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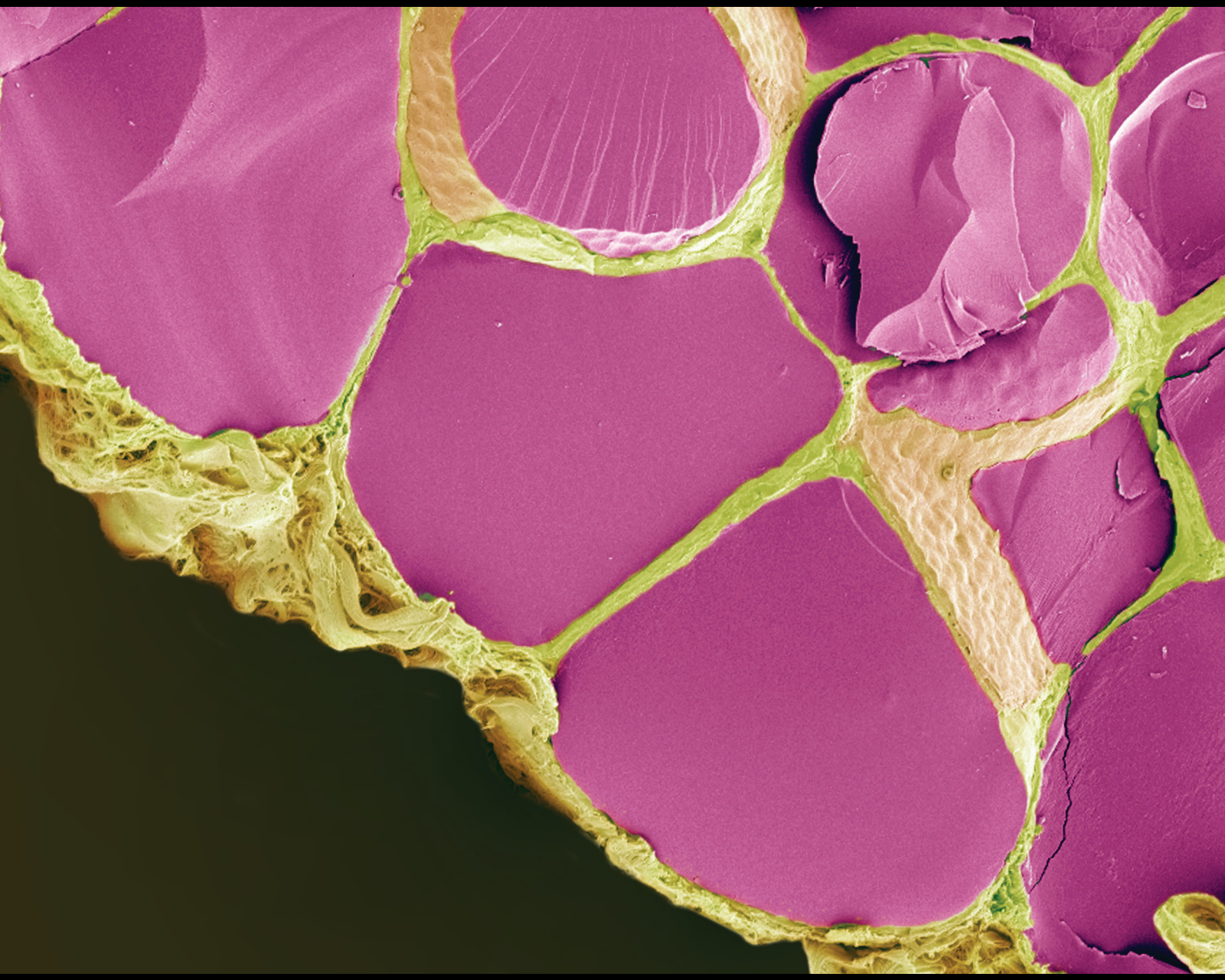
Fat, Muscle, and Bone Interactions in Obesity and the Metabolic Syndrome

Guest Editors: Reina Armamento-Villareal, Nicola Napoli, Debra Waters, and Dennis Villareal



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Editorial Board

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Editorial

Fat, Muscle, and Bone Interactions in Obesity and the Metabolic Syndrome

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The increasing numbers of individuals diagnosed with obesity have led to burgeoning health care cost directed not only at the accompanying metabolic abnormality but also on the care of the associated physical disability particularly in the elderly population [1, 2]. The latter has resulted in a growing interest in the interactions between fat, muscle, and bone in the context of obesity and modalities to address obesity-related limitations in physical function. Obesity and the metabolic syndrome are associated with fatty infiltration in the muscles which leads to poor muscle quality, poor strength, and poor physical function most especially in the elderly [3]. In addition, adipose tissue secretes inflammatory cytokines that may cause skeletal muscle inflammation and may contribute to poor muscle quality [4, 5]. Furthermore, for bone, the traditional concept that a high body weight is osteoprotective is now challenged by recent findings of an increased prevalence of fractures in obese patients [6–8]. It is possible that the increase in fractures in these patients could be related to frequent falls from physical frailty. However, recent scientific advances have shown that cell signals that promote mesenchymal stem cells to differentiate into the adipogenic pathway are associated with suppression of signaling in the myogenic and osteogenic pathways [9, 10], both detrimental to muscle and bone. Knowledge and understanding of these interactions have led to the development of animal models, identification of novel targets for possible new therapies, and development of modalities that may alleviate the negative consequences of obesity and the metabolic syndrome. Aside from improving the associated metabolic derangement, reversal of poor muscle function and prevention of fractures constitute the ultimate goal for care in these patients.

In this issue, the authors tackle this important relationship between muscle, fat, and bone in the context of obesity and the metabolic syndrome ranging from basic physiology to the more complex interrelationship of these organs in disorders related to excess body weight. In the MrOS study, although the incidence of fractures goes down as body mass index (BMI) goes up from normal to overweight and lower obese category, when adjusted for bone mineral density (BMD), an increase in the incidence of fractures has been reported as BMI increases further to the higher obese category [6]. In the animal study by H. Fehrendt et al., the authors showed that, compared to controls, animals fed a high fat diet have reduced cancellous bone mass, collagen expression, amount of osteoid, and cell-to-cell contacts, despite the absence of differences in BMD and the number of osteoblasts and osteoclasts. In addition, there was an increase in the number of apoptotic osteocytes in high fat diet mice compared to controls. Taken together, these findings provided some mechanistic insights into the increased risk of fractures associated with obesity even in the presence of adequate bone

mass. The review paper by N. Napoli et al. also provided a pathophysiologic basis for diabetes mellitus-associated bone disease and the role of mesenchymal differentiation and the different circulating factors in the alteration in bone metabolism in these patients. Given the close association between obesity and type 2 diabetes, it would not be surprising if both shared the same bone findings.

The important relationship between vitamin D metabolism and obesity is addressed by the paper by C. Cipriani et al. In this article, the authors reviewed the different mechanisms for low circulating vitamin D levels in obese patients. Conversely, the article also presented data from reports raising the possibility that vitamin D could promote adiposity by enhancing adipogenesis in low vitamin D states but not in vitamin D replete states. However, on the topic of vitamin D, an original report from N. Napoli and colleagues illustrated the altered bone turnover in patients with low vitamin D. Since hypovitaminosis D is common in obese patients and may contribute to the increased fracture prevalence reported in these patients, their findings underscored the need for vitamin D supplementation to normalize circulating vitamin D.

Frailty, which is common in obese older adults, is multifactorial and due to a combination of increased circulating adipokines (some of them proinflammatory) and poor muscle quality and from extra weight to carry with day to day activities. In the article by L. E. Aguirre et al., the authors demonstrated the individual and combined influence of the different hormones and cytokines on physical function among obese older adults. Among the circulating factors studied, it appears that testosterone, leptin, and adiponectin are important predictors of strength and endurance; however, TNF-α appears to be the only predictor of physical function. Along this line of research, O. Addison and colleagues reviewed the implications of increased intramuscular adipose tissue in metabolic, muscle, and mobility function. Increase in fatty deposition in the muscles contributes to poor muscle quality both from replacing muscles with fat and also from increased production of inflammatory cytokines from adipose tissues within the muscles. A review on the potential therapeutic targets and the effect of caloric restriction and exercise to mitigate fatty deposition in the skeletal muscles are also discussed in this article. There is evidence that lifestyle intervention by weight loss and exercise improves frailty in obese older adults, and although weight loss alone results in significant muscle and bone loss, the addition of exercise attenuates both muscle and bone loss [11]. The study by the group of G. Colaianni suggested that the myokine irisin may mediate the cross talk between muscle and bone. In their study, conditioned medium containing irisin from skeletal muscles of exercising animals induced a higher degree of osteoblastic differentiation compared to that coming from control animals. Aside from reenforcing the benefits of exercise on bone, it also suggests that anabolic effect of exercise on bone is mediated by the myokine irisin.

The degree of visceral fat accumulation in an individual may be dependent on the number of mitochondrial copies as suggested by the results of the study by J.-Y. Lee et al. Higher mitochondrial copy is associated with lower BMI, lower waist circumference, and reduced visceral fat emphasizing the genetic component of obesity.

There is evidence of an association between increasing BMI and susceptibility to musculoskeletal diseases in addition to frailty. The association between tendinopathies and obesity/diabetes was reviewed in the meta-analysis by the group of F. Franceschi et al. These authors analyzed the data from 15 papers showing higher odds ratios among obese individuals to develop tendinopathies. Another original study by U. G. Longo et al. investigated the relationship between high fibrinogen levels and rotator cuff injury (both of which are common in patients with metabolic disorders) but found no association. Finally, a study by U. Tarantino et al. evaluated the clinical and histomorphometric features of 80 patients undergoing hip arthroplasty for severe osteoarthritis (a common problem in obese patients) or osteoporosis-related femoral neck fractures. They found that bone volume fraction was lower in subjects with femoral neck fractures than in subjects with osteoarthritis and normal or osteopenic BMD. However, bone volume fraction in patients with combined osteoarthritis and osteoporosis is similar to that of patients with femoral neck fractures. The authors suggested that the limited mobility from hip osteoarthritis (likely severe) in some patients could contribute to the risk for developing osteoporosis.

The manuscripts in this issue highlight the interrelationships between fat, muscle, and bone in obesity and the metabolic syndrome and the disorders associated with derangement in this interaction. In this context, frailty appears to be a major consequence most especially in the elderly leading to loss of independence and increased nursing home admissions. Although the attainment of ideal body weight from lifestyle measures is highly unlikely in obese subjects, our group was able to show that a 10% weight loss with the addition of exercise improved physical function and attenuated the weight loss-associated muscle and bone loss [11]. Since not everyone is a candidate for lifestyle intervention, further studies are needed to identify potential targets to reverse frailty in the growing population of obese older adults.

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Nicola Napoli
Debra Waters
Dennis Villareal

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Review Article

Obesity as a Risk Factor for Tendinopathy: A Systematic Review

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Purpose. In the last few years, evidence has emerged to support the possible association between increased BMI and susceptibility to some musculoskeletal diseases. We systematically review the literature to clarify whether obesity is a risk factor for the onset of tendinopathy. *Methods.* We searched PubMed, Cochrane Central, and Embase Biomedical databases using the keywords "obesity," "overweight," and "body mass index" linked in different combinations with the terms "tendinopathy," "tendinitis," "tendinosis," "rotator cuff," "epicondylitis," "wrist," "patellar," "quadriceps," "Achilles," "Plantar Fascia," and "tendon." *Results.* Fifteen studies were included. No level I study on this subject was available, and the results provided are ambiguous. However, all the 5 level II studies report the association between obesity measured in terms of BMI and tendon conditions, with OR ranging between 1.9 (95% CI: 1.1–2.2) and 5.6 (1.9–16.6). *Conclusions.* The best evidence available to date indicates that obesity is a risk factor for tendinopathy. Nevertheless, further studies should be performed to establish the real strength of the association for each type of tendinopathy, especially because the design of the published studies does not allow identifying a precise cause-effect relationship and the specific role of obesity independently of other metabolic conditions.

1. Introduction

Tendinopathies are common musculoskeletal diseases affecting the tendons. The term tendinopathy describes a range of clinical conditions related to tendons and surrounding structures [1, 2].

Although tendinopathies also include conditions of damage to the tendon in absence of symptoms, these pathologies often occur with pain in the injured tendon, which is accentuated or appears during palpation of the affected area or during active and passive movements involving the tendon.

Pain is often associated with a reduction in the strength of the muscles attached to the tendons involved in the pathological process [3, 4]. Chronic tendinopathies are a common problem for patients whose activities require repetitive movements; for this reason, they are particularly widespread among sportsmen. These conditions can also occur after an acute injury, when the healing process of the injured tendon fails [4]. In the past, the terms "tendinitis" and "tendinosis" were widely and indiscriminately used in place of tendinopathy, often considering this condition as an inflammatory pathology, but such definitions should be imposed only after a histological study [5]. Actually, histological samples from chronic tendinopathies have confirmed that there is no acute inflammatory condition, but rather a failure of the tendon repair associated with angiofibroblastic degeneration [4, 6, 7]. In fact, histologically, the findings are more typical of a "failed healing response," with a haphazard proliferation of tenocytes, intracellular abnormalities in tenocytes, disruption of collagen fibers, and subsequent increase in noncollagenous matrix.

However, factors that predispose to tendinopathies have not yet been clarified, although there is evidence to support a role for biomechanical factors, functional alterations, aging, and metabolic disorders [7, 8].

Particularly, obesity has recently been indicated as important but potentially modifiable risk factor in the onset and progression of some tendinopathies [9]. In fact, in contrast to other conditions, the advantage from studying obesity lies in the possibility of preventing and treating this risk factor.

Obesity is already a well-known risk factor for many other diseases of the musculoskeletal system [9]. The prevalence of obesity in industrialized countries has increased steadily in recent decades. In the United States, between 2007 and 2008, the prevalence of obesity in adults was estimated to be 32.2% in men and 35.5% in women, reaching percentages of 72.3% in men and 64.1% in women if both obesity and overweight are considered together. In Europe, the prevalence of obesity appears tripled since 1980 and each year four million people become obese [10, 11].

The World Health Organization recommends a standard classification of adult overweight and obesity using the following body mass index (BMI) calculations: a BMI of 25.0 to 29.9 kg per m^{-2} is defined as overweight; a BMI of 30.0 kg per m^{-2} or more is defined as obesity [10, 11].

Other measurements are also used to identify a pathological fat distribution, such as waist circumference (cm) or waist/hip ratio.

The purpose of this review was to summarise the current literature reporting data on the relationship between obesity and tendons diseases to verify the hypothesis that obesity is a risk factor for the development of tendinopathy.

2. Methods

The systematic review was performed following the PRISMA (preferred reporting items for systematic reviews and meta-analyses) statement [12, 13]. The PRISMA search algorithm is shown in Figure 1.

We searched PubMed, Cochrane Central, and Embase Biomedical databases using the keywords "obesity," "overweight," and "body mass index" linked in different combinations with the terms "tendinopathy," "tendinitis," "rotator cuff," "epicondylitis," "wrist," "patellar," "quadriceps," "Achilles," "plantar fascia," and "tendon." No limit regarding the year of publication and the study design was imposed. We selected articles in English, Spanish, French, and Italian, according to the authors' skills. All peer-reviewed journals were evaluated and all relevant articles were retrieved. Three authors (Francesco Franceschi, Edoardo Franceschetti, and Michele Paciotti) independently reviewed the text of each abstract. Full-text versions were obtained to include or exclude the studies. Clinical studies investigating, as declared aim of the study, the association between obesity and one or more types of tendinopathy were selected. The definition of obesity had to be based on instrumental evaluation through body mass index (BMI) or waist circumference (WC) or waist-to-hip ratio (WHR). We screened the references lists of the studies found in order to find additional relevant publications.

