2. Materials and Methods

2.1. Subjects. In total, 2,799 individuals aged 20–79 yrs (790 men and 2,009 women) were recruited in March 2009 from the local population in communities of Shanghai. The inclusion criteria were as follows: (1) healthy men and women according to the following excluding criteria; (2) blood tests in this study were accorded with the laboratory normal reference range. The following criteria were used to exclude individuals from the study: (1) serious effects from cerebrovascular disease; (2) diabetes mellitus; (3) chronic renal disease and chronic liver disease; (4) evidence of other metabolic or inherited bone diseases; (5) rheumatoid arthritis or collagen disease; (6) recent major gastrointestinal disease; (7) significant disease of any endocrine organ that would affect bone mass; (8) hyperthyroidism; (9) any neurological or musculoskeletal condition; and (10) any form of calcium and vitamin-D therapy in three months or taking anti-osteoporotic drugs (e.g., bisphosphonates, selective estrogen receptor modulators, and calcitonin). Postmenopausal women who had experienced early menopause (before 45 yrs of age) were excluded. This study was approved by the Ethics Committee of the Shanghai Sixth People's Hospital, Shanghai Jiao Tong University.

In addition, 520 healthy postmenopausal women with normal liver and kidney function were collected in communities of Shanghai. We carried BMD and the following BTMs in 520 postmenopausal women.

Age, body weight, height, and age of menarche and amenorrhea were recorded. All subjects were medically examined and interviewed using a standardized questionnaire to collect information on life style, smoking habits, the level of physical activity during leisure time, and use of vitamins and medications. All healthy subjects included in the present study had (1) normal blood counts and (2) normal results for liver and kidney function tests.

2.2. Biochemical Markers. All biochemical markers were measured at the same time point using a single lot of reagents in one batch, following both the manufacturer's protocol and specialized assay laboratory quality control procedures. Fasting blood samples were collected for the measurement of the serum levels of calcium, phosphate, albumin, glucose, insulin, cholesterol, triglycerides (TG), blood urea nitrogen (BUN), creatinine (Cr), alanine aminotransferase (ALT), aspartate transaminase (AST), r-glutamyl transpeptidase (r-GT), alkaline phosphatase (ALP), and B ultrasound of the spleen, kidneys, hepatobiliary system, and pancreas.

Serum 25-hydroxyvitamin D [25(OH)D], PTH, β-CTX, OC in form of N-MID, and P1NP were measured using an automated Roche electro-chemiluminescence system (Roche Diagnostic Gmbh). The intra- and inter-assay coefficients of variation (CVs) for 25(OH)D were 5.6% and 8.0%, respectively. The lower limit of detection for 25(OH)D was 4 ng/mL (10 mmol/L). The intra- and interassay CVs were 1.4% and 2.9% for PTH, 2.5% and 3.5% for β-CTX, 2.9% and 4.0% for OC, and 2.3% and 2.8% for P1NP, respectively.

2.3. BMD Measurement. 520 postmenopausal women's BMD (g/cm^2) of the left proximal femur including the total hip, the femoral neck, the trochanter, and Ward's triangle was measured using dual-energy X-ray absorptiometry (DXA) on a Lunar Prodigy GE densitometer (Lunar Corp, Madison, WI, USA). Both of the scanners were calibrated daily, and the coefficient of variability (CV) values for the DXA measurements at L1-4, the total hip, and the femoral neck were 1.39%, 0.7%, and 2.22%, respectively, for the Lunar Prodigy. The long-term reproducibility of the DXA data during the trial, which was based on phantom measurements that were repeated weekly, was 0.45%.

2.4. Statistical Analyses. The anthropometric characteristics were presented as the means ± SD, and BTMs were presented as geometric means with 95% confidence intervals (95% CI). Data were analyzed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). The BTMs ALP, OC, P1NP, PTH, and β-CTX were determined to be not normally distributed by the Kolmogorov-Smirnov test. The 95% reference interval for each BTM examined was calculated as the mean ± 1.96 SD. The CI for the lower and upper bounds of the reference intervals was computed as the boundary ± 1.96 standard error (SE). We performed multiple regression analysis between BTMs and BMD, controlling age, height, weight, and years since menopause (YSM).

Table 1: Anthropometric characteristics and other variables in the men and women aged 35–45 yr used to calculate the reference intervals of the bone turnover markers.

Characteristics	Men (n = 226)	Women (n = 406)
Age (year)	39.8 ± 3.2 (35–45)	39.4 ± 3.2 (35–45)
Height (cm)	173.2 ± 5.9 (165.8–180.1)	162.0 ± 4.9 (153.7–172.5)
Weight (kg)	75.0 ± 12.5 (61.6–85.5)	58.3 ± 7.1 (44.7–68.3)
BMI (kg/m^2)	25.4 ± 3.1 (20.8–26.4)	22.2 ± 2.6 (17.9–24.6)

*Data are presented as the mean ± SD. Parenthesis for minimum and maximum.

3. Results

3.1. Subject Characteristics. A total of 2,799 subjects were screened for entry into the study by measuring indicators of liver and kidney function and blood calcium and phosphorus: 148 failed the initial screening, leaving 2,651 subjects. 25(OH)D concentrations were divided into four subgroups according to the following criteria: severely deficient (<10 ng/mL), deficient (10–20 ng/mL), insufficient (20-30 ng/mL), and sufficient (≥30 ng/mL). The prevalence of vitamin D insufficiency was 84% in males and 89% in females. The prevalence of vitamin D deficiency was 30% in males and 46% in females. Based on 25(OH)D concentrations, we excluded the subject whose 25(OH)D concentrations were <10 ng/mL in 2651 cases, leaving 705 males and 1836 females last.

3.2. Reference Intervals. Recent studies containing data from larger cohorts of healthy premenopausal women have reported reference intervals for which the age range was 30–45 yr [13–15]. Many factors affect bone turnover markers, including age, weight, and gender. Therefore, we used the age range of 35- to 45-year-olds year to minimize the effects of such factors associated with preanalytical variability, and using this cutoff, we were left with 406 women and 226 men from which to calculate reference intervals. 25(OH)D concentrations of these subjects were 10 ng/mL and more. The anthropometric characteristics of this group of subjects are presented in Table 1. Table 2 reports the reference intervals for the BTMs studied and includes the medians, geometric means, and 95% reference intervals. The lower half of the reference range (values falling between the lower limit of the reference interval and the median) has been proposed as a target for treatment [7]. This range for OC was 4.91 to 13.90 ng/mL in women and 5.58 to 16.57 ng/mL in men; for P1NP, it was 13.72 to 32.90 ng/mL in women and 16.89 to 42.43 ng/mL in men; for β-CTX, it was 0.112 to 0.210 ng/mL in women and 0.100 to 0.378 ng/mL in men. The geometric means and 95% confidence intervals (95% CI) for age-specific BTM values in healthy women and men are shown in Tables 3 and 4.

3.3. Effect of Age on Biochemical BTMs. Table 3 shows the geometric means and SDs for the biochemical BTMs in the Chinese women stratified by 5 yr increments. Age-related changes in all biochemical BTMs were calculated by comparison with the BTM values for the reference intervals group. The bone formation markers OC and P1NP decreased with increasing age until the age of 44 and then abruptly increased for women aged 50–59 yr. The bone resorption marker β-CTX also decreased with increasing age until the age of 44 before sharply increasing in women > 50 yrs old. All BTMs began to decrease in women aged 70–74 yr.

The geometric means and 95% confidence intervals (95% CI) of the age-specific BTMs in healthy men are shown in Table 4. Age-related changes in all biochemical BTMs were compared with the BTM values of the reference intervals group. The bone formation markers OC and P1NP decreased with increasing age until the age of 59 and then quickly increased in men aged 60–69 yr. The bone resorption marker β-CTX also decreased with increasing age until the age of 54, at which point it sharply increased in men > 60 yrs old.

3.4. Correlations between BTMs and BMD. In 520 postmenopausal women correlation between BTMs and BMD is given in Table 5. All markers correlated with BMD in lumbar spine, femoral neck, and total hip ($\text{Beta}_{\text{std}} = -0.155 \sim -0.220$, $P < 0.001$). When adjusted for age, height, body weight, and YSM, all the three OC, β-CTX, and P1NP also correlated with BMD ($\text{Beta}_{\text{std}} = -0.157 \sim -0.217$, $P < 0.001$).

4. Discussion

It is important to establish reference intervals for BTMs from a sample of the healthy young population to use such markers to correctly assess bone turnover in subjects of various ages. In this study, we chose women aged 35–45 yrs for reference interval calculations because these women have achieved peak bone mass and are not yet perimenopausal. Women under 30 yrs of age were excluded to reduce the chance of including women with elevated bone turnover due to skeletal immaturity. In addition, women over the age of 45 were excluded because several studies have demonstrated that BTMs are increased in perimenopausal women because of the estrogen deficiency that occurs after spontaneous menopause in an increase in bone remodeling [16, 17]. It has been recommended that normal reference ranges of BTMs be established in large cohorts (approximately 100–200 subjects) of healthy 35- to 45-year-old premenopausal women with normal BMD [11]. The present study has provided the reference intervals of BTMs were in Chinese men; there are no previous studies of these markers in healthy men, and an age range for reference intervals has not been established. According to Tables 3 and 4, which presents the means for the BTMs within each 5 yr age group, we found that BTMs were stable in the group aged 35 to 45 yr.

In the present study, the age-related analysis of all subjects revealed a significant negative correlation between each of the bone turnover markers and age. In women, the bone formation markers OC and P1NP decreased with increasing age until the age of 44 before sharply increasing in the 50- to 59-year-old age group. The bone resorption marker β-CTX also decreased with increasing age until the age of 44 and abruptly increased in women > 50 yrs old. We found

Table 2: Medians, geometric means, and 95% reference intervals for the bone turnover markers in 35–45-year-old subjects.

Measurement	Women (n = 406)			Men (n = 226)		
	Median	Geometric mean	95% reference interval	Median	Geometric mean	95% reference interval
ALP (U/L)	53.00	54.80	29.55–82.04	63.00	63.87	35.52–94.16
OC (ng/mL)	13.90	15.05	4.91–22.31	16.57	18.01	5.58–28.62
P1NP (ng/mL)	32.90	35.22	13.72–58.67	42.43	44.01	16.89–65.49
β-CTX (ng/mL)	0.210	0.241	0.112–0.497	0.378	0.400	0.100–0.612
25(OH)D (ng/mL)	20.58	20.98	10.97–32.15	22.89	23.55	11.11–34.43
PTH (pg/mL)	35.02	37.30	15.52–66.78	30.39	33.92	14.61–63.22

Values were obtained from 35–45-year-old healthy men and women.

Table 3: Geometric means and 95% confidence intervals (95% CI) for the age-specific measurements of bone turnover markers in healthy women.

Age	Number	β–CTX (ng/mL)	P1NP (ng/mL)	OC (ng/mL)	PTH (pg/mL)
20–24	145	0.412* (0.354–0.470)	54.97* (47.87–62.06)	21.23* (18.72–22.47)	37.20 (35.07–39.33)
25–29	386	0.335* (0.304–0.367)	45.94* (41.38–50.49)	18.48* (17.19–19.77)	35.70 (34.29–37.10)
30–34	247	0.280* (0.230–0.313)	40.21* (35.78–44.63)	16.80* (15.33–18.26)	36.45 (34.76–38.13)
35–39	210	0.250 (0.221–0.284)	35.93 (31.72–40.13)	15.37 (13.95–16.78)	38.10 (35.99–40.21)
40–44	178	0.239 (0.201–0.271)	34.27 (30.50–38.04)	13.84 (12.48–15.27)	38.55 (36.17–40.94)
45–49	104	0.278* (0.221–0.310)	36.35* (31.10–41.58)	14.84* (12.88–16.79)	38.99 (36.09–41.89)
50–54	138	0.470* (0.399–0.541)	52.72* (45.54–56.90)	23.46* (20.44–26.46)	35.83 (33.58–38.08)
55–59	184	0.474* (0.407–0.540)	50.21* (43.85–56.57)	24.94* (22.19–27.68)	36.50 (34.76–38.24)
60–64	51	0.441* (0.307–0.584)	47.07* (34.78–59.36)	26.40* (20.28–32.60)	37.92 (32.71–43.13)
65–69	70	0.471* (0.356–0.585)	50.61* (38.11–63.11)	25.57* (22.90–28.22)	36.13 (32.72–39.55)
70–74	77	0.431* (0.297–0.565)	50.04* (37.94–62.13)	26.50* (20.82–32.17)	35.57 (30.94–38.21)
75–79	66	0.384* (0.284–0.482)	45.44* (35.60–54.47)	24.11* (18.85–29.37)	36.25 (29.70–38.81)
Reference value[a]	406	0.242 (0.218–0.297)	35.22 (30.67–40.10)	15.05 (13.81–16.59)	37.30 (33.18–40.92)

All values are presented as the geometric means (95% CI).
* $P < 0.05$ versus reference values.
[a]Reference values were calculated from the 35–45-year-old cohort as the geometric mean (95% CI).

that women aged 30 to 34 yrs had higher levels of β-CTX, OC, and P1NP, which may suggest that they had not yet reached skeletal maturity; therefore, we excluded this age group when establishing the reference intervals. Previous studies have found that bone turnover markers are negatively associated with age [10, 18]; however, these particular studies analyzed women aged 20 to 50 yrs and 20 to 40 yrs and therefore did not include postmenopausal women or elderly women. Glover et al. [19] reported that the median values of serum P1NP and β-CTX were lower than those reported as reference values in European premenopausal women. The measured reference values for P1NP in the present study were similar to those reported by de Papp et al. [14] in American premenopausal women and by Adami et al. [20] in Italian premenopausal women aged 35 to 45 yrs. In contrast, the reference values for β-CTX in our study were lower than those reported for American and Italian premenopausal women [14, 20]. Wu et al. [21] also examined serum β-CTX, serum bone ALP, and urinary BTMs in 289 premenopausal Chinese women aged 30–49 yrs; the reported means were 5.18 ng/mL for OC, 17.8 U/L for bone ALP, and 0.250 ng/mL for β-CTX [21]. Compared with Wu et al., we measured higher levels of OC. As measured in our current study, the OC levels in premenopausal (17.01 ng/mL) and postmenopausal women (24.34 ng/mL) were remarkably higher than those reported by Wu et al. (4.32 μg/L and 9.14 μg/L, resp.), whose study included 555 women aged 35 to 60 yrs [22]. The different results between the two studies were perhaps due to the season during which they collected blood (May to August). The P1NP level in the postmenopausal women of our population was close to that in a previous study (52.0 (10.5–492.7) ng/mL) in Beijing that included 1,724 postmenopausal women (aged 47 to 108 yr) [23]. However, the serum β-CTX level measured in our study was higher than that in the same previous study (0.407 (0.036–2.140) ng/mL) [23]. The dissimilar results from within the same country may be due to differences in the age range of the subjects used to calculate reference intervals, smoking habits, physical activities, or other factors affecting BTMs.

In the cohort of men we studied, age-related changes in BTMs were discovered. The bone formation markers OC and P1NP decreased with increasing age until the age of 59 and then abruptly increased in the 60- to 69-year-old group. The bone resorption marker β-CTX also decreased with increasing age until the age of 54 and then sharply increased in men > 60 yrs old. Elevated levels of bone markers in young men reflect the active bone turnover that occurs during the formation of peak BMD. We found that the age

Table 4: Geometric means and (95% CI) for the age-specific measurements of bone turnover markers in healthy men.

Age	Number	β-CTX (ng/mL)	P1NP (ng/mL)	OC (ng/mL)	PTH (pg/mL)
20–24	43	0.745* (0.464–1.024)	64.44* (41.81–87.05)	25.21* (17.57–28.86)	31.77 (25.61–39.94)
25–29	72	0.504* (0.415–0.551)	51.81* (43.42–60.20)	21.08* (18.12–24.06)	34.73 (31.14–38.33)
30–34	93	0.419* (0.353–0.482)	44.68* (38.25–51.10)	18.51* (15.97–21.05)	34.06 (30.46–37.67)
35–39	102	0.402 (0.333–0.470)	44.22 (37.64–57.01)	18.34 (15.66–21.02)	35.51 (32.40–38.62)
40–44	117	0.402 (0.318–0.485)	43.98 (36.78–51.78)	17.85 (14.89–20.79)	35.09 (31.89–38.29)
45–49	45	0.340* (0.252–0.428)	36.62* (29.37–43.88)	16.02* (11.97–20.05)	30.01 (25.92–34.20)
50–54	53	0.342* (0.267–0.414)	39.59* (32.23–46.93)	16.69* (13.72–19.65)	34.60 (30.86–38.35)
55–59	64	0.353* (0.278–0.427)	36.93* (30.62–43.23)	15.58* (13.07–18.09)	35.80 (31.57–40.02)
60–64	45	0.393* (0.276–0.508)	41.19* (31.63–50.13)	20.95* (16.32–25.66)	36.94* (31.27–42.60)
65–69	26	0.352* (0.218–0.485)	39.39* (27.48–50.87)	19.43* (12.28–25.51)	37.44* (30.58–44.31)
70–74	21	0.383* (0.167–0.598)	40.00* (26.65–53.33)	21.84* (15.95–27.73)	37.45* (30.52–44.38)
75–79	24	0.355* (0.208–0.501)	40.10* (25.60–54.69)	20.86* (14.72–27.00)	36.34* (29.98–42.70)
Reference value[a]	226	0.400 (0.310–0.479)	44.01 (37.27–52.97)	18.01 (14.91–20.97)	33.92 (30.21–38.17)

All values are presented as the geometric means (95% CI).
* $P < 0.05$ versus reference values.
[a]Reference values were calculated from the 35–45-year-old cohort as the geometric mean (95% CI).

Table 5: Standardized correlation coefficients ($Beta_{std}$) between BTMS and BMD in 520 postmenopausal women.

BTMs	L1-4 BMD		Femoral neck BMD		Total hip BMD	
	Not adjusted	Adjusted	Not adjusted	Adjusted	Not adjusted	Adjusted
β-CTX	−0.155**	−0.157**	−0.188**	−0.182**	−0.207**	−0.195**
P1NP	−0.197**	−0.201**	−0.169**	−0.193**	−0.220**	−0.217**
OC	−0.191**	−0.188**	−0.178**	−0.176**	−0.209**	−0.199**

Data adjusted age, height, body weight, and YSM are given.
** $P < 0.01$.

at which BTMs decreased in men was about 10 yrs later than in women. This finding is consistent with the fact that the age of onset of osteoporosis in men is 10 yrs later than in women, perhaps because women finish growing and achieve peak BMD earlier than men [24]. The results of a study by Szulc et al. were similar to ours: before 25 yrs of age, markers of bone formation (OC, BAP, and P1NP) were high, but they decreased and reached their lowest levels at the age of 56–60 yrs [25]. To date, most studies have found a decrease in BTMs in men until the age of 50–60 yrs [26]. However, the number of young men studied was small and the age range investigated began after peak values were achieved [27].

Previous reports have indicated that 25(OH)D levels are negatively correlated with serum PTH, OC, P1NP, and β-CTX in women and men [28, 29]. These reports noted that patients with 25(OH)D insufficiency exhibit higher levels of bone turnover than those with sufficient 25(OH)D levels [29, 30]. However, most studies were carried out in elderly osteoporotic women or less cared for young men and women. Vitamin D insufficiency is associated with high levels of BTMs (including OC, β-CTX, P1NP, and PTH) in young individuals, so we also examined the 25(OH)D levels in our 35- to 45-year-old subjects to avoid its effect on BTMs. We found that the level of 25(OH)D was 20.58 ng/mL in women aged 35–45 yrs and 22.89 ng/mL in men of the same age. No differences were found in 25(OH)D levels between the two groups. The levels of 25(OH)D in 35- to 45-yr-old subjects reflected vitamin D status in healthy Chinese adults.

The study found that OC, β-CTX, and P1NP and the BMD of lumbar spine, femoral neck, and total hip were significantly negatively correlated. The effects remained after being adjusted for age, height, weight, and YSM. After menopause, an increased bone turnover is related to bone loss by an imbalance between bone formation and bone resorption. A number of studies have found significant relationship between BTMs and bone mass [31, 32]. In contrast, Kawana et al. [33] failed to report significant correlations between ultrasound parameters and biochemical markers of bone remodeling (osteocalcin, Pyr, and D-Pyr) in postmenopausal women aged 48–57 years. Several studies have indicated a potential relationship between BTMs and QUS in postmenopausal women [34]. This means that the combination of bone resorption and bone formation markers is a good predictor for loss of bone mass.

Our study has the following strengths: (1) the sample size was large enough (greater than 600 men and 1,000 women) to avoid sample error; (2) the random selection of women from the local population, thereby covering a wide age-range and being representative of the Chinese population; and (3) the participants were well characterized and the exclusion criteria were detailed and evidence based to ensure a more precisely characterized sample population. Thus, numerous

factors to minimize biological variability were considered and controlled.

Certain limitations of this study must also be acknowledged. First, we did not measure BMD in 2,799 subjects (790 males and 2,009 females). Second, the purpose of this study was to establish the reference intervals for BTMs, for which we analyzed a sufficient number of young men and women; however, compared with the number of young subjects, the sample size of elderly subjects was not adequate, particularly when examining elderly men. Finally, the cross-sectional nature of the study design is a limitation.

5. Conclusion

The present study provides reference interval values for BTMs in healthy men and women. These reference ranges are based on data showing that BTMs are stable between the ages of 35 and 45 yrs. These results will contribute to the appropriate assessment of bone turnover in Shanghai area population and offer a comparison to measurements obtained in other populations. This study also found that BTMs correlated with BMD in Chinese postmenopausal women and suggested that BTMs were the key determining factors of early BMD decreases.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Acknowledgments

The authors acknowledge the excellent assistances of Yao-Hua Ke and Wen-Zhen Fu with blood extraction and analysis. This study was supported by the National Science Foundation of China (NSFC) (Grants nos. 81170803, 30800387, 81070692, and 81000360), the Program of Shanghai Chief Scientists (Project no.: 08XD1403000), STCSM10DZ1950100, and Academic Leaders in Health Sciences in Shanghai (XBR 2011014). The authors would like to thank all of the patients who participated in this study.

References

[1] P. Garnero, E. Sornay-Rendu, B. Claustrat, and P. D. Delmas, "Biochemical markers of bone turnover, endogenous hormones and the risk of fractures in postmenopausal women: the OFELY study," *Journal of Bone and Mineral Research*, vol. 15, no. 8, pp. 1526–1536, 2000.

[2] S. C. E. Schuit, M. van der Klift, A. E. A. M. Weel et al., "Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study," *Bone*, vol. 34, no. 1, pp. 195–202, 2004.

[3] S. A. Wainwright, L. M. Marshall, K. E. Ensrud et al., "Hip fracture in women without osteoporosis," *Journal of Clinical Endocrinology and Metabolism*, vol. 90, no. 5, pp. 2787–2793, 2005.

[4] S. C. C. M. van Coeverden, J. C. Netelenbos, C. M. de Ridder, J. C. Roos, C. Popp-Snijders, and H. A. Delemarre-van De Waal, "Bone metabolism markers and bone mass in healthy pubertal boys and girls," *Clinical Endocrinology*, vol. 57, no. 1, pp. 107–116, 2002.

[5] J. Léger, I. Mercat, C. Alberti et al., "The relationship between the GH/IGF-I axis and serum markers of bone turnover metabolism in healthy children," *European Journal of Endocrinology*, vol. 157, no. 5, pp. 685–692, 2007.

[6] D. Yilmaz, B. Ersoy, E. Bilgin, G. Gümüşer, E. Onur, and E. D. Pinar, "Bone mineral density in girls and boys at different pubertal stages: relation with gonadal steroids, bone formation markers, and growth parameters," *Journal of Bone and Mineral Metabolism*, vol. 23, no. 6, pp. 476–482, 2005.

[7] R. Eastell, I. Barton, R. A. Hannon, A. Chines, P. Garnero, and P. D. Delmas, "Relationship of early changes in bone resorption to the reduction in fracture risk with risedronate," *Journal of Bone and Mineral Research*, vol. 18, no. 6, pp. 1051–1056, 2003.

[8] P. Gerdhem, K. K. Ivaska, S. L. Alatalo et al., "Biochemical markers of bone metabolism and prediction of fracture in elderly women," *Journal of Bone and Mineral Research*, vol. 19, no. 3, pp. 386–393, 2004.

[9] P. Garnero, E. Hausherr, M. C. Chapuy et al., "Markers of bone resorption predict hip fracture in elderly women: the EPIDOS prospective study," *Journal of Bone and Mineral Research*, vol. 11, no. 10, pp. 1531–1538, 1996.

[10] L. J. Melton III, S. Khosla, E. J. Atkinson, W. M. O'Fallon, and B. L. Riggs, "Relationship of bone turnover to bone density and fractures," *Journal of Bone and Mineral Research*, vol. 12, no. 7, pp. 1083–1091, 1997.

[11] P. D. Delmas, R. Eastell, P. Garnero, M. J. Seibel, and J. Stepan, "The use of biochemical markets of bone turnover in osteoporosis," *Osteoporosis International*, vol. 11, supplement 6, pp. S2–S17, 2000.

[12] X. P. Wu, E. Y. Liao, G. Huang, R. C. Dai, and H. Zhang, "A comparison study of the reference curves of bone mineral density at different skeletal sites in native Chinese, Japanese, and American Caucasian women," *Calcified Tissue International*, vol. 73, no. 2, pp. 122–132, 2003.

[13] M. S. M. Ardawi, A. A. Maimani, T. A. Bahksh, A. A. Rouzi, M. H. Qari, and R. M. Raddadi, "Reference intervals of biochemical bone turnover markers for Saudi Arabian women: a cross-sectional study," *Bone*, vol. 47, no. 4, pp. 804–814, 2010.

[14] A. E. de Papp, H. G. Bone, M. P. Caulfield et al., "A cross-sectional study of bone turnover markers in healthy premenopausal women," *Bone*, vol. 40, no. 5, pp. 1222–1230, 2007.

[15] S. J. Glover, P. Garnero, K. Naylor, A. Rogers, and R. Eastell, "Establishing a reference range for bone turnover markers in young, healthy women," *Bone*, vol. 42, no. 4, pp. 623–630, 2008.

[16] P. R. Ebeling, L. M. Atley, J. R. Guthrie et al., "Bone turnover markers and bone density across the menopausal transition," *Journal of Clinical Endocrinology and Metabolism*, vol. 81, no. 9, pp. 3366–3371, 1996.

[17] H. Hoshino, K. Kushida, M. Takahashi et al., "Changes in levels of biochemical markers and ultrasound indices of os calcis across the menopausal transition," *Osteoporosis International*, vol. 11, no. 2, pp. 128–133, 2000.

[18] K. M. Chiu, J. Ju, D. Mayes, P. Bacchetti, S. Weitz, and C. D. Arnaud, "Changes in bone resorption during the menstrual cycle," *Journal of Bone and Mineral Research*, vol. 14, no. 4, pp. 609–615, 1999.

[19] S. J. Glover, M. Gall, O. Schoenborn-Kellenberger et al., "Establishing a reference interval for bone turnover markers in 637 healthy, young, premenopausal women from the United

Kingdom, France, Belgium, and the United States," *Journal of Bone and Mineral Research*, vol. 24, no. 3, pp. 389–397, 2009.

[20] S. Adami, G. Bianchi, M. L. Brandi et al., "Determinants of bone turnover markers in healthy premenopausal women," *Calcified Tissue International*, vol. 82, no. 5, pp. 341–347, 2008.

[21] X. Y. Wu, X. P. Wu, H. Xie et al., "Age-related changes in biochemical markers of bone turnover and gonadotropin levels and their relationship among Chinese adult women," *Osteoporosis International*, vol. 21, no. 2, pp. 275–285, 2010.

[22] X. W. Zhou, X. Y. Wu, L. Luo et al., "The relationship between bone turnover markers and BMD decreasing rates in Chinese middle-aged women," *Clinica Chimica Acta*, vol. 412, no. 17-18, pp. 1648–1657, 2011.

[23] J. Zhao, W. Xia, M. Nie et al., "The levels of bone turnover markers in Chinese postmenopausal women: peking vertebral fracture study," *Menopause*, vol. 18, no. 11, pp. 1237–1243, 2011.

[24] C. M. Gundberg, A. C. Looker, S. D. Nieman, and M. S. Calvo, "Patterns of osteocalcin and bone specific alkaline phosphatase by age, gender, and race or ethnicity," *Bone*, vol. 31, no. 6, pp. 703–708, 2002.

[25] P. Szulc, P. Garnero, F. Munoz, F. Marchand, and P. D. Delmas, "Cross-sectional evaluation of bone metabolism in men," *Journal of Bone and Mineral Research*, vol. 16, no. 9, pp. 1642–1650, 2001.

[26] J. M. Wishart, A. G. Need, M. Horowitz, H. A. Morris, and B. E. C. Nordin, "Effect of age on bone density and bone turnover in men," *Clinical Endocrinology*, vol. 42, no. 2, pp. 141–146, 1995.

[27] D. A. Hanson, M. A. E. Weis, A. M. Bollen, S. L. Maslan, F. R. Singer, and D. R. Eyre, "A specific immunoassay for monitoring human bone resorption: quantitation of type I collagen cross-linked N-telopeptides in urine," *Journal of Bone and Mineral Research*, vol. 7, no. 11, pp. 1251–1258, 1992.