Demographics data, diagnosis, design of the study, objective means of measuring the weight, and main findings concerning the statistical association between increased weight and tendinopathy were independently extracted by all the investigators.

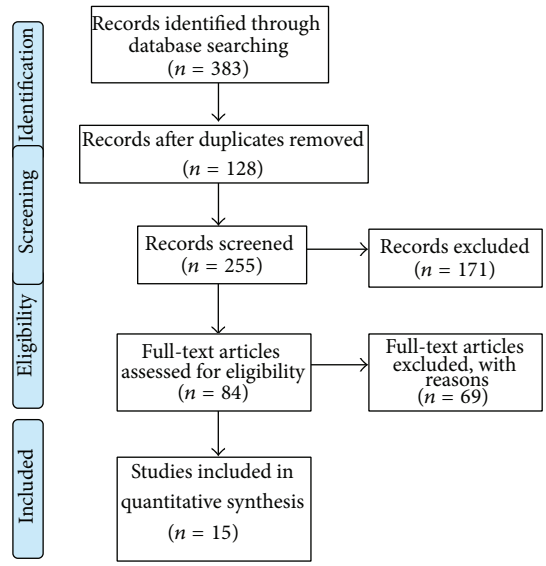

Figure 1: PRISMA 2009 flow diagram.

Biomechanical studies, case reports, literature reviews, technical notes, and instructional courses were excluded. We also excluded articles reporting data of subjects of less than 18 years of age. To avoid bias, all the included articles were reviewed and discussed by all the authors.

3. Results

The literature search and cross-referencing resulted in 383 references, of which 299 were rejected due to off topic abstract and/or duplication of the results (Figure 1). After reading the remaining full-text articles, another 69 articles were excluded for failing to fulfil the inclusion criteria. The remaining 15 articles, including 5 frequency-matched case-control studies [14–18], 4 cross-sectional studies [19–22], 5 retrospective case-control studies [23–27], and 1 case-series study [28], were included in the present study.

The total number of patients in the included studies was 36,843, of which 9,002 were the subjects affected by tendinopathy.

All the characteristics of the studies are shown in Table 1.

4. Discussion of the Results

In this study, we reviewed all the data provided by published studies that focused on analysing the association between obesity and the development of the most frequent kind of tendinopathy.

4.1. Obesity and Rotator Cuff Tendinopathy. Rotator cuff (RC) tendinopathy is most frequently observed.

Rotator cuff disease, which includes a range of clinical and pathological characteristics, is a multifactorial condition, the origin of which is unclear, but the failed healing response typically seen in other tendinopathies is the end result [29]. In fact, the theory, common in the past, based on the mechanical impingement of the rotator cuff has not been demonstrated and does not explain the clinical manifestations of the pathology.

Table 1: Summary of the studies.

Authors	Year	Tendinopathy	Study design	Number of patients	Age in years (range)	Measures of obesity	Relevant results	Conclusions of the study	Association
Wendelboe et al. [14]	2004	Rotator cuff	Frequency-matched case-control study Prognostic study, Level II	311 RCR cases versus 993 controls	(53–77)	BMI	OR: 1.9 (95% CI: 1.1–2.2) for males and 2.4 (1.4–4.2) for females with BMI ≥ 35. OR: 3.1 (95% CI: 1.3–7.6) for males and 3.5 (1.8–6.9) for females with BMI ≥ 35. Risk directly correlated with the grade of obesity for both men ($P = .002$) and women ($P < .001$)	Obesity increases risk to need RCR	Yes
Rechardt et al. [19]	2010	Rotator cuff	Cross-sectional study (population study)	6,237 of which chronic 28 (2.8%) with RC tendinitis	50.8 for men, 52.9 for women (>30)	BMI, waist circumference, waist-to-hip ratio	WC 94.0–101.9 cm and RC tendinitis OR: 2.0 (1.1–3.5) in men	Increased WC is associated with chronic RC tendinitis in men	Partial
Titchener et al. [23]	2014	Rotator cuff	Retrospective case-control, treatment study Level III	5,000 cases of RC disease versus 5,000 controls (BMI calculated in 3,385 of cases (67.7%) and 3,050 (61.0%) of controls)	55 (interquartile range 44–65)	BMI	BMI 25.1–30 (overweight) and RC disease OR: 1.23 (1.10–1.38) BMI 30.1–40 (obese) and RC disease OR: 1.25 (1.09–1.44) BMI > 40 (morbidly obese) no increased risk After adjustment for consultation rate, the effect persisted only in the BMI 25.1–30 (overweight) group OR: 1.15 (1.02–1.31)	Significant association only for patients who are slightly overweight (BMI 25–30) Impossible to differentiate comorbid factors such as diabetes mellitus, atherosclerosis, and hyperlipidemia	Partial
Titchener et al. [24]	2013	Epicondylitis (lateral and medial)	Retrospective case-control, treatment study Level III	4998 versus 4998 controls (BMI calculated in 3,449 of cases (69%) and 3,049 (61.0%) of controls)	49 (interquartile range 42–56)	BMI	BMI > 40 and lateral epicondylitis OR: 1.41 (1.01–1.97) The effect disappeared when BMI was adjusted for consultation rate	Obesity is not associated with epicondylitis	No
Descatha et al. [28]	2013	Epicondylitis (lateral and medial)	Case-series (longitudinal study) Level IV	699 workers with no symptoms at baseline. At 36 months: 48 suffered from medial or lateral epicondylitis (6.9%), 34 from lateral epicondylitis (4.9%), 30 from medial epicondylitis (4.3%), and 16 from both	38.1 ± 9.3 (20–66)	BMI	BMI > 30 and lateral epicondylitis: univariate analyses OR: 2.4 (1.2–4.8), multivariate analyses OR: 1.8 (0.8–3.9). No association for medial epicondylitis	Obesity is associated only with lateral epicondylitis	Partial
Shiri et al. [20]	2006	Epicondylitis (lateral and medial)	Cross-sectional study (population study)	4,783 of the initial 5,871 (81.5%) 67 with lateral epicondylitis (1.3%) and 19 with medial epicondylitis (0.4%)	46.3 ± 9.6 (30–64)	BMI, waist circumference	Only in women WC > 100 cm and medial epicondylitis OR: 2.7 (1.2–6.0) BMI > 30 kg/m^2 and medial epicondylitis OR: 1.9 (1.0–2.7) No association for lateral epicondylitis	Obesity is associated with medial epicondylitis.	Partial
Alvarez-Nemegyei [25]	2007	Pes anserinus	Retrospective case-control study Level III	22 cases of tendinopathy versus 38 controls	62.1 ± 11.5 for cases, 59.8 ± 9.4 for controls	BMI	Obesity: case 16/22 (72.7), controls 21/38 (55.3), $P = .28$ nonsignificant	No association	No

Table 1: Continued.

Authors	Year	Tendinopathy	Study design	Number of patients	Age in years (range)	Measures of obesity	Relevant results	Conclusions of the study	Association
Taunton et al. [27]	2002	Patellar tendon	Retrospective case-control study Level III	96 cases versus 1906 controls	34.3	Weight, BMI	No association	No association	No
Frey and Zamora [21]	2007	Achilles, posterior tibial, and peroneal tendon	Cross-sectional study (population study)	1411 of which 208 with tendinitis/tendinosis	>18	BMI	123 (65.4%) of the overweight/obese subjects had a diagnosis of tendinitis compared to 65 (34.6%) normal subjects. BMI > 25 and tendinitis OR: 1.923 (1.39–2.66) $P < .0001$	Being overweight or obese significantly increased the chances of tendinitis	Yes
Holmes and Lin [26]	2006	Achilles	Retrospective case-control study, Level III	82 cases	49.5 (27–77)	BMI	Obesity was statistically associated with Achilles tendinopathy $P = .025$ for women and $P = .001$ for men, respectively	Obesity is one of the etiological factors of the Achilles tendinopathy	Yes
Gaida et al. [22]	2010	Achilles	Population-based study (cross-sectional study)	298 cases (127 men, 171 women) asymptomatic Achilles tendinopathy in 17 men (13%) and 8 women (5%) ($P = .007$).	Men 38.3 ± 12.2 Women 36.5 ± 10.5	Fat distribution (android/gynoid fat mass ratio and upper body/lower body fat mass ratio) determined using WC, WHR, and dual-energy X-ray absorptiometry	Men with Achilles tendinopathy had greater WHR (0.926 ± 0.091, 0.875 ± 0.065, $P = .039$), higher android/gynoid fat mass ratio (0.616 ± 0.186, 0.519 ± 0.142, $P = .014$), and higher upper body/lower body fat mass ratio (2.346 ± 0.630, 2.022 ± 0.467, $P = .013$). Women with tendinopathy had less total fat (17196 ± 3173 g, 21626 ± 7882 g, $P = .009$), trunk fat (7367 ± 1662 g, 10087 ± 4152 g, $P = .003$), and android fat (1117 ± 324 g, 1616 ± 811 g, $P = .005$). They had lower central/peripheral fat mass ratios (0.711 ± 0.321 g, 0.922 ± 0.194 g, $P = .004$) than women with normal tendons	Men with Achilles tendinopathy had a central fat distribution. Women had a peripheral fat distribution.	Yes
Scott et al. [15]	2013	Achilles	Frequency-matched case-control study Prognostic study, Level II	197 cases versus 100 controls	Cases: 52.77 ± 11.8 (21–82) Controls: 42.74 ± 12.1 (21–78)	BMI	Significant difference in BMI: $P < .001$ 34.69 ± 7.54 (17.9–75.9) versus 30.56 ± 7.55 (19.7–61-5)	Patients with Achilles tendinopathy exhibited a significant higher BMI than controls	Yes
Klein et al. [16]	2013	Achilles	Frequency-matched case-control study Prognostic study, Level II	472 cases versus 472 controls	Cases: 51.2 ± 13.5 (16–88) Controls: 52.0 ± 14.3 (18–88)	BMI	OR: 2.60, 95% CI = 1.87–3.61; 3.81, 95% CI = 2.57–5.63; 3.77, 95% CI = 2.24–6.34; 6.56, 95% CI = 3.18–13.55 For BMI: 25.0–29.9, 30.0–34.9, 35.0–39.9, and >40.0, respectively	BMI plays a role in the development of Achilles tendinopathy	Yes

Table 1: Continued.

Authors	Year	Tendinopathy	Study design	Number of patients	Age in years (range)	Measures of obesity	Relevant results	Conclusions of the study	Association
Taunton et al. [27]	2002	Achilles	Retrospective case-control study, Level III	96 cases versus 1906 controls	40.7	Weight, BMI	No association	No association	Np
Taunton et al. [27]	2002	Plantar fascia	Retrospective case-control study, Level III	158 cases versus 1846 controls	41.8	Weight, BMI	Weight >60 kg in female OR: 0.378 (0.203–0.706)	Women with a body weight greater than 60 kg were at increased risk of experiencing plantar fasciitis	Partial
Irving et al. [17]	2007	Chronic plantar heel	Frequency-matched case-control study Prognostic study, Level II	80 cases versus 80 controls	52.3 ± 11.7	BMI	Significantly greater BMI for CPHP group (29.8 ± 5.4 kg/m^2 versus 27.5 ± 4.9 kg/m^2; $P < .01$) CPHP were more likely to be obese (OR: 2.9, CI: 1.4–6.1, $P < .01$)	Obesity is associated with chronic plantar heel pain	Yes
Frey and Zamora [21]	2007	Plantar fascia	Cross-sectional study (population study)	1411 of which 189 with plantar fasciitis	>18	BMI	208 affected by plantar fasciitis BMI > 25 and plantar fasciitis OR: 1.4 (1.016–1.93) $P < .040$	If the subjects were overweight or obese, there was an increased likelihood, although not significant, of plantar fasciitis	No
Riddle et al. [18]	2003	Plantar fascia	Frequency-matched case-control study Prognostic study, Level II	50 cases versus 100 controls	49 ± 11 (31–85)	BMI	BMI > 30 OR: 5.6 (CI: 1.9–16.6) compared with the BMI ≤ 25 kg/m^2	Obesity appears to be independent risk factor for plantar fasciitis.	Yes

BMI: body mass index; WC: waist circumference; WHR: waist-to-hip ratio; OG: group of obese patients; CG: control group; RC: rotator cuff; RCR: rotator cuff repair; CPHP: chronic plantar heel pain; OR: odds ratio; CI: confidence interval; and n.r.: not reported.

Several studies tried to identify the risk factors involved in this condition.

Some studies suggested a link between shoulder disorders and metabolic factors, such as diabetes mellitus [11].

Diabetes was clearly demonstrated to be associated with RC tendinopathy, increasing incidence and affecting postinjury healing process [30]. Regarding obesity, we did not find the same amount of evidence.

In particular, there are very few studies in literature; none of them is a level I study, and each study has a different design.

The prognostic study performed by Wendelboe et al. [14] in 2004, analysing 311 participants, showed that individuals with a BMI ≥35.0 had an increased risk to require rotator cuff repair with an odd ratio of 3.1 (CI 1.3–7.6) for males and 3.5 (1.8–6.9) for females. Moreover, this risk was directly correlated with the grade of obesity for both men ($P = .002$) and women ($P < .001$).

In 2010, Rechardt and colleagues [19] carried out a cross-sectional study investigating the national Finnish Health Survey. They evaluated if smoking, waist circumference, and waist-to-hip ratio were related to an increased prevalence of shoulder pain in both men and women. Metabolic syndrome, type 2 diabetes mellitus, and carotid intima-media thickness were associated with shoulder pain in men, whereas high level of C-reactive protein was associated with shoulder pain in women. Increased waist circumference and type 1 diabetes mellitus were associated with chronic rotator cuff tendinitis in men.

A large case-control study was performed by Titchener et al. [23] using The Health Improvement Network database to assess and to quantify the relative contributions of some constitutional and environmental risk factors for rotator cuff disease in the community. Their data included 5000 patients with rotator cuff disease who were individually matched with a single control by age, sex, and general practice (primary care practice). Multivariate analysis showed that only "overweight" body mass index of 25.1 to 30 (OR = 1.15) was significantly associated with rotator cuff disease, and, contrarily, mass index greater than 30 was not found to be associated with rotator cuff disease. However, the authors declared the impossibility to differentiate comorbid factors such as diabetes mellitus, atherosclerosis, and hyperlipidemia.

4.2. Obesity and Elbow Tendinopathies. Lateral and medial epicondylitis, also known as "tennis elbow" and "golf elbow," respectively, are the most common tendinopathies of the elbow. They are pathological conditions of the proximal insertion of the forearm muscles at the humeral epicondyles, which mostly involve the common wrist extensor muscle (lateral epicondylitis) and the common wrist flexor muscle (medial epicondylitis) [20].

The cause of epicondylitis is unknown; it is hypothesized that the lesions occur because of a combination of mechanical overloading and abnormal microvascular responses. Consequently, also the risk factors for this pathology are not well identified. In 2013, Titchener and coworkers [24] matched 4998 participants with controls to evaluate different environmental and constitutional risk factors for epicondylitis. Their results showed that patients with a BMI over 40 were at higher risk of being affected by lateral epicondylitis than those with normal BMI [OR 1.41 (1.01–1.97)]. However, this association disappeared when BMI was adjusted for consultation rate using multivariate conditional logistic regression [OR 0.94 (0.66–1.34)].

In the level IV study performed by Descatha and colleagues [28], designed to assess the incidence of epicondylitis in workers exposed physically, the stratification of the risk factors, made by univariate analysis, showed how subjects with BMI >30 kg/m^2 had higher incidence rates for the disease (OR: 2.4, CI: 1.2–4.8). No significant association was instead found for medial epicondylitis.

Conversely, the cross-sectional study by Shiri et al. [20], developed to primarily investigate the prevalence and the risk factors associated with lateral and medial epicondylitis, assessed a causal relationship only between medial epicondylitis in women and both waist circumference >100 cm (OR: 2.7 CI: 1.2–6.0) and BMI >30 kg/m^2 (OR: 1.9 CI: 1.0–2.7), with no increased risk as regards lateral epicondylitis.

4.3. Obesity and Knee Tendinopathies. Knee pathologies such as arthritis are known to be particularly common among obese patients [31, 32].

Regarding knee tendinopathies, a nosological distinction should be made between extensor apparatus tendinopathies and pes anserinus tendinopathies. Diseases of the extensor apparatus, commonly observed among sportsmen, affect quadriceps tendon and patellar tendon at their bony attachments. Pes anserinus tendinopathies are characterized by the presence of pain under load, standing, walking, or taking the stairs, at the insertion of muscles semimembranosus, semitendinosus, gracilis, and sartorius in the superomedial surface of the tibia. It is very frequent in obese women with valgus knee because the hamstring tendons rub against the medial condyle of the knee during every movement. The anserine bursa, which lies between the tendons footprint and the posterior surface of the tibia, may be involved in the inflammatory process and leads to the so-called anserine bursitis which is part of the tendinopathy [33].

A case-control study by Alvarez-Nemegyei [25], performed in 2007 and involving 22 cases and 38 controls, failed to find a relationship between pes anserinus tendinitis/bursitis and diabetes or obesity.

Likewise, Taunton et al. [27], retrospectively analysing 96 cases of patellar tendinopathy among a total of 2002 running related injuries, found neither an increased weight nor an increased BMI in those kinds of patients. Panasiuk and Groblewski [34] presented a case report on a patient with BMI =41, without other comorbidities, who atraumatically injured his patellar tendon. According to the authors, the increased load played a crucial role as cofactor in the mechanism of this spontaneous tendon rupture.

They underlined the dangerousness of such a high load and how individuals with important obesity have a potential risk of acute tendon injuries due to the increased weight and mass.

In literature, there are other case reports [35, 36] dealing with the spontaneous ruptures of patellar tendon or quadriceps tendon, and an increased weight of the patient is often reported among the risk factors.

However, given the rarity of these conditions, no sufficient clinical studies of high level of evidence have been made on this topic; therefore, there are no statistically valid information on the possible association between obesity and quadriceps tendon rupture.

4.4. Obesity and Achilles Tendinopathy. Micro-traumatic tendinopathies of the Achilles tendon are functional overload pathologies that can lead to rupture of the tendon, the ultimate result of a long standing process of failed healing response. In Achilles tendon rupture patients, this failed healing response process is most often entirely asymptomatic, and, involving the tendon in variable extension, determines a decrease in mechanical strength of the tendon, which can be overcome by a sudden strain, and result in a tear [37]. Holmes and Lin [26] in 2006 studied some metabolic risk factors (obesity, diabetes, hypertension, use of oestrogen, and exposure to steroids) to define and quantify their possible etiological role in Achilles tendinopathy.

Using Chi-square analysis to compare observed and expected prevalence in a group of 82 participants versus published national data, they found a statistically significant association for all these conditions and Achilles tendinopathy. In particular, as regards obesity, it was associated with Achilles tendinopathy for both men and women subjects ($P = .001$ and $.0025$). Since the microcirculation is the common denominator between all of these metabolic diseases, alterations of blood flow were suspected to underlie the onset of Achilles tendinopathy.

A 2007 cross-sectional study by Frey and Zamora [21] showed a high BMI (both in overweight and in obese range) significantly increased the chances of Achilles, posterior tibial, and peroneal tendinitis. In particular, 123 (65.4%) of the overweight/obese subjects had a diagnosis of tendinitis compared to 65 (34.6%) normal subjects, and having a BMI >25 increases the risk of being affected by tendinitis [OR: 1.923 (1.39–2.66) $P < .0001$].

Also, Gaida and coworkers in 2010 [22] investigated the relationship between adiposity and asymptomatic Achilles tendinopathy through a cross-sectional study. Examining 298 individuals, they found that men with Achilles tendon pathology had a central fat distribution, while women with tendon pathology had a peripheral fat distribution. These opposite findings, seemingly paradoxical, according to the authors depend on the effect that oestrogens have on the deposition of fat in women. They concluded that the asymptomatic condition of the participants is a clear and important proof that differences in adipose tissue distribution precede tendon pain. In 2013, Scott and colleagues [15] compared 197 patients affected by Achilles tendinopathy versus 100 controls to investigate the relationship between Achilles tendinopathy and body mass index. They found a statistically significant difference in terms of BMI (34.69 ± 7.54 (17.9–75.9) versus 30.56 ± 7.55 (19.7–61.5), $P < .001$) and mean age between the two groups.

Similarly, in the 10-year retrospective analysis performed by Klein et al. [16] on 944 subjects, mean BMI was significantly higher in the group of patients with Achilles tendonitis compared to the control group (30.2 ± 6.5 versus 25.9 ± 5.3, $P < .001$). Overweight and obese patients were 2.6 to 6.6 times more likely than patients with normal BMI to be affected by Achilles tendonitis ($P < .001$).

Taunton et al. [27] carried out a retrospective case-control analysis of 2002 running related injuries. Comparing the 96 cases of Achilles tendinopathy they recorded, with the other 1906 patients, they found no statistically significant association between obesity and this tendinopathy.

4.5. Obesity and Plantar Fasciitis. It is intuitive to speculate that an excess of body weight may be a determinant factor in the common feet pain. In obese subjects, the baropodometric examination reveals very often the loss of the transverse foot arch, resulting in discharging the body weight on the central metatarsal heads with pain on walking [38]. Riddle and colleagues [18], in their level II prognostic study, matched 50 patients affected by plantar fasciitis with 100 controls. They obtained that participants with a BMI >30 kg/m^2 are 5.6 times (CI: 1.9–16.6) more likely to be affected when compared with subjects with BMI ≤25 kg/m^2. Taunton et al. [27] carried out a retrospective case-control analysis of 2002 running related injuries and reported that a high body weight in women (>60 kg) was associated with plantar fasciitis (OR: 0.378, CI: 0.203–0.706).

The Australian group of Frey and Zamora [21], in 2007, identified obesity (along with the pronated foot) as independent and modifiable risk factor for chronic plantar heel pain, through a univariate analysis performed on 80 patients and 80 controls.

Considering that this study cannot establish causality, it is unclear whether increased BMI existed in the case group participants prior to the development of CPHP or whether the pain associated with the condition caused participants to reduce their physical activity, thereby leading to an increase in BMI. However, it is plausible that increased BMI may be a risk factor for CPHP as individuals with increased BMI experience higher vertical forces under the heel during gait [39], leading to higher internal stresses within the heel [40], which may lead to damage of soft tissue structures and the development of symptoms.

An increased incidence of chronic plantar heel pain in individuals with a BMI >25 Kg/m^{-2} was also demonstrated by a study by Irving et al. [17]. They also found an increased chance, although not significant, to be affected by plantar fasciitis if overweight or obese. The authors proposed to relate these data to the effect of an increased weight on musculoskeletal disorders of the lower district (feet and ankles), which are known to be caused by overuse and stress, which are factors made worse by weight.

A recent review [41] established that adult obese individuals are three times more affected by chronic plantar heel pain and foot pain compared to normal weight subjects.

This association includes plantar fasciitis, a condition closely associated with obesity. However, the authors did not exclude the existence of a reverse causality, whereby the presence of plantar pain intervenes by limiting the mobility, thus favouring being overweight. Another discussion point involved the effectiveness of weight loss on the pain symptoms reduction. In fact, the studies reviewed did not provide

evidence of a recovery from the distressing symptoms following bariatric surgery or other weight-loss strategies.

4.6. Comments. Most of the published articles that we analysed in this review are observational studies. However, to date, no level I study was performed about this topic. All the 5 frequency-matched case-control studies (level II), 14–18 published on this matter, agree to report the association between obesity measured in terms of BMI (BMI $\geq$ 30 kg/m^2) and tendon diseases, with odds ratios ranging from 1.9 (95% CI: 1.1–2.2) to 5.6 (1.9–16.6). All the 4 cross-sectional studies, included in this review, also indicate an association between these two conditions, with the exception of the part of the study by Frey and Zamora [21] concerning plantar fasciitis, which however finds a correlation, although not significant.

Nevertheless, such study designs do not allow a precise identification of a cause-effect relationship between pathological body mass index and each type of tendinopathy. At present, in fact, not enough works focused on the mechanism through which excess weight may be responsible for tendinopathies. The largest amount of studies investigating pathophysiological mechanisms focused on Achilles tendinopathy. A 2013 murine study by Boivin et al. [42] examined both the potential negative effect of obesity on Achilles tendon and quadriceps muscle and the potential mitigating effect of exercise and branched-chain amino acid (BCAA) on the same structures. After subjecting the mice to a high fat diet (and its resulting obesity), they found significant alterations in the structure of the Achilles tendon removed from the mouse, with increased tendon cross-sectional area and decreased modulus. Exercises and BCAA integration improved only partially the outcomes, decreasing the stiffness of the Achilles tendon. The authors speculated that the exceeding fat intake, causing the enlargement of the diameter of the fibers and the shortening of the modulus of the tendon, actually leads to a stiffer tendon, less able to withstand the loads.

In 2012, another study [43] focused attention on the Achilles tendon, using 20 healthy adult males divided into low normal weight and overweight based on BMI. The authors measured, by ultrasound, the thickness of the Achilles tendon before and after a session of calf training with ankle weights on. Their aim was to assess the cumulative transverse tendon strain defined as the natural log of the ratio of post- to preexercise tendon thickness. While the thickness, in absolute terms, was greater, both before and after training, for the group classified as "overweight," in fact, the acute transverse strain response was significantly higher in the group of healthy subjects (−11% versus −20%, $P = .0004$). Their pathophysiological explanation of the obtained findings is based on the harmful effect that the tensile load exerts on cell matrix and particularly on morphology and disposition of collagen fibers; this would alter the physiological movement of interstitial fluids, not allowing a proper and normal response to exercise.

A similar study was carried out by Abate et al. [3] in 2012 recruiting a sample of athletes (runners) and a control sample of nonrunning subjects and dividing both groups further into two groups: normal weight and overweight. The results obtained by US (ultrasound) showed a statistically significant difference in terms of the thickness of the Achilles tendon only between runners and nonrunners among normal weight subjects ($P = .002$), indicating that the physiological hypertrophy of the tendon occurs only in normal subjects. Conversely, both US abnormalities and intratendinous microvessels were observed more frequently in overweight participants tendons ($P = .0007$ and $P = .0003$) and, within this group, were significantly prevalent in runners ($P = .001$ and $P = .004$). The authors attribute these findings to the significantly lower ability of the tendon of an obese subject to resist the stress (such as running) and to repair the damage caused by the stress. According to some other hypothesis, a prolonged state of systemic, low-grade inflammation, such as in obesity and states of impaired insulin sensitivity, may act as a risk factor for a "failed healing response" after an acute tendon insult, thus predisposing affected individuals to development of chronic overuse tendinopathies [1, 6]. However, it should be necessary to distinguish what could be the real burden of obesity in the pathophysiological process that leads to tendinopathy. Obesity is present in a number of metabolic diseases such as diabetes which have been associated with tendinopathy on cardiovascular grounds, not the obesity per se. It is known that obesity is associated with alterations in glucose metabolism and conditions as dyslipidemia, hypertension, glucose intolerance, and insulin resistance. These were found both in obese patients and in patients affected by tendinopathy [44].

Future studies should carry out clinical observations on obese patients affected by tendinopathy, distinguishing when obesity is associated with other metabolic diseases and when it is not.

In addition to metabolic pathophysiological mechanisms, mechanical factors are supposed to play a role in the onset of tendinopathy. In particular, among the studies evaluated in this review, there is a stronger association between lower limb tendinopathies and obesity, compared to upper limbs, which seems to prove the hypothesis that higher loading force can be an important risk factor.

Our results are consistent with those obtained from the systematic review performed by Gaida et al. [44] in 2009, which analysed studies published until March 2007. By means of the sensitive analysis, they found 81% of positive association between increased adiposity and tendinopathies considering trials including clinical patients and 77% considering case-control studies.

They also reported poorer outcomes among obese individuals after the treatment of a tendon injury.

Analysing the long-term results and effects of a pathological BMI on the tendinopathy healing process and on the surgical outcomes is certainly an area of research that can provide useful findings, especially if further research will be particularly focused on the differences in results between subjects with obesity compared to those affected by multiple metabolic diseases.

5. Conclusions

Obesity is widespread and, therefore, it is very easy to run into patients with tendinous pathologies who are also overweight.

The best evidence available to date indicates obesity as a risk factor for tendinopathy. In particular, this association seems strong for Achilles tendinopathy and for plantar fasciopathy, in which the increased weight creates an increased load for the tendons, stressing these structures.

Nevertheless, given the low number of high-level studies on the subject, the relationship between obesity and tendinopathies is still enigmatic. Much remains to be studied on this matter and further studies should be performed to establish the real strength of the association between each type of tendinopathy and the obesity per se, isolated from all other metabolic diseases.

Future research will have to go in two directions: clinically, analysing clinical data to confirm and quantify the correlation of obesity with tendinopathies, and experimentally, examining the possible pathophysiological mechanisms underlying this causal relationship.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Vitamin D and Its Relationship with Obesity and Muscle

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The skin synthesis of vitamin D represents the first step of a metabolic pathway whose features have been extensively studied and clarified in the last decades. In particular, the production of active and inactive forms of the hormone and the actions of the corresponding enzymes have offered new insights into the knowledge of vitamin D metabolism. Additionally, the description of the different organs and tissues expressing the vitamin D receptor and its possible functions, as well as its genetic determinants, have allowed focusing on the interrelationship between vitamin D and many physiological and pathological functions. In this context, many studies reported the association between vitamin D and adipose tissue metabolism, as well as the possible role of the hormone in obesity, weight, and fat mass distribution. Finally, many reports focused on the vitamin D-related effects on skeletal muscle, particularly on the mechanisms by which vitamin D could directly affect muscle mass and strength. This paper is mainly aimed to review vitamin D metabolism and its relationship with obesity and skeletal muscle function.

1. Metabolism of Vitamin D

It is an old knowledge that skin exposure to sunlight is the main source of vitamin D production [1, 2]; in fact more than 80% of systemic vitamin D_3 derives from epidermis and the other 20% is obtained through the diet from animal, cholecalciferol (D_3), or plant, ergocalciferol (D_2), and through drug supplementations [3].

Vitamin D_3 skin production depends on a photochemical process in which epidermal 7-dehydrocholesterol (7DHC or provitamin D3) is converted to previtamin-D3 (pre-D3) by ultraviolet radiation (UVR) [4] (Figure 1). The so formed pre-D_3 isomerizes to D_3 in a thermosensitive but noncatalytic process [5]. To prime sunlight reaction this biochemical process requires specific UVB wavelengths, between 290 and 315 nm, present only for limited number of hours also varying with respect to latitude and season. Therefore, a number of personal and environmental factors are important to maximize the formation of pre-D_3, like skin pigmentation, clothes, and sunscreen use [1]. However, prolonged exposure to sunlight does not produce toxic amounts of vitamin D_3 because of the pre-D_3 conversion to the biologically inactive compounds called lumisterol and tachysterol [6].

In addition to this classical way of vitamin D production, research over the last decade has revealed that numerous pathways for metabolism of vitamin D exist with the production of at least 40 metabolites whose role is only partially known [3, 7].