[28] O. Sahota, T. Masud, P. San, and D. J. Hosking, "Vitamin D insufficiency increases bone turnover markers and enhances bone loss at the hip in patients with established vertebral osteoporosis," *Clinical Endocrinology*, vol. 51, no. 2, pp. 217–221, 1999.

[29] S. H. Scharla, C. Scheidt-Nave, G. Leidig et al., "Lower serum 25-hydroxyvitamin D is associated with increased bone resorption markers and lower bone density at the proximal femur in normal females: a population-based study," *Experimental and Clinical Endocrinology and Diabetes*, vol. 104, no. 3, pp. 289–292, 1996.

[30] M. Brazier, S. Kamel, M. Maamer et al., "Markers of bone remodeling in the elderly subject: effects of vitamin D insufficiency and its correction," *Journal of Bone and Mineral Research*, vol. 10, no. 11, pp. 1753–1761, 1995.

[31] P. Garnero, E. Hausherr, M. C. Chapuy et al., "Markers of bone resorption predict hip fracture in elderly women: the EPIDOS prospective study," *Journal of Bone and Mineral Research*, vol. 11, no. 10, pp. 1531–1538, 1996.

[32] L. J. Melton, S. Khosla, E. J. Atkinson, W. M. O'Fallon, and B. L. Riggs, "Relationship of bone turnover to bone density and fractures," *Journal of Bone and Mineral Research*, vol. 12, no. 7, pp. 1083–1091, 1997.

[33] K. Kawana, K. Kushida, M. Takahashi et al., "The effect of menopause on biochemical markers and ultrasound densitometry in healthy females," *Calcified Tissue International*, vol. 55, no. 6, pp. 420–425, 1994.

[34] H. Hoshino, K. Kushida, M. Takahashi et al., "Changes in levels of biochemical markers and ultrasound indices of os calcis across the menopausal transition," *Osteoporosis International*, vol. 11, no. 2, pp. 128–133, 2000.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 727164, 8 pages
http://dx.doi.org/10.1155/2013/727164

Research Article

Relationship between Serum Levels of OPG and TGF-β with Decreasing Rate of BMD in Native Chinese Women

Xi-Yu Wu, Yi-Qun Peng, Hong Zhang, Hui Xie, Zhi-Feng Sheng, Xiang-Hang Luo, Ru-Chun Dai, Hou-De Zhou, Xian-Ping Wu, and Er-Yuan Liao

Institute of Metabolism and Endocrinology, The Second Xiangya Hospital of Central South University, 139 Renmin-Zhong Road, Changsha, Hunan 410011, China

Correspondence should be addressed to Xian-Ping Wu; wuxp39@sohu.com and Er-Yuan Liao; eyliao1207@21cn.com

Received 27 October 2012; Revised 30 December 2012; Accepted 2 January 2013

Academic Editor: Guang-Da Xiang

The objective of this study was to investigate the relationship between serum levels of OPG, TGF-β1, and TGF-β2 and BMD decrease rate (BDR) in native Chinese women. This cross-sectional study was performed on 465 healthy native Chinese women aged 35–80 years. Serum levels of OPG, TGF-β1, and TGF-β2 were determined. BDR was measured by DXA at the posteroanterior spine, hip, and distal forearm. At all skeletal sites tested, there was a negative correlation between BDR and serum levels of both OPG ($r = -0.122$ to -0.230, all $P = 0.007$–0.000) and TGF-β2 ($r = -0.100$ to -0.173, all $P = 0.029$–0.000) and a positive correlation between BDR and serum TGF-β1 ($r = 0.245 - 0.365$, all $P = 0.000$). After adjustment for age and BMI, there were no statistically significant correlations between serum levels of OPG or TGF-β2 and BDR. However, statistically significant correlations between serum TGF-β1 and BDR at the lumbar spine and ultradistal forearm remained. Multiple linear regression stepwise analysis showed that serum OPG could explain 1.4–3.7% of BDR variation. Serum TGF-β1 was a positive determinant of BDR and could explain 5.3–13.3% of BDR variation.

1. Introduction

Osteoprotegerin (OPG), transforming growth factor-β1 (TGF-β1) and TGF-β2 are cytokines closely associated with bone metabolism [1, 2]. OPG is one of the key cytokines secreted by osteoblasts and plays an important role in the bone remodeling balance [3]. OPG knockout can result in severe osteoporosis in mice [4], and overexpression of OPG can lead to osteopetrosis in OPG-transgenic mice [2, 5]. In postmenopausal women, the circulating OPG level affects bone turnover, changes in bone mass, and the prevalence of vertebral fractures [6, 7]. TGF-β is very rich in bone tissue [8, 9]. The ratio of TGF-β1 and TGF-β2 is about 4 : 1, and there is 70% sequence homology between the two forms [10]. TGF-β could not only promote osteoblasts proliferation and differentiation [11, 12], but also inhibit osteoclasts activity [13, 14]. TGF-β1 gene knockout cause osteopenia in mice [15], while the mice with osteoporosis treated by TGF-β could increase BMD [16, 17]. TGF-β1 gene mutation can lead to low bone mass in human [18]. The studies demonstrated that both TGF-β1 and TGF-β2 stimulate bone formation and bone mineralization [19–21], but TGF-β2 was more active than TGF-β1 in stimulating formation of a mass [19]. *In vivo*, TGF-β2 stimulated synthesis of TGF-β1 in chondrocytes and osteoblasts [19]. TGF-beta may act as a bone-coupling factor linking bone resorption to bone formation [10]. TGF-β is expressed by osteoblasts; it not only stimulates the differentiation of osteoclasts and maintains their survival [22], but also regulates bone formation and resorption [23]. After menopause, serum TGF-β2 is increased in women with osteoporosis and shows a positive correlation with bone resorption markers [24].

Bone mineral density (BMD) decrease rate (BDR) [25] is an important parameter measured by dual-energy X-ray absorptiometry (DXA). When we used the Hologic DXA bone densitometer to measure BMD; BDR can be calculated by a peak reference (PR%) value in measurement report. Using the GE-Lunar DXA bone densitometer, the young adult mean (YAM%) represents the BMD. BDR means that compared to the peak BMD in reference databases, the

percentage of BMD reducing or bone loss percentage in subjects. BDR is an important index closely related to the diagnosis of osteoporosis in Chinese [26] and Japanese [27]. The relationship between the cytokines OPG, TGF-β1 and TGF-β2 and BDR in native Chinese women remains unknown. To investigate this relationship, DXA was used to measure both BMD and BDR at the lumbar spine, proximal femur, and distal forearm in 465 healthy native Chinese women aged 35–80 years. Serum levels of OPG, TGF-β1, and TGF-β2 were also determined, and their relationships with BDR were analyzed.

2. Material and Methods

2.1. Study Participants. Four hundred and sixty-five healthy Chinese women aged 35–80 years were randomly selected between September 2007 and May 2010. These volunteers, all residents of Changsha and surrounding regions, were recruited by public health organizations (i.e., health stations/clinics) that provide health care for local residents. All subjects were screened by a detailed questionnaire, history, and physical examination. Women were excluded from the study if they had a condition affecting bone metabolism such as disease of the kidney, liver, parathyroid or thyroid, diabetes mellitus, oligomenorrhea or menopause at <40 years, hyperprolactinemia, oophorectomy, rheumatoid arthritis, ankylosing spondylitis, a malabsorption syndrome, a malignant tumor, hematologic disease or previous pathological fracture. Subjects were also excluded if they had been receiving glucocorticoids, estrogens, thyroid hormone, fluoride, bisphosphonate, calcitonin, thiazide diuretics, barbiturates, antiseizure medications, vitamin D- or calcium-containing drugs, as were those who smoked or consumed alcohol or caffeine. The study involved 142 premenopausal women, 58 perimenopausal women (last menses <12 months before the study started) and 265 postmenopausal women (last menses >12 months before the study started); of the latter, the mean (± SD) age at menopause was 48.3 ± 3.83 years (range 41–57 years) and the median duration of menopause was 11.0 years (range 1–40 years). The study was approved of the Ethical Committee of Xiangya Medical College, Central South University, China, and all participants provided written consent to participate.

2.2. Markers Measurement. Fasting morning (7–9 AM) blood samples were collected and centrifuged within 1 h and stored at −70°C until analysis. We measured serums TGF-β1 and TGF-β2 concentrations with a sensitive enzyme-linked immunosorbent assay (ELISA) kit (DRG International, Inc., Highway, Mountainside, NJ, USA) and OPG with an ELISA kit (Biomedica Gruppe, Vienna, Austria) and quantified the results using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT, USA). The minimum detectable concentration was 0.14 pmol/L for OPG, 0.002 μg/L for TGF-β1 and 0.01 μg/L for TGF-β2. The intra- and interassay CVs were 6.4% and 8.2% for OPG, 3.8% and 8.8% for TGF-β1, and 5.9% and 8.9% for TGF-β2. BMD and BDR were measured with a DXA fan-beam bone densitometer (Hologic Delphi A; Hologic, Bedford, MA, USA) at the posteroanterior (PA) spine (L1–L4); the left hip, including the femoral neck (FN) and total hip; and the radius + ulna ultradistal (RUUD) of the nondominant forearm. The *in vivo* deviations in precision of two repeated BMD measurements in 33 subjects, determined by the root mean square coefficient of variation method [28], were 0.83% for the PA spine, 1.88% for the FN, 0.88% for the total hip, and 2.21% for the RUUD. A control spine phantom scan performed each day had a long-term (>13 years) coefficient of variation (CV) of <0.45%.

2.3. Statistical Analysis. All calculations were performed using SPSS V17.0 for Windows software (SPSS, Inc., Chicago, IL, USA). The geometric mean and SD were used for serums OPG, TGF-β1, and TGF-β2 because these did not follow a logarithmic normal distribution. All subjects were stratified by 10-year age groups, and serums OPG, TGF-β1, and TGF-β2, and BDR at the various skeletal sites were reported as the mean ± SD for each group. The mean values of the different parameters from each group were compared for significant differences and assessed using one-way analysis of variance whenever significant. The relationship between serums OPG, TGF-β1, and TGF-β2 with BDR at the various skeletal sites was evaluated by linear regression and Pearson's correlation analysis. BDR was calculated using the formula BDR(%) = subjects' PR(%) − 100% [25]. Peak BMD was calculated from the BMD reference database previously established and continuously improved by us [29]. Multiple linear regression was used to determine the influence of OPG, TGF-β1, and TGF-β2 on BDR.

3. Results

3.1. Basic Characteristics of the Subjects. The characteristics of the subjects are summarized in Table 1. Height and TGF-β1 were significantly higher in the premenopausal period than in the perimenopausal period and after menopause, whereas serum levels of OPG and TGF-β2 were markedly lower in the premenopausal period than in the perimenopausal period and after menopause. Height and weight were higher in the perimenopausal period than after menopause. Table 2 shows the serum levels of the age-related cytokines and the BDR at the various skeletal sites. From 35 to 44 years, serums OPG and TGF-β2 were at their lowest levels, whereas TGF-β1 was at its highest. From 45 to 54 years, serum OPG was at its highest level. Above 45 years, there were no significant differences in TGF-β1 or TGF-β2 between any age group. Compared with the 35–44-year group, there was a significant increasing trend of BDR with age in subjects above 45 years of age. In subjects aged ≥65 years, the mean BDR at the RUUD was −34.1%, which was markedly higher than that at the other skeletal sites.

3.2. Correlations between BDR and Cytokines. Figure 1 shows scatter plots and correlations between the cytokine levels and the BDR at the different skeletal sites. There were obvious negative correlations between serum levels of both OPG and TGF-β2 and BDR, and marked positive correlations between serum TGF-β1 and BDR. Table 3 shows Pearson's

Table 1: Demographics and clinical characteristics of the study subjects.

Factor measured	Premenopausal (n = 142)	Perimenopausal (n = 58)	Postmenopausal (n = 265)
Age (years)	40.8 ± 3.50	48.0 ± 2.95	59.7 ± 7.30
Height (cm)	156.2 ± 5.38[b]	154.2 ± 5.05[c]	151.9 ± 4.54
Weight (kg)	56.9 ± 7.38[c]	57.1 ± 8.03[c]	54.3 ± 8.46
BMI (kg/m^2)	23.3 ± 2.68	24.0 ± 3.03	23.5 ± 3.34
OPG (pmol/L)[a]	2.67 ± 1.86[b]	4.11 ± 1.96	4.40 ± 1.85
TGF-β1 (μg/L)[a]	39.0 ± 1.60[b]	27.1 ± 1.67	24.0 ± 1.52
TGF-β2 (μg/L)[a]	11.5 ± 1.28[b]	13.0 ± 1.36	14.3 ± 1.32

BMI: body mass index, OPG: osteoprotegerin, and TGF-β: transforming growth factor-beta.
[a]Values are geometric mean ± SD.
[b]P = 0.045–0.000 compared with perimenopausal and postmenopausal.
[c]P = 0.002–0.000 compared with postmenopausal.

Table 2: Age-related serums OPG, TGF-β1, and TGF-β2 and BDR at different skeletal sites in native Chinese women.

Parameter	Age (year)			
	35–44 (n = 128)	45–54 (n = 146)	55–64 (n = 117)	≥65 (n = 74)
OPG (pmol/L)[a]	2.61 ± 1.88[b]	4.76 ± 1.84[c]	4.09 ± 1.87	3.77 ± 1.88
TGF-β1 (μg/L)[a]	38.9 ± 1.64[b]	25.2 ± 1.63	25.0 ± 1.52	24.9 ± 1.44
TGF-β2 (μg/L)[a]	11.9 ± 1.32[b]	14.2 ± 1.33	14.0 ± 1.34	13.6 ± 1.31
PA-BDR (%)	−1.72 ± 11.3[b]	−13.1 ± 13.8[c]	−23.6 ± 10.8	−26.3 ± 11.4
FN-BDR (%)	−3.07 ± 12.5[b]	−10.2 ± 14.5[c]	−21.1 ± 11.1[d]	−27.4 ± 9.85
Hip-BDR (%)	−2.34 ± 12.2[b]	−9.96 ± 13.7[c]	−18.6 ± 10.8[d]	−25.0 ± 10.1
RUUD-BDR (%)	−1.02 ± 9.62[b]	−9.61 ± 13.6[c]	−24.1 ± 10.9[d]	−34.1 ± 10.3[e]

OPG: osteoprotegerin, TGF-β: transforming growth factor-beta, BDR: bone mineral density decrease rate, PA: posteroanterior spine, FN: femoral neck, Hip: total hip, and RUUD: radius + ulna ultradistal.
[a]Values are geometric mean ± SD.
[b]P = 0.012–0.000 compared with 45–54, 55–64 and ≥65-year age groups.
[c]P = 0.049–0.000 compared with 55–64 and ≥65-year age groups.
[d]P = 0.011–0.000 compared with ≥65-year age group.
[e]P = 0.003–0.000 compared with other sites in the same age group.

correlation coefficients and partial correlation coefficients for the cytokines and BDRs at the different skeletal sites. There were notable positive correlations between serums OPG and TGF-β2, and marked negative correlations between serum TGF-β1 and serum levels of both OPG and TGF-β2. After controlling for age and body mass index (BMI), the partial correlation coefficients for both OPG and TGF-β2 with BDR were no longer statistically significant. However, the partial correlation coefficients for serum TGF-β1 with BDR at the PA spine and RUUD remained statistically significant.

3.3. Association between BDR and Cytokines. Figure 2 display comparisons between the cytokines. When serum OPG was grouped by quartile, the BDRs at the PA spine, hip, and RUUD in Q1 and Q2 were significantly higher than those in Q3 and Q4. At the FN, the mean BDR was lowest in Q3 and markedly lower than in Q1 and Q2. When serum TGF-β1 was grouped by quartile, the BDR in Q4 was notably higher than that in Q1, Q2, and Q3. The BDRs in Q1 and Q2 were lower, but there were no significant differences between them. When serum TGF-β2 was grouped according to quartile, the BDR was maximal in Q1, markedly higher than in Q2, Q3, and Q4.

Using serum levels of OPG, TGF-β1, and TGF-β2 as independent variables and the BDRs at the different skeletal sites as dependent variables, multiple linear regression stepwise analysis was conducted (Table 4). The results show that OPG could explain 1.4–3.7% of the variation in BDR at each skeletal sites. The influence of OPG on BDR was lowest at the FN (1.4%) and greatest at the RUUD (3.7%). TGF-β1 was a positive determinant of BDR at each skeletal site, explaining about 5.3–13.3% of BDR variation. The influence of TGF-β1 was lowest at the FN (5.3%) and greatest at the PA spine (13.3%). TGF-β2 had no influence on BDR at any skeletal site according to this analysis.

4. Discussion

Our research confirmed the presence of marked negative correlations between serum levels of both OPG and TGF-β2 and BDR in native Chinese women; thus, the BDR was lower with

Table 3: Correlation coefficients (r) for serums OPG, TGF-β1, and TGF-β2 with BDR at various skeletal sites in native Chinese women.

Marker	OPG		TGF-β1		TGF-β2	
	r	P-r	r	P-r	r	P-r
TGF-β1	−0.270[a]	−0.215[a]				
TGF-β2	0.433[a]	0.399[a]	−0.237[a]	−0.172[a]		
PA-BDR	−0.230[a]	−0.044	0.365[a]	0.183[a]	−0.173[a]	−0.030
FN-BDR	−0.122[a]	0.066	0.245[a]	0.014	−0.110[a]	0.026
Hip-BDR	−0.148[a]	0.040	0.276[a]	0.080	−0.100[a]	0.040
RUUD-BDR	−0.205[a]	−0.024	0.344[a]	0.104[a]	−0.145[a]	0.020

Pearson's correlation coefficients (r) and partial correlation coefficients (P-r) after adjustment for age and body mass index are shown.
OPG: osteoprotegerin, TGF-β: transforming growth factor-beta, BDR: bone mineral density decrease rate, PA: posteroanterior spine, FN: femoral neck, Hip: total hip, and RUUD: radius + ulna ultradistal.
[a] $P = 0.036$–0.000.

Table 4: Multiple linear regression analysis of OPG, TGF-β1, and TGF-β2 with BDR at various skeletal sites in native Chinese women.

Dependent	OPG		TGF-β1		TGF-β2	
	β	R^2C (%)	β	R^2C (%)	β	R^2C (%)
PA-BDR	−0.179[a]	2.9	0.309[a]	13.3	—[b]	—[b]
FN-BDR	−0.127[a]	1.4	0.190[a]	5.3	—[b]	—[b]
Hip-BDR	−0.138[a]	1.7	0.229[a]	7.4	—[b]	—[b]
RUUD-BDR	−0.202[a]	3.7	0.266[a]	10.8	—[b]	—[b]

Serums OPG, TGF-β1, and TGF-β2 were independent variables; BDRs were dependent variables.
OPG: osteoprotegerin, TGF-β: transforming growth factor-beta, BDR: bone mineral density decrease rate, R^2C: R square change, PA: posteroanterior spine, FN: femoral neck, Hip: total hip, and RUUD: radius + ulna ultradistal.
[a] $P = 0.012$–0.000.
[b] Independent was excluded in this analysis.

higher circulating levels of OPG or TGF-β2 and higher with lower levels of these cytokines. There was a notably positive correlation between serum TGF-β1 and BDR, indicating that the BDR was higher with higher circulating levels of TGF-β1 and lower with lower levels of this cytokine. The partial correlation coefficients for OPG and TGF-β2 levels with BDR were insignificant at all skeletal sites, suggesting that these correlations are affected by both age and BMI and weaken or disappear when these influences are excluded. The partial correlation coefficients for TGF-β1 and BDR at the PA spine and RUUD remained statistically significant, demonstrating that, though the correlations between TGF-β1 and BDR at these skeletal sites were affected by both age and BMI, they remained close. These findings also imply that the correlation between circulating TGF-β1 and BDR differed between the various skeletal sites.

The results illustrate that the serum levels of OPG were the highest in women aged 45–54 years because they are in the rapid bone loss period of early postmenopause (the average age of menopause is 48.3 ± 3.83 years in this group) (Table 2). The increasing serum levels of OPG may be a compensatory defense mechanism for resistance to rapid bone loss [30]. Previous research on the general population has shown that, after menopause, increased serum OPG is related to increased risks for osteoporosis and vertebral fracture in women [6]. However, Ueland et al. [31] found no correlations between OPG genetic polymorphisms or changes in serum OPG and morbidity from osteoporosis in elderly Australian women. Another study showed that serum OPG in women was positively correlated with bone turnover markers including TRACP-5b, osteocalcin, and C-terminal cross-linked telopeptide [32]. The authors suggest that circulating OPG might help to prevent bone mass loss in women. After menopause, the bone mass loss rate might be lower in women with higher OPG levels, thereby increasing the strength of the hip [33]. A longitudinal study with a large sample size found that bone loss rate in women was related to circulating OPG levels; similar to the results of our study, the higher the serum OPG, the greater the bone loss rate [34]. The main reason for inconsistent results demonstrated from different research groups may be related to the difference of race, age, and the sample quantity of subjects. Other studies have demonstrated that, in patients with hyperthyroidism [35] or enteritis [36], increased serum OPG might prevent excessive bone mass loss. Considering the finding that serum OPG is significantly decreased in adipose women in the perimenopausal period [37], the authors suggested that circulating OPG might have no protective effect on bone mass loss in these patients. Research on large samples of the general female population verified that common TGF-β1 genetic polymorphisms had no influence on various bone turnover markers, BMD, or bone mass loss [38]. Nonetheless, many studies have shown that changes of both TGF-β1 [18, 39–44] and TGF-β2 [24] are related to bone turnover velocity. For instance, serum TGF-β1 was increased and bone loss rate decreased in women with the TT genotype of the TGF-β1 gene [39], while the

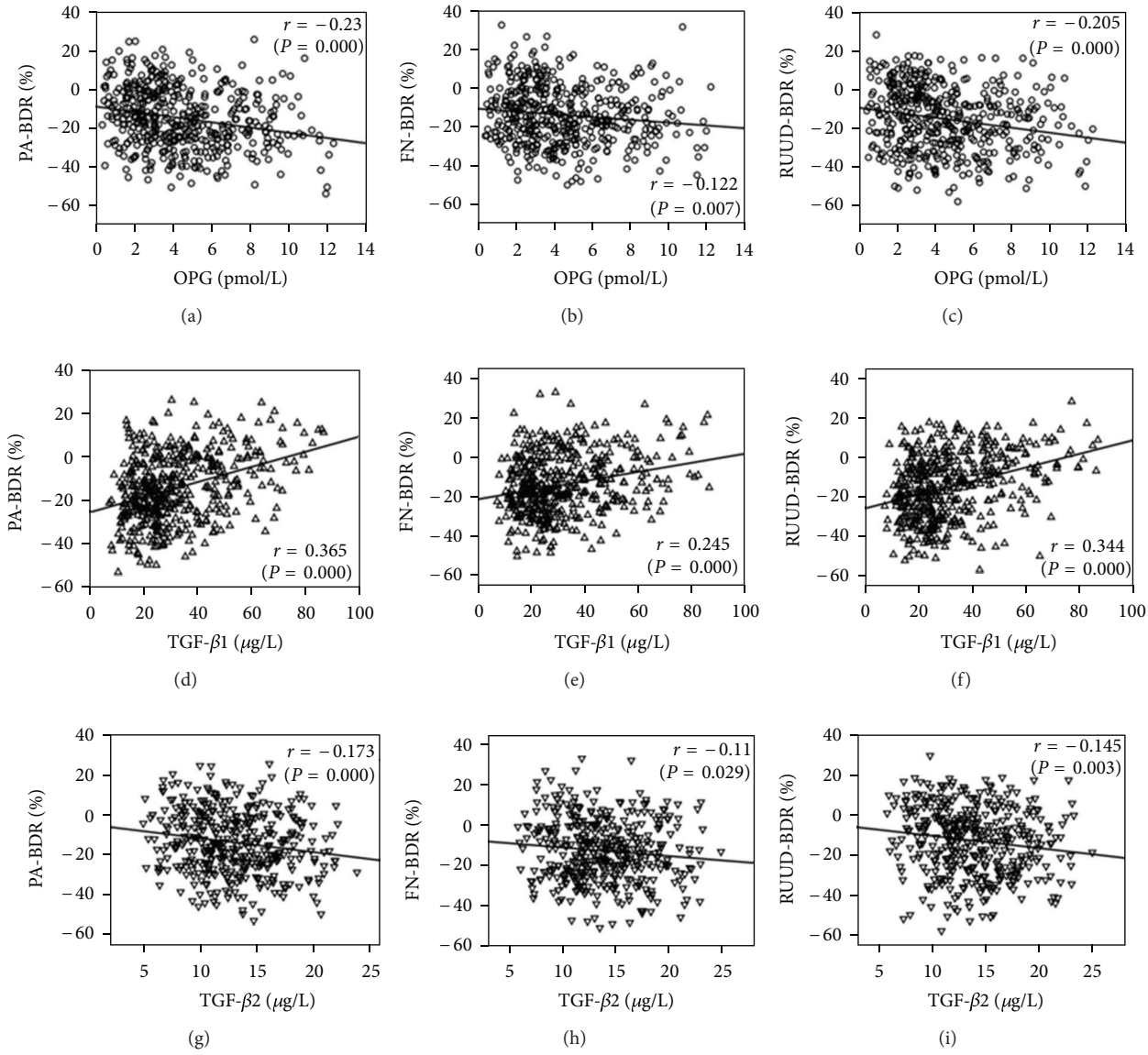


Figure 1: Scatter plots of serums OPG, TGF-β1, and TGF-β2 concentration versus BDR at various skeletal sites in native Chinese women. OPG: osteoprotegerin, TGF-β: transforming growth factor-beta, BDR: bone mineral density decrease rate, PA: posteroanterior spine, FN: femoral neck, and RUUD: radius + ulna ultradistal.

prevalence of brittle fracture was increased in women with the TC genotype [40]. Early studies demonstrated that TGF-β1 is a downstream factor of estrogen [13] and that TGF-β1 is involved in the vitamin D signaling pathway [45], which plays an important role in the local regulation of bone metabolism. New research suggests that TGF-β1 genetic polymorphism is associated with vitamin D and has an important effect on the incidence of osteoporotic vertebral fracture after menopause [46].

Our research demonstrated obvious differences in BDR related to cytokine levels. For serum OPG (Figure 2), BDRs at the PA spine, hip, and RUUD were significantly higher in Q1 and Q2 than in Q3 and Q4. For serum TGF-β1, the pattern of BDR was opposite to that for OPG; namely, BDR was minimal in Q1 and Q2 (lower TGF-β1) and maximal in Q4 (higher TGF-β1) at every skeletal site. The BDR decrease with TGF-β1 level increase is similar to other research results [15]. In TGF-β1 knockout mice, the bone mass in tibia metaphysis decreased by 30%, and BMD and bone strength decreased markedly [15]. Furthermore, the regulation of TGF-β1 levels in bone cells exhibited biphasic characteristics. Low concentrations of TGF-β1 and TGF-β2 increased the RANKL/OPG ratio due to the upregulation of these proteins in the osteoblasts/stromal cells and increased the differentiation of osteoclasts [47]. However, opposite effects were observed in the presence of high concentrations of TGF-β1 and TGF-β2 [47]. Recent studies have demonstrated that TGF-β1 is mainly expressed by differentiated osteoblasts and that it is deposited in the bone matrix [48]. TGF-β2, in contrast, is mainly expressed by the precursors of osteoblasts. These

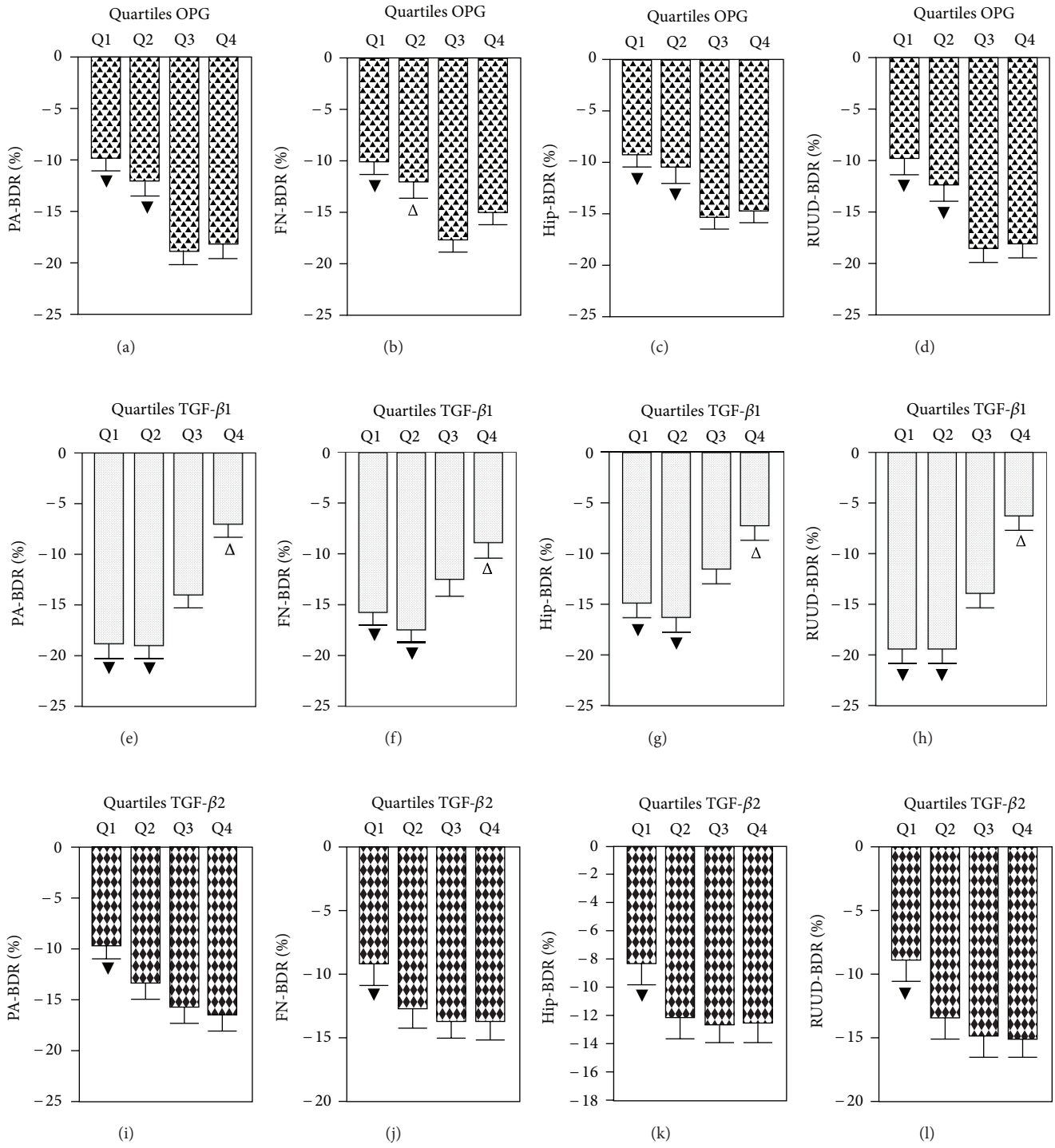


Figure 2: BDRs at different skeletal sites in native Chinese women displayed by quartiles of serums OPG, TGF-β1, and TGF-β2 concentrations. OPG: osteoprotegerin, TGF-β: transforming growth factor-beta, Q1: first quartile, Q2: second quartile, Q3: third quartile, Q4: fourth quartile, PA: posteroanterior spine, BDR: bone mineral density decreased rate, FN: femoral neck, Hip: total hip, and RUUD: radius + ulna ultradistal. $^{\blacktriangledown}P = 0.045$–$0.000$ compared with Q3 and Q4; $^{\Delta}P = 0.010$–0.000 compared with Q3.

findings suggest that BDR in native Chinese women might be affected by changes in the circulating levels of cytokines including OPG, TGF-β1, and TGF-β2 and variations between different parts of the skeleton. We still found that TGF-β1 was independent determinant of BDR in our study population. TGF-β1 level could explain of 5.3–13.3% of the BDR variation. The influence of TGF-β1 on BDR was 2.9–4.6 times than that of OPG, with the two having opposite effects.