According to the classical pathway, to become fully active, vitamin D (referred to as either vitamin D_2 or vitamin D_3) must be hydroxylated on carbon 25, forming 25-hydroxyvitamin D [25(OH)D] in the liver, and then on carbon 1, forming 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] in the kidney [8]. 25(OH)D is the major circulating metabolite of vitamin D because it has a half-life of 21–30 days [9], so its serum concentration is the most reliable biochemical index of vitamin repletion. 1,25$(OH)_2$D is the most potent physiologically active circulating metabolite produced by humans [3]; it has a half-life of 4–15 h [10, 11] and is responsible for serum calcium and phosphate homeostasis via coordinate effects on the kidney, small intestine, and bone [12]. Indeed, it regulates intestinal calcium and phosphorus absorption [13],

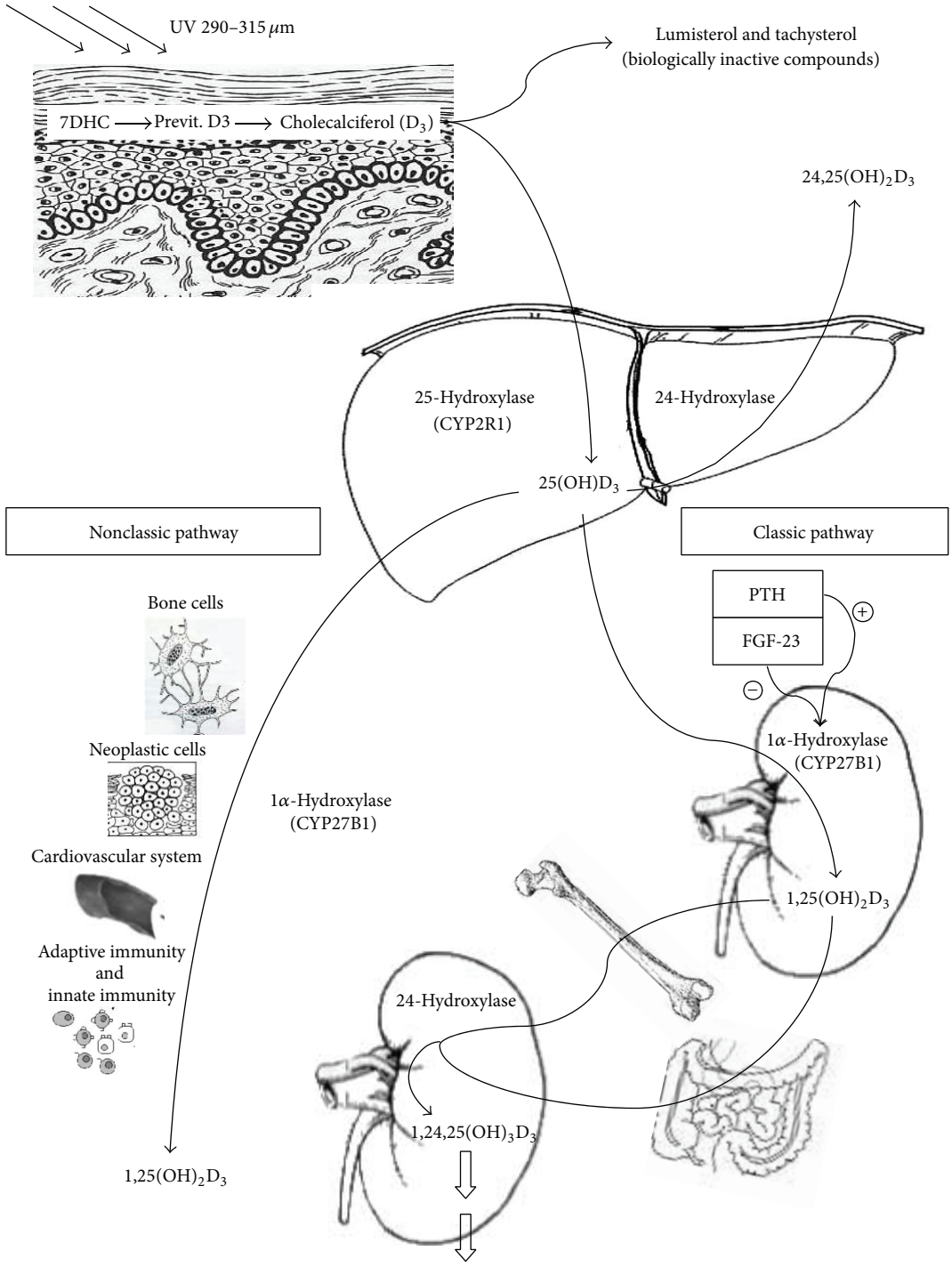

Figure 1: Overview of vitamin D metabolism.

calcium mobilization from bone, and renal reabsorption of calcium and phosphorus [14].

The conversion of 25(OH)D to $1,25(OH)_2D$ depends on the action of the cytochrome P450 enzyme (CYP450), 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase), in the kidney. However, a cytochrome P27B1 enzyme (CYP27B1), 1α-hydroxylase, activity has also been demonstrated in bone cells, both osteoblasts and osteocytes [15, 16]; it leads to a local production of $1,25(OH)_2D$ within the osteocytes and directly affects autocrine activities promoting osteoblast and osteocyte maturation and bone remodelling [16, 17]. In recent years 1α-hydroxylase activity has been found in other tissues, such as placenta, skin, immune system, and granuloma tissue [18]. Synthesis of $1,25(OH)_2D$ in the kidney is directly stimulated by PTH integrating the role of vitamin D in maintaining mineral homeostasis. In fact hypocalcemia,

hyperphosphatemia, or reduction in serum fibroblast growth factor 23 (FGF23) results in increased production of PTH that stimulates hydroxylation of 25(OH)D [19]. Conversely, when $1,25(OH)_2D$ levels increase, FGF-23 inhibits CYP27B1 in the proximal renal tubule [20]. Additionally, $1,25(OH)_2D$ is capable of inversely regulating its own levels by inducing the synthesis of 25-hydroxyvitamin D-24-hydroxylase (24-OHase) [21]. This enzyme is located essentially ubiquitously in all kinds of cells including renal and intestinal cells. The enzyme is also a mixed-function oxidase cytochrome P450 molecule and catalyzes the hydroxylation on carbon 24 leading to the production of 1,24,25-hydroxyvitamin D, the first step in the 24 oxidation pathway that leads to the formation of an inactive water soluble metabolite, calcitroic acid, which is excreted in the urine [22]. 24-hydroxylase produces metabolite also from 25(OH)D leading to the production of 24,25-dihydroxyvitamin D $[24,25(OH)_2D]$. Showing the intriguing mechanism in vitamin D metabolism, recently we demonstrated that the administration of high doses of vitamin D leads to a rapid conversion of 25(OH)D in both active and inactive $[24,25(OH)_2D]$ metabolites [23].

The role and the mechanism of action of these metabolites are not well defined [3]; it could be only hypothesized that if a $24,25(OH)_2D$ receptor exists, it would be a member of the nuclear hormone receptor family by analogy with the vitamin D receptor (VDR) [8]. In fact, as a fat-soluble secosteroid hormone, $1,25(OH)_2D$ carries out its mechanism of action binding an intracellular receptor that is a member of the superfamily of nuclear receptors. VDR forms a heterodimer with the retinoid X receptor acting as a transcription factor that binds to vitamin D response elements in the promoter region of target genes. This interaction with specific DNA sequences results in the activation or repression of transcription processes. In addition, other ligand-recruited complexes appear to act more directly on the transcriptional apparatus, known as steroid receptor activator complex (SRC) [24]. VDR is expressed both in classical target organs of vitamin D involved in mineral homeostasis and in most tissues and cells of the human body explaining the molecular basis of the pleiotropic effect of vitamin D endocrine-system and its *nonclassical actions* [25]. This system regulates cell proliferation and differentiation and has immunomodulatory, anti-inflammatory, and antifibrotic properties. VDR polymorphisms and different vitamin D metabolisms, involving numerous cytochromes and cytokines, are also considered to be implicated in pathogenetic mechanisms involving numerous systems, for example, cardiovascular [26], metabolic [27], neurological [28], immunological [29], and neoplastic [30] tissues.

2. Vitamin D and Obesity

A number of studies have shown that obesity, defined as a body mass index (BMI) $\geq 30\ kg/m^2$ [31, 32], is associated with low serum 25(OH)D levels [33, 34]. A bidirectional genetic study, which limits confounding, has suggested that higher BMI leads to lower 25(OH)D, each unit increase in BMI being associated with 1.15% lower concentration of 25(OH)D, after adjusting for age, sex, laboratory batch, and month of measurement [35].

The basis of low vitamin D concentration in obesity is still under debate and could be the result of several mechanisms. One hypothesis is that the high content of body fat acts as a reservoir for lipid soluble vitamin D and increases its sequestration, thus determining its low bioavailability [36]. It has also been reported that fat content is inversely related to serum 25(OH)D concentration and that this association is stronger than that between 25(OH)D and BMI [35]. In obese subjects, not only fat mass is increased but also lean body mass, as an adaptative response to greater body weight. In animal studies it has been shown that 25(OH)D was stored 33% in fat and 20% in muscle [37], suggesting that muscle could be also another reservoir of vitamin D in humans. Other authors have theorized that obesity is associated with decreased sunlight exposure, limited outdoor activity, or clothing habits that limits cutaneous vitamin D synthesis [38]. Another hypothesis is that the synthesis of 25-hydroxyvitamin D by the liver may occur at a lower rate in obese subjects due to hepatic steatosis [39]. An alternative explanation is that higher leptin and interleukin 6 circulating levels, mostly secreted by adipose tissue, may have inhibitory effects on 25(OH)D synthesis via their receptors [40]. Even though these previously reported hypotheses may have a role in explaining the reasons for the high prevalence of hypovitaminosis D in obesity, a recent study addresses the question by taking into consideration not only BMI but also body size. This study showed that a volumetric dilutional model accounted for essentially all the variability in serum 25(OH)D concentrations attributable to obesity; in fact once serum 25(OH)D concentrations in obese individuals are adjusted for body size, there is no longer a difference between obese and nonobese individuals [41].

A difference that certainly characterizes obese subjects is the higher fat mass and researchers are now focusing on the interplay between fat mass and vitamin D. Adipose tissue is nowadays considered as a major active endocrine organ secreting heterogeneous bioactive factors, the so-called adipokines [42]. Humans have two major anatomically distinct types of adipose tissues, white and brown which are derived from different cell lineages and exert opposite roles on lipid metabolism. The white fat stores energy and the brown fat dissipates it by using lipids as fuel for thermogenesis. Fat cells are extremely plastic, able to rapidly expand in size and number. In obesity, adipocytes become enlarged with increased macrophage infiltration and a switch towards the proinflammatory phenotype. Interestingly, the ability to both recruit and differentiate new adipocytes is impaired in individuals with hypertrophic adipose tissue [43]. Differentiation into adipocytes requires key transcription factors like the nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) and the CCAAT-enhancer-binding proteins [44].

It has been clearly shown that adipose tissue may both regulate and be regulated by vitamin D [45]. The expression of the vitamin D receptor, 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1) genes, and 24-hydroxylase enzyme has been shown in human adipocytes [46]. There are some experimental data suggesting that vitamin D could promote greater

adiposity, leading to elevated parathyroid hormone, which may promote calcium influx into adipocytes thereby enhancing lipogenesis [47]. Also 1,25-hydroxyvitamin D modulates adipogenesis through vitamin D receptor-dependent inhibition of critical molecular components of adipogenesis such as peroxisome proliferator-activated receptor γ [48]. Data on $1,25(OH)_2D$ level are controversial in obese subjects; they are reported to be increased or decreased, probably due to the heterogeneity of the technique used in measuring $1,25(OH)_2D$ by immunoassay, which is not totally specific and measures other vitamin D metabolites in serum [49, 50].

The complex biochemical interactions between adipose tissue and vitamin D *in vitro* raise the question as to whether hypovitaminosis D, itself, may contribute to obesity or inhibit weight loss *in vivo*. A few studies have shown that vitamin D, with or without calcium, appears not to have a definite effect on weight, but that it may affect fat mass and distribution. This effect was seen when 25(OH)D level was less than 50 nmol/L; it was not observed when 25(OH)D was above this threshold [51–54]. This demonstrated that giving supplemental vitamin D to those who were replete has no additional effect.

An unresolved question is what dose of vitamin D should be used in obese subjects to replete vitamin D stores and how to maintain normal 25(OH)D levels after repletion. The Institute of Medicine (IOM) guidelines suggest that there is no evidence that increases in vitamin D intake beyond the requirements for nonobese persons can affect bone health or other health conditions among obese persons [55], while Endocrine Society guidelines suggest two to three times more vitamin D in obese people for their age group to satisfy their body's vitamin D requirement [56].

These conclusions are supported by a recent randomized study of seven doses of vitamin D_3 (from 400 IU/d to 4800 IU/d) showing how the response to vitamin D supplementations was dependent on body size. After vitamin D supplementation, all obese women reached adequate levels of serum 25(OH)D, but women with BMI $< 25\,kg/m^2$ reached much higher levels of 25(OH)D with the same dose, suggesting that "one size does not fit all": the dose depends on the threshold of vitamin D to be achieved and on body size [57–61].

However, if the goal is to affect the number of comorbid conditions commonly associated with obesity, where it has been speculated that vitamin D insufficiency may play a role, such as type 2 diabetes [62], cardiovascular disease [63], and hypertension [64], it is likely that the dose of vitamin D required to affect these comorbidities may be different from that needed to suppress PTH [57]. It has been suggested that PTH is suppressed at a lower serum 25(OH)D in obese women compared to the entire population [54]. It is possible that there may be a different set-point for the calcium PTH relationship in the obese, as demonstrated in a calcium-citrate clamp that showed an exaggerated PTH response to hypocalcemia as compared to normal subjects [65]. The etiology for the above is unknown, as well as the dose of vitamin D needed to suppress PTH. Likewise, the dose required to affect comorbidities associated with obesity is uncertain. Considering the effect of vitamin D supplementation on glycaemic indices in obese, 1000 UI/d had no effect [66], while 4,000 to 10,000 IU/d had beneficial effect [67, 68]. Considering the effect on hypertension, a high dose of vitamin D_3 (15,000 IU/d), in obese hypertensive patients, was demonstrated to reduce tissue-renin angiotensin system activity [69]. Regarding cardiovascular disease risk markers in overweight subjects, a vitamin D supplement of 3332 IU/d was able to significantly reduce triglyceride levels and proinflammatory cytokines [70]. However, Jorde et al. demonstrated that a dose of vitamin D 40 000 IU per week had no positive effect on glucose tolerance, blood pressure, or serum lipids in a sample of subjects with sufficient vitamin D baseline levels [71]. These studies emphasize that only patients with an insufficient vitamin D level would benefit from vitamin D supplements, with a dosage that would appear to be higher than the dose needed to obtain only vitamin D sufficiency and thus PTH suppression. However the mechanisms to explain these results are still largely unknown.

This consideration should be extended also to obese patients who undergo bariatric surgery, which is used with an increasing frequency for weight reduction. Indeed, bariatric surgical procedures may induce malabsorption; therefore, the combination of both low preoperative vitamin concentration and malabsorption may render these patients more prone to severe vitamin D deficiencies. Supplementation with vitamin D should be considered before and after surgery [71]. In any case, clinical studies to determine optimal treatment guidelines for the surgical and nonsurgical population with obesity are warranted.