5. Conclusions

This study investigated correlations between serum levels of OPG, TGF-β1, and TGF-β2 and BDR at various skeletal sites in native Chinese women, with results indicating that changes in circulating TGF-β1 and OPG are related to BDR. TGF-β1 was a positive determinant of BDR.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by the China National Natural Scientific Foundation (81070246). The authors thank Ms. Hong Liu, Ru-Yi Zhong and Xing- Zhi Cao and Dr. Peng-Fei Shan and Tuan-Yu Fang for their assistance.

References

[1] K. Janssens, P. Ten Dijke, S. Janssens, and W. Van Hul, "Transforming growth factor-β1 to the bone," *Endocrine Reviews*, vol. 26, no. 6, pp. 743–774, 2005.

[2] W. S. Simonet, D. L. Lacey, C. R. Dunstan et al., "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density," *Cell*, vol. 89, no. 2, pp. 309–319, 1997.

[3] L. C. Hofbauer, S. Khosla, C. R. Dunstan, D. L. Lacey, W. J. Boyle, and B. L. Riggs, "The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption," *Journal of Bone and Mineral Research*, vol. 15, no. 1, pp. 2–12, 2000.

[4] A. Mizuno, N. Amizuka, K. Irie et al., "Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin," *Biochemical and Biophysical Research Communications*, vol. 247, no. 3, pp. 610–615, 1998.

[5] H. Yasuda, N. Shima, N. Nakagawa et al., "Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 7, pp. 3597–3602, 1998.

[6] P. Mezquita-Raya, M. de la Higuera, D. F. García et al., "The contribution of serum osteoprotegerin to bone mass and vertebral fractures in postmenopausal women," *Osteoporosis International* , vol. 16, no. 11, pp. 1368–1374, 2005.

[7] A. Rogers, G. Saleh, R. A. Hannon, D. Greenfield, and R. Eastell, "Circulating estradiol and osteoprotegerin as determinants of bone turnover and bone density in postmenopausal women," *Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 10, pp. 4470–4475, 2002.

[8] Y. Ogawa and S. M. Seyedin, "Purification of transforming growth factors β1 and β2 from bovine bone and cell culture assays," *Methods in Enzymology*, vol. 198, pp. 317–327, 1991.

[9] B. H. Thorp, I. Anderson, and S. B. Jakowlew, "Transforming growth factor-B1, -β2 and -β3 in cartilage and bone cells during endochondral ossification in the chick," *Development*, vol. 114, no. 4, pp. 907–911, 1992.

[10] L. F. Bonewald and G. R. Mundy, "Role of transforming growth factor-beta in bone remodeling," *Clinical Orthopaedics and Related Research*, no. 250, pp. 261–276, 1990.

[11] S. Mohan and D. J. Baylink, "Bone growth factors," *Clinical Orthopaedics and Related Research*, no. 263, pp. 30–48, 1991.

[12] M. Centrella, S. Casinghino, R. Ignotz, and T. L. McCarthy, "Multiple regulatory effects by transforming growth factor-β on type I collagen levels in osteoblast-enriched cultures from fetal rat bone," *Endocrinology*, vol. 131, no. 6, pp. 2863–2872, 1992.

[13] D. E. Hughes, A. Dai, J. C. Tiffee, H. H. Li, G. R. Munoy, and B. F. Boyce, "Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-," *Nature Medicine*, vol. 2, no. 10, pp. 1132–1135, 1996.

[14] J. Beaudreuil, G. Mbalaviele, M. Cohen-Solal, C. Morieux, M. C. De Vernejoul, and P. Orcel, "Short-term local injections of transforming growth factor-β1 decrease ovariectomy-stimulated osteoclastic resorption in vivo in rats," *Journal of Bone and Mineral Research*, vol. 10, no. 6, pp. 971–977, 1995.

[15] A. G. Geiser, Q. Q. Zeng, M. Sato, L. M. Helvering, T. Hirano, and C. H. Turner, "Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-β1 gene," *Bone*, vol. 23, no. 2, pp. 87–93, 1998.

[16] D. Gazit, Y. Zilberman, G. Turgeman, S. Zhou, and A. Kahn, "Recombinant TGF-beta1 stimulates bone marrow osteoprogenitor cell activity and bone matrix synthesis in osteopenic, old male mice," *Journal of Cellular Biochemistry*, vol. 73, no. 3, pp. 379–389, 1999.

[17] M. Machwate, E. Zerath, X. Holy et al., "Systemic administration of transforming growth factor-β2 prevents the impaired bone formation and osteopenia induced by unloading in rats," *Journal of Clinical Investigation*, vol. 96, no. 3, pp. 1245–1253, 1995.

[18] B. L. Langdahl, J. Y. Knudsen, H. K. Jensen, N. Gregersen, and E. F. Eriksen, "A sequence variation: 713-8delC in the transforming growth factor-beta 1 gene has higher prevalence in osteoporotic women than in normal women and is associated with very low bone mass in osteoporotic women and increased bone turnover in both osteoporotic and normal women," *Bone*, vol. 20, no. 3, pp. 289–294, 1997.

[19] M. E. Joyce, A. B. Roberts, M. B. Sporn, and M. E. Bolander, "Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur," *Journal of Cellular Biochemistry*, vol. 110, no. 6, pp. 2195–2207, 1990.

[20] M. Noda and J. J. Camilliere, "In vivo stimulation of bone formation by transforming growth factor-β," *Endocrinology*, vol. 124, no. 6, pp. 2991–2994, 1989.

[21] F. L. Cals, C. A. Hellingman, W. Koevoet, J. R. J. D. Baatenburg, and G. J. Osch, "Effects of transforming growth factor-β subtypes on in vitro cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 6, no. 1, pp. 68–76, 2012.

[22] K. Fuller, J. M. Lean, K. E. Bayley, M. R. Wani, and T. J. Chambers, "A role for TGFbeta(1) in osteoclast differentiation and survival," *Journal of Cell Science*, vol. 113, no. 13, pp. 2445–2453, 2000.

[23] C. Chenu, J. Pfeilschifter, G. R. Mundy, and G. D. Roodman, "Transforming growth factor β inhibits formation of osteoclast-like cells in long-term human marrow cultures," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 85, no. 15, pp. 5683–5687, 1988.

[24] M. Shaarawy, S. Zaki, M. Sheiba, and A. M. El-Minawi, "Circulating levels of osteoclast activating cytokines, interleukin-11 and transforming growth factor-β2, as valuable biomarkers for

the assessment of bone turnover in postmenopausal osteoporosis," *Clinical Laboratory*, vol. 49, no. 11-12, pp. 625–636, 2003.

[25] X. W. Zhou, X. Y. Wu, L. Luo et al., "The relationship between bone turnover markers and BMD decreasing rates in Chinese middle-aged women," *Clinica Chimica Acta*, vol. 412, no. 17-18, pp. 1648–1657, 2011.

[26] Z. Liu, J. Piao, L. Pang et al., "The diagnostic criteria for primary osteoporosis and the incidence of osteoporosis in China," *Journal of Bone and Mineral Metabolism*, vol. 20, no. 4, pp. 181–189, 2002.

[27] H. Orimo, Y. Hayashi, M. Fukunaga et al., "Diagnostic criteria for primary osteoporosis: year 2000 revision," *Journal of Bone and Mineral Metabolism*, vol. 19, no. 6, pp. 331–337, 2001.

[28] S. L. Bonnick, C. C. Johnston Jr., M. Kleerekoper et al., "Importance of precision in bone density measurements," *Journal of Clinical Densitometry*, vol. 4, no. 2, pp. 105–110, 2001.

[29] X. P. Wu, E. Y. Liao, H. Zhang, P. F. Shan, X. Z. Cao, and S. P. Liu, "Establishment of BMD reference plots and determination of peak BMD at multiple skeletal regions in mainland Chinese women and the diagnosis of osteoporosis," *Osteoporosis International*, vol. 15, no. 1, pp. 71–79, 2004.

[30] S. Kudlacek, B. Schneider, W. Woloszczuk, P. Pietschmann, and R. Willvonseder, "Serum levels of osteoprotegerin increase with age in a healthy adult population," *Bone*, vol. 32, no. 6, pp. 681–686, 2003.

[31] T. Ueland, J. Bollerslev, S. G. Wilson et al., "No associations between OPG gene polymorphisms or serum levels and measures of osteoporosis in elderly Australian women," *Bone*, vol. 40, no. 1, pp. 175–181, 2007.

[32] O. S. Indridason, L. Franzson, and G. Sigurdsson, "Serum osteoprotegerin and its relationship with bone mineral density and markers of bone turnover," *Osteoporosis International*, vol. 16, no. 4, pp. 417–423, 2005.

[33] E. J. Samelson, K. E. Broe, S. Demissie et al., "Increased plasma osteoprotegerin concentrations are associated with indices of bone strength of the hip," *Journal of Clinical Endocrinology & Metabolism* , vol. 93, no. 5, pp. 1789–1795, 2008.

[34] L. Jørgensen, A. Vik, N. Emaus et al., "Bone loss in relation to serum levels of osteoprotegerin and nuclear factor-kappaB ligand: the Tromsø Study," *Osteoporosis International* , vol. 21, no. 6, pp. 931–938, 2010.

[35] Y. Mochizuki, N. Banba, Y. Hattori, and T. Monden, "Correlation between serum osteoprotegerin and biomarkers of bone metabolism during anti-thyroid treatment in patients with Graves' disease," *Hormone Research*, vol. 66, no. 5, pp. 236–239, 2006.

[36] A. R. Moschen, A. Kaser, B. Enrich et al., "The RANKL/OPG system is activated in inflammatory bowel diseases and relates to the state or bone loss," *Gut*, vol. 54, no. 4, pp. 479–487, 2005.

[37] M. Holecki, B. Zahorska-Markiewicz, J. Janowska et al., "osteoprotegerin—does it play a protective role in the pathogenesis of bone loss in obese perimenopausal women?" *Endokrynologia Polska*, vol. 58, no. 1, pp. 7–10, 2007.

[38] F. E. A. McGuigan, H. M. Macdonald, A. Bassiti et al., "Large-scale population-based study shows no association between common polymorphisms of the TGFB1 gene and BMD in women," *Journal of Bone and Mineral Research*, vol. 22, no. 2, pp. 195–202, 2007.

[39] V. Hinke, T. Seck, C. Clanget, C. Scheidt-Nave, R. Ziegler, and J. Pfeilschifter, "Association of transforming growth factor-β1 (TGFβ1) T29 → C gene polymorphism with bone mineral density (BMD), changes in BMD, and serum concentrations of TGF-β1 in a population-based sample of postmenopausal German women," *Calcified Tissue International*, vol. 69, no. 6, pp. 315–320, 2001.

[40] H. H. Lau, A. Y. Ho, K. D. Luk, and A. W. Kung, "Transforming growth factor-beta1 gene polymorphisms and bone turnover, bone mineral density and fracture risk in southern Chinese women," *Calcified Tissue International* , vol. 74, no. 6, pp. 516–521, 2004.

[41] Y. Yamada, A. Miyauchi, J. Goto et al., "Association of a polymorphism of the transforming growth factor-beta1 gene with genetic susceptibility to osteoporosis in postmenopausal Japanese women," *Journal of Bone and Mineral Research*, vol. 13, no. 10, pp. 1569–1576, 1998.

[42] Y. Yamada, A. Miyauchi, Y. Takagi, M. Tanaka, M. Mizuno, and A. Harada, "Association of the C-509 → T polymorphism, alone or in combination with the T869 → C polymorphism, of the transforming growth factor-beta1 gene with bone mineral density and genetic susceptibility to osteoporosis in Japanese women," *Journal of Molecular Medicine*, vol. 79, no. 2, pp. 149–156, 2001.

[43] B. Akinci, F. Bayraktar, A. Saklamaz et al., "Low transforming growth factor-beta1 serum levels in idiopathic male osteoporosis," *Journal of Endocrinological Investigation*, vol. 30, no. 5, pp. 350–355, 2007.

[44] A. Wiercinska-Drapalo, J. Jaroszewicz, E. Tarasow, R. Flisiak, and D. Prokopowicz, "Transforming growth factor beta(1) and prostaglandin E2 concentrations are associated with bone formation markers in ulcerative colitis patients," *Prostaglandins and Other Lipid Mediators*, vol. 78, no. 1–4, pp. 160–168, 2005.

[45] J. Yanagisawa, Y. Yanagi, Y. Masuhiro et al., "Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators," *Science*, vol. 283, no. 5406, pp. 1317–1321, 1999.

[46] S. Mori, N. Fuku, Y. Chiba et al., "Cooperative effect of serum 25-hydroxyvitamin D concentration and a polymorphism of transforming growth factor-β1 gene on the prevalence of vertebral fractures in postmenopausal osteoporosis," *Journal of Bone and Mineral Metabolism*, vol. 28, no. 4, pp. 446–450, 2010.

[47] M. Krast, G. Gorny, R. J. Sells Galvin, and M. J. Oursler, "Roles of stromal cell RANKL, OPG, and M-CSF expression in biphasic TGF-β regulation of osteoclast differentiation," *Journal of Cellular Physiology*, vol. 200, no. 1, pp. 99–106, 2004.

[48] A. Toom, A. Arend, D. Gunnarsson et al., "Bone formation zones in heterotopic ossifications: histologic findings and increased expression of bone morphogenetic protein 2 and transforming growth factors β2 and β3," *Calcified Tissue International*, vol. 80, no. 4, pp. 259–267, 2007.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 895045, 9 pages
http://dx.doi.org/10.1155/2013/895045

Review Article

Novel Adipokines and Bone Metabolism

Yuan Liu,[1,2] Cheng-Yuan Song,[3] Shan-Shan Wu,[1] Qiu-Hua Liang,[1] Ling-Qing Yuan,[1] and Er-Yuan Liao[1]

[1] *Institute of Metabolism and Endocrinology, Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, China*
[2] *Geriatric Department, Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, China*
[3] *Neurology Department, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China*

Correspondence should be addressed to Er-Yuan Liao; eyliao007@yahoo.com.cn and Ling-Qing Yuan; allenylq@hotmail.com

Received 15 December 2012; Accepted 2 January 2013

Academic Editor: Guang-Da Xiang

Osteoporosis is a serious social issue nowadays. Both the high morbidity and its common complication osteoporotic fracture load a heavy burden on the whole society. The adipose tissue is the biggest endocrinology organ that has a different function on the bone. The adipocytes are differentiated from the same cell lineage with osteoblast, and they can secrete multiple adipokines with various functions on bone remolding. Recently, several novel adipokines have been identified and investigated thoroughly. In this paper, we would like to highlight the complicated relation between the bone metabolism and the novel adipokines, and it may provide us with a new target for prediction and treatment of osteoporosis.

1. Introduction

Bone is the principal structural connective tissue that supports the whole body and protects the delicate organs inside. It also serves as an endocrine organ that plays a pivotal role in the regulation of the mineral homeostasis. Being modulated by multiple factors, the bone tissue keeps continuously undergoing biological processes of regeneration by osteoblast and absorption by osteoclast. Respectively, osteoblasts originate from the bone marrow mesenchymal stem cells (MSCs) which are a series of polypotential cells while osteoclasts are raised from the hematopoietic lineage mainly located in the blood and the bone marrow. The normal differentiation, proliferation and maturation of these two cells are the prerequisite of bone homeostasis. Actually, in a healthy bone, there is an elaborate balance between the number and activity of these two cells which is maintained by various systemic hormones and local factors such as the parathyroid hormone, 1,25-dihydroxy vitamin D3, and loading effect. This balance promises the normal bone turn over and remolding, and once the balance is broken down and the absorption overrides the regeneration, osteoporosis occurs.

Fat is the biggest endocrine organ in human body, it severs as an important part in the energy restoration and nutrition metabolism. It also has a complex connection with the bone. A common phenomenon is that, with aging, the fat tissue in the bone marrow increased while the bone mass decreased. Actually, both the adipocyte and osteoblast are originated from the same cell lineage: MSCs. The differentiation trend to preadipocytes or preosteoblasts is competitive and being inhibited by each other. However, growing evidence suggests the fat tissue may have a more complicated effect on the bone tissue which is realized by adipokines. Previously, several studies have demonstrated that TNF-α and IL-6 were involved in bone metabolism through mutiple pathways like the TGF-β, RANKL/RANK pathway, STATs, and so forth [1–3]. Recently, lots of grand new adipokines were discovered, such as adiponectin, leptin, and vaspin. These adipokines might participate in the bone metabolism through different mechanisms. Here, we get a review on the relationship between this novel adipokines and bone metabolism.

2. Adipokines and Bone Metabolism

Novel adipokines, including adiponectin, leptin, resistin, chemerin, omentin, vaspin, and visfatin, participate in wildly

physiological and pathophysiological procedure ranging from the eating behavior modulation, fatty acid oxidation, and energy expenditure, and so forth [4]. And also the relationship between the adipokines and the bone metabolism is very close and received a highly attention in both the clinical and biological researches.

2.1. Adiponectin and Bone Metabolism. Adiponectin, also known as GBP28 (gelatin-binding protein of 28 kDa) or apM1 (adipose most abundant gene transcript 1), is secreted predominantly by differentiated adipocytes and widely participated in multiple metabolic procedure including bone metabolism.

It has a great effect on promoting the bone regeneration by affecting both of the two processes in osteogenesis: the differentiation of MSCs to preosteoblasts as well as the osteoblasts proliferation and maturation.

MSCs are a series of pluripotency and self-renewing cell; it can competitively differentiate into osteoblast, adipose cells, and chondrocyte under different conditions. Studies showed that adiponectin could promote the osteogenic differentiation while it could inhibit the adipocyte formation. In this procedure, cyclooxygenase-2 (COX2) is a key factor. Lee and his colleagues found that, through the COX2-dependent manner, adiponectin could promote the osteogenic differentiation of the MSCs by increasing the expression of alkaline phosphatase (ALP), osteocalcin (OC), and type 1 collage (Col-I) [5]. Meanwhile Yokota found that also by the COX2-dependent manner, adiponectin could inhibit the adipose differentiation of both MSCs and cloned stromal preadipocytes [6]. To some extent, reducing the number of adipocyte could increase that of osteoblast due to the competitive relation of these two cells during differentiation. So, adiponectin could promote the MSCs osteogenic differentiation both directly and indirectly. Moreover, adiponectin stimulates the proliferation, maturation, and mineralization of osteoblasts. Kanazawa and colleague found that after 24-hour incubation with adiponectin, BrdU incorporation was significantly enhanced in MC3T3-E1 cells, this result showed a positive effect of adiponectin on proliferation [7]. Also, Huang and colleagues found that incubating with adiponectin could induce the expression of bone morphogenetic protein-2 (BMP-2) in osteoblasts by the p38 signal pathway [8]. Moreover, Oshima and colleagues found that MC3T3-E1 cells incubated with 3 μg/mL recombinant adiponectin showed a dose-dependent increasing expression of Col-I, OC mRNA and ALP activities in a 12-day culture [9].

Besides the positive modulation on osteoblasts, adiponectin also has a negative effect on osteoclasts. In Oshima's study, they found that adiponectin inhibited the M-CSF and RANKL induced osteoclastic differentiation of both mouse macrophages and human $CD14^+$ mononuclear cells and therefore suppressed the bone-resorption activity of osteoclasts. In the osteoclastic precursor RAW264 cells, adiponectin was able to inhibit the TLR4-mediated NF-κB activity and the subsequent osteoclastogenesis [10]. Interestingly, Luo et al. found that the suppressed effect was time depended. The sustained release of adiponectin by matrigel controlled-release system, instead of short-term administration, had the ability to suppress the osteoclastic activity, and this effect worked in both in vitro and in vivo studies [11]. Another in vivo study showed that the increase of adiponectin expression by adenovirus could increase trabecular bone mass, accompanied with reduction in the number of osteoclasts and plasma cross-linked N-telopeptide of type I collagen (NTx) level [9]. In summary, adiponectin can increase the bone mass by promoting bone formation and impeding bone absorption.

Also, adiponectin has a promoting effect on chondrocytes. Adiponectin at 0.5 μg/mL can increase chondrocyte proliferation, proteoglycan synthesis, and matrix mineralization which were reflected by the upregulation of type II collagen, aggrecan, Runx2, and ALP activity [12].

Adiponectin has a great impact on promoting bone mass in vitro; however, during the clinical epidemiological survey, the results are contradictory as whether serum adiponectin concentration and bone mineral density (BMD) are associated. Several researches showed that the serum adiponectin level had no relation with the BMD both in premenopausal and postmenopausal woman. Also they found that serum adiponectin levels were not significantly different between the patients with osteoporotic fractures and nonosteoporotic fractures [13–16]. However, most of the researches showed a negative correlation between adiponectin and BMD [17–23]. In a META analysis including 59 studies, a great inverse correlation between adiponectin levels and BMD independent of gender and menopausal status was found. Moreover, overexpression of adiponectin can be recognized as a high-risk predictor for vertebral fractures in men [24].

It is a little tricky that there is a positive relationship between adiponectin and bone metabolism in the biological research while there is a negative correlation in the clinical investigation. The following reasons may account for these discrepancies.

First, the serum adiponectin concentration is affected by multiple factors, like the age, gender, race, smoking, diabetes status, hormone levels, and so on [19, 25–28]. For instance, in healthy people, the serum adiponectin concentration is 14.2 ± 8.6 μg/mL [29], but in diabetic men is 6.31 ± 4.10 μg/mL and in diabetic women is 9.08 ± 7.32 μg/mL [27]. Although the researches had adjusted for the age, gender, and other elements, they all have their own limitations that may influence the results.

What's more, the convincing way to study the relation between adiponectin and bone cells is measuring the adiponectin concentration in the bone marrow plasma instead of serum. The effect of the cytokines and hormones in bone marrow microenvironment exerts influence directly on MSCs and osteoblast. Several findings suggested that adiponectin had a paracrine role on bone marrow and mesenchymal progenitor cells. However, all the studies we mentioned before were talking about the serum adiponectin concentration and BMD, but the relationship between bone marrow plasma adiponectin and BMD is still not clear. We cannot simply consider that the serum adiponectin concentration is the same as that in bone marrow. In fact, in Berner's study, the adiponectin protein level in bone marrow plasma

was much higher than that in serum or bone marrow cells, and the difference between these two levels diminished with aging [30]. However, Modder showed in his research that the adiponectin levels were higher in peripheral when compared to bone marrow plasma in healthy postmenopausal women [31]. Unfortunately, the relation between normal people or osteoporosis patients still needs to be elucidated. So, if we are able to measure the adiponectin in the bone marrow plasma and analyze its correlation with BMD, we may get a consistent result. Neverthless, according to the previous investigation, serum adiponectin concentration was negatively correlated with the BMD. This negative relation has profound clinical significance as serum adiponectin levels still can work as a predictor of osteoporosis in some certain population. However, further researches are still needed.

2.2. Leptin and Bone Metabolism. Leptin is a 16-kDa protein hormone that is primary being secreted by the white fat tissue. As early as 1994, the researchers in Rockefeller University had already found and cloned this adipokine. During the past 20 years, groundbreaking studies about leptin had been finished.

Now we can tell various cells such as the undifferentiated bone marrow MSCs, hematopoietic cells, adipocytes, and osteoblasts, osteoclast express leptin receptor. Also, researches have already demonstrated its important role in appetite modulation, energy consumption, and body weight regulation. As early as 1999, Thomas et al. had already found that leptin could promote osteogenesis differentiation in bone marrow MSCs [32]; however, the function of leptin on bone formation is still controversial.

According to the in vitro studies and in vivo investigation, leptin acts a protective role on bone mass. On one hand, Xu et al. found that leptin could stimulate the proliferation and osteoblastic differentiation of both BMSCs and dental stem cells [33, 34]. And for the osteoblast and chondrocyte, leptin also was a promoter in the proliferation [35]. Moreover, in the fracture rat, the serum leptin is greatly increased after the injury indicated a positive relationship between the leptin and bone regeneration [36, 37]. On the other hand, incubating with leptin could decrease the osteoclastic activity of both human peripheral blood mononuclear cells (PBMCs) and murine spleen cells [38]. And during the fetal growth and development, there was a negative correlation between the concentration of serum leptin and bone resorption marker cross-linked carboxy-terminal telopeptide of type I collagen (ICTP) [39]. Moreover, van der found that, in the leptin deficient mice, the number of osteoclasts in peripheral blood was increased while cortical bone parameters were decreased in an age-related way [40]. These results all indicated that leptin might increase bone formation and decrease bone resorption with the overall effect of increasing bone mass.

However, in vivo interventional studies, the situation is much more complicated. Unlike the other adipokines which lay effect on bone only through endocrinology way, leptin affect the bone metabolism through two mechanisms: the endocrinology system and nervous system. Correspondingly, there were two administration ways to study the signaling pathway involved: subcutaneously injection and intracerebroventricular (ICV) injection.

In the subcutaneously model, studies indicated a stimulative role of leptin exerted on bone formation. Giving leptin to pregnant mice at the early stage of pregnancy could greatly increase the baby mice's ossification centers by activating the differentiation and proliferation of both chondrocyte and osteoblast [41]. Also, in the leptin receptor-deficient db/db mice, a decline in bone growth, osteoblast-lined bone perimeter and bone formation rate were observed, and those phenoma could be reversed after following subcutaneous administration of leptin [42]. Leptin also can increase the bone mineral apposition rate and bone mineral content in mice [43]. These all illuminated a positive association between the leptin and the bone formation.

Nevertheless, in the ICV model, the effect of leptin is still unclear. Although Bartell found that culture of isolated bone marrow MSCs from the mice after ICV leptin injection showed an increase of the bone formation and BMD along with decrease of osteoclastogenesis [44], it is more widely accepted that the nervous system exerts negative effect on bone downstream of leptin administration. Ducy et al. found that the leptin inhibited bone formation through the nervous system [45]. Moreover, because leptin did not have receptors on hypothalamus, its function was mainly realized by decreasing the serotonin level. Serotonin is a neurotransmitter which is synthesized and released by brainstem, and it could bind to its receptor Htr2c and led to the activation of the sympathetic nervous system [46]. And also in Elefteriou's study, they found that the sympathetic nervous system would release noradrenaline after the administration of leptin. The noradrenaline would bind to its receptor Adrb2 (beta2-adrenergic receptors) which would lead to the production of RANKL (Receptor Activator of Nuclear Factor Kappa B Ligand) in osteoblasts and then activate the osteoclasts and decrease bone mass as the final result [47]. However, about how the nervous system works downstream of leptin is still insufficiently elucidated.

According to 20 papers about the relation between serum leptin and BMD published during 2007–2012 [23, 48–66], 17 of them showed no correlation between this two elements after adjusted for the age, gender, or hormonal level among others. For the rest of the three papers, one paper showed a positive correlation among prepubertal girls (48 cases) [54] while another showed that leptin is inversely associated to BMD in Brazilian obese adolescents (109 cases) [61]. And the last one showed that Leptin was positively related with the whole body and femoral BMD in postmenopausal nondiabetic elderly women (63 cases) [23]. However, they are not very trustful for their limited cases and the certain group they faced. So simply from the epidemiological aspect, there is no correlation between leptin and BMD, and also leptin is not an independent predictor for fracture risk.

In summary, the leptin exerts a rather complicated and inconsistent effect on bone metabolism from both biological and epidemical aspects, so we still need to carry more researches to unveil the truth.

2.3. Resisten and Bone Metabolism. Resisten, also known as adipose tissue-specific secretory factor (ADSF) or C/EBP-epsilon-regulated myeloid-specific secreted cysteine-rich protein (XCP1), was initially identified as a factor produced by adipocytes and named for its ability to induce insulin resistance [67]. Although it is widely expressed in myocytes, hepatocytes, adipocytes, MSCs, preosteoclasts, and osteoblasts, the expression level is much higher in bone marrow compared to other tissues [68]. That indicates that the resistin may play an important role in the bone metabolism. Actually, according to the recent researches, the answer is positive.

It can stimulate the proliferation of osteoblasts through the PKC and PKA-dependent way [69]. However, on the maturation of the osteoblasts, resisten only has a weak effect. It only can mildly increase the mRNA expressions of Col-I after the administration of 7 days whereas those of Runx2, osterix, OC, and ALP were no significant difference from untreated cells. Although its effect on osteoblasts is weak, resistin has a strong effect on the osteoclastogenesis by greatly increasing the number of osteoclast and activating the NF-κB promoter [69]. So, resisten is a negative factor for the bone mass which is consistent with the epidemiological studies that the serum resisten concentration is negatively correlated with the whole body BMD in Chinese men and postmenopausal women as well as the lumbar spine BMD in male adult [17, 52].

2.4. Visfatin and Bone Metabolism. Visfatin, which is also known as pre-B cell colony-enhancing factor (PBEF) or nicotinamide phosphoric acid RNA transferase (Nampt), is isolated as a novel adipokine preferentially expressed in visceral adipose tissue when compared to subcutaneous fat. Researchers also found a high expression of visfatin in bone marrow plasma, and this finding demonstrated a potential possibility that visfatin might modulate bone metabolism [70]. Later, this assumption was proved by our previous study: Visfatin could stimulate human osteoblast proliferation and increase the expression of osteogenic marker Col-I in a dose- and time-dependent manner. And it also could cause an increase in mineralization of osteoblasts [71]. On the other side, treatment with visfatin inhibitor FK866 reduced osteogenesis by reducing ALP activity and bone nodule formation in primary cultured BMSC [72]. Moreover, visfatin might be a negative regulator in osteoclastogenesis. Visfatin suppresses the differentiation of $CD14^+$ monocytes into multinucleated $TRAP^+$ osteoclasts dose dependently [73]. Nevertheless, there is no relation with bone mineral density that was found during the clinical investigation [52, 63, 74, 75]. However, in Anastasilakis' research, they found that visfatin could be a predictor of acute phase reaction (APR) caused by intravenous zoledronate treatment, for instance, lower visfatin levels at baseline accompanied with higher risk for APR [76]. This founding is very profound for the clinical usage of diphosphate.

2.5. Vaspin and Bone Metabolism. Vaspin was a recently identified adipokine, playing a protective role in many metabolic diseases, ranging from diabetes mellitus to atherosclerosis. The researches about vaspin were mainly focused on the insulin resistance [77], hepatitis disease [78, 79], and cardiovascular disease [80, 81]. Presently, our previous study showed that the vaspin could inhibit the apoptosis of the osteoblasts [82]. However, no report about its function in osteogenesis, osteoclastogenesis as well as the relation with BMD was published.

2.6. Chemerin and Bone Metabolism. Chemerin is also a kind of adipokines that can modulate the adipogenesis and osteogenesis. Its modulation function mainly stands on the adipogenesis aspect. Expression and secretion of chemerin dramatically increased during adipocytes differentiation. And knock-down chemerin or chemerin like receptor 1 (CMKLR1) expression could abrogate adipocytes differentiation and increased osteoblast marker gene expression and mineralization after osteoblastic stimulations [83]. So, chemerin actually is a negative regulator during osteogenesis for its facilitating role in adipogenesis.

2.7. Omentin-1 and Bone Metabolism. Omentin-1 is a novel 34 kDa adipokine with a great function on the glucose metabolism and insulin resistance, and it is recognized as a biomarker of metabolic disorders [84, 85]. Also, it has an important function on bone metabolism.

Our previous study found that omentin-1 inhibited osteoblastic differentiation while it had no direct effect on osteoclastic differentiation in vitro. What's more, in the ovary ectomized mice, overexpression of omentin-1 by adenovirus led to a decline of the serum OC, tartrate-resistant acid phosphatase-5b, and RANKL/OPG ratios, and a lower BMD was observed too [86].

Also, the negative relation was found in epidemic survey. Omentin-1 level was negatively correlated with BMD in the anorexia nervosa girls [87] and Iranian postmenopausal women [74]. However, since this conclusion was deduced from limited groups instead of large population, we still need more data to confirm this potential negative relation.

3. Future

Osteoporosis is now a worldwide problem that bothers a lot of patients. According to the investigation, the estimated number of people with osteoporosis is approximately 10–12 million in the United States [88]. And in Austria about 5.9% of men and 22.8% of women over 50 years old face the osteoporosis problem [89]. This situation is even more serious in developing countries. The FRACTURK study showed that the prevalence of osteoporosis at the femoral neck was 7.5% and 33.3% in men and women aged 50 years or more in Turkey [90]. And in China, the data showed that the prevalence of osteoporosis at least one site in Chinese women was 23.9 ± 13.3% in the age between 50 and 59 while the prevalence increased with aging [91]. So, osteoporosis becomes a serious social problem and called for more attention.

Current drugs admitted by the U.S. Food and Drug Administration (FDA) for osteoporotic treatment include

Table 1: Relation between adipokines and bone metabolism.

Adipokines	Osteogenic	Osteoclastic	Relationship with BMD
Adiponectin	Promotes the MSCs osteogenesis and osteoblast maturation [5–9]	Inhibits the osteoclastogenesis and osteoclastic activity [9–11]	Negative related with the BMD; a predictor of osteoporotic fractures in certain population [11–18]
Leptin	Stimulate osteogensis both in vitro studies and subcutaneous injection animal model [19–25]	Inhibits the osteoclastogenesis and osteoclastic activity [26–31]	No relationship was found after adjust for age, gender and hormone [17, 32–50]
Resistin	Stimulates proliferation and weakly promotes the osteoblasts maturation [51]	Strongly increases the number and activity of osteoclast [51]	Negative related with certain population [11, 36]
Visfatin	Stimulates human osteoblast proliferation, maturation, and mineralization [52, 53]	Inhibits the formation of osteoclast [54]	No relationship was found [36, 47, 55, 56]
Vaspin	Inhibits the apoptosis of osteoblast [57]	No paper published	No paper published
Chemerin	Negative effect by greatly promoting the adipogenesis of the MSCs [58]	No paper published	No paper published
Omentin-1	Inhibits the osteoblastic differentiation [59]	No paper published	Not clear, need more data [56, 60]

Alendronate, Risedronate, Zoledronic acid, and Teriparatide. The first three drugs belong to the diphosphonate, the first line therapy of osteoporosis. The function of diphosphonate is mainly focused on the inhibition of the osteoclast and decrease of the bone resorption. It can only slow down the bone decline instead of reversing it. And Teriparatide, the human recombinant parathyroid hormone 1–34, has been demonstrated to be a new and promising way for severe osteoporosis treatment for its function on bone regeneration. It can increase the BMD, decrease the fracture risk, and improve the clinical symptoms such as the back pain in osteoporosis patients [92–94]. So, it is recommended for patients with severe osteoporosis. According to the study, Diphosphonate reduced vertebral fractures by 40% to 70% and nonvertebral fractures by 20% to 35% while Teriparatide reduced vertebral fractures 65% and nonvertebral fractures 53% [95].

What's more, there are some other second line drugs for osteoporosis treatment such as selective estrogen receptor modulators (SERMs), calcitonin and denosumab. Although we have plenty of drugs in using, the situation of osteoporosis treatment that is not optimistic. According to the investigation, 15 million osteoporosis patients in Japan were bothered by osteoporosis while only 20% of them were under treatment [96]. Situation in Switzerland is even worse: every other woman and every fifth man aged 50 years or older will suffer an osteoporotic fracture during her or his remaining lifetime [97]. The low under treatment rate and high osteoporotic fracture rate are warning us that there is a long way for osteoporosis prevention and treatment.

Fat tissue, as the biggest endocrinology organ, draws an important impact on the bone metabolism not only by the loading effect but also the adipokines. Those adipokines form a complex network to regulate the bone regeneration and resorption as we mentioned before (see in Table 1). Most of them have a positive effect on bone formation while some of them could also inhibit or stimulate the osteoclast activation as well. That provides us a future possible target for new osteoporosis drugs.

Moreover, during the clinical investigation, some adipokines, such as adiponectin or resisten, were found to have a positive or negative relation between the serum concentration and BMD, and this provides us with a future predictor of osteoporosis risk or osteoporotic fracture risk in some certain population. And the negative correlation between visfatin and acute phase reaction (APR) caused by intravenous zoledronate treatment is of great clinical importance too.

In summary, it has a great meaning to study the adipokine and the relation with bone metabolism. Although to date some results may be conflicting and most of the study is still in the lab, there is a promising future for the adipokines, the new therapeutic target and predictor.

Conflict of Interests

The authors have declared that no competing interests exist.

Acknowledgment

This work was supported by funding from The National Natural Science Foundation of China (Grant nos. 81270962 and 81070246) and the National Science Foundation for Postdoctoral Scientists of China (Grant no. 2012M521568).

References

[1] N. Kim, Y. Kadono, M. Takami et al., "Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis," *Journal of Experimental Medicine*, vol. 202, no. 5, pp. 589–595, 2005.

[2] Y. Li, A. Li, K. Strait, H. Zhang, M. S. Nanes, and M. N. Weitzmann, "Endogenous TNFα lowers maximum peak bone mass and inhibits osteoblastic smad activation through NF-κB," *Journal of Bone and Mineral Research*, vol. 22, no. 5, pp. 646–655, 2007.

[3] S. Itoh, N. Udagawa, N. Takahashi et al., "A critical role for interleukin-6 family-mediated Stat3 activation in osteoblast differentiation and bone formation," *Bone*, vol. 39, no. 3, pp. 505–512, 2006.

[4] P. E. Scherer, "Adipose tissue: from lipid storage compartment to endocrine organ," *Diabetes*, vol. 55, no. 6, pp. 1537–1545, 2006.

[5] W. L. Hyun, Y. K. Sang, A. Y. Kim, J. L. Eun, J. Y. Choi, and B. K. Jae, "Adiponectin stimulates osteoblast differentiation through induction of COX2 in mesenchymal progenitor cells," *Stem Cells*, vol. 27, no. 9, pp. 2254–2262, 2009.

[6] T. Yokota, C. S. Reddy Meka, K. L. Medina et al., "Paracrine regulation of fat cell formation in bone marrow cultures via adiponectin and prostaglandins," *Journal of Clinical Investigation*, vol. 109, no. 10, pp. 1303–1310, 2002.

[7] I. Kanazawa, T. Yamaguchi, S. Yano, M. Yamauchi, M. Yamamoto, and T. Sugimoto, "Adiponectin and AMP kinase activator stimulate proliferation, differentiation, and mineralization of osteoblastic MC3T3-E1 cells," *BMC Cell Biology*, vol. 8, article 51, 2007.

[8] C. Y. Huang, C. Y. Lee, M. Y. Chen, H. C. Tsai, H. C. Hsu, and C. H. Tang, "Adiponectin increases BMP-2 expression in osteoblasts via AdipoR receptor signaling pathway," *Journal of Cellular Physiology*, vol. 224, no. 2, pp. 475–483, 2010.

[9] K. Oshima, A. Nampei, M. Matsuda et al., "Adiponectin increases bone mass by suppressing osteoclast and activating osteoblast," *Biochemical and Biophysical Research Communications*, vol. 331, no. 2, pp. 520–526, 2005.

[10] N. Yamaguchi, T. Kukita, Y. J. Li et al., "Adiponectin inhibits osteoclast formation stimulated by lipopolysaccharide from Actinobacillus actinomycetemcomitans," *FEMS Immunology and Medical Microbiology*, vol. 49, no. 1, pp. 28–34, 2007.

[11] E. Luo, J. Hu, C. Bao et al., "Sustained release of adiponectin improves osteogenesis around hydroxyapatite implants by suppressing osteoclast activity in ovariectomized rabbits," *Acta Biomaterialia*, vol. 8, no. 2, pp. 734–743, 2012.

[12] T. D. Challa, Y. Rais, and E. M. Ornan, "Effect of adiponectin on ATDC5 proliferation, differentiation and signaling pathways," *Molecular and Cellular Endocrinology*, vol. 323, no. 2, pp. 282–291, 2010.

[13] L. Lenchik, T. C. Register, F. C. Hsu et al., "Adiponectin as a novel determinant of bone mineral density and visceral fat," *Bone*, vol. 33, no. 4, pp. 646–651, 2003.

[14] J. Jürimäe, K. Rembel, T. Jürimäe, and M. Rehand, "Adiponectin is associated with bone mineral density in perimenopausal women," *Hormone and Metabolic Research*, vol. 37, no. 5, pp. 297–302, 2005.

[15] B. Özkurt, Z. N. Özkurt, M. Altay, C. N. Aktekin, O. Çağlayan, and Y. Tabak, "The relationship between serum adiponectin level and anthropometry, bone mass, osteoporotic fracture risk in postmenopausal women," *Eklem Hastaliklari ve Cerrahisi*, vol. 20, no. 2, pp. 78–84, 2009.

[16] R. Sodi, M. J. Hazell, B. H. Durham, C. Rees, L. R. Ranganath, and W. D. Fraser, "The circulating concentration and ratio of total and high molecular weight adiponectin in postmenopausal women with and without osteoporosis and its association with body mass index and biochemical markers of bone metabolism," *Clinical Biochemistry*, vol. 42, no. 13-14, pp. 1375–1380, 2009.

[17] K. W. Oh, W. Y. Lee, E. J. Rhee et al., "The relationship between serum resistin, leptin, adiponectin, ghrelin levels and bone mineral density in middle-aged men," *Clinical Endocrinology*, vol. 63, no. 2, pp. 131–138, 2005.

[18] M. D. Kontogianni, U. G. Dafni, J. G. Routsias, and F. N. Skopouli, "Blood leptin and adiponectin as possible mediators of the relation between fat mass and BMD in perimenopausal women," *Journal of Bone and Mineral Research*, vol. 19, no. 4, pp. 546–551, 2004.

[19] M. R. G. Araneta, D. Von Mühlen, and E. Barrett-Connor, "Sex differences in the association between adiponectin and BMD, bone loss, and fractures: the rancho bernardo study," *Journal of Bone and Mineral Research*, vol. 24, no. 12, pp. 2016–2022, 2009.

[20] L. Basurto, R. Galván, N. Cordova et al., "Adiponectin is associated with low bone mineral density in elderly men," *European Journal of Endocrinology*, vol. 160, no. 2, pp. 289–293, 2009.

[21] J. B. Richards, A. M. Valdes, K. Burling, U. C. Perks, and T. D. Spector, "Serum adiponectin and bone mineral density in women," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 4, pp. 1517–1523, 2007.

[22] J. Jürimäe and T. Jürimäe, "Plasma adiponectin concentration in healthy pre- and postmenopausal women: relationship with body composition, bone mineral, and metabolic variables," *American Journal of Physiology*, vol. 293, no. 1, pp. E42–E47, 2007.

[23] E. Zoico, M. Zamboni, V. Di Francesco et al., "Relation between adiponectin and bone mineral density in elderly post-menopausal women: role of body composition, leptin, insulin resistance, and dehydroepiandrosterone sulfate," *Journal of Endocrinological Investigation*, vol. 31, no. 4, pp. 297–302, 2008.

[24] E. Biver, C. Salliot, C. Combescure et al., "Influence of adipokines and ghrelin on bone mineral density and fracture risk: a systematic review and meta-analysis," *Journal of Clinical Endocrinology & Metabolism*, vol. 96, no. 9, pp. 2703–2713, 2011.

[25] K. Ağbaht, A. Gürlek, J. Karakaya, and M. Bayraktar, "Circulating adiponectin represents a biomarker of the association between adiposity and bone mineral density," *Endocrine*, vol. 35, no. 3, pp. 371–379, 2009.

[26] J. Jürimäe, T. Kums, and T. Jürimäe, "Adipocytokine and ghrelin levels in relation to bone mineral density in physically active older women: longitudinal associations," *European Journal of Endocrinology*, vol. 160, no. 3, pp. 381–385, 2009.

[27] I. Kanazawa, T. Yamaguchi, M. Yamamoto, M. Yamauchi, S. Yano, and T. Sugimoto, "Relationships between serum adiponectin levels versus bone mineral density, bone metabolic markers, and vertebral fractures in type 2 diabetes mellitus," *European Journal of Endocrinology*, vol. 160, no. 2, pp. 265–273, 2009.

[28] J. Komatsu, M. Fujiwara, M. Matsuura, S. Ito, and M. Oritsu, "Study on the usefulness of high-molecular-weight (HMW)-Adiponectin level check of Japanese general population upon health check: comparison of carotid ultrasonography measurement," *Clinical Biochemistry*, vol. 45, no. 1-2, pp. 72–76, 2012.

[29] T. Imatoh, M. Miyazaki, K. Kadowaki, S. Tanihara, C. Akashi, and H. Une, "Measurement of high-molecular-weight adiponectin is not useful in assessing coronary stenosis," *Clinical Chemistry and Laboratory Medicine*, vol. 50, no. 4, pp. 741–745, 2012.

[30] H. S. Berner, S. P. Lyngstadaas, A. Spahr et al., "Adiponectin and its receptors are expressed in bone-forming cells," *Bone*, vol. 35, no. 4, pp. 842–849, 2004.

[31] U. I. Mödder, M. M. Roforth, K. Hoey et al., "Effects of estrogen on osteoprogenitor cells and cytokines/bone-regulatory factors in postmenopausal women," *Bone*, vol. 49, no. 2, pp. 202–207, 2011.

[32] T. Thomas, F. Gori, S. Khosla, M. D. Jensen, B. Burguera, and B. L. Riggs, "Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes," *Endocrinology*, vol. 140, no. 4, pp. 1630–1638, 1999.

[33] J. Xu, T. Wu, Z. Zhong, C. Zhao, Y. Tang, and J. Chen, "Effect and mechanism of leptin on osteoblastic differentiation of hBMSCs," *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi*, vol. 23, no. 2, pp. 140–144, 2009.

[34] S. Um, J. R. Choi, J. H. Lee, Q. Zhang, and B. Seo, "Effect of leptin on differentiation of human dental stem cells," *Oral Diseases*, vol. 17, no. 7, pp. 662–669, 2011.

[35] J. Cornish, K. E. Callon, U. Bava et al., "Leptin directly regulates bone cell function in vitro and reduces bone fragility in vivo," *Journal of Endocrinology*, vol. 175, no. 2, pp. 405–415, 2002.

[36] L. Wang, J. S. Yuan, H. X. Zhang, H. Ding, X. G. Tang, and Y. Z. Wei, "Effect of leptin on bone metabolism in rat model of traumatic brain injury and femoral fracture," *Chinese Journal of Traumatology*, vol. 14, no. 1, pp. 7–13, 2011.

[37] L. Wang, X. Tang, H. Zhang, J. Yuan, H. Ding, and Y. Wei, "Elevated leptin expression in rat model of traumatic spinal cord injury and femoral fracture," *Journal of Spinal Cord Medicine*, vol. 34, no. 5, pp. 501–509, 2011.

[38] W. R. Holloway, F. M. L. Collier, C. J. Aitken et al., "Leptin inhibits osteoclast generation," *Journal of Bone and Mineral Research*, vol. 17, no. 2, pp. 200–209, 2002.

[39] O. Ogueh, S. Sooranna, K. H. Nicolaides, and M. R. Johnson, "The relationship between leptin concentration and bone metabolism in the human fetus," *Journal of Clinical Endocrinology and Metabolism*, vol. 85, no. 5, pp. 1997–1999, 2000.

[40] M. van der Velde, B. C. van der Eerden, Y. Sun et al., "An age-dependent interaction with leptin unmasks ghrelin's bone-protective effects," *Endocrinology*, vol. 153, no. 8, pp. 3593–3602, 2012.

[41] L. Bertoni, M. Ferretti, F. Cavani et al., "Leptin increases growth of primary ossification centers in fetal mice," *Journal of Anatomy*, vol. 215, no. 5, pp. 577–583, 2009.

[42] R. T. Turner, S. P. Kalra, C. P. Wong et al., "Peripheral leptin regulates bone formation," *Journal of Bone and Mineral Research*, vol. 28, no. 1, pp. 22–34, 2013.

[43] M. W. Hamrick, M. A. Della-Fera, Y. H. Choi, C. Pennington, D. Hartzell, and C. A. Baile, "Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice," *Journal of Bone and Mineral Research*, vol. 20, no. 6, pp. 994–1001, 2005.

[44] S. M. Bartell, S. Rayalam, S. Ambati et al., "Central (ICV) leptin injection increases bone formation, bone mineral density, muscle mass, serum IGF-1, and the expression of osteogenic genes in leptin-deficient ob/ob mice," *Journal of Bone and Mineral Research*, vol. 26, no. 8, pp. 1710–1720, 2011.

[45] P. Ducy, M. Amling, S. Takeda et al., "Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass," *Cell*, vol. 100, no. 2, pp. 197–207, 2000.

[46] V. K. Yadav, F. Oury, N. Suda et al., "A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure," *Cell*, vol. 138, no. 5, pp. 976–989, 2009.

[47] F. Elefteriou, J. D. Ahn, S. Takeda et al., "Leptin regulation of bone resorption by the sympathetic nervous system and CART," *Nature*, vol. 434, no. 7032, pp. 514–520, 2005.

[48] K. E. Barbour, J. M. Zmuda, R. Boudreau et al., "The effects of adiponectin and leptin on changes in bone mineral density," *Osteoporosis International*, vol. 23, no. 6, pp. 1699–1710, 2012.

[49] V. D. Sherk, S. P. Malone, M. G. Bemben, A. W. Knehans, I. J. Palmer, and D. A. Bemben, "Leptin, fat mass, and bone mineral density in healthy pre- and postmenopausal women," *Journal of Clinical Densitometry*, vol. 14, no. 3, pp. 321–325, 2011.

[50] B. K. Koroglu, F. Kiris, I. H. Ersoy et al., "Relation of leptin, adiponectin and insulin resistance to bone mineral density in type 2 diabetic postmenopausal women," *Endokrynologia Polska*, vol. 62, no. 5, pp. 429–435, 2011.

[51] T. Iida, T. Domoto, A. Takigawa et al., "Relationships among blood leptin and adiponectin levels, fat mass, and bone mineral density in Japanese pre- and postmenopausal women," *Hiroshima Journal of Medical*, vol. 60, no. 4, pp. 71–78, 2011.

[52] H. Zhang, H. Xie, Q. Zhao et al., "Relationships between serum adiponectin, apelin, leptin, resistin, visfatin levels and bone mineral density, and bone biochemical markers in postmenopausal Chinese women," *Journal of Endocrinological Investigation*, vol. 33, no. 10, pp. 707–711, 2010.

[53] N. Wu, Q. P. Wang, H. Li, X. P. Wu, Z. Q. Sun, and X. H. Luo, "Relationships between serum adiponectin, leptin concentrations and bone mineral density, and bone biochemical markers in Chinese women," *Clinica Chimica Acta*, vol. 411, no. 9-10, pp. 771–775, 2010.

[54] Y. J. Rhie, K. H. Lee, S. C. Chung, H. S. Kim, and D. H. Kim, "Effects of body composition, leptin, and adiponectin on bone mineral density in prepubertal girls," *Journal of Korean Medical Science*, vol. 25, no. 8, pp. 1187–1190, 2010.

[55] G. A. King, S. E. Deemer, and D. L. Thompson, "Relationship between leptin, adiponectin, bone mineral density, and measures of adiposity among pre-menopausal hispanic and caucasian women," *Endocrine Research*, vol. 35, no. 3, pp. 106–117, 2010.

[56] A. Yilmaz, N. Nur, and B. Turgut, "Leptin and bone mineral density in haemodialysis patients," *Annals of the Academy of Medicine Singapore*, vol. 38, no. 4, pp. 374–377, 2009.

[57] L. C. Yang, Y. Lan, J. Hu, Y. H. Yang, Q. Zhang, and J. H. Piao, "Correlation of serum leptin level with bone mineral density and bone turnover markers in Chinese adolescent dancers," *Biomedical and Environmental Sciences*, vol. 22, no. 5, pp. 369–373, 2009.

[58] S. Oguz, O. L. Tapisiz, H. Aytan et al., "Is leptin a significant predictor of bone mineral density in postmenopausal Turkish women?" *Rheumatology International*, vol. 29, no. 4, pp. 393–396, 2009.

[59] X. Lei, X. Peng, N. Wu, M. Hu, and Z. Sun, "Serum adiponectin, leptin level, and bone mineral density in postmenopausal women," *Zhong Nan da Xue Xue Bao*, vol. 34, no. 6, pp. 559–562, 2009.

[60] R. Filip and G. Raszewski, "Bone mineral density and bone turnover in relation to serum leptin, α-ketoglutarate and sex

steroids in overweight and obese postmenopausal women," *Clinical Endocrinology*, vol. 70, no. 2, pp. 214–220, 2009.

[61] W. L. Do Prado, A. De Piano, M. Lazaretti-Castro et al., "Relationship between bone mineral density, leptin and insulin concentration in Brazilian obese adolescents," *Journal of Bone and Mineral Metabolism*, vol. 27, no. 5, pp. 613–619, 2009.

[62] L. F. Yang, H. Xie, L. Q. Yuan, and X. P. Wu, "Serum adiponectin and leptin levels and bone mineral density in 232 men," *Journal of Central South University*, vol. 33, no. 6, pp. 523–526, 2008.

[63] X. D. Peng, H. Xie, Q. Zhao, X. P. Wu, Z. Q. Sun, and E. Y. Liao, "Relationships between serum adiponectin, leptin, resistin, visfatin levels and bone mineral density, and bone biochemical markers in Chinese men," *Clinica Chimica Acta*, vol. 387, no. 1-2, pp. 31–35, 2008.

[64] T. Tamura, M. Yoneda, K. Yamane et al., "Serum leptin and adiponectin are positively associated with bone mineral density at the distal radius in patients with type 2 diabetes mellitus," *Metabolism*, vol. 56, no. 5, pp. 623–628, 2007.

[65] Y. X. Jiang, S. Y. Tang, X. P. Wu, L. F. Yang, and E. Y. Liao, "Serum leptin with bone mineral density and body fat mass in males," *Journal of Central South University*, vol. 32, no. 6, pp. 1075–1079, 2007.

[66] C. Di Carlo, G. A. Tommaselli, A. Di Spiezio Sardo et al., "Longitudinal evaluation of serum leptin and bone mineral density in early postmenopausal women," *Menopause*, vol. 14, no. 3, pp. 450–454, 2007.

[67] C. M. Steppan, S. T. Bailey, S. Bhat et al., "The hormone resistin links obesity to diabetes," *Nature*, vol. 409, no. 6818, pp. 307–312, 2001.

[68] L. Patel, A. C. Buckels, I. J. Kinghorn et al., "Resistin is expressed in human macrophages and directly regulated by PPARγ activators," *Biochemical and Biophysical Research Communications*, vol. 300, no. 2, pp. 472–476, 2003.

[69] L. Thommesen, A. K. Stunes, M. Monjo et al., "Expression and regulation of resistin in osteoblasts and osteoclasts indicate a role in bone metabolism," *Journal of Cellular Biochemistry*, vol. 99, no. 3, pp. 824–834, 2006.

[70] S. M. Krzysik-Walker, J. A. Hadley, J. E. Pesall, D. C. McFarland, R. Vasilatos-Younken, and R. Ramachandran, "Nampt/visfatin/PBEF affects expression of myogenic regulatory factors and is regulated by interleukin-6 in chicken skeletal muscle cells," *Comparative Biochemistry and Physiology*, vol. 159, no. 4, pp. 413–421, 2011.

[71] H. Xie, S. Y. Tang, X. H. Luo et al., "Insulin-like effects of visfatin on human osteoblasts," *Calcified Tissue International*, vol. 80, no. 3, pp. 201–210, 2007.

[72] Y. Li, X. He, J. He, B. Anderstam, G. Andersson, and U. Lindgren, "Nicotinamide phosphoribosyltransferase (Nampt) affects the lineage fate determination of mesenchymal stem cells: a possible cause for reduced osteogenesis and increased adipogenesis in older individuals," *Journal of Bone and Mineral Research*, vol. 26, no. 11, pp. 2656–2664, 2011.

[73] A. R. Moschen, S. Geiger, R. Gerner, and H. Tilg, "Pre-B cell colony enhancing factor/NAMPT/visfatin and its role in inflammation-related bone disease," *Mutation Research*, vol. 690, no. 1-2, pp. 95–101, 2010.