3. Vitamin D and Skeletal Muscle

Vitamin D depletion has been frequently associated with worse physical performance, increased risk of falls, and impaired muscle strength, particularly in the elderly [72–81]. While muscle weakness and pain represent the typical pattern of osteomalacic-associated muscle disease, even atypical clinical presentations are frequent. They include hypotonia, waddling gait, impaired physical function, and uniform generalized muscle wasting and bone pain [82].

Vitamin D exerts an important role in the regulation of skeletal muscle tropism and contraction. As for bone, it has been proposed that vitamin D acts on muscle tissue through both a direct and an indirect effect. The proposed mechanisms include proximal muscle atrophy, loss of type II muscle fibers, and secondary hyperparathyroidism [83–87]. Indeed, vitamin D acts to maintain the function of type II muscle fibers [83, 84]. The histopathological findings showed atrophy of type II skeletal muscle fibers in adults with vitamin D deficiency [85]. This finding is of utmost importance because type II muscle fibers are the first to be recruited when preventing a fall [86].

As far as secondary hyperparathyroidism is concerned, it has been shown that parathyroid hormone negatively affects skeletal muscle function in animal models through proteolysis of muscle proteins and by reducing inorganic phosphate, creatine phosphate, and Ca-ATPase in muscle cells [82].

The direct effects of vitamin D on muscle have to be connected with VDR. Since it was identified in skeletal muscle cells, several reports stated that vitamin D affects muscle function through the binding of $1,25(OH)_2D$ to its receptor, resulting in muscle growth, as well as other adaptations [74]. Hence, the role of vitamin D on muscle seems to be connected to the induction of genomic effects, leading to the synthesis of new proteins affecting muscle cell contractility, proliferation, and differentiation and to the regulation of calcium transport in the sarcoplasmic reticulum [87, 88]. Nevertheless, the underlying mechanism is actually not well understood. Data from literature demonstrated that, during development, $1,25(OH)_2D$ decreases cell proliferation and enhances myogenic cell differentiation in the mesodermal stem cells by modulating the expression of key pro- and antimyogenic factors, such as IGF-I, IGF-II, follistatin, and myostatin [88]. Hence, $1,25(OH)_2D$ can affect myogenic differentiation of skeletal muscle cell lines through an upregulation of *IGF-II* and *follistatin* and a downregulation of *IGF-I* and *myostatin* expression [88]. Garcia et al. demonstrated that the addition of 1,25-dihydroxyvitamin D_3 to skeletal muscle cells enhanced the expression of myogenic markers and transcription factors at different stages of differentiation [88]. Moreover, after 10 days of incubation of the cells with 1,25-dihydroxyvitamin D_3, muscle fibers turned to be positive for MHC type II, a late myogenic marker, and showed an increase in the mean diameter and in the width, compared to the controls [88].

Recently, the presence of a functional vitamin D system in muscle, including a CYP27B1 bioactivity, has been demonstrated [86, 87]. This system has been described to act by inhibiting muscle cells proliferation and myotube formation and increasing myotubes size, thus suggesting a direct effect of the hormone on muscle [87]. Conversely, data from Wang and DeLuca demonstrated the absence of vitamin D receptor on skeletal muscle suggesting that the effect of vitamin D in muscle function is most likely indirect [89]. These authors also speculated that the muscle impairment of osteomalacia might depend on associated metabolic changes such as hypocalcemia, hypophosphatemia, and elevated PTH levels [89].

A number of clinical studies have reported that a low vitamin D status is associated with loss of handgrip strength and impaired lower extremity function with increased risk of falls [73–80] (Table 1). Moreover, the effect of vitamin D administration on physical performance, falls, and muscle strength has been widely investigated. Short- and long-term studies collectively demonstrate a relationship between vitamin D status and fall prevention and improvement in muscle strength in community-dwelling older individuals receiving a long-term supplementation with calcium and vitamin D [90–92] (Table 1). Nevertheless, data are still conflicting [74, 81, 93–95]. A meta-analysis of eight randomized controlled trials showed that doses of 700 IU to 1000 IU supplemental vitamin D_3 a day could reduce falls by 19% or by up to 26% in the elderly [96]. This benefit was significant within 2–5 months and beyond 12 months of treatment; in addition it may not depend on additional calcium supplementation [96]. Active forms of vitamin D were not found to be more effective and vitamin D_3 has been reported as possibly better than vitamin D_2 in preventing falls [96]. Finally, based on the possible better efficacy of higher doses of vitamin D, the authors pointed out the need for future research exploring such doses [96]. On the contrary, a double-blind, placebo-controlled trial of 2256 community-dwelling women, aged 70 years or older, considered to be at high risk of fracture, concluded that an annual oral administration of high dose cholecalciferol (500,000 IU) resulted in an increased risk of falls and fractures [97]. Nevertheless, these results were observed early after dosing, being the RR of falls in the vitamin D group 1.31 in the first 3 months (95% CI, 1.12–1.54), but only 1.13 (95% CI, 0.99–1.29) during the remaining months of the year [94].

Other authors found no significant effect of vitamin D supplementation on muscle strength [94, 95]. A more recent study, by Knutsen et al., reported the absence of any improvement in muscle strength or power (as assessed by jump, handgrip, or chair-rising test) after sixteen weeks of daily supplementation with 1,000 IU of vitamin D_3 in a healthy adult population aged 18–50 years with hypovitaminosis D [98]. Such discrepancies could be due to the lack of homogeneity among the populations studied and the different doses of vitamin D used [93–95]. Indeed, some works actually focused on deficient and others on nondeficient patients and the dose scheme was not adequate in some instances to significantly increase vitamin D serum levels above the threshold of sufficiency [93–95]. On the other hand, the last point is in turn relatedto the fact that the optimal dose and frequency of vitamin D supplementation to achieve and maintain adequate vitamin D serum levels are still debated.

General muscle strength is often evaluated by handgrip strength and/or thigh muscle strength measured by a dynamometer. Gupta et al. reported enhanced handgrip strength in vitamin D deficient Indians aged 20–40 years treated with 60,000 IU per week for 8 weeks followed by 60,000 IU/month for 4 months of cholecalciferol, combined with calcium [90]. In contrast, Goswami et al. reported no improvement in skeletal muscle strength with such a scheduled supplementation [99]. A recent study from our group represents one of the few ones dealing with the issue of muscle strength and vitamin D supplementation in young chronically D-deficient/insufficient people. We evaluated the effect of a single oral dose of 600,000 IU of cholecalciferol on the handgrip strength in young women with vitamin D deficiency [100]. The results showed rapidly improved vitamin D status, while we did not observe any changes in muscle strength parameters in the whole cohort over 3 months, or in a subgroup of women followed up for 6 months. Moreover, 25(OH)D and PTH did not correlate with the two parameters of muscle strength studied at any time point. Finally, we found an increase of serum phosphate in response to vitamin D administration, which could be the most important mechanism of vitamin D effect on muscle, as also suggested by the significant correlation between serum phosphorus levels and muscle strength we found after supplementation both in the whole sample and in the subgroup of women followed up for 6 months [100]. However, the small sample size did not allow concluding the possible

Table 1: Effect of vitamin D on muscle strength and falls.

Author, year, and study type	Patients, age	Endpoints/tools	Result
Visser et al., 2003 [73]; prospective observational study	1008 for grip strength evaluation; 331 for muscle mass evaluation; 55–85 yrs	Grip strength; appendicular skeletal muscle mass (using dual-energy X-ray absorptiometry)	(i) Persons with baseline 25-OHD levels <25 nmol/liter were 2.57 (based on grip strength) and 2.14 (based on muscle mass) times more likely to experience sarcopenia, compared with those with levels >50 nmol/liter (ii) PTH >4.0 pmol/liter was associated with an increased risk of sarcopenia
Latham et al., 2003 [101]; multicenter, RCT*	243 hospitalized patients; 65 yrs or older	Falls, physical performance (isometric knee extensor strength), and self-rated function	No effect of vitamin D (calciferol, 300,000 IU) on physical health, falls, and physical performance, even in patients with baseline vitamin D levels <12 ng/mL
Kenny et al., 2003 [95]; RCT*	65 healthy, community-dwelling men; 65–87 yrs	Upper and lower extremity muscle strength and power (using a leg press and handgrip strength), physical performance (specific tests), and activity (using questionnaires)	(i) Baseline 25OHD correlated with baseline single-leg stance time and physical activity score. Baseline PTH levels correlated with baseline 8-foot walk time and physical activity score (ii) No significant difference in strength, power, and physical performance between groups (cholecalciferol 1,000 IU/d or placebo for 6 months, all received 500 mg of calcium)
Broe et al., 2007 [75]; secondary data analysis of a previous RCT*	124 nursing-home residents; 68–104 yrs	Falls	Supplementation with 800 IU of cholecalciferol reduced the adjusted-incidence rate ratio of falls by 72%, compared to placebo; no differences for the 200, 400, and 600 IU dose
Bischoff-Ferrari et al., 2004 [78]; population-based survey	Ambulatory population; 60–90 yrs	Lower-extremity function; timed 8-foot walk test; and repeated sit-to-stand test	The group in the highest quintiles of 25(OH)D had an average decrease of 0.27 s in the 8-foot walk test and an average decrease of 0.67 s in the sit-to-stand test
Gerdhem et al., 2005 [77]; prospective observational study	986; 75.0–75.9 yrs	Gait, balance, and self-estimated activity level thigh muscle strength	25OHD correlated with gait speed ($P < 0.001$), balance test ($P < 0.001$), self-estimated activity level ($P < 0.001$), and thigh muscle strength ($P = 0.02$)
Houston et al., 2007 [81]; post hoc analysis of a prospective population-based study	976; 65 yrs or older	Short physical performance battery (SPPB) and handgrip strength	(i) Vitamin D levels were significantly associated with SPPB score in men ($P = 0.04$) and handgrip strength in men ($P = 0.004$) and women ($P = 0.01$) (ii) Men and women with serum 25OHD <25.0 nmol/L had significantly lower SPPB score; and those with serum 25OHD <50 nmol/L had significantly lower handgrip strength than those with serum 25OHD ≥25 and ≥50 nmol/L, respectively, ($P < 0.05$) (iii) PTH was significantly associated with handgrip strength only ($P = 0.01$)
Pfeifer et al., 2009 [91]; double-blind, controlled trial	242 community-dwelling people; 70 yrs or older	Falls, body sway, timed-up-and-go test, and maximum isometric leg extensor strength (assessed with a strain gauge dynamometer)	(i) Calcium plus vitamin D significantly decreased the number of subjects with first falls of 27% at month 12 and 39% at month 20, compared to calcium alone (ii) Significant improvements in quadriceps strength of 8%, a decrease in body sway of 28%, and a decrease in time needed to perform the TUG test of 11%
Moreira-Pfrimer et al., 2009 [92]; prospective, double-blind, placebo-controlled, randomized trial	46 patients in long-stay geriatric care, 62–94 years	Maximum isometric strength of hip flexors (SHF) and knee extensors (SKE), measured by a portable mechanical dynamometer	SHF was increased in the calcium/vitamin D group (1 g calcium + cholecalciferol 150,000 IU once a month for the first 2 months and then 90,000 IU once a month for the last 4 months) by 16.4% ($P = 0.0001$) and SKE by 24.6% ($P = 0.0007$), no improvement in the calcium + placebo group

Table 1: Continued.

Author, year, and study type	Patients, age	Endpoints/tools	Result
Kukuljan et al., 2009 [93]; RCT*	180 healthy men, 50–79 yrs	Total body lean and fat mass (DXA^), midfemur muscle cross-sectional area (quantitative computed tomography), muscle strength, and physical function	Daily consumption of low-fat fortified milk (providing 1000 mg calcium and 800 IU vitamin D_3, per day) does not enhance the effects of resistance training exercise on skeletal muscle size, strength, or function
Bischoff-Ferrari et al., 2009 [96]; meta-analysis of RCT*	2426 patients from 8 RCT	Falls	(i) High dose supplemental vitamin D reduced fall risk by 19% (ii) Achieved serum 25 (OH)D concentrations of 60 nmol/L or more resulted in a 23% fall reduction
Lips et al., 2010 [94]; double-blind, placebo-controlled trial	126 patients with vitamin D insufficiency; 70 yrs or older	Mediolateral body sway and short physical performance battery (SPPB)	(i) After 16 wk, mediolateral sway and SPPB did not differ significantly between treatment groups (vitamin D_3 8400 IU/week versus placebo) (ii) In the post hoc analysis treatment with vitamin D_3 significantly reduced sway compared with placebo ($P = 0.047$) in patients with elevated baseline sway
Gupta et al., 2010 [90]; double-blind, randomized trial	40 healthy volunteers; 20–40 yrs	Handgrip and gastrosoleus dynamometry, pinch-grip strength, respiratory pressures, 6-minute walk test, and muscle energy Metabolism on ^{31P} magnetic resonance spectroscopy	The supplemented group (60,000 IU D_3/week for 8 weeks followed by 60,000 IU/month for 4 months + 1 g of calcium daily) gained a handgrip strength of 2Æ4 kg; gastrosoleus strength of 3Æ0 Nm; and walking distance of 15Æ9 m over the placebo group
Murad et al., 2011 [76]; meta-analysis	45,782 participants from 26 trials	Falls	Vitamin D use was associated with statistically significant reduction in the risk of falls (odds ratio for suffering at least one fall, 0.86; 95% confidence interval, 0.77–0.96)
Goswami et al., 2012 [99]; RCT*	173 healthy females, mean age 21.7 + 4.4 yrs	Handgrip and pinch grip strength and distance walked in 6 min	Mean handgrip strength and its increase were comparable in 4 groups (double placebo, calcium/placebo, cholecalciferol/placebo, and cholecalciferol/calcium at 6 months)
Cipriani et al., 2013 [100]; prospective intervention study	18 women with vitamin D deficiency (25–39 yrs)	Handgrip strength (using a dynamometer and evaluating maximal voluntary contraction (MVC) and speed of contraction (S))	(i) No significant change in MVC and S values after vitamin D supplementation (cholecalciferol 600,000 IU) (ii) A significant correlation between MVC and S and serum phosphorus after supplementation ($P < 0.02$ and $P < 0.05$, resp.)
Knutsen et al., 2014 [98]; RCT*	251 healthy adults with vitamin D deficiency (18–50 yrs)	Jump height, handgrip strength, and chair-rising test	(i) Percentage change in jump height did not differ between the group receiving vitamin D_3 (1000 IU daily) and placebo ($P = 0.44$) (ii) No significant effect of vitamin D on handgrip strength or the chair-rising test

*Randomized controlled trial.
^Dual-energy X-ray absorptiometry.

mechanisms underlying our results, particularly those related to the effect of high $1,25(OH)_2$ levels on muscle tissue [100].

As discussed in experimental data, clinical studies reported conflicting results, demonstrating that the effect of vitamin D on muscle strength and performance still presents many controversial issues and open questions [101, 102] that need to be addressed also in relation to the reported variation in vitamin D receptor gene [103]. The discordant results are substantially connected to the high variability in terms of study design and muscle parameters considered as outcomes and also reflect the discordant findings on the mechanisms underlying vitamin D and muscle function. Among all, the possible direct effect of the hormone on muscle tissue is still controversial, since opposite data are available on VDR expre ssion on skeletal muscle [83, 89]. Moreover, the metabolic changes associated with vitamin D deficiency have

been suggested to be related to a muscle strength improvement after vitamin D supplementation [100]. Hence, given the important action of vitamin D on skeletal muscle tissue, a better understanding of the mechanisms involved is needed, as it will give a new insight into the clinical management of deficient patients.