[74] M. Tohidi, S. Akbarzadeh, B. Larijani et al., "Omentin-1, visfatin and adiponectin levels in relation to bone mineral density in Iranian postmenopausal women," *Bone*, vol. 51, no. 5, pp. 876–881, 2012.

[75] G. Iacobellis, M. Iorio, N. Napoli et al., "Relation of adiponectin, visfatin and bone mineral density in patients with metabolic syndrome," *Journal of Endocrinological Investigation*, vol. 34, no. 1, pp. e12–15, 2011.

[76] A. D. Anastasilakis, S. A. Polyzos, S. Delaroudis et al., "The role of cytokines and adipocytokines in zoledronate-induced acute phase reaction in postmenopausal women with low bone mass," *Clinical Endocrinology*, vol. 77, no. 6, pp. 816–822, 2012.

[77] E. Koiou, K. Tziomalos, K. Dinas et al., "The effect of weight loss and treatment with metformin on serum vaspin levels in women with polycystic ovary syndrome," *Endocrine Journal*, vol. 58, no. 4, pp. 237–246, 2011.

[78] F. Cekmez, F. E. Canpolat, O. Pirgon et al., "Apelin, vaspin, visfatin and adiponectin in large for gestational age infants with insulin resistance," *Cytokine*, vol. 56, no. 2, pp. 387–391, 2011.

[79] M. Kukla, W. Mazur, R. J. Buldak, and K. Zwirska-Korczala, "Potential role of leptin, adiponectin and three novel adipokines—visfatin, chemerin and vaspin—in chronic hepatitis," *Molecular Medicine*, vol. 17, no. 11-12, pp. 1397–1410, 2011.

[80] S. Phalitakul, M. Okada, Y. Hara, and H. Yamawaki, "Vaspin prevents TNF-alpha-induced intracellular adhesion molecule-1 via inhibiting reactive oxygen species-dependent NF-kappaB and PKCtheta activation in cultured rat vascular smooth muscle cells," *Pharmacological Research*, vol. 64, no. 5, pp. 493–500, 2011.

[81] G. Aust, O. Richter, S. Rohm et al., "Vaspin serum concentrations in patients with carotid stenosis," *Atherosclerosis*, vol. 204, no. 1, pp. 262–266, 2009.

[82] X. Zhu, Y. Jiang, P. F. Shan et al., "Vaspin attenuates the apoptosis of human osteoblasts through ERK signaling pathway," *Amino Acids*. In press.

[83] S. Muruganandan, A. A. Roman, and C. J. Sinal, "Role of chemerin/CMKLR1 signaling in adipogenesis and osteoblastogenesis of bone marrow stem cells," *Journal of Bone and Mineral Research*, vol. 25, no. 2, pp. 222–234, 2010.

[84] M. Assadi, H. Salimipour, S. Akbarzadeh et al., "Correlation of circulating omentin-1 with bone mineral density in multiple sclerosis: the crosstalk between bone and adipose tissue," *PLoS ONE*, vol. 6, no. 9, Article ID e24240, 2011.

[85] R. Shibata, N. Ouchi, R. Takahashi et al., "Omentin as a novel biomarker of metabolic risk factors," *Diabetology & Metabolic Syndrome*, vol. 4, no. 1, p. 37, 2012.

[86] H. Xie, P. L. Xie, X. H. Luo et al., "Omentin-1 exerts bone-sparing effect in ovariectomized mice," *Osteoporosis International*, vol. 23, no. 4, pp. 1425–1436, 2012.

[87] L. J. Guo, T. J. Jiang, L. Liao, H. Liu, and H. B. He, "Relationship between serum omentin-1 level and bone mineral density in girls with anorexianervosa," *Journal of Endocrinological Investigation*. In press.

[88] C. Holroyd, C. Cooper, and E. Dennison, "Epidemiology of osteoporosis," *Best Practice and Research*, vol. 22, no. 5, pp. 671–685, 2008.

[89] M. J. Henry, J. A. Pasco, G. C. Nicholson, and M. A. Kotowicz, "Prevalence of osteoporosis in Australian men and women: Geelong Osteoporosis Study," *Medical Journal of Australia*, vol. 195, no. 6, pp. 321–322, 2011.

[90] S. Tuzun, N. Eskiyurt, U. Akarirmak et al., "Incidence of hip fracture and prevalence of osteoporosis in Turkey: the FRACTURK study," *Osteoporosis International*, vol. 23, no. 3, pp. 949–955, 2012.

[91] E. Y. Liao, X. P. Wu, X. G. Deng et al., "Age-related bone mineral density, accumulated bone loss rate and prevalence of osteoporosis at multiple skeletal sites in Chinese women," *Osteoporosis International*, vol. 13, no. 8, pp. 669–676, 2002.

[92] A. E. Moore, G. M. Blake, K. A. Taylor et al., "Changes observed in radionuclide bone scans during and after teriparatide treatment for osteoporosis," *European Journal of Nuclear Medicine and Molecular Imaging*, vol. 39, no. 2, pp. 326–336, 2012.

[93] J. B. Walsh, W. F. Lems et al., "Effectiveness of Teriparatide in women over 75 years of age with severe osteoporosis: 36-month results from the European Forsteo Observational Study (EFOS)," *Calcified Tissue International*, vol. 90, no. 5, pp. 373–383, 2012.

[94] S. Yu, R. T. Burge, S. A. Foster, S. Gelwicks, and E. S. Meadows, "The impact of teriparatide adherence and persistence on fracture outcomes," *Osteoporosis International*, vol. 23, no. 3, pp. 1103–1113, 2012.

[95] E. S. Christenson, X. Jiang, R. Kagan, and P. Schnatz, "Osteoporosis management in post-menopausal women," *Minerva Ginecologica*, vol. 64, no. 3, pp. 181–194, 2012.

[96] M. Iki, "Epidemiology of osteoporosis in Japan," *Clinical Calcium*, vol. 22, no. 6, pp. 797–803, 2012.

[97] K. Lippuner, "Epidemiology and burden of osteoporosis in Switzerland," *Therapeutische Umschau*, vol. 69, no. 3, pp. 137–144, 2012.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2013, Article ID 850735, 8 pages
http://dx.doi.org/10.1155/2013/850735

Research Article

Comparison of Classification Algorithms with Wrapper-Based Feature Selection for Predicting Osteoporosis Outcome Based on Genetic Factors in a Taiwanese Women Population

Hsueh-Wei Chang,[1] Yu-Hsien Chiu,[2] Hao-Yun Kao,[2] Cheng-Hong Yang,[3] and Wen-Hsien Ho[2]

[1] *Department of Biomedical Science and Environmental Biology, Graduate Institute of Natural Products, College of Pharmacy, Cancer Center, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 807, Taiwan*
[2] *Department of Healthcare Administration and Medical Informatics, Kaohsiung Medical University, Kaohsiung 807, Taiwan*
[3] *Department of Electronic Engineering, National Kaohsiung University of Applied Sciences, Kaohsiung 807, Taiwan*

Correspondence should be addressed to Wen-Hsien Ho; whho@kmu.edu.tw

Received 26 October 2012; Revised 21 December 2012; Accepted 27 December 2012

Academic Editor: Guang-Da Xiang

An essential task in a genomic analysis of a human disease is limiting the number of strongly associated genes when studying susceptibility to the disease. The goal of this study was to compare computational tools with and without feature selection for predicting osteoporosis outcome in Taiwanese women based on genetic factors such as single nucleotide polymorphisms (SNPs). To elucidate relationships between osteoporosis and SNPs in this population, three classification algorithms were applied: multilayer feedforward neural network (MFNN), naive Bayes, and logistic regression. A wrapper-based feature selection method was also used to identify a subset of major SNPs. Experimental results showed that the MFNN model with the wrapper-based approach was the best predictive model for inferring disease susceptibility based on the complex relationship between osteoporosis and SNPs in Taiwanese women. The findings suggest that patients and doctors can use the proposed tool to enhance decision making based on clinical factors such as SNP genotyping data.

1. Introduction

The World Health Organization (WHO) has defined osteoporosis as a skeletal disorder characterized by diminished bone strength resulting in increased fracture risk [1]. Bone strength is determined by interacting somatic and genetic factors [2]. Reported somatic factors include aging [2–5], menopause [5, 6], and body mass index (BMI) [4, 5, 7, 8]. To identify the genetic determinants of osteoporosis, an earlier study by the first author of this paper [9] investigated how the incidence of low bone mineral density (BMD) in Taiwanese women is affected by interactions among eleven single nucleotide polymorphisms (SNPs) in nine genes known to be involved in osteoporosis [10–16], including tumor necrosis factor-alpha (TNFα), transforming growth factor-beta 1 (TGFB1; TGFβ1), Osteocalcin, parathyroid hormone (PTH), interleukin 1 receptor antagonist (IL1_ra), heat shock 70 kDa protein 1-like (HSPA1L; HSP70 hom), heat shock 70 kDa protein 1B (HSPA1B; HSP70-2), calcitonin receptor (CTR), and bone morphogenetic protein-4 (BMP-4). Generally, several hormones, cytokines, and cell signaling-related proteins were chosen. For example, CTR, which is a receptor for the linear polypeptide hormone calcitonin, reduces blood calcium and suppresses the effects of PTH [17]. The hormonal function of osteocalcin is to release insulin from the pancreas [18]. The cytokine family includes TNFα, TGF-β, BMP4 (protein of TGF-β superfamily), and IL-1RA (protein of interleukin 1 cytokine family) whereas cell-signaling proteins include HSP70 hom and HSP70-2. Studies of the interactions among these hormones (e.g., [9] and references therein) indicate that osteoporosis is an endocrinological problem.

Several gene polymorphisms may cooperatively contribute to the development of osteoporosis in Taiwanese women. Accumulating evidence reveals that SNPs are

Table 1: Panel of 11 SNPs [9].

SNP	Gene	rs number	Genotype		
			1	2	3
1	TNFα-857	rs1799724	TT	TC	CC
2	TGFβ1-509	rs1800469	TT	TC	CC
3	Osteocalcin	rs1800247	CC	CT	TT
4	TNFα-308	rs1800629	AA	AG	GG
5	PTH (BstB I)	rs6254	GG	AG	AA
6	PTH (Dra II)	rs6256	AA	AC	CC
7	IL1_ra[b]	VNTR[a]	A1A1[b]	A1A2	A1A4
8	HSP70 hom	rs2227956	CC	CT	TT
9	HSP 70-2	rs1061581	GG	AG	AA
10	CTR	rs1801197	CC	CT	TT
11	BMP-4	rs17563	CC	CT	TT

[a]VNTR: various number of tandem repeat.
[b]IL1_ra genotype: A1: 410 bp; A2: 240 bp; A4: 325 bp.

potential genetic markers for predicting osteoporosis outcome in Taiwanese women [9]. Chang et al. [19] also proposed a novel odds ratio-based genetic algorithm (OR-GA) method of using odds ratios for quantitatively measuring the disease risk associated with various SNP combinations to determine the susceptibility to osteoporosis in Taiwanese women. Taiwanese women who are carriers of risk alleles in two or more of these SNPs are likely to be at increased risk of osteoporosis because several partial deficiencies in these pathways may severely diminish bone density. Therefore, SNPs may indicate risk of osteoporosis in Taiwanese women and may be useful in clinical association studies to determine the genetic basis of disease susceptibility.

The risk of osteoporosis is likely to be higher than normal in carriers of risk alleles in two or more of these SNPs because several partial deficiencies in these pathways may substantially decrease bone density. Therefore, interacting polymorphisms may affect osteoporosis risk. In [9], the effects of age, BMI, and genetic factors on BMD were evaluated in pre- and postmenopausal Taiwanese women were evaluated. Eleven interacting polymorphisms in nine genes were studied in terms of their effects on the incidence of low BMD (Table 1). Combinations of SNPs were evaluated for genotype associations in women with osteoporosis. The findings showed that specific SNP combinations may be risk factors for postmenopausal osteoporosis in Taiwanese women. In addition to these specific SNP combinations, BMI and age also showed independent associations with BMD in postmenopausal Taiwanese women.

Although an apparent association between SNPs and osteoporosis has been identified in Taiwanese women, a continuing challenge in genomics studies of Taiwanese women populations lies in identifying significant genes. Exhaustive computation over the model space is infeasible if the model space is very large, as there are 2^p models with p SNPs [20, 21]. Feature selection techniques are designed to find responsible genes and SNPs for certain diseases. By selecting a small number of SNPs with significantly larger effects compared to other SNPs and by disregarding SNPs of lesser significance, researchers can focus on the most promising candidate genes and SNPs for use in diagnosis and therapy [21, 22].

In [9], combined polymorphisms in different genomic regions were evaluated for associations with BMD variation. The findings showed that a combination of several gene polymorphisms contributes to the development of osteoporosis in Taiwanese women. However, that study did not report a subset of SNPs that can be used to predict osteoporosis outcome in this population. Therefore, the current study used the same dataset used in [9] to elucidate the relationship between osteoporosis and SNPs in Taiwanese women in a performance comparison of three different classification algorithms with wrapper-based feature selection [23]: multilayer feedforward neural network (MFNN) [24–28], naive Bayes [29], and logistic regression [30]. The MFNNs have proven particularly effective for nonlinear mapping based on human knowledge and are now attracting interest for use in solving complex classification problems [24]. An MFNN containing layers of simple computing nodes, which is analogous to brain neural networks, has proven effective for approximating nonlinear continuous functions and for revealing previously unknown relationships between given input and output variables [25, 26]. The unique structure of MFNNs enables them to learn by using algorithms such as backpropagation and evolutionary algorithms [31, 32]. Potential medical applications of MFNNs include solving problems in which the relationship between independent variables and clinical outcome are poorly understood [33]. Because MFNNs are capable of self-training with minimal human intervention, many studies of large epidemiology databases have, in addition to conventional statistical methods, used MFNNs for further insight into the interrelationships among variables. A naive Bayes classifier assumes that the presence (or absence) of a particular feature of a class is unrelated to the presence (or absence) of any other feature, given the class variable. Depending on the precise nature of the probability model, naive Bayes classifiers can be trained very efficiently in a supervised learning setting. The classifier obtained by using this set of discriminant functions and by estimating the relevant probabilities from the training set is often called the naive Bayesian classifier because, if the the attributes are "naively" assumed to be independent given the class, direct application of the Bayes theorem easily confirms that this classifier is optimal in terms of minimizing the misclassification rate or zero-one loss [34, 35]. Logistic regression is a statistical method of predicting the outcome of a variable that is categorical (i.e., it can have several different categories) and is dependent on one or more predictor variables. A logistic function can be used to model the probabilities describing the possible outcome of a single trial as a function of explanatory variables. Logistic regression is typically used to measure the relationship between a categorical dependent variable and one or more continuous independent variables by converting the dependent variable to probability scores [36].

The wrapper-based feature selection method [23], in which the feature selection algorithm acts as a wrapper around the classification algorithm, was also used to identify an SNP subset with sufficient predictive power to distinguish between high- and low-risk alleles. In the wrapper-based

Table 2: Demographic data for study subjects.

Factor	Range	Descriptive statistics
Age (year)	27–83	$\mu = 56.38; \sigma = 10.37$
Menopause	Postpremenopausal/ Prepremenopausal	247 (83.73%)/48 (16.27%)
BMI (kg/m^2)	17.22–35.49	$\mu = 23.53; \sigma = 2.874$
BMD	High/low	112 (37.97%)/183 (62.03%)

BMI: body mass index; BMD: bone mineral density.

approach, the function used to evaluate feature subsets uses the classification algorithm itself to perform a best-first search for a good subset [23]. Starting from an empty feature set, it searches forward for potential feature subsets by performing greedy hillclimbing augmented with a backtracking technique [37]. The wrapper-based feature selection method is applied here because Huang et al. [21] showed that it may be superior to hybrid approaches combining chi-square and information-gain methods reported in the literature. A comprehensive literature review shows no attempts to predict osteoporosis outcome in Taiwanese women using genetic factors (SNPs) and the three above mentioned classification algorithms with wrapper-based feature selection method.

This study therefore compared performance in three classification algorithms: MFNN, naive Bayes, and logistic regression, with and without wrapper-based feature selection techniques. Identifying the genes and SNPs associated with Taiwan population of women with osteoporosis would enable researchers to focus on the candidate genes and SNPs that are most promising for use in diagnosis and therapy. The results of our studies could be generalized to SNP searches in genetic studies of human disorders and to development of new molecular diagnostic/prognostic tools. However, before routine application of genomic analysis in clinical practice, genetic markers must be validated in prospective clinical trials.

2. Materials and Methods

2.1. Subjects. The dataset in this study, which included SNPs, age, menopause, and BMI, was the same dataset used in a previous study by the first author of this paper [9]. The T-score was calculated according to WHO classifications using a locally derived reference range provided by the manufacturer. The subjects were divided into two BMD groups according to T-score [38–40]. Subjects with T-score > -1 were enrolled in the high BMD group, and those with T-scores ≤ -1 were enrolled in the low BMD group. The overall dataset was derived from 295 cases, including (i) 247 postmenopausal cases (83.73%) and 48 prepremenopausal cases (16.27%); (ii) 112 high BMD cases (37.97%) and 183 low BMD cases (62.03%). Table 2 presents the demographic characteristics of the study subjects. Post-menopause was defined as the absence of menstruation for >6 months or age ≥ 50 years [9]. Clinical data used for diagnosis were further converted into numerical form, that is, 1 for "high BMD" and 0 for "low BMD."

2.2. Candidate Genes. Table 1 shows the 22 SNPs analyzed in this study, which were the same as those analyzed previously by the first author of this paper [9]. Table 1 shows that the nine candidate genes included TNFα, transforming growth factor-beta 1 (TGFβ1), osteocalcin, parathyroid hormone (PTH), interleukin 1 receptor antagonist (IL1_ra), HSP, calcitonin receptor (CTR), bone morphogenetic protein-4 (BMP-4), and three genotypes per locus.

2.3. Classification Algorithms. The three families of classification algorithms used as the basis for comparisons in this study were MFNN, naive Bayes, and logistic regression. These classifiers were implemented using the Waikato Environment for Knowledge Analysis (WEKA) software [37].

An MFNN is an artificial neural network (ANN) model in which connections between the units do not form a directed cycle [24–28, 30]. From an algorithmic perspective, the underlying process of an MFNN can be divided into retrieving and learning phases [24]. Assume an L-layer feedforward neural network with N_l units at the lth layer. In the retrieving phase, the MFNN iterates through all layers to produce the retrieval response $\{a_i(L),\ i = 1, 2, \ldots, N_L\}$ at the output layer based on test pattern inputs $\{a_i(0),\ i = 1, 2, \ldots, N_0\}$, the known weights w_{ij} of the network, and the nonlinear activation function f_i (e.g., sigmoid function). In the learning phase of this MFNN, the backpropagation algorithm [30] and evolutionary algorithms [31, 32] are used in the learning scheme. The backpropagation algorithm is used as a simple gradient descent approach. The weight updating mechanism is a backpropagation of corrective signals from the output layer to the hidden layers. The goal is iteratively selecting a set of weights $w_{ij}(l)$ for all layers such that the squared error function E can be minimized by a pair of input training patterns $\{a_i(0),\ i = 1, 2, \ldots, N_0\}$ and target training patterns $\{t_j,\ j = 1, 2, \ldots, N_L\}$.

Mathematically, the iterative gradient descent formulation for updating each specific weight $w_{ij}(l)$ can be expressed by the following equation:

$$w_{ij}(l) \longleftarrow w_{ij}(l) - \eta \frac{\partial E}{\partial w_{ij}(l)}, \tag{1}$$

where η is the learning rate and $\partial E/\partial w_{ij}(l)$ can be effectively calculated through a numerical chain rule by backpropagating the error signal from the output layer to the input layer.

Structurally, however, an MFNN is a spatial and iterative neural network with several layers of hidden neuron units between the input and output neuron layers. The basic function of each neuron is the linear basis function, and activation is modeled with a non-decreasing and differentiable sigmoid function. This approach uses an MFNN to model osteoporosis outcome. Inputs contain the information about clinical factors, for example, SNPs, that are needed for the database. Outputs contain the information about the osteoporosis outcome.

In summary, the MFNN is trained first by repeatedly providing input-output training pairs and by executing the backpropagation learning algorithm. After this training process is complete, the MFNN is tested by sending testing data

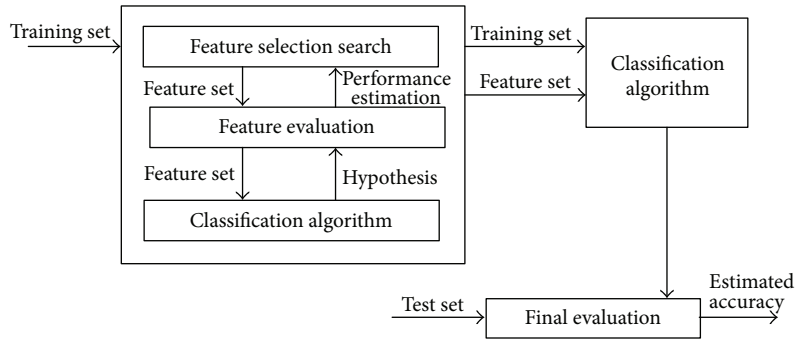


Figure 1: Flowchart of wrapper-based approach to feature subset selection [23].

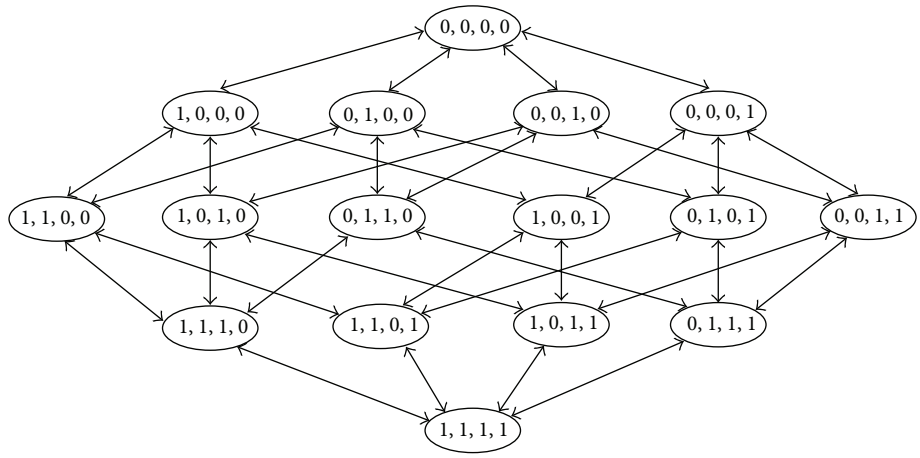


Figure 2: State space search for feature subset selection [23].

inputs (i.e., SNPs) to the network. The forward propagation of the MFNN reveals the osteoporosis outcome for a specific case so that causes can be inferred from effects. Here, the default WEKA parameters were used, that is, hidden layer neurons = 6, learning rate = 0.3, momentum variable = 0.2, and training time = 500.

Second, all features in naive Bayes, which is the simplest Bayesian network, are assumed to be conditionally independent [34]. Let $(X_1, X_2, \ldots, X_p)$ be features (i.e., SNPs) used to predict class C (i.e., disease status, 1 = high BMD or 0 = low BMD). Given a data instance with genotype $(x_1, x_2, \ldots, x_p)$, the best prediction of the disease class is given by class c, which maximizes the conditional probability $\Pr(C = c \mid X_1 = x_1, X_2 = x_2, \ldots, X_p = x_p)$. Bayes theorem is used to estimate the conditional probability $\Pr(C = c \mid X_1 = x_1, X_2 = x_2, \ldots, X_p = x_p)$, which is decomposed into a product of conditional probabilities.

Third, the logistic regression generates the coefficients for the following formula used for logit transformation of the probability of a patient having a characteristic of interest: $\text{logit}(p) = b_0 + b_1x_1 + b_2x_2 + \cdots + b_kx_k$ [41]. The formula used to calculate the probability of the characteristic of interest in this study is $p = 1/(1 + e^{-\text{logit}(p)})$, where 1 = high BMD and 0 = low BMD.

2.4. Feature Selection. The wrapper-based feature selection approach [23], in which a feature selection algorithm acts as a wrapper around a classification algorithm, was used to find a subset of SNPs that maximizes the performance of the prediction model. Figure 1 shows that, in the wrapper approach, the feature subset is selected by using a black box classification algorithm (i.e., selection is performed using the interface alone and does not require knowledge of the algorithm). To search for a good subset, the feature subset selection algorithm includes the classification algorithm itself in the evaluation function. The accuracy of the deduced classifiers is estimated using accuracy estimation techniques. The search space is organized such that each state represents a feature subset. For n features, each state has n bits, and each bit indicates whether a feature is present (1) or absent (0). To determine the connectivity between the states, this study used operators that add or delete a single feature from each state, where the states correspond to the search space commonly used in stepwise method [23]. Figure 2 shows an example of the state space and operators obtained by stepwise method in a four-feature problem. The size of the search space for n features is $O(2^n)$ [23]. The classification algorithms are used to calculate a performance measure for each of 16 different subsets.

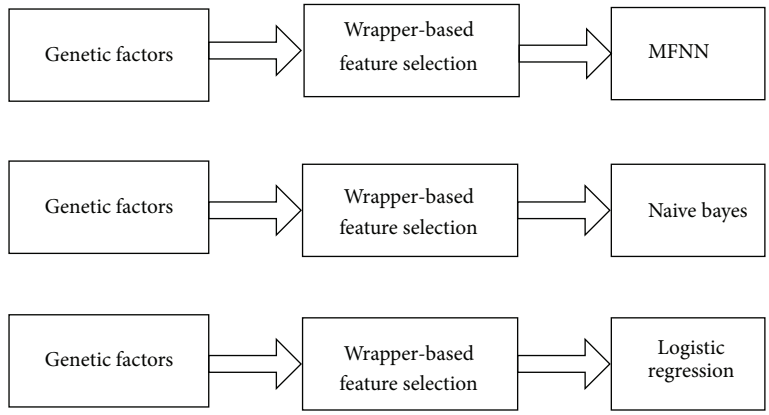


Figure 3: In the wrapper-based feature selection approach, genetic factors are evaluated independently of multilayer feedforward neural network (MFNN), naive Bayes, and logistic regression.

Table 3: Results of repeated 10-fold cross-validation experiment using multilayer feedforward neural network (MFNN), naive Bayes, and logistic regression without feature selection.

Algorithm	AUC	Sensitivity	Specificity	Number of SNPs
MFNN	0.489	0.400	0.629	11
Naive Bayes	0.462	0.296	0.612	11
Logistic regression	0.485	0.333	0.615	11

AUC: area under the ROC curve.

Therefore, the wrapper-based approach conducts a best-first search for a good subset by including the classification algorithm itself (MFNN, naive Bayes, or logistic regression) in the feature subset evaluation [23]. To search for potential feature subsets, the best-first search starts from an empty feature set and searches forward by greedy hillclimbing augmented with a backtracking technique [37]. Figure 3 shows how MFNN, naive Bayes, and logistic regression were applied in the wrapper-based approach.

2.5. Evaluating Predictive Performance. The performance of the prediction models was measured in terms of receiver operating characteristic (ROC) and area under the ROC curve (AUC) [42]. The AUC of a classifier can be interpreted as the probability of the classifier ranking a randomly chosen positive example higher than a randomly chosen negative one [42]. Most researchers have now adopted AUC for evaluating the predictive capability of classifiers since AUC is a better performance metric compared to accuracy [42]. This study used the AUC value for performance comparison of different prediction models using the same dataset. The higher the AUC, the better the learning performance [43]. Other calculations included sensitivity, the proportion of correctly predicted responders out of all tested responders, and specificity, the proportion of correctly predicted nonresponders out of all tested nonresponders.

To investigate the generalization of the prediction models produced by the above algorithms, the repeated 10-fold cross-validation method was used [44]. First, the whole dataset was randomly divided into ten distinct parts. The model was then trained with nine-tenths of the data and tested by the remaining tenth of data to estimate its predictive performance. This procedure was repeated nine more times. Each time, a different tenth of the data was used as testing data, and a different nine-tenths of the data were used as training data. Finally, the average estimate over all runs was reported by running the above regular 10-fold cross-validation 100 times with different splits of data. In repeated 10-fold cross-validation testing, the performance of all models was evaluated with and without feature selection.

3. Results

Tables 3 and 4 summarize the results of the repeated 10-fold cross-validation experiments for MFNN, naive Bayes, and logistic regression using SNPs with and without feature selection. First, the AUC, sensitivity, and specificity were calculated for the three predictive models without wrapper-based feature selection. Table 3 shows that the average AUC values for the MFNN, the naive Bayes, and the logistic regression prediction models were 0.489, 0.462 and 0.485, respectively. In terms of AUC, the the MFNN model (AUC = 0.489) outperformed the naive Bayes (AUC = 0.462) and logistic regression (AUC = 0.485) models.

A repeated 10-fold cross-validation experiment was performed to compare performance in the three wrapper-based predictive algorithms. Table 4 shows that the MFNN, the naive Bayes, and the logistic regression models had average AUC values of 0.631, 0.569, and 0.620, respectively. In terms of AUC, the MFNN model (AUC = 0.631) outperformed both the naive Bayes model (AUC = 0.569) and the logistic regression model (AUC = 0.620). Each wrapper-based model selected 3 to 8 SNPs (Table 4). Out of 11 SNPs, the wrapper-based MFNN model identified only 4: rs1800469 (TGFβ1-509), VNTR (IL1_ra), rs2227956 (HSP70 hom), and rs1801197 (CTR).

The classifiers were also compared with and without feature selection. Feature selection using the wrapper-based approach clearly improved performance in the MFNN,

Table 4: Results of repeated 10-fold cross-validation experiment using multilayer feedforward neural network (MFNN), naive Bayes, and logistic regression with wrapper-based feature selection approach.

Algorithm	AUC	Sensitivity	Specificity	Number of SNPs
MFNN	0.631	0.579	0.689	4 (rs1800469, VNTR, rs2227956, rs1801197)
Naive Bayes	0.569	0	0.620	3 (rs1800469, rs1800247, rs1801197)
Logistic regression	0.620	0.407	0.623	8 (rs1800469, rs1800629, rs6254, rs6256, rs2227956, rs1061581, rs1801197, rs17563)

AUC: area under the ROC curve.

the naive Bayes, and the logistic regression. Overall, the MFNN classifier with the wrapper-based approach demonstrated superior prediction performance (AUC = 0.631) compared to the other models. Additionally, the MFNN classifier with wrapper-based feature selection required fewer SNPs ($n = 4$) compared to the MFNN classifier without feature selection ($n = 11$).

Table 4 shows that the AUCs did not significantly differ between the MFNN model with wrapper-based feature selection (AUC = 0.631) and the logistic regression model with wrapper-based feature selection (AUC = 0.620). However, the MFNN classifier with wrapper-based feature selection required fewer SNPs ($n = 4$) compared to the logistic regression classifier with wrapper-based feature selection ($n = 8$), that is, by selecting a small number of SNPs with significantly larger effects compared to other SNPs and by disregarding relatively insignificant SNPs, the MFNN model with wrapper-based feature selection successfully identified a subset of four major SNPs that could be used to predict osteoporosis outcome in the study population (rs1800469 (TGFβ1-509), VNTR (IL1_ra), rs2227956 (HSP70 hom), and rs1801197 (CTR)). After confirming that the MFNN model outperforms the logistic regression model, the next objective was finding the candidate genes and SNPs that are most promising for diagnosing osteoporosis, designing therapies, and predicting outcome in the studied population of Taiwanese women with osteoporosis.

4. Discussion

This study compared three classification algorithms, including MFNN, naive Bayes, and logistic regression with and without feature selection in terms of accuracy in predicting osteoporosis outcome in a population of Taiwanese women. Accounting for models is not a trivial task because even a relatively small set of candidate genes obtains a large number of possible models [20]. For example, the 11 candidate SNPs studied yielded 2^{11} possible models. The three classifiers were chosen for comparison because they cover varying techniques with different representational models such as probabilistic MFNN, naive Bayes, and logistic regression models [43]. The proposed procedures can also be implemented using the publicly available software WEKA [37] and are thus easily applicable in genomic studies. To the best of our knowledge, this study is the first to propose the use of three classification algorithms, including MFNN, naive Bayes, and logistic regression, and wrapper-based feature selection method for modeling osteoporosis outcome in Taiwanese women based on genetic factors such as SNPs.

In this paper, the wrapper-based feature selection approach was used to find a subset of SNPs that maximizes the performance of the prediction model according to how feature selection search is incorporated in the classification algorithms. The results showed that the MFNN classifier with wrapper-based approach was superior to the other tested algorithms and achieved the greatest AUC with the smallest number of SNPs when distinguishing between high and low BMD in Taiwanese women. These results suggest that MFNN model is a good method of modeling complex nonlinear relationships among clinical factors and the responsiveness of osteoporosis outcome in Taiwanese women. The wrapper-based approach does not require knowledge of the classification algorithm used in the feature selection process, in which features are optimized by using the classification algorithm as part of the evaluation function [21, 23]. Another advantage of the wrapper-based method is its inclusion of the interaction between feature subset search and the classification model [21]. However, the risk of over-fitting is high when using the wrapper-based method [21, 45]. In the current study, use of the wrapper-based feature selection approach to assess high and low BMD individuals revealed a panel of genetic markers, including TGFβ1-509, IL1_ra, HSP70 hom, and CTR, which were more prominent compared to other markers observed in the examined Taiwanese women population with osteoporosis.

A noted limitation of this study is that, due to the small sample size, the AUC values were too low (<0.7) to obtain good dataset classifications. A dataset based on a larger sample size is needed for improved accuracy. Therefore, further prospective clinical trials are recommended to determine whether the observed outcome associations with these candidate genes are reproducible in a larger population of Taiwanese women with osteoporosis.

5. Conclusion

This study used an MFNN methodology with wrapper-based feature selection method to predict osteoporosis outcome in Taiwanese women based on clinical factors such as SNPs. The trained MFNN model showed good responsiveness in inferring osteoporosis outcome. The findings suggest that patients and doctors can use the proposed tool to enhance decision making based on clinical factors such as SNP genotyping data. However, genetic markers require validation in further prospective clinical trials before routine clinical use of genomic analysis for predicting osteoporosis outcome.

Acknowledgments

This work was partly supported by the National Science Council, Taiwan, under Grant no. NSC 101-2320-B-037-022 and by Grants DOH102-TD-C-111-002 and DOH101-TD-C-111-002 and that of the Kaohsiung Medical University Research Foundation (KMUER014).

References

[1] A. Klibanski, L. Adams-Campbell, T. Bassford et al., "Osteoporosis prevention, diagnosis, and therapy," *Journal of the American Medical Association*, vol. 285, no. 6, pp. 785–795, 2001.

[2] P. Mezquita-Raya, M. Muñoz-Torres, G. Alonso et al., "Susceptibility for postmenopausal osteoporosis: interaction between genetic, hormonal and lifestyle factors," *Calcified Tissue International*, vol. 75, no. 5, pp. 373–379, 2004.

[3] S. L. Hui, C. W. Slemenda, and C. C. Johnston, "Age and bone mass as predictors of fracture in a prospective study," *Journal of Clinical Investigation*, vol. 81, no. 6, pp. 1804–1809, 1988.

[4] M. A. Osmanagaoglu, B. Okumuş, T. Osmanagaoglu, and H. Bozkaya, "The relationship between serum dehydroepiandrosterone sulfate concentration and bone mineral density, lipids, and hormone replacement therapy in premenopausal and postmenopausal women," *Journal of Women's Health*, vol. 13, no. 9, pp. 993–999, 2004.

[5] M. Sadat-Ali, I. M. Al-Habdan, F. A. Al-Mulhim, and A. Y. El-Hassan, "Bone mineral density among postmenopausal Saudi women," *Saudi Medical Journal*, vol. 25, no. 11, pp. 1623–1625, 2004.

[6] L. J. Melton, S. H. Kan, M. A. Frye, H. W. Wahner, W. M. O'Fallon, and B. L. Riggs, "Epidemiology of vertebral fractures in women," *American Journal of Epidemiology*, vol. 129, no. 5, pp. 1000–1011, 1989.

[7] V. Camozzi, V. Carraro, M. Zangari, F. Fallo, F. Mantero, and G. Luisetto, "Use of quantitative ultrasound of the hand phalanges in the diagnosis of two different osteoporotic syndromes: Cushing's syndrome and postmenopausal osteoporosis," *Journal of Endocrinological Investigation*, vol. 27, no. 6, pp. 510–515, 2004.

[8] H. Xu, D. H. Xiong, F. H. Xu, Y. Y. Zhang, S. F. Lei, and H. W. Deng, "Association between VDR ApaI polymorphism and hip bone mineral density can be modified by body mass index: a study on postmenopausal Chinese women," *Acta Biochimica et Biophysica Sinica*, vol. 37, no. 1, pp. 61–67, 2005.

[9] G. T. Lin, H. F. Tseng, C. K. Chang et al., "SNP combinations in chromosome-wide genes are associated with bone mineral density in Taiwanese women," *Chinese Journal of Physiology*, vol. 51, no. 1, pp. 32–41, 2008.

[10] B. L. M. Hogan, "Bone morphogenetic proteins: multifunctional regulators of vertebrate development," *Genes and Development*, vol. 10, no. 13, pp. 1580–1594, 1996.

[11] D. E. Hughes, A. Dai, J. C. Tiffee, H. H. Li, G. R. Munoy, and B. F. Boyce, "Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β," *Nature Medicine*, vol. 2, no. 10, pp. 1132–1135, 1996.

[12] E. Phelps, O. Bezouglaia, S. Tetradis, and J. M. Nervina, "Parathyroid hormone induces receptor activity modifying protein-3 (RAMP3) expression primarily via $3^{'},5^{'}$-cyclic adenosine monophosphate signaling in osteoblasts," *Calcified Tissue International*, vol. 77, no. 2, pp. 96–103, 2005.

[13] X. Feng, S. Bonni, and K. Riabowol, "HSP70 induction by ING proteins sensitizes cells to tumor necrosis factor alpha receptor-mediated apoptosis," *Molecular and Cellular Biology*, vol. 26, no. 24, pp. 9244–9255, 2006.

[14] Y. Ishida, T. Kondo, A. Kimura, K. Matsushima, and N. Mukaida, "Absence of IL-1 receptor antagonist impaired wound healing along with aberrant NF-κB activation and a reciprocal suppression of TGF-β signal pathway," *Journal of Immunology*, vol. 176, no. 9, pp. 5598–5606, 2006.

[15] S. Hong, S. Lim, A. G. Li et al., "Smad7 binds to the adaptors TAB2 and TAB3 to block recruitment of the kinase TAK1 to the adaptor TRAF2," *Nature Immunology*, vol. 8, no. 5, pp. 504–513, 2007.

[16] T. Mukai, F. Otsuka, H. Otani et al., "TNFα inhibits BMP-induced osteoblast differentiation through activating SAPK/JNK signaling," *Biochemical and Biophysical Research Communications*, vol. 356, no. 4, pp. 1004–1010, 2007.

[17] W. F. Boron and E. L. Boulpaep, *Medical Physiology: A Cellular and Molecular Approach*, Elsevier Saunders, New York, NY, USA, 2004.

[18] N. K. Lee, H. Sowa, E. Hinoi et al., "Endocrine regulation of energy metabolism by the skeleton," *Cell*, vol. 130, no. 3, pp. 456–469, 2007.

[19] H. W. Chang, L. Y. Chuang, C. H. Ho, P. L. Chang, and C. H. Yang, "Odds ratio-based genetic algorithms for generating SNP barcodes of genotypes to predict disease susceptibility," *OMICS: A Journal of Integrative Biology*, vol. 12, no. 1, pp. 71–81, 2008.

[20] K. E. Lee, N. Sha, E. R. Dougherty, M. Vannucci, and B. K. Mallick, "Gene selection: a Bayesian variable selection approach," *Bioinformatics*, vol. 19, no. 1, pp. 90–97, 2003.

[21] L. C. Huang, S. Y. Hsu, and E. Lin, "A comparison of classification methods for predicting Chronic Fatigue Syndrome based on genetic data," *Journal of Translational Medicine*, vol. 7, p. 81, 2009.

[22] E. Lin, Y. Hwang, K. H. Liang, and E. Y. Chen, "Pattern-recognition techniques with haplotype analysis in pharmacogenomics," *Pharmacogenomics*, vol. 8, no. 1, pp. 75–83, 2007.

[23] R. Kohavi and G. H. John, "Wrappers for feature subset selection," *Artificial Intelligence*, vol. 97, no. 1-2, pp. 273–324, 1997.

[24] J. T. Tsai, J. H. Chou, and T. K. Liu, "Tuning the structure and parameters of a neural network by using hybrid Taguchi-genetic algorithm," *IEEE Transactions on Neural Networks*, vol. 17, no. 1, pp. 69–80, 2006.

[25] W. H. Ho and C. S. Chang, "Genetic-algorithm-based artificial neural network modeling for platelet transfusion requirements on acute myeloblastic leukemia patients," *Expert Systems with Applications*, vol. 38, no. 5, pp. 6319–6323, 2011.

[26] W. H. Ho, J. X. Chen, I. N. Lee, and H. C. Su, "An ANFIS-based model for predicting adequacy of vancomycin regimen using improved genetic algorithm," *Expert Systems with Applications*, vol. 38, no. 10, pp. 13050–13056, 2011.

[27] Z. Zhang, Y. Ge, D. Zhang, and X. Zhou, "High-content analysis in monastrol suppressor screens: a neural network-based classification approach," *Methods of Information in Medicine*, vol. 50, no. 3, pp. 265–272, 2011.

[28] W. H. Ho, K. T. Lee, H. Y. Chen, T. W. Ho, and H. C. Chiu, "Disease-free survival after hepatic resection in hepatocellular carcinoma patients: a prediction approach using artificial neural network," *PLoS ONE*, vol. 7, no. 1, Article ID e29179, 2012.

[29] E. Lin and L. C. Huang, "Identification of significant genes in genomics using Bayesian variable selection methods," *Advances and Applications in Bioinformatics and Chemistry*, vol. 1, pp. 13–18, 2008.

[30] W. S. Ke, Y. Hwang, and E. Lin, "Pharmacogenomics of drug efficacy in the interferon treatment of chronic hepatitis C using classification algorithms," *Advances and Applications in Bioinformatics and Chemistry*, vol. 3, pp. 39–44, 2010.

[31] J. T. Tsai, T. K. Liu, and J. H. Chou, "Hybrid Taguchi-genetic algorithm for global numerical optimization," *IEEE Transactions on Evolutionary Computation*, vol. 8, no. 4, pp. 365–377, 2004.

[32] W. H. Ho, J. H. Chou, and C. Y. Guo, "Parameter identification of chaotic systems using improved differential evolution algorithm," *Nonlinear Dynamics*, vol. 61, no. 1-2, pp. 29–41, 2010.

[33] C. J. Robinson, S. Swift, D. D. Johnson, and J. S. Almeida, "Prediction of pelvic organ prolapse using an artificial neural network," *American Journal of Obstetrics and Gynecology*, vol. 199, no. 2, pp. 193–e1, 2008.

[34] P. Domingos and M. Pazzani, "On the optimality of the simple Bayesian classifier under zero-one loss," *Machine Learning*, vol. 29, no. 2-3, pp. 103–130, 1997.

[35] Q. Wang, G. M. Garrity, J. M. Tiedje, and J. R. Cole, "Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy," *Applied and Environmental Microbiology*, vol. 73, no. 16, pp. 5261–5267, 2007.

[36] M. Bhandari and A. Joensson, *Clinical Research for Surgeons*, Thieme, New York, NY, USA, 2009.

[37] I. H. Witten and E. Frank, *Data Mining: Practical Machine Learning Tools and Techniques*, Morgan Kaufmann, San Francisco, Calif, USA, 2005.

[38] J. M. Koh, Y. H. Khang, C. H. Jung et al., "Higher circulating hsCRP levels are associated with lower bone mineral density in healthy pre- and postmenopausal women: evidence for a link between systemic inflammation and osteoporosis," *Osteoporosis International*, vol. 16, no. 10, pp. 1263–1271, 2005.

[39] J. Y. Y. Leung, A. Y. Y. Ho, T. P. Ip, G. Lee, and A. W. C. Kung, "The efficacy and tolerability of risedronate on bone mineral density and bone turnover markers in osteoporotic Chinese women: a randomized placebo-controlled study," *Bone*, vol. 36, no. 2, pp. 358–364, 2005.

[40] K. M. Summers and T. P. Brock, "Impact of pharmacist-led community bone mineral density screenings," *Annals of Pharmacotherapy*, vol. 39, no. 2, pp. 243–248, 2005.

[41] A. Cucchetti, F. Piscaglia, A. D. Grigioni et al., "Preoperative prediction of hepatocellular carcinoma tumour grade and micro-vascular invasion by means of artificial neural network: a pilot study," *Journal of Hepatology*, vol. 52, no. 6, pp. 880–888, 2010.

[42] T. Fawcett, "An introduction to ROC analysis," *Pattern Recognition Letters*, vol. 27, no. 8, pp. 861–874, 2006.

[43] R. Hewett and P. Kijsanayothin, "Tumor classification ranking from microarray data," *BMC Genomics*, vol. 9, no. 2, article S21, 2008.

[44] C. F. Aliferis, A. Statnikov, I. Tsamardinos, J. S. Schildcrout, B. E. Shepherd, and F. E. Harrell Jr., "Factors influencing the statistical power of complex data analysis protocols for molecular signature development from microarray data," *PLoS ONE*, vol. 4, no. 3, Article ID e4922, 2009.

[45] Y. Saeys, I. Inza, and P. Larrañaga, "A review of feature selection techniques in bioinformatics," *Bioinformatics*, vol. 23, no. 19, pp. 2507–2517, 2007.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2012, Article ID 208719, 7 pages
doi:10.1155/2012/208719

Review Article

Sex Steroids and Bone Health Status in Men

Kok-Yong Chin and Soelaiman Ima-Nirwana

Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

Correspondence should be addressed to Soelaiman Ima-Nirwana, imasoel@medic.ukm.my

Received 18 August 2012; Accepted 27 September 2012

Academic Editor: Ling-Qing Yuan

Male osteoporosis is a health problem which deserves more attention as nearly 30% of osteoporotic fractures happen in men aged 50 years and above. Although men do not experience an accelerated bone loss phase and testosterone deficiency is not a universal characteristic for aged men, osteoporosis due to age-related testosterone deficiency does have a negative impact on bone health status of men. Observations from epidemiological studies indicate that elderly men with higher testosterone can preserve their BMD better and thus are less prone to fracture. Observations on men with estrogen resistance or aromatase deficiency indicate that estrogen is equally important in the maintenance of bone health status. This had been validated in several epidemiological studies which found that the relationships between estrogen and bone health indices are significant and sometimes stronger than testosterone. Studies on the relationship between quantitative ultrasound and bone remodeling markers suggest that testosterone and estrogen may have differential effects on bone, but further evidence was needed. In conclusion, both testosterone and estrogen are important in the maintenance of bone health in men.

1. Introduction

Male osteoporosis is a health issue that deserves more attention. Although men do not experience a phase of accelerated bone loss similar to menopause of women, their bone health status declines gradually with age [1, 2]. The incidence of osteoporotic fracture in men increased exponentially after their seventies, which was relatively late compared to women in which the increase transpired as early as in their fifties [3, 4]. As a result of this late manifestation of osteoporosis and a relatively shorter life span compared to their female counterpart, it was generally thought that male osteoporosis was a rare disease condition [5]. However, the incidence of male osteoporosis proved otherwise. According to an estimation by Johnell and Kanis, in the year 2000, 39% of the global incidence of osteoporotic fractures happened in men. The incidence in men aged 50 years and above was 39% vertebral fractures, 30% hip fractures and 25% wrist fractures [6].

Apart from that, male fracture patients seldom receive treatment for osteoporosis. The morbidity and mortality of male fracture patients are higher compared to female patients. The Dubbo Osteoporosis Study indicated that the 5-year mortality rate for male fracture patients aged 60 years and above was higher compared to females, and the mortality rate after a second fracture also exceeded that of females [7]. In the Canadian Multicenter Osteoporosis Study, it was reported that male fracture patients suffered from inability to take care of themselves and immobility [8]. Male osteoporosis also causes substantial economic burden to the health care system. According to estimation by Burge et al., men contributed to 24% of osteoporotic fractures in the United States, and it translated to an economic loss of 4.1 billion USD. The cost of osteoporosis will continue to rise as a result of increase in life span and in fracture incidence [9].

2. Testosterone and Bone Health Status in Men

Androgens are C-19 steroids produced by the testes and adrenal glands, while testosterone (T) is the most abundant androgen found in the body of men. Testosterone is bound by sex hormone-binding globulin (SHBG) and albumin in the blood. The bond between T and albumin is weak and

dissociates readily when T reaches target cells. Hence, the amount of T bound to albumin and the truly unbound T (free fraction) is termed bioavailable T. The bond between T and SHBG is strong, thus preventing the entry of T into target cells. Testosterone can be converted into 5a-dihydrotestosterone (DHT) found in peripheral tissues. It can be catalyzed by the aromatase enzyme estradiol. Testosterone and DHT bind with androgen receptors while estradiol binds with estrogen receptors. Both of these receptors can be found in bone tissue [1, 10].

In vitro studies demonstrated that androgen could increase the proliferation and decrease the apoptosis of osteoblast via regulation of protein kinase B [11]. It also played a vital role in the process of mineralization, which is the late differentiation stage of osteoblast [12, 13]. Androgen also prevented parathyroid-induced osteoclast formation [14] and decreased bone resorption activity of osteoclast via deactivation of lysosomal enzymes [15]. Interleukin-1 (IL-1) and parathyroid-induced prostaglandin E-2 production was also hindered by androgen [16]. It exhibited similar effects on IL-6 production in bone marrow stromal cells [17].

The importance of androgen on bone health in men can be observed in orchidectomized and genetically modified (androgen receptor knockout/ARKO) rat model. A study showed that the femoral bones for both rat models were characterized by low bone mineral density (BMD) and low trabecular bone volume. The rats also had lower cross-sectional midfemoral shaft area, cortical area, cortical thickness, and periosteal perimeter compared to normal rats. This could contribute to low bone formation rate, mineralizing rate, and periosteal osteoblast number in these rats [18]. In a study by Yarrow et al., orchidectomized young male rats exhibited reduced trabecular volume, number, and width, increased osteoid surface, and trabecular separation one month after orchidectomy, and the condition was reversed by supraphysiological testosterone injection [19]. In ARKO mice, thinning of trabecular bone and lowering of mineralization rate happened in young mice [20]. This indicated that androgen acted through the androgen receptor to exhibit its anabolic effects on bone. These animal studies consolidate the theory that androgen and its receptors are important in the maintenance of the male skeletal system.

Osteoporosis is also a common characteristic for hypogonadal men, regardless of their age and the type of hypogonadism. Testosterone was proven to increase the BMD of hypogonadal men, regardless of the type of hypogonadism [21, 22]. Suppression of bone resorption markers in patients receiving testosterone treatment was seen after a 6-month treatment period [21]. A study by Benito et al. indicated that apart from BMD, improvements in micromagnetic imaging indices such as bone volume fraction, trabecular thickness, topological erosion index, and surface-to-curve ratio were observed in hypogonadal males receiving T therapy [23]. The significance of androgen on bone health in men was also seen in prostate cancer patients who have undergone androgen deprivation therapy (ADT). A review by Diamond et al. reported that the BMD of patients receiving ADT showed greater reduction rate compared to normal males. For example, the BMD reduction rate at the femoral neck for patients receiving ADT was 1.8%–2.3% per year, which was higher compared to normal men at 0.7% per year [24]. Prostate cancer patients who received ADT and survived five years following treatment had higher bone fracture risk compared to patients who did not receive ADT [25]. Besides that, the prevalence rates of osteoporosis and osteopenia are also higher in prostate cancer patients who received ADT compared to normal men. In a study by Bruder et al., 53% of the ADT-receiving patients with a mean age of 77 years were osteoporotic [26]. Judging from these observations on T-deprived men, it was evident that T was important in maintaining the integrity of the male skeletal system.

3. Age-Related Testosterone Deficiency and Bone Health Status of Men

The decline of T in men is gradual, and subnormal T level is not a universal characteristic for aged men. There are aged men who are in their eighth decade but still possessing bioavailable T level within the normal reference range of young men [27]. Thus, terms such as partial androgen deficiency, late-onset hypogonadism, and testosterone deficiency syndrome have been used to describe hypogonadism that developed in aged men [28]. The Baltimore Longitudinal Aging Study found that the prevalence of hypogonadism among men aged 50 years and above was 12% for the age group 50–59 years, 19% for the age group 60–69 years, 28% for the age group 70–79 years, and 49% for the age group 80 years and above (reference range for T was based on total T value of men aged 21–45 years) [29]. In studies comparing middle-aged men to elderly men or young men to elderly men, a linear decline in T levels, especially free and bioavailable T, was a common finding regardless of study types (cross-sectional or longitudinal) (refer to Table 1) [30–32].

The age-related decline in testosterone level was attributed to two factors, which were the degeneration of Leydig's cells and the increase of SHBG level with age. In rat models, age increment was related to the decline of weight of the testes, volume of Leydig's cells, and T production by Leydig's cells [45]. Leydig's cells in aged rats were found to be less responsive towards luteinizing hormone (LH) stimulation and possess less hormone receptors compared to control rats [46]. The binding of LH to its receptor also produced relatively less cAMP in old rats compared to young rats [46]. A genetic study revealed that expression of genes responsible for cholesterol metabolism (Scavenger Receptor class B member 1 (SR-B1) and carboxylesterase ES-10), steroidogenesis (cytochrome P450scc and cytochrome P450c17), and antioxidant enzymes (copper-zinc superoxide dismutase and glutathione transferase) was reduced in aged rats compared to young rats [47]. From these results, it is reasonable to envisage that Leydig's cell degeneration is the result of a combination of various factors such as failure of the oxidative stress barrier, which leads to suppression of cAMP generation and damage to T producing enzymes. This causes a decreased response towards LH stimulation and subsequently the lowering of testosterone production.

Table 1: Epidemiological studies on the relationship between sex hormones and bone health status in men.

Researcher	Study and subject involved	Findings
Khosla et al. (1998) [33]	Rochester Epidemiology Study. 280 men aged 25–85 years.	Testosterone and estrogen (estradiol and estrone) correlated significantly with BMD of subjects at multiple sites. In a multiple stepwise regression model, estrogen was the only significant predictor of proximal femoral BMD.
van den Beld et al. (2000) [34]	403 men aged 73–94 years.	Testosterone and estradiol (total, free, and bioavailable fractions) were significantly associated with total body, femoral neck, ward and trochanteric BMD.
Amin et al. (2000) [35]	Framingham Study. 405 men aged 68–96 years.	Total estradiol had a positive and significant relationship with BMD of subjects while the relationship between testosterone and BMD was not significant.
Szulc et al. (2003) [36]	MINOS Study. 792 men consisting of two groups. Group 1 aged 19–40 years and Group 2 aged 51–85 years.	The reduction of free testosterone and free testosterone index showed a significant relationship with inability to perform functional tests and risk of fall. Men with lower BMD also had lower free testosterone.
Khosla et al. (2005) [37]	Rochester Epidemiology Study. 314 men aged 22–91 years.	Sex hormones (bioavailable testosterone and estradiol) had significant relationship with several volumetric BMD and structural indices as assessed using pQCT. Subendocortical area showed significant and negative relationship with testosterone. This indicated that testosterone exhibited antiresorptive effect in subendocortical area. Estrogen exhibited significant relationship with vBMD and structural indices particularly at cortical sites.
Mellström et al. (2006) [38]	MrOS Study Sweden. 2908 men with a mean age of 75.4 years.	Free testosterone level below the overall median was predictive of fracture after 50 years of age while free estradiol level at the lowest 10th percentile could predict fracture. Free testosterone and estradiol had significant relationship with BMD of subjects at multiple sites.
Araujo et al. (2008) [39]	Boston Area Health Study. 832 men aged 30–79 years.	Only estradiol levels correlated significantly with femoral neck, hip, ultradistal radius, and spine BMD after adjustment for confounders was performed. Testosterone did not correlate with BMD significantly.
LeBlanc et al. (2009) [40]	MrOS Study USA. 1436 Caucasian and 446 minorities aged 65 years and above.	Men with low bioavailable estradiol, low bioavailable testosterone, and high SHBG had significantly higher risk for nonvertebral fracture.
Travison et al. (2009) [41]	Boston Area Health Study. 808 men aged 30–79 years.	Estradiol levels had significant relationship with several hip strength parameters, especially cross-sectional area. It was suggested that anthropometric factors were the mediating factor in this relationship.
Paller et al. (2009) [42]	The Third National Health and Nutrition Survey USA. 623 men aged 20-90 years.	Men with the lowest quartile of free testosterone had 4-time odds of suffering from osteopenia. Subjects with the lowest quartile of free estradiol had 70% odds of suffering from osteopenia. The relationship between testosterone and bone health status was more obvious in aged men, but the relationship between estradiol and bone health status was apparent in younger men.
Venkat et al. (2009) [43]	350 Indian male military recruits aged 21–55 years.	Spine BMD had significant relationships with estradiol and testosterone (total, free, and bioavailable fraction) although the strength of the relationship was lower for testosterone. Femoral BMD was associated with estradiol but not testosterone levels.
Woo et al. (2011) [44]	MrOS Hong Kong. 1158 Chinese men aged 65 years and above.	Free testosterone level correlated significantly with femoral and hip BMD, but the percentage of changes of BMD in 4 years was significantly associated with estradiol, not testosterone. Men in the lowest quartile for bioavailable and free testosterone and estrogen were more prone to fracture.

Epidemiological observations showed that age-related decline of free and bioavailable T was greater than total T [30]. This is direct result of the increase of SHBG level with age. In normal young men, a rise in SHBG is usually followed by a feedback mechanism, in which the body would increase the T production to maintain the optimal level of free T. However, this feedback mechanism fails in aged men because their Leydig's cells are unable to synthesize sufficient T. As a result, the level of unbound T drops gradually [1, 27]. The increase of SHBG had been linked to age-related decline

of growth hormone and insulin-like growth factor-1, as a negative association was found between these factors in cross-sectional studies [48, 49]. However, a direct link has not been validated.