4. Conclusion

Vitamin D represents one of the most studied and discussed topics in the field of bone and mineral metabolism diseases worldwide. The metabolism of the hormone has been extensively clarified, particularly the role of the different enzymes involved, as well as the active and inactive metabolites and the vitamin D receptor. Taken together, these data have also allowed best investigating the pleiotropic and multiorgan-targeted effects of vitamin D. In particular, several studies described the interrelationship between the hormone and the adipose tissue, both considering obesity as a predisposing condition to hypovitaminosis D and vitamin D as a cofactor in the pathogenesis of obesity. Moreover, direct and indirect effects of the hormone on the skeletal muscle tissue lead to a better understanding of the clinical features associated with vitamin D deficiency.

As many efforts have been made in the understanding of vitamin D metabolism and functions, several mechanisms still need to be covered, particularly in relation to many genetic factors involved. Additionally, notwithstanding the whole amount of data on the field, no consensus currently exists on definition and treatment regimen of hypovitaminosis D, mostly as far as particular conditions (such as obesity) and targeting functions (as muscle strength) are concerned.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Alliance of Mesenchymal Stem Cells, Bone, and Diabetes

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Bone fragility has emerged as a new complication of diabetes. Several mechanisms in diabetes may influence bone homeostasis by impairing the action between osteoblasts, osteoclasts, and osteocytes and/or changing the structural properties of the bone tissue. Some of these mechanisms can potentially alter the fate of mesenchymal stem cells, the initial precursor of the osteoblast. In this review, we describe the main factors that impair bone health in diabetic patients and their clinical impact.

1. Introduction

Bone fragility has emerged as a new complication of Type 2 Diabetes (T2D). The pathophysiological link between bone fragility and diabetes is not completely understood. Several mechanisms may influence bone homeostasis by impairing the function of osteoblasts, osteoclasts, and osteocytes and/or changing the structural properties of the bone tissue. Notably, adipocytes and osteoblasts are derived from a common precursor, the mesenchymal stem cell (MSC), and the differentiation is modulated by several interacting pathways that may be disrupted in diabetes. Other organs and endocrine systems such as the gut, kidney, and cardiovascular and vitamin D systems are altered in diabetes and, therefore, may also affect bone metabolism. As a result, fractures are an added burden in diabetes. However, while bone mineral density (BMD) is decreased in patients with Type 1 diabetes (T1D), it is normal or even increased in T2D patients.

In this review, we describe the main factors that impair bone health in diabetic patients and their clinical impact.

2. The Mesenchymal Stem Cell Fate: To Be or Not to Be

Although fat cells primarily compose the adipose tissue, they also populate bone marrow in coexistence with osteoblasts and their common mesenchymal progenitor [1]. A fine balance exists between adipogenesis and osteoblastogenesis that rely mainly on the activity and interdependence of two systems, the WNT signaling and the Peroxisome proliferator-activated receptors-γ (PPAR-γ) pathways [2]. Activation of Wnt signaling pathway promotes osteogenesis [3–5], while inhibiting adipogenesis [6, 7]; on the other hand, PPAR-γ favors the differentiation of mesenchymal stem cells into adipocytes over osteoblasts [8]. The reciprocal activity of these pathways may determine the prevalence of one lineage over the other, leading, for example, to impaired bone formation in case of prevailing adipogenesis. In fact, marrow adipogenesis have been associated with reduced, bone formation [9–11], and BMD [12, 13], the latter being a strong predictor of fracture risk [14].

2.1. An Osteoblast: The WNT Signaling Pathway. Wnt glycoproteins are a large family of growth factors (19 secreted proteins) that mediate crucial biological processes like embryogenesis, organogenesis, and tumorigenesis. The WNT signaling consists of the canonical (or Wnt/β-catenin) and the noncanonical pathways (reviewed in [15]). The Wnt/β-catenin pathway is activated when canonical Wnt ligands signal through the Frizzled receptors and the low-density lipoprotein receptor-related protein- (LRP-) 5 or LRP-6 coreceptors. The signal leads to inhibition of the glycogen synthase kinase (GSK) 3β. This serine/threonine kinase is a main regulator of β-catenin degradation and therefore activity. WNT activity is tightly regulated by several molecules acting locally. Those that interact with Wnt coreceptors LRP (e.g., sclerostin and Dickkoff or Dkk-1) [16–18] selectively inhibit the Wnt canonical pathway; others bind to Wnts, thus inhibiting both canonical and noncanonical signalling.

WNT canonical pathway controls MSC differentiation to three specific lineages, adipocytes, osteoblasts, and chondrocytes. For example, WNT canonical pathway represses chondrocyte [19] and adipocytes differentiation [20], but it is required for chondrocyte hypertrophy [19]. Moreover, the activation of the Wnt/β-catenin (primarily Wnt10b) promotes osteoblast differentiation and proliferation from MSC, through stimulation of osteogenic transcription factors, such as Runx2 and osterix [21]. This process activates a negative feedback control with Dkk-1 and sclerostin production by osteocytes. At the same time osteoblasts produce osteoprotegerin (OPG) which decreases osteoclast differentiation and maintains bone homeostasis [22]. Inhibition of adipogenesis by Wnt signaling pathway appears to be mainly mediated by β-catenin which inhibits expression of selected PPARγ2 targets genes [23].

The relevance of WNT is also proved by the fact that mutations in the LRP-5 Wnt coreceptor are associated with changes in BMD [3, 4, 24]. For example, loss-of-function LRP-5 knock-out mice present reduced bone mass [3, 25], while point mutations in the LRP-5 gene are related to increase bone mass in humans [4] and mice [26, 27]. On the other hand, variants at the LRP-5 gene are strongly associated with obesity and LRP-5 knock-out mice showed increased plasma cholesterol and impaired glucose tolerance [28].

2.2. An Adipocyte: The PPAR-γ2 Pathway. Adipogenesis is under the control of the master regulator PPAR-γ. PPARs are transcriptional regulators that form part of the ligand activated nuclear hormone receptor superfamily [29]. PPAR-γ is expressed as two protein isoforms produced from a single gene [30, 31]. While the expression of PPAR-γ1 takes place in many different cell-types, PPAR-γ2 is expressed only in adipocytes and bone marrow stromal cell [32, 33].

PPAR-γ2 acts as a molecular switch that regulates the fate of pluripotent MSC. PPAR-γ2 upregulation during the early phases of adipogenesis directly relates with the presence of CCAAT/enhancer binding protein (C/EBP) family of transcription factors, which are stimulated by adipogenic hormones [29]. Growth factors signaling through the MAP kinase (MAPK) cascade may regulate PPAR-γ2 activity [34]. On the other hand, osteogenic stimuli that enhance Wnt signaling may inhibit PPAR-γ2 by formation of a corepressor complex [35]; reciprocally, PPAR-γ2 suppresses Wnt signaling by stimulating the degradation of β-catenin by proteasome [36].

This pathway is relevant and also being target of some antidiabetic drugs like Thiazolidinediones (TZDs) which are effective in lowering blood glucose but also increase the risk of fractures [37]. In fact, activation of PPAR-γ2 in bone marrow enhances MSC differentiation to adipocytes while inhibiting osteogenesis. Some studies have demonstrated an accumulation of lipids in bone marrow [38] and an increase of PPAR-γ2 expression associated with bone loss and aging [39]. Adipocyte-specific transcription factors such as PPARγ2 and C/EBPa are more represented in old bone marrow than in adult marrow [39]. Similarly, mice with T1D display increased PPAR-γ2 in bone tissue, reduced bone formation, and increased marrow adiposity [40]. Abnormal expression of these factors increased accumulation of lipids in sites outside of adipose tissues such as the bone marrow by inducing differentiation of mesenchymal cells toward a mesenchymal adipocyte-like default cell (MAD cell) [39].

3. Osteoclasts and the RANKL System

Marrow stromal cells and osteoblasts are required for the formation and activation of osteoclasts. Osteoclast precursors come from the haematopoietic stem cell compartment and need to directly interact with molecules produced by osteoblasts such as M-CFS and the receptor activator of nuclear factor-κB ligand (RANKL) [41]. RANKL is a member of the tumor necrosis factor (TNF) family which, upon binding to RANK, activates downstream signaling molecules in osteoclasts such as nuclear factor-κB (NF-κB), c-fos, MAPK, and TNFRAF6 that are involved in differentiation and activation [42]. RANKL signal induces also the activation of small GTP-ases that modulate cytoskeletal activity and thus function and survival of osteoclasts. Osteoclastogenesis is also downregulated by OPG, another osteoblast derived protein which works as decoy receptor for RANKL and prevents the RANK/RANKL interaction [43]. Glass et al. showed that OPG expression by osteoblasts was dependent on the activity of β-catenin, the main WNT signaling effector [44], while Wnt3a inhibits osteoclastogenesis at later stages [45]. Conversely, activation of noncanonical pathway by Wnt5a stimulates osteoclast differentiation [46].

The RANK/RANKL/OPG system is targeted by several hormones and inflammatory cytokines which can participate in the bone loss associated with inflammatory diseases, including obesity and diabetes. Indeed, levels of OPG are associated with fat mass [47] and atherosclerosis parameters in diabetes [47–49], while soluble RANKL have been shown to predict T2D in humans and to participate in the genesis of insulin resistance in this disease (see Section 5.6) [50].

4. Bone-Fat Interaction

Although an impaired balance between adipogenesis and osteogenesis may alter the number of bone forming cells, fat interferes with bone homeostasis trough several additional factors, which are often difficult to discern.

Epidemiological evidences have shown a positive correlation between bone mass and BMI suggesting fat to be protective against bone loss [51–53]. Notably, weight loss can increase bone turnover and decrease BMD [54–56], the latter effect partly mediated by increased levels of the WNT antagonist sclerostin [57]. Epidemiological studies have also suggested that weight loss-induced bone loss increases the risk for osteoporotic fractures in older adults [58, 59]. A main factor driving bone loss induced by weight loss is the reduction in mechanical stress on the weight-bearing skeleton mediated by changes in local bone factors (e.g., prostaglandins) and the mechanostat [60, 61], which is thought to be the osteocyte. Interestingly, in a recent trial on older obese adults, it was found that the addition of exercise training to weight-loss therapy prevented weight-loss-induced increase in bone turnover and attenuates weight-loss-induced reduction in hip BMD [62, 63], together with a maintenance of muscle strength [64] and lean mass [62]. The positive effect on BMD was shown despite weight-loss-induced decrease in bone-active hormones such as estradiol and leptin [63] and prevented the weight loss-induced increase in sclerostin levels [57].

However, when mechanical loading effect of total body weight is statistically removed, the association between BMI and bone mass becomes negative [65]. This is consistent with the recent evidence that obese people may have an increased risk of osteoporotic and hip fractures which is independent of BMD [66, 67], suggesting an independent effect of obesity and factors related to this disease on fracture risk. Fat is a source of a number of biologically active molecules that regulate metabolic homeostasis but also interact with bone metabolism [2].

4.1. Adipokines

4.1.1. Leptin. It is produced primarily in the adipocytes of white adipose tissue, but it is also expressed in bone marrow adipocytes and osteoblastic cells. Leptin is involved in appetite and weight regulation and affects osteoblast proliferation and differentiation *in vitro* [68–70] and osteoclasts [68, 71, 72]. Leptin receptors are expressed in hypothalamus where their activation suppresses appetite. This hormone has also a peripheral action by targeting metabolically active cells such as insulin producing β-cells, osteoblasts, chondrocytes, and bone marrow stromal cells. The effect of leptin on bone metabolism is complex and data on rodents have yielded contradictory results. Mice deficient in either leptin (*ob*/*ob*) or the leptin receptor (*db*/*db*) displayed low trabecular bone volume and BMD in femur and tibia [73, 74]. Leptin deficiency was associated with an increase in the number and size of adipocytes in the femoral marrow [73], supporting *in vitro* data showing that this adipokine stimulated osteoblastogenesis while suppressing adipogenesis [69]. Leptin administration may improve bone formation and BMD in leptin-deficient mice but this effect is not evident when leptin levels are normal. Interestingly, leptin prevented bone marrow adiposity in T1D mice although it did not improve bone loss in this model [40]. On the other hand, Karsenty lab showed that leptin-deficient *ob*/*ob* mice exhibit increased vertebral bone mass [75]. Selective deletion of leptin receptor in osteoblast did not affect bone mass [76], while hypothalamic deletion of leptin receptor leads to increased bone mass that was reverted after intracerebroventricular infusion of leptin [75]. Taken together, these studies suggest that leptin has a direct effect on osteoblasts and bone marrow stromal cells but is also part of a very complex mechanism that regulates bone mass through a hypothalamic relay. Centrally, leptin inhibits bone formation, while peripherally it can decrease bone resorption and RANKL activity and increase formation enhancing the commitment of marrow-derived MSC to osteoblasts rather than adipocytes. Clinical studies also have provided conflicting evidences. According to some studies, but not all, leptin appears to be positively correlated with BMD [77]. The higher correlation is showed in postmenopausal women [77]. Women with vertebral fractures have significantly lower plasma leptin levels but not fat mass percentage [78], and increased leptin levels have been suggested to be protective against non-traumatic fractures independent of body weight [79]. Yet, other studies have found no relationship of leptin with either BMD or fractures [80, 81]. Thus, in summary, the role of leptin in clinical bone disease states is complex and needs clarification.

4.1.2. Adiponectin. Exclusively produced by fat tissue, adiponectin circulates in much higher concentrations than other adipokines. In contrast to leptin, adiponectin is negatively correlated with visceral fat mass and BMI in humans, and low levels are described in patients affected by diabetes or myocardial infarction [82–85]. Adiponectin is structurally similar to TNF and RANKL [85]. *In vitro* studies on the effect of adiponectin on bone cells yielded contradictory results. The majority of available data, however, suggest that adiponectin has an anabolic effect on osteoblasts and inhibits osteoclastogenesis, likely independently of RANKL and OPG [86, 87]. These actions would be expected to result in a positive effect of adiponectin on bone mass *in vivo*. In contrast, animal studies have found that adiponectin knock-out mice have increased both bone mass and trabecular number and lower bone fragility [86] and clinical results relative to the effect of adiponectin on BMD have been conflicting in humans. Some studies have reported an inverse association between serum adiponectin and BMD [88, 89], while others failed to detect any relationship in middle-aged men or women [90–93]. Among the most robust studies, that by Richards on 1,735 nondiabetic women found a strong negative correlation with BMD in postmenopausal women but not in the premenopausal ones, demonstrating the importance of menopausal status [94].

Similar results were recently described by Michaëlsson et al. [88] in a large cohort of elderly subjects and by Araneta et al. [95]. An inverse, but not statistically significant relationship was described also by Gonnelli et al., who studied elderly Italian men [93]. Recently, using more robust techniques that take into account bone geometry, such as peripheral quantitative computed tomography (pQCT), it was shown that adiponectin was inversely associated with bone mass in women but not in men [96]. A recent study have shown that, despite a positive association between BMI and BMD, a higher fat mass and lower lean mass were correlated with lower BMD in elderly adults. Inflammatory status (IL-6 and hs-CRP) and levels of adipokines leptin and adiponectin increased with increasing fat mass. In this study, adiponectin was the principal mediator of the apparent negative effect of fat mass on BMD [97]. Thus, in summary, the main body of evidence albeit not definitive appears to favor an inverse relationship.

4.1.3. Resistin. It is a recently discovered adipocyte-secreted factor [98]. Resistin has rarely been found to be produced by fat tissue [99]. It is expressed by bone marrow and produced by peripheral mononuclear cells as an inflammatory cytokine [100]. Resistin is involved in the atherogenic process and serum levels are higher in diabetic and obese patients [98, 101, 102]. Recent studies observed that resistin can influence bone remodeling. This adipokine is expressed by MSC and promotes both osteoblast and osteoclast differentiation [103]. The effect of resistin on BMD is not clear, although a small inverse relationship between serum resistin and lumbar spine BMD in adult men has been reported [92] and higher resistin levels have been related to low total and cortical bone density, measured by CT, in older age [104].