The age-related decline in testosterone levels poses negative implications on bone health status of aged men. A study on American men aged 20–90 years by Paller et al. found that those with free T level at the lowest quartile had the highest probability to be osteopenic [42]. A study by Van Den Beld et al. also found that there was a positive and significant association between T levels (total, free, and bioavailable fraction and androgen index) and BMD in a population of aged men [34]. This suggested that aged men with higher T levels were better able to maintain their BMD. The Swedish Osteoporotic Fractures in Men (MrOS) Study discovered that there was a negative relationship between free testosterone and previous fracture history after the fifth decade of life in a population of aged men [38]. These findings were also found in non-Western populations. The Hong Kong MrOS study found a positive and significant relationship between femoral and vertebral BMD and free T in a population of Chinese men aged 65 years and above. The researchers also suggested that free T level at the lowest quartile was predictive of bone fracture [44]. This could be due to an increased incidence of fall and a decrease in body functional capacity related to T level [36]. A study by Khosla et al. found that bioavailable T was associated with the subendocortical area in men, which was an index of endocortical resorption. The relationship was strongest at the femoral neck and in the aged population compared to the young population [37]. However, discrepancies existed among different studies, in which some found no relationship between bone health status of men and total T level [42, 44]. There were even some studies which stated that all T levels were not related to male BMD [39]. Besides, the relationship between T and BMD was not consistent at different testing sites. For example, Venkat et al. found that T was related to BMD at the vertebrae but not at the femora [43].

4. Estrogen and the Male Skeletal System

The estrogen level in men was shown to be higher than in postmenopausal women [33, 50]. The estrogen hormone in men is produced via conversion of T to estrogen via the aromatase enzyme (cytochrome 19) [1]. About 15% of the estrogen in men originates from the testes while the other 85% comes from peripheral tissue inclusive of bone. Furthermore, aromatase enzyme was found in osteoblasts, osteocytes, chondrocytes, and adipocytes but not in osteoclasts [51]. Therefore, it is reasonable to postulate that estrogen produced in the bone of men has paracrine or intracrine function. Estrogen had been found to reduce apoptosis, oxidative stress, and nuclear factor kappa B (NF-κB) activity in osteoblasts and increase apoptosis and hinder differentiation of osteoclasts [52].

The traditional notion that estrogen is only important in maintenance of the female skeletal system while testosterone is vital for the male skeletal system is now challenged by several experiments of nature. Smith et al. reported a young man with a rare condition of estrogen resistance due to mutation in the gene coding for the estrogen receptor. The patient also had low BMD and incomplete closure of the epiphyseal plate [53]. Later, Morishima et al. reported a young osteoporotic man with elevated bone remodeling makers who suffered from aromatase deficiency due to a mutation in the genes coded for CYP19 [54]. Estrogen replacement was reported to successfully increase the BMD and T-score of patients with aromatase deficiency and to normalize their bone remodeling marker levels, which subsequently led to closure of the epiphyseal plate and increase in bone age [55–58]. In a report by Bouillon et al., the BMD increment in a young man with aromatase enzyme deficiency after receiving estrogen replacement therapy was due to increase in bone size, without any significant changes in volumetric BMD [59]. This shows that estrogen is as important as androgen in inducing periosteal apposition.

Animal studies also proved that estrogen was important in the maintenance of bone health. A study in male mice with deletion of the aromatase gene showed that after maturation, the male mice had a lower femoral bone mass compared to wild type mice. This was attributed to increased bone resorption in the trabecular bone of the genetically modified mice. In aged animals, significant reductions in cortical and trabecular bone mass were observed in genetically modified mice compared to wild type [60]. Other researchers also showed that deletion of the aromatase gene in mice resulted in higher endosteal bone resorption, higher osteoclast number, and lower trabecular bone volume [61].

The age trend for estrogen in men is inconclusive. Several epidemiological studies found a decreasing trend with age [33, 34] while other studies found no significant changes [62] or a significant elevation with age [63]. Regardless of this, estrogen was found to be associated significantly with bone health status of elderly men in several large epidemiological studies. The Framingham Study discovered that aged men with higher estradiol level had higher BMD, and the difference in BMD between the first quartile and the fourth quartile was equivalent to 10 years of aging on bone [35]. Positive and significant relationships between estradiol level and several hip strength parameters, especially cross-sectional area of bone, were also observed in the Boston Bone Health Study [41]. Using QCT techniques, Khosla et al. discovered that E was significantly correlated with volumetric BMD and structural parameters, especially at cortical sites [37]. The American MrOS study also discovered that aged men with estradiol levels at the lowest quartile had significantly higher risk for nonvertebral fracture [40]. According to The Third National Health and Nutrition Survey of the United States, estradiol levels were found to be associated with BMD in older male population but not in the young population [42]. Hence, from these observations, estrogen seems to prevent deterioration of bone health status in elderly men but not in young men, particularly at the cortical sites. The findings of these epidemiological studies are summarized in Table 1.

5. Differential Effects of Testosterone and Estrogen

Bone health status can also be examined using methods other than BMD and QCT measurements. In a study using calcaneal quantitative ultrasound parameters as determinants of bone health status, both bioavailable T and bioavailable E were found to be associated with bone health status of men, but the effects of association of bioavailable E were stronger than bioavailable T. In the same study, bioavailable E had significant relationship with broadband attenuation of sound (BUA) but not with speed of sound (SOS) [64]. Another study by Chin et al. on Malaysian men found that the relationships between T measurements and SOS were significant but the relationships between E measurements and SOS were not [65]. Since BUA and SOS reflected different physical properties of bone [66], this might imply T and E influenced different bone properties. However, this hypothesis is yet to be validated.

In a remarkable experimental study by Falahati-Nini et al., relative contributions of T and E in regulating bone remodeling in elderly men were assessed. It was found that E significantly contributed to suppression of bone resorption, but the contribution of T was insignificant. On the other hand, both T and E were found to have significant contribution towards bone formation [67]. This was in line with the cross-sectional observation of Fatayerji and Eastell in which T and E were significantly correlated with bone formation markers in a group of men aged 20–79 years [68].

6. Conclusion

Both E and T are indispensable for the maintenance of bone health status in men. The significance of these sex hormones on bone health was clearly depicted in male patients who suffered from deficiency of either hormones due to hormone deprivation therapy or genetic mutation. These observations challenge the traditional view that testosterone is the sole determinant of bone health status in men. It is still unclear whether they act synergistically or independently in the maintenance of skeletal integrity in men. More studies should be conducted to establish the role of T and E in regulating bone health in men.

Conflict of Interests

There is no conflict of interests among the authors.

Acknowledgment

The authors thank Universiti Kebangsaan Malaysia for providing the research Grant DIP-2012-07 to enable them to carry out this research.

References

[1] B. L. Riggs, S. Khosla, and L. J. Melton, "Sex steroids and the construction and conservation of the adult skeleton," *Endocrine Reviews*, vol. 23, no. 3, pp. 279–302, 2002.

[2] K.-Y. Chin, S. Ima-Nirwana, M. Isa Naina et al., "Calcaneal quantitative ultrasound value for middle-aged and elderly malaysian Chinese men and its association with age and body anthropometry," *Journal of Clinical Densitometry*, vol. 15, no. 1, pp. 86–91, 2012.

[3] L. J. Donaldson, I. P. Reckless, S. Scholes, J. S. Mindell, and N. J. Shelton, "The epidemiology of fractures in England," *Journal of Epidemiology and Community Health*, vol. 62, no. 2, pp. 174–180, 2008.

[4] T. P. Van Staa, E. M. Dennison, H. G. M. Leufkens, and C. Cooper, "Epidemiology of fractures in England and Wales," *Bone*, vol. 29, no. 6, pp. 517–522, 2001.

[5] E. Gielen, D. Vanderschueren, F. Callewaert, and S. Boonen, "Osteoporosis in men," *Best Practice and Research*, vol. 25, no. 2, pp. 321–335, 2011.

[6] O. Johnell and J. A. Kanis, "An estimate of the worldwide prevalence and disability associated with osteoporotic fractures," *Osteoporosis International*, vol. 17, no. 12, pp. 1726–1733, 2006.

[7] D. Bliuc, N. D. Nguyen, V. E. Milch, T. V. Nguyen, J. A. Eisman, and J. R. Center, "Mortality risk associated with low-trauma osteoporotic fracture and subsequent fracture in men and women," *JAMA*, vol. 301, no. 5, pp. 513–521, 2009.

[8] A. Papaioannou, C. C. Kennedy, G. Ioannidis et al., "The impact of incident fractures on health-related quality of life: 5 years of data from the Canadian Multicentre Osteoporosis Study," *Osteoporosis International*, vol. 20, no. 5, pp. 703–714, 2009.

[9] R. Burge, B. Dawson-Hughes, D. H. Solomon, J. B. Wong, A. King, and A. Tosteson, "Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025," *Journal of Bone and Mineral Research*, vol. 22, no. 3, pp. 465–475, 2007.

[10] D. Vanderschueren, L. Vandenput, S. Boonen, M. K. Lindberg, R. Bouillon, and C. Ohlsson, "Androgens and bone," *Endocrine Reviews*, vol. 25, no. 3, pp. 389–425, 2004.

[11] H. Y. Kang, C. L. Cho, K. L. Huang et al., "Nongenomic androgen activation of phosphatidylinositol 3-kinase/Akt signaling pathway in MC3T3-E1 osteoblasts," *Journal of Bone and Mineral Research*, vol. 19, no. 7, pp. 1181–1190, 2004.

[12] W. Balkan, K. L. Burnstein, P. C. Schiller et al., "Androgen-induced mineralization by MC3T3-E1 osteoblastic cells reveals a critical window of hormone responsiveness," *Biochemical and Biophysical Research Communications*, vol. 328, no. 3, pp. 783–789, 2005.

[13] K. M. Wiren, A. C. Evans, and X. W. Zhang, "Osteoblast differentiation influences androgen and estrogen receptor-α and -β expression," *Journal of Endocrinology*, vol. 175, no. 3, pp. 683–694, 2002.

[14] Q. Chen, H. Kaji, T. Sugimoto, and K. Chihara, "Testosterone inhibits osteoclast formation stimulated by parathyroid hormone through androgen receptor," *FEBS Letters*, vol. 491, no. 1-2, pp. 91–93, 2001.

[15] L. Pederson, M. Kremer, J. Judd et al., "Androgens regulate bone resorption activity of isolated osteoclasts *in vitro*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 2, pp. 505–510, 1999.

[16] C. C. Pilbeam and L. G. Raisz, "Effects of androgens on parathyroid hormone and interleukin-1-stimulated prostaglandin production in cultured neonatal mouse calvariae," *Journal of Bone and Mineral Research*, vol. 5, no. 11, pp. 1183–1188, 1990.

[17] T. Bellido, R. L. Jilka, B. F. Boyce et al., "Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the androgen receptor," *The Journal of Clinical Investigation*, vol. 95, no. 6, pp. 2886–2895, 1995.

[18] K. Venken, K. De Gendt, S. Boonen et al., "Relative impact of androgen and estrogen receptor activation in the effects of androgens on trabecular and cortical bone in growing male mice: a study in the androgen receptor knockout mouse model," *Journal of Bone and Mineral Research*, vol. 21, no. 4, pp. 576–585, 2006.

[19] J. F. Yarrow, C. F. Conover, A. V. Purandare et al., "Supraphysiological testosterone enanthate administration prevents bone loss and augments bone strength in gonadectomized male and female rats," *American Journal of Physiology*, vol. 295, no. 5, pp. E1213–E1222, 2008.

[20] C. Chiang, M. Chiu, A. J. Moore et al., "Mineralization and bone resorption are regulated by the androgen receptor in male mice," *Journal of Bone and Mineral Research*, vol. 24, no. 4, pp. 621–631, 2009.

[21] J. K. Amory, N. B. Watts, K. A. Easley et al., "Exogenous testosterone or testosterone with finasteride increases bone mineral density in older men with low serum testosterone," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 2, pp. 503–510, 2004.

[22] C. Wang, G. Cunningham, A. Dobs et al., "Long-Term Testosterone Gel (AndroGel) treatment maintains beneficial effects on sexual function and mood, lean and fat mass, and bone mineral density in hypogonadal men," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 5, pp. 2085–2098, 2004.

[23] M. Benito, B. Vasilic, F. W. Wehrli et al., "Effect of testosterone replacement on trabecular architecture in hypogonadal men," *Journal of Bone and Mineral Research*, vol. 20, no. 10, pp. 1785–1791, 2005.

[24] T. H. Diamond, C. S. Higano, M. R. Smith, T. A. Guise, and F. R. Singer, "Osteoporosis in men with prostate carcinoma receiving androgen-deprivation therapy: recommendations for diagnosis and therapies," *Cancer*, vol. 100, no. 5, pp. 892–899, 2004.

[25] V. B. Shahinian, Y. F. Kuo, J. L. Freeman, and J. S. Goodwin, "Risk of fracture after androgen deprivation for prostate cancer," *The New England Journal of Medicine*, vol. 352, no. 2, pp. 154–164, 2005.

[26] J. M. Bruder, J. Z. Ma, J. W. Basler, and M. D. Welch, "Prevalence of osteopenia and osteoporosis by central and peripheral bone mineral density in men with prostate cancer during androgen-deprivation therapy," *Urology*, vol. 67, no. 1, pp. 152–155, 2006.

[27] J. M. Kaufman and A. Vermeulen, "The decline of androgen levels in elderly men and its clinical and therapeutic implications," *Endocrine Reviews*, vol. 26, no. 6, pp. 833–876, 2005.

[28] A. Morales, C. C. Schulman, J. Tostain, and F. C.W. Wu F., "Testosterone Deficiency Syndrome (TDS) needs to be named appropriately—the importance of accurate terminology," *European Urology*, vol. 50, no. 3, pp. 407–409, 2006.

[29] S. M. Harman, E. J. Metter, J. D. Tobin, J. Pearson, and M. R. Blackman, "Longitudinal effects of aging on serum total and free testosterone levels in healthy men," *Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 2, pp. 724–731, 2001.

[30] H. A. Feldman, C. Longcope, C. A. Derby et al., "Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts Male Aging Study," *Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 2, pp. 589–598, 2002.

[31] P. Y. Liu, J. Beilin, C. Meier et al., "Age-related changes in serum testosterone and sex hormone binding globulin in Australian men: longitudinal analyses of two geographically separate regional cohorts," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 9, pp. 3599–3603, 2007.

[32] T. G. Travison, A. B. Araujo, V. Kupelian, A. B. O'Donnell, and J. B. McKinlay, "The relative contributions of aging, health, and lifestyle factors to serum testosterone decline in men," *Journal of Clinical Endocrinology and Metabolism*, vol. 92, no. 2, pp. 549–555, 2007.

[33] S. Khosla, L. J. Melton, E. J. Atkinson, W. M. O'Fallon, G. G. Klee, and B. L. Riggs, "Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen," *Journal of Clinical Endocrinology and Metabolism*, vol. 83, no. 7, pp. 2266–2274, 1998.

[34] A. W. Van Den Beld, F. H. De Jong, D. E. Grobbee, H. A. P. Pols, and S. W. J. Lamberts, "Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men," *Journal of Clinical Endocrinology and Metabolism*, vol. 85, no. 9, pp. 3276–3282, 2000.

[35] S. Amin, Y. Zhang, C. T. Sawin et al., "Association of hypogonadism and estradiol levels with bone mineral density in elderly men from the Framingham study," *Annals of Internal Medicine*, vol. 133, no. 12, pp. 951–963, 2000.

[36] P. Szulc, B. Claustrat, F. Marchand, and P. D. Delmas, "Increased risk of falls and increased bone resorption in elderly men with partial androgen deficiency: the MINOS study," *Journal of Clinical Endocrinology and Metabolism*, vol. 88, no. 11, pp. 5240–5247, 2003.

[37] S. Khosla, L. J. Melton, R. A. Robb et al., "Relationship of volumetric BMD and structural parameters at different skeletal sites to sex steroid levels in men," *Journal of Bone and Mineral Research*, vol. 20, no. 5, pp. 730–740, 2005.

[38] D. Mellström, O. Johnell, Ö. Ljunggren et al., "Free testosterone is an independent predictor of BMD and prevalent fractures in elderly men: MrOS Sweden," *Journal of Bone and Mineral Research*, vol. 21, no. 4, pp. 529–535, 2006.

[39] A. B. Araujo, T. G. Travison, B. Z. Leder, and J. B. McKinlay, "Correlations between serum testosterone, estradiol, and sex hormone-binding globulin and bone mineral density in a diverse sample of men," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 6, pp. 2135–2141, 2008.

[40] E. S. LeBlanc, C. M. Nielson, L. M. Marshall et al., "The effects of serum testosterone, estradiol, and sex hormone binding globulin levels on fracture risk in older men," *Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 9, pp. 3337–3346, 2009.

[41] T. G. Travison, A. B. Araujo, T. J. Beck et al., "Relation between serum testosterone, serum estradiol, sex hormone-binding globulin, and geometrical measures of adult male proximal femur strength," *Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 3, pp. 853–860, 2009.

[42] C. J. Paller, M. S. Shiels, S. Rohrmann et al., "Relationship of sex steroid hormones with bone mineral density (BMD) in a nationally representative sample of men," *Clinical Endocrinology*, vol. 70, no. 1, pp. 26–34, 2009.

[43] K. Venkat, M. Desai, M. M. Arora, P. Singh, and M. I. Khatkhatay, "Age-related changes in sex steroid levels influence bone mineral density in healthy Indian men,"

Osteoporosis International, vol. 20, no. 6, pp. 955–962, 2009.

[44] J. Woo, T. Kwok, J. C. S. Leung, C. Ohlsson, L. Vandenput, and P. C. Leung, "Sex steroids and bone health in older Chinese men," *Osteoporosis International*, pp. 1–10, 2011.

[45] H. Chen, M. P. Hardy, I. Huhtaniemi, and B. R. Zirkin, "Age-related decreased Leydig cell testosterone production in the Brown Norway rat," *Journal of Andrology*, vol. 15, no. 6, pp. 551–557, 1994.

[46] H. Chen, M. P. Hardy, and B. R. Zirkin, "Age-related decreases in Leydig cell testosterone production are not restored by exposure to LH *in vitro*," *Endocrinology*, vol. 143, no. 5, pp. 1637–1642, 2002.

[47] H. Chen, R. A. Irizarry, L. Luo, and B. R. Zirkin, "Leydig cell gene expression: effects of age and caloric restriction," *Experimental Gerontology*, vol. 39, no. 1, pp. 31–43, 2004.

[48] E. M. T. Erfurth, L. E. Hagmar, M. Sääf, and K. Hall, "Serum levels of insulin-like growth factor I and insulin-like growth factor-binding protein 1 correlate with serum free testosterone and sex hormone binding globulin levels in healthy young and middle-aged men," *Clinical Endocrinology*, vol. 44, no. 6, pp. 659–664, 1996.

[49] J. Pfeilschifter, C. Scheidt-Nave, G. Leidig-Bruckner et al., "Relationship between circulating insulin-like growth factor components and sex hormones in a population-based sample of 50- to 80-year-old men and women," *Journal of Clinical Endocrinology and Metabolism*, vol. 81, no. 7, pp. 2534–2540, 1996.

[50] S. Khosla, L. J. Melton, E. J. Atkinson, and W. M. O'Fallon, "Relationship of serum sex steroid levels to longitudinal changes in bone density in young versus elderly men," *Journal of Clinical Endocrinology and Metabolism*, vol. 86, no. 8, pp. 3555–3561, 2001.

[51] L. Gennari, R. Nuti, and J. P. Bilezikian, "Aromatase activity and bone homeostasis in men," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 12, pp. 5898–5907, 2004.

[52] S. Khosla, M. J. Oursler, and D. G. Monroe, "Estrogen and the skeleton," *Trends in Endocrinology & Metabolism*, vol. 23, no. 11, pp. 576–581, 2012.

[53] E. P. Smith, J. Boyd, G. R. Frank et al., "Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man," *The New England Journal of Medicine*, vol. 331, no. 16, pp. 1056–1061, 1994.

[54] A. Morishima, M. M. Grumbach, E. R. Simpson, C. Fisher, and K. Qin, "Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens," *Journal of Clinical Endocrinology and Metabolism*, vol. 80, no. 12, pp. 3689–3698, 1995.

[55] J. P. Bilezikian, A. Morishima, J. Bell, and M. M. Grumbach, "Increased bone mass as a result of estrogen therapy in a man with aromatase deficiency," *The New England Journal of Medicine*, vol. 339, no. 9, pp. 599–603, 1998.

[56] C. Carani, K. Qin, M. Simoni et al., "Effect of testosterone and estradiol in a man with aromatase deficiency," *The New England Journal of Medicine*, vol. 337, no. 2, pp. 91–95, 1997.

[57] B. L. Herrmann, B. Saller, O. E. Janssen et al., "Impact of estrogen replacement therapy in a male with congenital aromatase deficiency caused by a novel mutation in the CYP19 gene," *Journal of Clinical Endocrinology and Metabolism*, vol. 87, no. 12, pp. 5476–5484, 2002.

[58] F. Lanfranco, L. Zirilli, M. Baldi et al., "A novel mutation in the human aromatase gene: insights on the relationship among serum estradiol, longitudinal growth and bone mineral density in an adult man under estrogen replacement treatment," *Bone*, vol. 43, no. 3, pp. 628–635, 2008.

[59] R. Bouillon, M. Bex, D. Vanderschueren, and S. Boonen, "Estrogens are essential for male pubertal periosteal bone expansion," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 12, pp. 6025–6029, 2004.

[60] C. Miyaura, K. Toda, M. Inada et al., "Sex- and age-related response to aromatase deficiency in bone," *Biochemical and Biophysical Research Communications*, vol. 280, no. 4, pp. 1062–1068, 2001.

[61] C. Matsumoto, M. Inada, K. Toda, and C. Miyaura, "Estrogen and androgen play distinct roles in bone turnover in male mice before and after reaching sexual maturity," *Bone*, vol. 38, no. 2, pp. 220–226, 2006.

[62] V. Senanarong, S. Vannasaeng, N. Poungvarin et al., "Endogenous estradiol in elderly individuals: cognitive and noncognitive associations," *Archives of Neurology*, vol. 59, no. 3, pp. 385–389, 2002.

[63] Å. Bjørnerem, B. Straume, M. Midtby et al., "Endogenous sex hormones in relation to age, sex, lifestyle factors, and chronic diseases in a general population: the Tromsø study," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 12, pp. 6039–6047, 2004.

[64] N. O. Kuchuk, N. M. Van Schoor, S. M. F. Pluijm, J. H. Smit, W. De Ronde, and P. Lips, "The association of sex hormone levels with quantitative ultrasound, bone mineral density, bone turnover and osteoporotic fractures in older men and women," *Clinical Endocrinology*, vol. 67, no. 2, pp. 295–303, 2007.

[65] K.-Y. Chin, I.-N. Soelaiman, I. N. Mohamed, and W. Z. W. Ngah, "Serum testosterone, sex hormone-binding globulin and total calcium levels predict the calcaneal speed of sound in men," *Clinics*, vol. 67, no. 8, pp. 911–916, 2012.

[66] G. Guglielmi and F. de Terlizzi, "Quantitative Ultrasond in the assessment of Osteoporosis," *European Journal of Radiology*, vol. 71, no. 3, pp. 425–431, 2009.

[67] A. Falahati-Nini, B. L. Riggs, E. J. Atkinson, W. M. O'Fallon, R. Eastell, and S. Khosla, "Relative contributions of testosterone and estrogen in regulating bone resorption and formation in normal elderly men," *The Journal of Clinical Investigation*, vol. 106, no. 12, pp. 1553–1560, 2000.

[68] D. Fatayerji and R. Eastell, "Age-related changes in bone turnover in men," *Journal of Bone and Mineral Research*, vol. 14, no. 7, pp. 1203–1210, 1999.

Hindawi Publishing Corporation
International Journal of Endocrinology
Volume 2012, Article ID 532862, 7 pages
doi:10.1155/2012/532862

Research Article

Palm Tocotrienol Supplementation Enhanced Bone Formation in Oestrogen-Deficient Rats

Ima Nirwana Soelaiman, Wang Ming, Roshayati Abu Bakar, Nursyahrina Atiqah Hashnan, Hanif Mohd Ali, Norazlina Mohamed, Norliza Muhammad, and Ahmad Nazrun Shuid

Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

Correspondence should be addressed to Ima Nirwana Soelaiman, imasoel@medic.ukm.my

Received 24 July 2012; Revised 10 September 2012; Accepted 13 September 2012

Academic Editor: Ling-Qing Yuan

Postmenopausal osteoporosis is the commonest cause of osteoporosis. It is associated with increased free radical activity induced by the oestrogen-deficient state. Therefore, supplementation with palm-oil-derived tocotrienols, a potent antioxidant, should be able to prevent this bone loss. Our earlier studies have shown that tocotrienol was able to prevent and even reverse osteoporosis due to various factors, including oestrogen deficiency. In this study we compared the effects of supplementation with palm tocotrienol mixture or calcium on bone biomarkers and bone formation rate in ovariectomised (oestrogen-deficient) female rats. Our results showed that palm tocotrienols significantly increased bone formation in oestrogen-deficient rats, seen by increased double-labeled surface (dLS/Bs), reduced single-labeled surface (sLS/BS), increased mineralizing surface (MS/BS), increased mineral apposition rate (MAR), and an overall increase in bone formation rate (BFR/BS). These effects were not seen in the group supplemented with calcium. However, no significant changes were seen in the serum levels of the bone biomarkers, osteocalcin, and cross-linked C-telopeptide of type I collagen, CTX. In conclusion, palm tocotrienol is more effective than calcium in preventing oestrogen-deficient bone loss. Further studies are needed to determine the potential of tocotrienol as an antiosteoporotic agent.

1. Introduction

Osteoporosis is an important age-related disease constituting a major health problem especially among postmenopausal women. In women, the rate of bone loss increases dramatically after menopause or ovariectomy due to oestrogen deficiency. Thus, there is an increased rate of activation of remodeling sites resulting in the decrease of both trabecular and cortical bones. In addition, oestrogen deficiency has been associated with oxidative stress because oestrogen is an antioxidant with radical-scavenging properties. The levels of lipid peroxidation (LPO) and hydrogen peroxide (H_2O_2) were increased and enzymatic antioxidants like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S transferase (GST) were decreased in the femur of ovariectomized rats [1]. Oxidative stress has been shown to stimulate osteoclast differentiation and function as well as inhibit osteoblast differentiation. Glutathione peroxidase 1 (Gpx1) is the main antioxidant enzyme expressed in osteoclasts and is responsible for the degradation of hydrogen peroxide. Its overexpression in the osteoclastic cell line RAW264.7 prevents RANKL-induced osteoclastogenesis [2]. Hydrogen peroxide is essential for oestrogen deficiency bone loss and osteoclast formation, suggesting a crucial role for hydrogen peroxide in osteoclast formation. 17β-estradiol stimulates Gpx1 expression in bone-marrow-derived osteoclasts, and oestrogen deficiency was a key step in the reactive oxygen species (ROS-) mediated stimulation of TNFα expression, leading to enhanced osteoclastogenesis and bone resorption [3]. Plasma total oxidative status (TOS) and oxidative stress index (OSI) value were significantly higher, and plasma total antioxidant status (TAS) level was lower in patients than in healthy controls ($P < 0.001$ for all). There was a significant negative correlation between OSI and bone

mineral density (BMD) in lumbar and femoral neck region ($r = 0.63$, $P < 0.001$; $r = 0.40$, $P = 0.018$). These findings indicated that increased osteoclastic activity and decreased osteoblastic activity may be associated with an imbalance between oxidant and antioxidant status in postmenopausal osteoporosis [4]. Our own earlier studies showed that ferric nitrilotriacetate (Fe-NTA), a strong oxidising agent, increased osteoclast number and reduced osteoblast number in rat femoral bone. Trabecular thickness was also lower in the group treated with Fe-NTA, compared to the normal control [5]. In light of these findings, it is hypothesized that antioxidants can prevent postmenopausal bone loss.

Palm oil vitamin E consists of tocopherols and tocotrienols. Each of them has four stereoisomers respectively, namely, α-, β-, γ-, and δ-tocopherols (T) and tocotrienols (T3). Tocopherols contain a saturated phytol side chain in the chroman ring and a saturated hydrocarbon chain. On the other hand, tocotrienols contain three double bonds in the side chain and an unsaturated hydrocarbon chain. Palm tocotrienol at the dose of 100 mg/kg body weight significantly reduced the thiobarbituric acid reactive substances (TBARSs) level in the femur with a significant increase in glutathione peroxidase activity compared to the age-matched control group. These were not observed in the α-tocopherol group. This indicated that tocotrienol has better antioxidant activity in bone than α-tocopherol [6]. Our earlier studies have shown that tocotrienol was able to prevent and even reverse osteoporosis in oestrogen deficiency, testosterone deficiency, glucocorticoid excess, and nicotine exposure [7–13]. The dose of tocotrienol, 60 mg/kg, had been established as the optimum dose for bone protection which we have established in our previous studies [5, 13–15].

Calcium is the most abundant mineral in the body. It is highly consumed through diet. The richest source of calcium includes cheeses and low-fat dairy products such as milk, yogurt, and tau-fu. It is essential for the development and maintenance of strong bones and teeth. Calcium supplementation has been used as treatment for osteoporosis. In adults with a baseline calcium intake of 500–900 mg/day, increasing or supplementing this intake by a further 500–1000 mg/day has a beneficial effect on bone mineral density [16].