4.2. Inflammatory Cytokines. Adipose tissue is an important "factory" of cytokines like interleukin- (IL-) 1, IL-6, and TNF that have been associated with bone loss. Levels of these cytokines are elevated in obesity and T2D and are directly related to bone fat and insulin resistance. They are also increased in patients with T1D as a result of the autoimmune activation in this disease.

4.2.1. IL6. One-third of the circulating levels of IL-6 is produced by adipocytes and adipose tissue matrix [105]. This is consistent with the evidence that serum IL-6 is increased in overweight and obese individuals [106, 107]. IL-6 may affect glucose homeostasis and energy expenditure either directly or indirectly by acting on adipocytes, hepatocytes, skeletal muscle, and pancreatic β-cells [108]. T2D and obesity have been associated with a genetic polymorphism of IL-6 (−174 G/C) [109], supporting that level of expression of this molecule may influence metabolic homeostasis. High IL-6 levels have been associated with hyperlipidemia, hyperglycemia, and insulin resistance [110]. In contrast, intermittent exposure to IL-6 induces positive effect on blood glucose and energy expenditure [108] and administration in the central nervous system increases energy expenditure and decreases body fat in rats [110]. Similarly, the relationship between bone and IL-6 is ambivalent. As other inflammatory cytokines, IL-6 stimulates osteoclastogenesis [111] but may have also opposite effect by stimulating indirectly osteoblast proliferation or differentiation [112, 113].

4.2.2. TNF. The NF-κB pathway is the effector of TNF through the TNF receptor 1 (TNFR1) [114], expressed by macrophages and osteoclasts precursors. In the bone, TNF stimulates osteoclastogenesis enhancing expression of RANKL in several target cells including osteoblasts [115]. This promotes osteoclasts differentiations indirectly but also blocking osteoclasts apoptosis by acting via the mTOR/S6 kinase [116]. The main result is the increased lifespan of osteoclasts in proinflammatory environment [115]. TNF may also inhibit bone formation. In fact, a number of *in vitro* evidences have shown that high TNF levels can block both differentiation and proliferation of osteoblasts and their progenitors. NF-κB signaling transduction, enhanced by TNF, is a potent inhibitor of osteoblast differentiation and activity. Some of these effects seem to be mediated by reduced Runx2 expression [117, 118] and also by the activation of the MAPK cascade [119]. Expression of osterix, a critical regulator of the early osteoblastic differentiation, is also inhibited [120]. Recently, it has been suggested that TNF can inhibit the Wnt β-catenin pathway by upregulation of Wnt inhibitors Dkk-1 [121] and sclerostin [122]. Notably, both TNF and sclerostin are increased in obesity and diabetes. Consistent with these findings, recent clinical studies have shown that patients on anti-TNF treatments have a significant decrease in bone resorption and osteoclastic activity [123].

4.3. Oxidative Stress. Obesity and diabetes are associated with increased oxidative stress [124, 125]. A low grade inflammation present in obesity and the abnormal activation of resident macrophages in the adipose tissues increase the levels or reactive oxygen species (ROS). A main source of ROS is the increased exposure of target tissue to inflammatory cytokines such as IL-1, TNF, and IL-6 which are increased in obesity. ROS may also directly regulate the activity of transcription factors, such as NF-κB, thus controlling proinflammatory genes expression [126]. Moreover, dysglycemia frequently observed in obesity is associated with increased release of ROS by enhanced NADPH oxidase activity [124]. ROS have important direct effects on the differentiation and survival of osteoclasts, osteoblasts, and osteocytes [127, 128]. ROS disrupt the Wnt signaling pathway by altering the activity of FoxO transcription factors; under the ROS stimulus, these factors can decoy β-catenin preventing its binding to target genes necessary for osteoblast differentiation [129].

ROS have also an important action on the immune system and may indirectly promote osteoclastogenesis by altering the immunoskeletal interface. Superoxide upregulates the costimulatory molecules CD80 and CD86 [130]. CD80 promotes antigen-dependent T-cell activation and hence production of TNF by T cells. Another toxic effect of ROS is the induction of changes within the structure of protein. It has been suggested that this modifications can involve collagenous proteins and therefore potentially alter their properties.

4.4. Other Factors

4.4.1. Sex Hormones. The adipose tissue can contribute significantly to the circulating pool of estrogens. Aromatase expression in adipose tissue primarily accounts for the peripheral formation of estrogen and increases as a function of body weight and advancing age [131]. The positive effect of estrogen on bone is obvious and exemplified by the fact that estrogen deficiency is the main cause of bone loss in postmenopausal women. According to *in vitro* studies, estrogens have a proosteogenic effect while preventing adipogenic differentiation of bone marrow stromal cells [132]. This is consistent with evidence that estrogen deficiency is associated with marrow adiposity in postmenopausal women [132] and estrogen administration can reverse marrow adiposity in the ovariectomised rat model [133–135]. Estrogen deficiency has been associated with a decrease in SIRT1 [134], a longevity factor previously associated with increased expression of the osteogenic factor Runx2 in MSC [136]. Estrogen deficiency leads also to increased oxidative stress into bone tissue which is supposed to negatively affect the balance between osteogenesis and adipogenesis [127]. Interestingly, estrogen replacement therapy in postmenopausal women [137] and elderly men have been associated with reduced levels of the WNT antagonist sclerostin [138, 139]. Oxidative metabolism of estrogen is another important determinant of postmenopausal bone loss [140, 141], bone mineral density in men [142], and body composition. Observational studies support an association between estrogen metabolism and BMI [143], suggesting that obesity is associated with significant decreases in hydroxylation of estrone at C-2. This results in reduced production of less active or inactive estrogenic metabolites, which can possibly sustain bone mass in obesity. Indeed, a recent study showed that, in postmenopausal women, an increase in the metabolism of estrogen towards the inactive metabolites is associated with lower body fat and higher lean mass than those with predominance of the metabolism towards the active metabolites [144].

While both estrogen and testosterone are important in bone health in both sexes, estrogen is the predominant sex hormone in females and testosterone in males. Increased aromatase activity in the excessive fat tissue may also lead to low testosterone levels in obese or T2D males. Approximately one-third of T2D males are testosterone deficient [145]. An even greater proportion of men who are both diabetic and obese experience testosterone deficiency, and the likelihood of testosterone deficiency increases as T2D progresses or worsens. Testosterone has been showen to prevent osteoclastogenesis in a way that is osteoblasts-dependent [146]. *In vitro* data showed that exposure of human adipose-derived stem cells (hADSC) to testosterone or dihydrotestosterone in adipogenesis-inducing medium impaired lipid acquisition and decreased PPARγ, C/EBPα, and C/EBPβ gene expression [147]; another study found that such an effect may involve the WNT signaling pathway through the formation of a complex between the androgen, β-catenin, and the related transcription factor TCF4, thus involving the WNT signaling pathway [148]. Moreover, testosterone can prevent *in vitro* rosiglitazone-induced adipogenesis of MSC [149].

4.4.2. Amylin. It belongs to calcitonin family and it is secreted with insulin. Amylin has central and peripheral effect, inducing satiety and gastric empty and reducing body weight and fat [150, 151]. Obese people have higher blood levels of amylin and reduced sensitivity to its action [150]. In the skeleton, amylin may stimulate osteoblast proliferation [152] and high serum levels have been shown to correlate with high bone mass. Amylin osteogenic actions may present different efficacy depending on the diabetic status. For example, amylin treatment in streptozotocin-induced diabetic rats increased bone volume and osteocalcin (OCN) levels but was not able to ameliorate diabetic osteopenia [153]. More recently, Gutiérrez-Rojas et al. showed that in rats with streptozotocin-induced T2D amylin increased osteoblast number and OCN expression in long bone and normalized trabecular structure; on the contrary, insulin resistant rats treated with amylin did not present any apparent osteogenic effect in the femur, although both OCN and OPG/RANKL ratio were increased in the tibia. These findings demonstrate a different osteogenic efficacy of amylin in two diabetic settings [154].

4.4.3. Ghrelin. Ghrelin is a polypeptide mainly secreted from neuroendocrine cells of the fundus of the stomach and in smaller amounts from renal, pituitary, and hypothalamus cells [155, 156]. Ghrelin is believed to play an important role in energy balance and in food intake. Its serum concentration is inversely associated to BMI and increased in diet-induced weight loss [155, 156]. Recent studies showed that ghrelin may be produced by osteoblasts, and ghrelin receptors were detected in both rat and human bone cells [157]. Although ghrelin has a positive effect on osteoblast proliferation and differentiation [158–161], *in vivo* studies on animals have been contradictory showing either no association with bone mass in mice [158] or a positive effect in rats with increased osteoblast-like cells number, expression of osteoblast differentiation markers, and BMD [157].

The InChianti study has shown that serum ghrelin is positively correlated with trabecular BMD, measured by pQCT, in a cohort of 401 elderly healthy Italian women [162]. Similarly, Gonnelli et al. described a significant, positive effect of ghrelin on femoral neck BMD in elderly men [93]. On the contrary, using dual-energy X-ray absorptiometry (DEXA) measurement, no significant effects of ghrelin on BMD at any bone sites were found in 80 Korean middle aged males [92] or in the Rancho Bernardo cohort after adjusting for BMI and age [163].

4.4.4. Hydroxyl Steroid Dehydrogenase. Glucocorticoids serum level is connected to obesity and bone metabolism. 11-β-hydroxysteroid dehydrogenase (11b-HSD) 1, which converts inactive cortisone into active cortisol, is involved in adipocyte differentiation and obesity [164]. In fact 11b-HSD1 is expressed in adipocyte, while 11b-HSD2, which inactivates glucocorticoids, is not expressed [165]. 11b-HSD2 knock-out mice are protected from obesity with low levels of glucocorticoid [165, 166], and they are resistant to hyperglycemia induced by stress or high fat feeding [167]. In obese humans serum 11b-HSD is elevated [165, 168, 169] and

more active compared to nonobese individuals. 11b-HSD1 is also expressed by osteoblasts and osteoclasts [170, 171]. The expression of osteoblastic 11b-HSD1 determines the synthesis of active glucocorticoids. This has consequent effects on osteoblast proliferation and differentiation and the risk of induced osteoporosis increases with age and depends on autocrine actions of the enzyme 11b-HSD1 [172].

5. Diabetes-Bone Interaction

Obesity is prevalent in patients with diabetes. Although diabetes-induced bone loss is partially dependent on obesity related factors, there are other diabetes-specific elements that can increase further the deleterious effect on bone metabolism. Among these factors, hyperglycemia is the hallmark of diabetes which is ultimately associated with chronic complications. These complications are common both in T1D and T2D and can impact bone health directly or indirectly by increasing the risk of falls. Finally, T1D and T2D differ by the presence of some more specific pathophysiological elements, such as insulin deficiency in T1D compared with insulin-resistance or loss of incretin effect in T2D. All these factors can impact differently bone metabolism.

5.1. Impaired Calcium Balance and Vitamin D Deficiency. High blood glucose may increase urinary calcium excretion and generate several interactions with the parathyroid hormone (PTH)/vitamin D axis [173–175]. Increased calcium loss is associated with hypocalcemia and suppression of PTH secretion. Conversely, improvement of blood glucose control may reduce calcium and phosphate urinary excretion and $1,25(OH)_2$ vitamin D_3 ($1,25(OH)_2D_3$) levels and increase phosphate levels but without improving serum calcium or PTH [176]. Numerous studies have shown that both patients with T1D and T2D have impaired vitamin D status. A cross-sectional study on 5,677 patients with T2D and impaired glucose tolerance had significantly lower 25OH vitamin D (25OHD) levels compared with controls [177]. Obesity itself is also associated with altered vitamin D metabolism and impaired vitamin D status, which is likely due to the decreased bioavailability of vitamin D because of its deposition in body fat compartments [178]. Indeed, although higher levels of 25OHD have been associated with 13% reduced risk of diabetes, this effect was attenuated after adjustment for BMI [179]. Vitamin D supplementation aimed at raising 25OHD levels above 30 ng/mL had no effect on insulin secretion, insulin sensitivity, or the development of diabetes compared with placebo [180]. Similarly it is documented that new-onset T1D patients have reduced levels of $1,25(OH)_2D$ and 25OHD compared with healthy controls [181]. Here, a causative role of vitamin D deficiency in diabetes has been suggested although not proven by intervention trials thus far. Moreover, vitamin D supplementation in young patients with new-onset T1D did not improve markers of bone turnover [182]. However, no trials of vitamin D supplementation on BMD or fractures are available in patients with diabetes. A major complication of diabetes is renal impairment with osteodystrophy. Here disturbances of calcium, phosphate, FGF-23, and Vitamin D physiology may have a major impact on the bone health of diabetic patients. This topic is beyond the scope of this paper.

5.2. Hyperglycemia and Oxidative Stress. High blood glucose induces formation of advanced glycation end-products (AGE), with negative effects on structural proteins such as type I collagen, the main bone matrix protein. This nonenzymatic glycosylation is a multistep process. Glucose leads to the formation of a Schiff's base which is further degraded to a class of intermediate products containing highly reacting dicarbonyls. Reaction of these carbonyls with the NH_2 side chain containing amino acids (arginine, lysine, and hydroxylysine) leads to formation of irreversible AGE compounds such as pentosidine and N^{ε}-carboxymethyllysine (CML) [183]. AGEs may have damaging effects on collagens by forming irreversible cross-links between the fibers in the triple helix [184]. AGE involving collagen and other structural or circulating proteins are a source of ROS that can further induce structural changes by means of posttranslational modifications [185]. Since collagen is a structural protein with relatively slow turnover, the irreversible changes induced by glucose and ROS are retained within the tissues. These changes are linked to reduced strength and impaired biomechanical properties of both cancellous and cortical bone [186]. This is consistent with the clinical evidences that increased levels of pentosidine in patients with diabetes are associated with a more frequent history of spine fractures, independently of BMD measured by DEXA. It has been shown that high urinary pentosidine levels were associated with a 42% increase of clinical fractures incidence in patients with diabetes compared with controls [187]. Similarly, serum levels of pentosidine were higher in diabetics patients who experienced vertebral fractures [188].

AGE may also reduce bone strength by impairing bone formation. It has been shown that AGE disturb osteoblast function [189] and attachment to collagen matrix [190] and interfere with their normal development [191]. Circulating AGE can also bind specific receptors called RAGE located on osteoblasts and immune cells [192, 193]. These receptors enhance production of inflammatory cytokines and ROS, feeding a vicious circle of chronic inflammation [192] and increased bone resorption [193]. Hyperglycemia can be "toxic" to osteoblasts themselves. Botolin et al. have shown that acute (24 h) hyperglycemia and its associated hyperosmolality suppress expression of genes involved in osteoblast maturation [194] including OCN [195, 196]. Similarly, chronic hyperglycemia downregulates OCN expression [197] and calcium uptake in osteoblast cultures [198], while increasing PPAR-γ2 expression [199]. In mice, blood glucose levels are positively related with bone marrow induced osteoblast death and negatively related with OCN expression in bone [200].

Hyperglycemia and oxidative stress may also affect MSC differentiation. There is evidence that adipogenesis may prevail over bone formation when adipose tissue-and muscle-derived stem cells are exposed to high glucose concentration. Culturing MSC with high glucose media induced expression of adipogenesis markers such as PPAR-γ2, GLUT4, and

adiponectin but reduced osteogenic (OCN, osteopontin, osteonectin) or chondrogenic (type II collagen) markers. The adipogenic shift was mediated by ROS through enhanced signaling by PKC-β [201]. Diabetes is also linked to a generalized damage of blood vessels wall, which results in chronic micro- and macrovascular complications. Oxygen tension within the marrow microenvironment is physiologically lower than other tissues and the diabetes status may further alter cellular homeostasis. Indeed, some authors have shown reduced differentiation of MSC toward either adipose or osteoblast phenotype [202, 203], while others suggested an increased differentiation of MSC under hypoxia conditions [204, 205]. Finally, hyperglycemia-induced acidosis may enhance bone resorption and impair bone quality [128].