Quantitative changes in bone turnover can be assessed easily and noninvasively by the measurement of serum and urinary biochemical markers; the most sensitive markers include serum osteocalcin, bone specific alkaline phosphatase, the N-terminal propeptide of type I collagen for bone formation, and the crosslinked C-(CTX) and N-(NTX) telopeptides of type I collagen for bone resorption [17]. In this study we assayed serum osteocalcin and CTx. The serum cross-linked C-telopeptide of type I collagen (CTX) is a marker of osteoclast activity and is used to assess the level of bone resorption. These peptide fragments are detected by the radioimmunoassay technique. Serum osteocalcin is a major noncollagenous protein that is synthesized by osteoblasts. It plays an important role in the regulation of bone growth and in the correct deposition of the minerals in the matrix. Its expression follows the proliferative phase of osteoblastic differentiation, so it can be considered a marker of mature osteoblasts. Bone histomorphometry is the quantitative study of the microscopic organization and structure of bone tissue by computer-assisted analysis of images formed by a microscope. Bone histomorphometry contains three sets of parameters, namely, structural, static, and dynamic parameters. In this study we studied the dynamic bone histomorphometry which measures the rate of bone growth over time [18].

The aim of this study was to compare between the effects of palm tocotrienols and calcium supplementation on bone metabolism ovariectomised (oestrogen-deficient) rats. The results of this study will indicate whether the tocotrienols are better than calcium as supplements to prevent osteoporosis in the postmenopausal state.

2. Materials and Methods

2.1. Animals and Treatment. Thirty-two female Sprague-Dawley rats (four months old) weighing between 180 and 200 g were obtained from the Laboratory Animals Resource Unit, Universiti Kebangsaan Malaysia. The rats were randomly assigned into groups of sham-operated (SHAM), ovariectomised-control (OVC), ovariectomised and given $60\,\text{mg}\,\text{kg}^{-1}$ of tocotrienol mixture (OV+T), and ovariectomised and given 1% calcium in drinking water *ad libitum* (OV+Ca). Each group has eight rats. Two to three rats were kept in a cage under 12-hour natural light/dark cycles. All rats received normal rat chow from Gold Coin (Port Klang, Selangor, Malaysia). Calcium content of the rat chow as given by the supplier was between 0.8 1.2% [14].

All the groups except the Ovx+Ca group were given deionised water *ad libitum*. The palm tocotrienol mixture was a gift from Carotech Bhd. (Ipoh, Malaysia) consisting of α-tocotrienol 24.67%, γ-tocotrienol 38.955%, δ-tocotrienol 4.55%, and α-tocopherol 20.11%. It was diluted in olive oil (Bertolli, Italy) and given via oral gavage at the dose of $60\,\text{mg}\,\text{kg}^{-1}$ body weight daily at 9 am for 8 weeks. Calcium supplementation was administered as 1% calcium in drinking water ad libitum [19]. For measurement of dynamic histomorphometric parameters, the bone samples were double labelled by intraperitoneal injections of a fluorochrome, calcein, at 9 days and 2 days before sacrifice [18]. The fluorochrome was incorporated into the active formation sites in the bone and can be visualised as fluorescent lines by fluorescent microscopy.

A baseline control group (BC) of eight rats was given the calcein injections upon receipt and sacrificed without any treatment. The right femora were dissected and prepared for dynamic histomorphometric studies.

2.2. Blood and Bone Sampling. Blood samples were collected before the start and after eight weeks of treatment from the retroorbital vein after anesthetizing the rats with ether. After 3 hours in room temperature, the blood was centrifuged at 3000 rpm for 10 min and the serum was stored at -70°C. The left femora were dissected out and fixed with 70% alcohol.

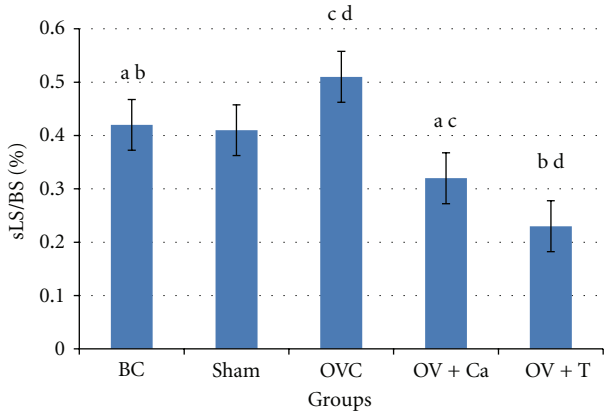


Figure 1: Single-labeled surface (sLS/BS). BC: baseline control group. Sham: sham-operated rats given deionised water and vehicle olive oil. OVC: ovariectomised rats given deionised water and vehicle olive oil. Ov+Ca: ovariectomised rats given 1% calcium in deionised water and vehicle olive oil. Ov+T: ovariectomised rats given deionised water and palm tocotrienol. Same alphabet indicates significant difference between the groups ($P < 0.05$).

2.3. Bone Biochemical Markers. Bone biochemical markers of serum osteocalcin and C-terminal telopeptide of type 1 collagen (CTX) were measured before and after the treatment using an ELISA reader (VERSAmax, Sunnyvale, USA). The kits used were rat osteocalcin ELISA (Biomedical Technologies, Herlev, Denmark) and Ratlaps ELISA CTX-1 (Nordic Biosciences, IDS, UK).

2.4. Bone Histomorphometry. The dynamic bone histomorphometric parameters were measured according to The American Society of Bone Mineral Research Histomorphometry Nomenclature Committee 1987 [18]. The left femora were dissected out and fixed with 70% ethanol. After one week, the femora were cut sagittally at the epiphyseal and metaphyseal area. The femora were then embedded in methyl methacrylate (Osteo-Bed Bone Embedding Kit; Polysciences, USA) and sectioned at 9 μm thickness using a microtome (Leica RM2155, Wetzlar, Germany). The unstained bones were analyzed using an image analyzer Pro-Plus (Media Cybernetics, Silver Spring, MD, USA) and a fluorescence microscope (Nikon Eclipse 80 μ, Japan). The dynamic bone histomorphometric parameters include single-labeled surface/bone surface (sLS/BS), double-labeled surface/bone surface (dLS/BS), mineralizing surface/bone surface (MS/BS), bone formation rate/bone surface (BFR/BS), and Mineral Apposition Rate (MAR). The primary parameters are the single-labeled surface (sLS) and double-labeled surface (dLS). The dLS represented bone growth in seven days, while the sLS was actually two calcein labels superimposed on each other due to insignificant bone growth. The sLS/BS and dLS/BS parameters are obtained using the Weibel grid as described by Freere and Weibel [20]. The other parameters were derived as follows: MS/BS = [dLS + 1/2 sLS]/BS and BFR/BS = [dLS + 1/2 sLS X MAR]/BS. The MAR was calculated by obtaining the mean interlabel distance using the Pro Plus image anaylzer and dividing it by seven days, which is the duration of time between the calcein injections. The measurements were performed at the metaphyseal region which is rich in trabecular bone. This secondary spongiosa area is located 3 to 7 mm from the lowest point of the growth plate and 1 mm from the lateral cortex, excluding the endocortical region [21].

2.5. Statistical Analyses. The Kolmogorov-Smirnoff tests were used for normality testing. Following that the Kruskal-Wallis andMann-Whitney U tests were used for comparison between treatment groups since the data were not normally distributed. To compare data between before and after treatment, the Wilcoxon Rank Sign test was used. Data analysis was carried out using the Statistical Package for Social Sciences (SPSSs) version 19.0 software. The results were expressed as mean values ± standard error of the mean (SEM).

This study was approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) certificate no: FP/FAR/2010/NAZRUN.

3. Results

3.1. Dynamic Bone Histomorphometry. sLS indicates that there was poor bone growth, such that the two calcein labels were superimposed on each other. Figure 1 showed that increased percentage of sLS in the OVC group, while both calcium and tocotrienol significantly reduced the percentage of sLS.

dLS indicates bone growth. The interlabel distance indicates bone growth in seven days. Ovariectomy did not significantly change the percentage of dLS compared to BC and Sham. However, tocotrienol significantly increased percentage of dLS more than all the other groups (Figure 2).

No significant differences were seen between groups in the percentage of MS/BS. (Figure 3).

The MAR was higher in the Ov+T group compared to all the other groups. Calcium supplementation to the ovariectomised rats (Ov+Ca) did not increase the MAR (Figure 4).

BFR was higher in the Ov+T group compared to all the other groups. The Ov+Ca group did not differ from the OVC group (Figure 5).

The CTX levels were significantly lower in all the groups after treatment. However, there were no significant differences before and after treatment for all the groups (Figure 6).

There were no significant differences in serum osteocalcin levels seen between groups as well as within groups (Figure 7).

4. Discussion

During early adulthood, bone mass is stable until menopause. For about the first five years after menopause, women lose bone mass at the rate of about 2% to 3% per year and then continue to lose about 1% of bone mass per year to the end of life. Hence, postmenopausal women are prone to osteoporosis [16].

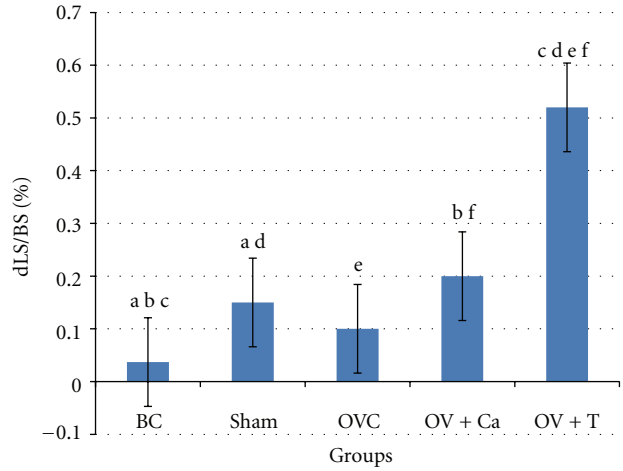


Figure 2: Double-labeled surface (dLS/BS). BC: baseline control group. Sham: sham-operated rats given deionised water and vehicle olive oil. OVC: ovariectomised rats given deionised water and vehicle olive oil. Ov+Ca: ovariectomised rats given 1% calcium in deionised water and vehicle olive oil. Ov+T: ovariectomised rats given deionised water and palm tocotrienol. Same alphabet indicates significant difference between the groups ($P < 0.05$).

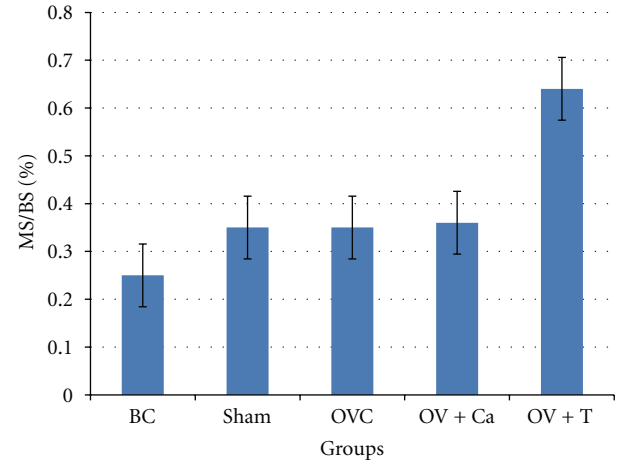


Figure 3: Mineralising surface (MS/BS). BC: baseline control group. Sham: sham-operated rats given deionised water and vehicle olive oil. OVC: ovariectomised rats given deionised water and vehicle olive oil. Ov+Ca: ovariectomised rats given 1% calcium in deionised water and vehicle olive oil. Ov+T: ovariectomised rats given deionised water and palm tocotrienol. No significant difference was seen between the groups at $P < 0.05$.

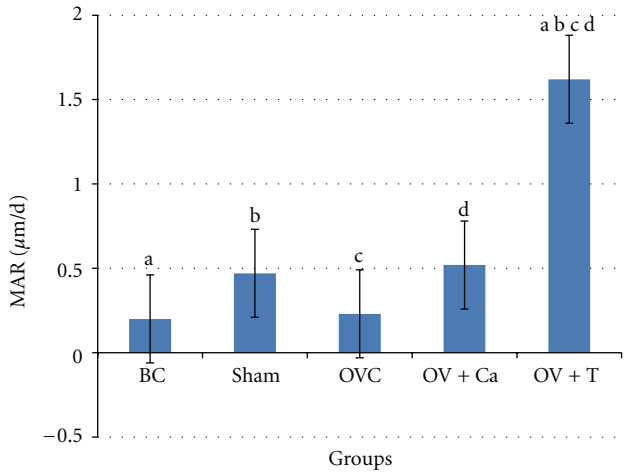


Figure 4: Mineral apposition rate (MAR). BC: baseline control group. Sham: sham-operated rats given deionised water and vehicle olive oil. OVC: ovariectomised rats given deionised water and vehicle olive oil. Ov+Ca: ovariectomised rats given 1% calcium in deionised water and vehicle olive oil. Ov+T: ovariectomised rats given deionised water and palm tocotrienol. Same alphabet indicates significant difference between the groups ($P < 0.05$).

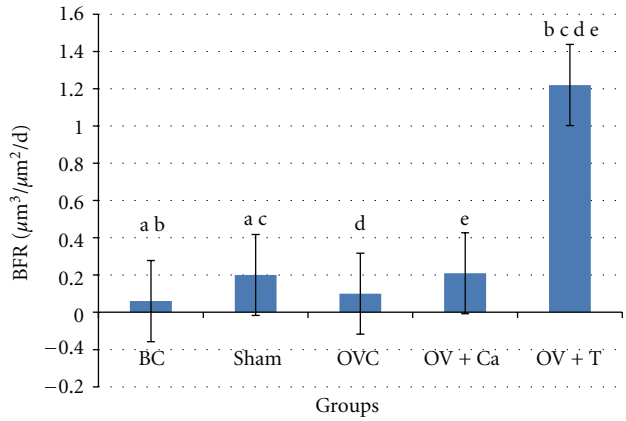


Figure 5: Bone formation rate (BFR/BS). BC: baseline control group. Sham: sham-operated rats given deionised water and vehicle olive oil. OVC: ovariectomised rats given deionised water and vehicle olive oil. Ov+Ca: ovariectomised rats given 1% calcium in deionised water and vehicle olive oil. Ov+T: ovariectomised rats given deionised water and palm tocotrienol. Same alphabet indicates significant difference between the groups ($P < 0.05$).

Early reports by others showed that calcium supplementation in postmenopausal women for two months was shown to be effective in preventing osteoporosis. Markers of bone turnover were reduced [22]. In the present study, calcium supplementation was given to ovariectomised rats. However, no improvement in the dynamic histomorphometry was observed. The position statement on calcium and bone health by the Australian and New Zealand Bone and Mineral Society, Osteoporosis Australia, and the Endocrine Society of Australia states that the effect of calcium supplementation on bone health is modest, as shown by increases in BMD and reductions in excessive bone turnover. However, the relative risk reduction for osteoporotic fracture is likely to be no more than 10%–20%. Although inadequate calcium intake is likely to be deleterious to bone, calcium intake significantly *above* the recommended level is unlikely to achieve additional benefit for bone health [16]. This is in agreement with our study which did not find any significant improvement in bone formation in rats supplemented with calcium.

Palm vitamin E contains a mixture of tocopherols and tocotrienols but with a higher percentage of tocotrienol.

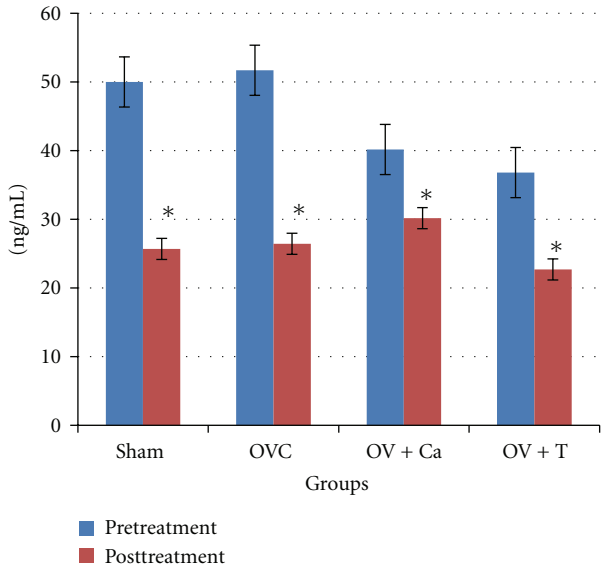


Figure 6: Serum C-terminal telopeptide of type 1 collagen (CTX). Sham: sham-operated rats given deionised water and vehicle olive oil. OVC: ovariectomised rats given deionised water and vehicle olive oil. Ov+Ca: ovariectomised rats given 1% calcium in deionised water and vehicle olive oil. Ov+T: ovariectomised rats given deionised water and palm tocotrienol. *indicates significant difference before and after treatment ($P < 0.05$).

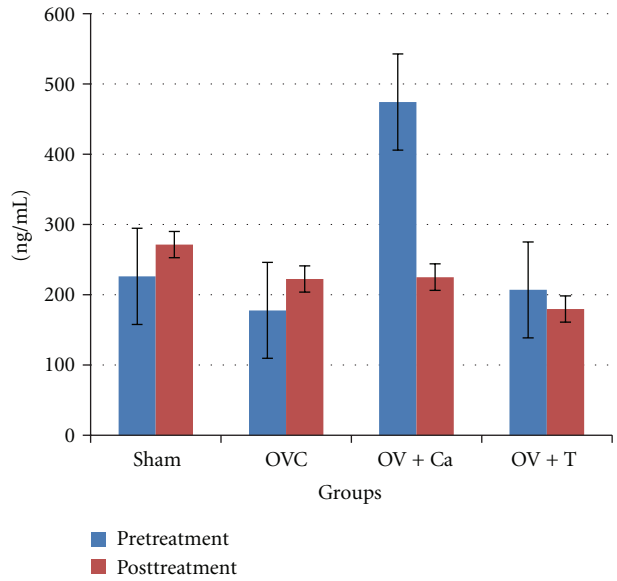


Figure 7: Serum osteocalcin. Sham: sham-operated rats given deionised water and vehicle olive oil. OVC: ovariectomised rats given deionised water and vehicle olive oil. Ov+Ca: ovariectomised rats given 1% calcium in deionised water and vehicle olive oil. Ov+T: ovariectomised rats given deionised water and palm tocotrienol. No significant difference was seen between the groups at $P < 0.05$.

Our preparation consisted of 80% tocotrienol and only 20% tocopherol. Our previous animal studies found that both tocotrienols and tocopherols were able to prevent the decline in bone density due to various factors. However, the tocotrienols were found to have better efficacy in some studies [10, 13, 23]. In fact, tocotrienol was also shown to improve bone biomechanical strength better than α-tocopherol [15]. This may be because tocotrienol demonstrated better antioxidant properties in bones compared to α-tocopherol [6]. A study by Fujita et al. [24] found that α-tocopherol actually increases bone resorption by stimulating osteoclast fusion, an effect that was not shown by α-tocotrienol. This effect of α-tocopherol was thought not to be due to its antioxidant properties, since other antioxidants, including α-tocotrienol, simultaneously tested, did not show that effect. In this study, our preparation was mainly tocotrienol, and it showed significantly better effects on bone formation in oestrogen-deficient rats compared to calcium. Therefore, we postulate that the beneficial effects were due to the tocotrienol component and not α-tocopherol, since our previous studies showed that α-tocopherol was less effective. Furthermore, the study of Fujita et al. [24] indicated that α-tocopherol was harmful to bone. Thus, it is important to remove the α-tocopherol component from palm vitamin E, leaving only the beneficial tocotrienols in place.

In this study, the dynamic histomorphometry clearly showed that tocotrienols increased bone formation and reduced bone resorption. However, no significant changes in the serum bone biomarkers were seen even though CTX was significantly lower in all the groups after treatment compared to before treatment was started. This may be because serum biomarkers are subject to circadian rhythm, therefore variation in serum levels may contribute to the insignificant results seen. Another reason may be that bone remodeling is a dynamic process, thus serum biomarkers are in a constant state of fluctuation. Furthermore, the technique may not be sensitive enough to detect small changes in nanograms per milliliter. The antiosteoporotic effects of tocotrienol are better illustrated in the dynamic histomorphometric parameters since findings in bone are more stable and lasting compared to serum findings. Similar biomarker findings were seen in our previous study [25].

There were several limitations in the current study which should be noted. The rats in group OV+Ca were treated with 1% calcium in drinking water, *ad libitum* daily for two months. One gram of calcium was powdered and mixed with 99 mL of deionised water which the rats drank when needed. In this way, the amount of calcium consumption could not be quantified. However, this method was selected since it did not cause any trauma to the animals. This is unavoidable since the tocotrienol and vehicle were administered by daily oral gavage. To administer calcium in this way will mean two oral gavages in a day which might traumatize the animal too much. We have successfully used this mode of administration of calcium in our earlier studies [14, 26, 27]. In addition, Sanders et al. [16] mentioned that the benefits of calcium treatment are more consistent in late postmenopausal women than in perimenopausal women, perhaps because of greater variation in the rate of bone loss amongst perimenopausal women. In the present study,

the menopausal state in rats was induced by ovariectomy. After two weeks of ovariectomy, treatment with calcium was started. Therefore these ovariectomised rats may be considered to be in the early postmenopausal state. This may contribute to the results seen in our study, which showed that calcium supplementation did not significantly affect bone resorption and formation.

In conclusion, tocotrienols increased bone formation rate in oestrogen-deficient ovariectomised rats, while calcium did not. Further studies are needed to determine the potential of tocotrienol as an antiosteoporotic agent.

Acknowledgments

The authors would like to thank Mr. Muhammad Arizi Aziz, Mrs. Sinar Suriya Muhammad, Mrs. Mazliadiyana Mazlan, Mrs. Azizah Osman, and the staff of the Pharmacology Department for their technical assistance. Special thanks to Carotech Berhad (Malaysia) for supplying the palm tocotrienols. The study was sponsored by the Faculty of Medicine, University Kebangsaan Malaysia Fundamental Research Grant no. FF-324-2010.

References

[1] S. Muthusami, I. Ramachandran, B. Muthusamy et al., "Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats," *Clinica Chimica Acta*, vol. 360, no. 1-2, pp. 81–86, 2005.

[2] J. M. Lean, C. J. Jagger, B. Kirstein, K. Fuller, and T. J. Chambers, "Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation," *Endocrinology*, vol. 146, no. 2, pp. 728–735, 2005.

[3] J. M. Lean, J. T. Davies, K. Fuller et al., "A crucial role for thiol antioxidants in estrogen-deficiency bone loss," *The Journal of Clinical Investigation*, vol. 112, no. 6, pp. 915–923, 2003.

[4] O. Altindag, O. Erel, N. Soran, H. Celik, and S. Selek, "Total oxidative/anti-oxidative status and relation to bone mineral density in osteoporosis," *Rheumatology International*, vol. 28, no. 4, pp. 317–321, 2008.

[5] A. S. Nazrun, D. A. Luke, B. A. K. Khalid, and S. Ima-Nirwana, "Vitamin E protects from free-radical damage on femur of rats treated with ferric nitrilotriacetate," *Current Topics in Pharmacology*, vol. 9, no. 2, pp. 107–115, 2005.

[6] S. Maniam, N. Mohamed, A. N. Shuid, and I. N. Soelaiman, "Palm tocotrienol exerted better antioxidant activities in bone than α-tocopherol," *Basic and Clinical Pharmacology and Toxicology*, vol. 103, no. 1, pp. 55–60, 2008.

[7] M. Norazlina, S. Ima-Nirwana, M. T. Gapor, and B. A. K. Khalid, "Palm vitamin E is comparable to α-tocopherol in maintaining bone mineral density in ovariectomised female rats," *Experimental and Clinical Endocrinology and Diabetes*, vol. 108, no. 4, pp. 305–310, 2000.

[8] S. Ima-Nirwana, A. Kiftiah, A. G. Zainal, M. Norazlina, M. T. Abd. Gapor, and B. A. K. Khalid, "Palm vitamin E prevents osteoporosis in orchidectomized growing male rats," *Natural Product Sciences*, vol. 6, no. 4, pp. 155–160, 2000.

[9] S. Ima-Nirwana and S. Suhaniza, "Effects of tocopherols and tocotrienols on body composition and bone calcium content in adrenalectomised rats replaced with Dexamethasone," *Journal of Medicinal Food*, vol. 7, no. 1, pp. 45–51, 2004.

[10] N. S. Ahmad, B. A. K. Khalid, D. A. Luke, and S. Ima-Nirwana, "Tocotrienol offers better protection than tocopherol from free radical-induced damage of rat bone," *Clinical and Experimental Pharmacology and Physiology*, vol. 32, no. 9, pp. 761–770, 2005.

[11] A. S. Nazrun, D. A. Luke, B. A. K. Khalid, and S. Ima-Nirwana, "Vitamin E protects from free-radical damage on femur of rats treated with ferric nitrilotriacetate," *Current Topics in Pharmacolog*, vol. 9, no. 2, pp. 107–115, 2005.

[12] M. Norazlina, P. L. Lee, H. I. Lukman, A. S. Nazrun, and S. Ima-Nirwana, "Effects of vitamin E supplementation on bone metabolism in nicotine-treated rats," *Singapore Medical Journal*, vol. 48, no. 3, pp. 195–199, 2007.

[13] H. Hermizi, O. Faizah, S. Ima-Nirwana, S. Ahmad Nazrun, and M. Norazlina, "Beneficial effects of tocotrienol and tocopherol on bone histomorphometric parameters in Sprague-Dawley male rats after nicotine cessation," *Calcified Tissue International*, vol. 84, no. 1, pp. 65–74, 2009.

[14] M. Norazlina, P. L. Lee, H. I. Lukman, A. S. Nazrun, and S. Ima-Nirwana, "Effects of vitamin E supplementation on bone metabolism in nicotine-treated rats," *Singapore Medical Journal*, vol. 48, no. 3, pp. 195–199, 2007.

[15] M. Z. Mehat, A. N. Shuid, N. Mohamed, N. Muhammad, and I. N. Soelaiman, "Beneficial effects of vitamin E isomer supplementation on static and dynamic bone histomorphometry parameters in normal male rats," *Journal of Bone and Mineral Metabolism*, vol. 28, no. 5, pp. 503–509, 2010.

[16] K. M. Sanders, C. A. Nowson, M. A. Kotowicz, K. Briffa, A. Devine, and I. R. Reid, "Calcium and bone health: position statement for the Australian and New Zealand Bone and Mineral Society, Osteoporosis Australia and the Endocrine Society of Australia," *Medical Journal of Australia*, vol. 190, no. 6, pp. 316–320, 2009.

[17] P. Garnero, "Biomarkers for osteoporosis management: utility in diagnosis, fracture risk prediction and therapy monitoring," *Molecular Diagnosis and Therapy*, vol. 12, no. 3, pp. 157–170, 2008.

[18] A. M. Parfitt, M. K. Drezner, F. H. Glorieux et al., "Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee," *Journal of Bone and Mineral Research*, vol. 2, no. 6, pp. 595–610, 1987.

[19] M. Norazlina, S. Ima-Nirwana, and B. A. K. Khalid, "Calcium supplementation improves bone density and lean mass in vitamin E deficient rats," *Journal of the ASEAN Federation of Endocrine Societies*, vol. 19, no. 1-2, pp. 23–29, 2001.

[20] R. H. Freere and E. R. Weibel, "Stereologic techniques in microscopy," *Journal of the Royal Microscopy Society*, vol. 87, no. 1, pp. 25–34, 1967.

[21] P. A. J. Baldock, H. A. Morris, A. G. Need, R. J. Moore, and T. C. Durbridge, "Variation in the short-term changes in bone cell activity in three regions of the distal femur immediately following ovariectomy," *Journal of Bone and Mineral Research*, vol. 13, no. 9, pp. 1451–1457, 1998.

[22] P. Fardellone, M. Brazier, S. Kamel et al., "Biochemical effects of calcium supplementation in postmenopausal women: influence of dietary calcium intake," *American Journal of Clinical Nutrition*, vol. 67, no. 6, pp. 1273–1278, 1998.

[23] A. S. Nazrun, M. Norazlina, M. Norliza, and S. Ima Nirwana, "Comparison of the effects of tocopherol and tocotrienol on osteoporosis in animal models," *International Journal of Pharmacology*, vol. 6, no. 5, pp. 561–568, 2010.

[24] K. Fujita, M. Iwasaki, H. Ochi et al., "Vitamin E decreases bone mass by stimulating osteoclast fusion," *Nature Medicine*, vol. 18, pp. 589–594, 2012.

[25] M. Norazlina, S. Ima-Nirwana, M. T. A. Gapor, and B. A. Kadir Khalid, "Tocotrienols are needed for normal bone calcification in growing female rats," *Asia Pacific Journal of Clinical Nutrition*, vol. 11, no. 3, pp. 194–199, 2002.

[26] M. Norazlina, S. Ima Nirwana, and B. A. K. Khalid, "Effects of palm vitamin E, vitamin D and calcium supplementation on bone metabolism in vitamin E deficient rats," *Medical Journal of Islamic Academy of Science*, vol. 12, no. 4, pp. 89–96, 1999.

[27] M. Norazlina, L. H. L. Rita, and S. Ima-Nirwana, "The effects of vitamin E or calcium supplementation on bone mineral composition in vitamin E deficient rats," *Malaysian Journal of Biochemistry and Molecular Biology*, vol. 7, pp. 1–5, 2002.