5.3. Insulin Deficiency. Clinical, *in vivo,* and *in vitro* studies suggest that insulin exerts a bone anabolic effect on osteoblasts [206]. In humans, insulin deficiency was associated with reduced bone mass in a study of 62 new-onset T1D subjects evaluated before insulin therapy. Treatment with insulin after 7 years improved substantially bone mass and markers of bone turnover [207]. On the other hand, hyperinsulinemia present in patients with T2D may contribute partially to the higher BMD [208].

Anabolic action of insulin in osteoblasts seems to be mediated by increased Runx2 activity through suppression of its inhibitor Twist2 [209]. Runx2 is a major factor involved in osteoblast differentiation and proliferation, and its expression is actually impaired in models of T1D [210]. In mice lacking the insulin receptor in their osteoblasts bone formation is impaired and associated with a reduced number of osteoblasts and reduced trabecular bone volume [209]. These mice have also a decreased osteoclast activity as showed by reduced osteoclast erosion depth and serum levels of cross-linked C-telopeptide (CTX) [209]. The insulin receptor signals through four insulin receptor substrates molecules (IRS-1 to IRS-4). Mice lacking these substrates showed abnormal bone phenotype [211, 212]. IRS-1 deficient mice showed impaired bone healing which was restored after its reexpression in the fracture site [213], and IRS-2 knockout mice had reduced bone formation over resorption [211]. Part of the insulin signaling through the IRS may be mediated by the IGF-I [214]. Levels and/or action of IGF-I and PTH, another bone anabolic hormone, are also impaired in insulin deficiency conditions [153, 214, 215].

Moreover, studies on T1D animal models confirm that insulin deficiency adversely affects skeletal homeostasis. Streptozotocin-induced diabetic mice and nonobese diabetic (NOD) mice have low-turnover osteopenia [216] associated with a disruption in osteoblast [153, 217] and its multipotential mesenchymal precursor [194, 199]. Analysis of gene expression in mesenchymal cells from NOD mice bones demonstrated a switch from genes associated with a mature osteoblast phenotype to genes associated with an adipocyte phenotype. PPAR-γ2 and aP2, known markers of adipocyte differentiation and maturation, were raised in diabetic NOD mice, together with increased number of adipocytes. In contrast, expression of OCN was significantly decreased in diabetic NOD mice. Suppression of osteoblast maturation, demonstrated by lowered OCN mRNA levels, was correlated with decreased bone density in both NOD and streptozotocin-treated mice [194, 199, 216].

5.4. Loss of Incretin Effect (Gut-Hormones Interaction). The incretin effect is the increase of glucose stimulated insulin secretion resulting from the release of intestinally derived peptides in response to glucose or nutrients in the gut. The incretin effect depends primarily on two peptides, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1). GIP and GLP-1 have short half-life and are rapidly degraded by dipeptidyl peptidase-4 (DPP-4). This pathway is attenuated in T2D [218] and the therapeutic target of drugs commonly used in T2D such as GLP-1 receptor analogues, which are resistant to DPP-4 degradation, and inhibitors of DPP-4, which extend the half-life or the native incretins [219].

GLP-1 receptors are expressed on bone marrow stromal cells and immature osteoblasts [220, 221], and GLP-1 stimulates proliferation of MSC and inhibits differentiation to adipocytes [221]. GLP-1 receptor knockout mice have decreased cortical bone mass due to increased osteoclast number and activity [222], impaired mechanical and material properties [223], and decreased calcitonin secretion from thyroid C cells [222]. In fact, GLP-1 receptors are also expressed on thyroid C cells and therefore increase the secretion of calcitonin [224], which could contribute to the postprandial decrease in bone resorption.

In animal models, administration of GLP-1 (3 days) increases bone formation in normal rats and rats with streptozotocin-induced diabetes or fructose-induced insulin resistance, suggesting an insulin-independent action [225]. The diabetic and insulin resistant rats also demonstrated improvements in trabecular bone mass and microarchitecture. Also, 3 days of continuous administration of the GLP-1 analogue exenatide increased markers of bone formation and may improve microarchitecture in normal rats and rats with streptozotocin-induced diabetes or fructose-induced insulin resistance [225]. The positive effects of GLP-1 treatment on trabecular bone mass and microarchitecture may be likely mediated by a positive effect on bone formation and on OPG/RANKL ratio [226].

Effects on bone formation may be mediated by interference with WNT signalling, considering that incretin treatment in type 2 diabetic rats has been reported to lower serum levels of sclerostin and increasing serum levels of OCN [227]. Wnt signaling and its elements are important for both the production and function of the incretin hormones [228]. Grant et al. showed that the TCF7L2 variant 7903146, the main T2D associated gene [229], modifies the effect of incretins on insulin secretion [230]. On the other hand, Wnt signalling cascade increased expression of proglucagon gene *gcg* and *gip* mRNA [231]. García-Martínez and colleagues showed that Wnt/β-catenin signaling or lithium, which mimics the Wnt signaling, enhanced GIP production by enteroendocrine cells through a conserved TCF binding site within the proximal region of the *gip* promoter [231].

5.5. Sclerostin and WNT Signaling Pathway. Recent studies have suggested that part of the negative effect of diabetes on bone quality may be mediated by disturbances in the WNT signaling pathways. The glycoprotein sclerostin acts as an antagonist for the WNT/β-catenin canonical signaling pathway; this molecule is produced by osteocytes and exerts its inhibitory effect by binding to LRP-5 and/or 6 on osteoblasts. In mice with streptozotocin-induced T1D, WNT signaling is suppressed but also sclerostin is downregulated [232]. In these mice an increased osteocyte apoptosis and lower total and nuclear β-catenin staining was shown [232]. In contrast, T2D rats presented enhanced expression of Dkk-1 and SOST/sclerostin gene; SOST overexpression related to increased mRNA levels of the WNT activator LRP-5 [225]. Clinical studies have also showed that levels of sclerostin are higher in patients with T2D compared with control subjects [233], inversely related with bone turnover markers [233, 234], and positively associated with spine and hip BMD [233]. In both diabetic and healthy subjects, sclerostin levels were higher in males than females. In another study, sclerostin was higher in T2D than either healthy controls or T1D [235]. These findings suggested that elevated sclerostin levels may influence bone fragility and bone quality associated with T2D. Indeed, there is increasing evidence that sclerostin levels may predict the risk of hip and other osteoporotic fractures both in postmenopausal women [236, 237] and T2D [238, 239] as well as that sclerostin antibody targeting can ameliorate diabetic bone loss in rodents [240]. However, it should be noted that the assay for sclerostin is relatively new and has not been universally standardized [241] and this may have affected the results from different studies.

5.6. RANKL System and Insulin Resistance. RANKL is a member of the TNF superfamily and, after binding to its cognate receptor RANK, acts as a potent stimulator of NF-κB. There are evidences supporting a role of this system in diabetes and associated diseases [242]. Both liver tissues [243] and β-cells express RANKL and RANK [50]. Moreover, levels of OPG, which competes for RANK/RANKL interaction, are elevated in T2D, especially in those with poor glycemic control, and relates with fat mass and atherosclerosis parameters [47–49, 244]. Recently, Kiechl et al. showed that increased levels of soluble RANKL are associated with the development of diabetes in 844 subjects from the Brunek study (OR = 3.37; 95%CI: 1.63–6.97). Besides the epidemiologic finding, the authors showed that RANKL interacted with glucose homeostasis by acting on hepatocytes function and insulin resistance. Indeed, mice selectively lacking RANK in the hepatocyte (*Rank*LKO) were protected by high-fat diet induced insulin resistance and showed fasting glucose and insulin concentrations similar to those of *Rank*WT mice fed a normal-fat diet [50]. These data suggest the RANKL involvement in the pathogenesis of hepatic insulin resistance and T2D and provide a link between inflammation and disrupted glucose homeostasis.

5.7. Osteocalcin and Glucose Homeostasis. Not only does abnormal glucose homeostasis have deleterious effects on bone metabolism, but also the rate of bone turnover may in turn regulate glucose homeostasis. Recent research has showed that the osteoblast-derived protein OCN has endocrine effects, acting on islet cells stimulating β-cells proliferation and insulin secretion. Karsenty group has indicated that OCN-knockout mice display decreased β-cells proliferation, glucose intolerance, and insulin resistance. In *ex vivo* studies, when pancreatic β-cells isolated from wild-type mice were cocultured with wild-type osteoblasts or in the presence of supernatants from cultured osteoblasts, insulin secretion increased, suggesting the presence of an osteoblast-derived circulating factor that regulates β-cell function. Administration of OCN significantly decreased glycaemia and increased insulin secretion. Furthermore, OCN function was exerted through adiponectin-coculture of wild-type osteoblasts with adipocytes increased adiponectin expression and action [245, 246]. It is well known that insulin is involved in OCN production. More recently, insulin was also shown to be a key factor in regulating its bioactivity; OCN expression is mediated by suppression of *Esp* signalling by insulin within the osteoblast [247]. According to animal studies, OCN is a prehormone which is active only in its undercarboxylated form (ucOCN) [245]. However, clinical studies did not show consistent difference between the two forms of OCN, and vitamin K administration, which is believed to increase the rate of OCN carboxylation, has been unexpectedly associated with improved insulin sensitivity [248–250]. OCN decarboxylation is a pH dependent mechanism permitted in presence of increased osteoclastic reabsorption under the insulin stimulus [247]. Recently, it has been shown that ucOCN can signal trough the specific receptor Gprc6a expressed on beta cells [251]. Animal studies have suggested that testis may be also intercalated into this fine loop whit OCN inducing testosterone production by the Leydig cells [252]. Clinical studies did not confirm that this finding where subjects administered a bisphosphonate for osteoporosis had very low OCN values but reports of diabetes induction or worsening have been reported from the trials.

A number of human studies have also explored the relationship between OCN and glucose homeostasis. OCN levels have been reported to be lower in T2D compared to healthy subjects [253], inversely related to body mass index, fat mass, and plasma glucose [254–257] but also to atherosclerosis and inflammatory parameters such as high sensitive C-reactive protein and IL-6 [258]. However, most of these studies were conducted in healthy subjects or T2D only and limited by the cross-sectional design. On the other hand, studies evaluating treatments or conditions which are able to change OCN levels have shown opposite results questioning the OCN-glucose relationship in humans. For example, alendronate therapy, which decrease OCN levels, was associated with reduced diabetes risk [259]; treatment with vitamin K, which reduces ucOCN/OCN rate, improved insulin resistance [248–250]; chronic hyperparathyroidism, which is characterized by increased OCN release, was associated with increased insulin resistance and impaired glucose regulation [260].

Less evidence is available for T1D. A recent study showed no association between OCN and β-cell function in subjects

with new-onset T1D [182]. Although human studies in patients with T2D and in animals support a positive feedback between osteoblasts and β-cells, authors speculated that, in a condition of continuous autoimmune damage against β-cell such as in T1D, the OCN may be ineffective on controlling β-cell function. On the other hand, Thraikill et al. reported a positive effect of OCN on endogenous insulin production (assessed by authors as C-peptide/glucose ratio) [261] in subjects with long standing disease.

Thus at this stage it appears that the relationship of OCN and glucose homeostasis appears to be most robust from *in vitro* and animal studies but its role in humans is less clear and requires further investigation.

6. Diabetes and Fractures in Clinical Practice

The occurrence of fractures in diabetes mellitus is increased but the evidence for fracture occurrence has been reported from cross-sectional observational cohorts which have inherent weaknesses. Most of the risks have largely been derived from surrogate markers, for example, bone turnover markers, bone histology, DEXA, and other imaging modalities, showing predominantly a bone quality defect. Randomized prospective trials designed to assess fracture risk specifically and/or treatment are not available. The section below will describe epidemiology of and clinical factors associated with fractures in both T1D and T2D.

6.1. Bone Turnover Markers. A recent study has demonstrated that in patients affected by T1D, OCN levels are 4 or 5 times lower than in control subjects, while in T2D patients OCN is 2 or 3 times lower than in controls [262]. Other studies have been performed to individuate a possible link between glucose balance and bone markers. In particular, it has been demonstrated that, both in T1D and in T2D, higher levels of HbA1c are associated with lower levels of OCN [262, 263]. Scientific evidences have demonstrated that Type 1 diabetic children present a severe reduction of OCN levels mainly during sexual maturation. This represents a very significant cause of the missed goal of age-related peak bone mass, which takes place generally between 18 and 30 years. Bone markers belonging to protein products of collagen breakdown, including CTX (bone resorption) or type 1 procollagen N-terminal peptides P1NP (bone formation), and the complex pathway directed by OPG and RANKL, seem to be associated negatively with glucose balance. Studies have shown that higher levels of HbA1c correspond to lower levels of bone apposition markers including lower levels of OCN and OPG [262].

A recent study by Rubin group [216] has identified an alteration in circulating osteogenic precursor cell (COP) in T2D. These circulating cells arrive at bone formation site through blood vessels and form osteoblast-like cells [206, 209]. COP is characterized using antibodies against OCN. Subjects with T2D have a decrease in circulating OCN-positive cells, while the same subjects show an increase of immature OCN-positive cells with early markers CD146 (a marker of bone cells progenitors) and CD34 (which identify cells that can increase osteoblast function), which consequently mean small pool of immature COP cells. Moreover T2D subjects with HbA1c higher than 7.9% had higher levels of these immature cells [216]. Although questions exist about the role of circulating OCN cells in bone formation, there is evidence that osteoblast progenitor cell maturation in T2D is inhibited. OCN+/CD146+ cells presented lower expression of the Runx2 master marker of osteoblast differentiation and increased markers of oxidative stress. In the same study, bone formation markers such as P1NP and OCN were significantly lower in T2D. Similarly, bone resorption marker serum CTX was lower in T2D, although the difference in tartrate-resistant acid phosphatase (TRAP)-5b levels was not significant, suggesting low bone turnover. Moreover, the differences in turnover markers correlated with blood glucose control as showed by an inverse relationship between HbA1c and bone markers P1NP, OCN, and CTX [216].

6.2. Histology

6.2.1. Type 1 Diabetes. Microstructural defects or alterations in bone turnover may play a central role in diabetic osteopathy. Verhaeghe et al. [264] performed two studies on diabetic mice after 3 or 4 weeks after the onset of the disease. Serum OCN levels, osteoblast/osteoclast and osteoid surface percentages, and the daily mineral apposition rate were reduced in diabetic mice (mineral apposition rate in the tibia 1.0 ± 0.4 versus 5.6 ± 0.6 μ/day and vertebra 0.2 ± 0.1 versus 2.3 ± 0.2 μ/day), proving that osteoblast function is compromised with a consequent low bone turnover [264]. In the second study diabetic mice presented 25% less stiffness and strength in the femur than nondiabetic mice and a lower resistance to physical exercise. Finally, a 50% increased collagen cross-linking to the AGE Pentosidine was observed [265].

Armas et al. [210] compared bone histomorphometric and μ-CT results from iliac biopsies from 18 subjects with T1D on insulin treatment without complications and a good metabolic control. They found no differences in terms of histomorphometric or μCT parameters between diabetics and controls. However, fractured patients had a trend for abnormalities in structural and dynamic variables, such as lower BT/TV%, suggesting defects in their skeletal microarchitecture [210].

In conclusion, data obtained by the described studies seem to suggest that in T1D low bone formation delays bone apposition during growth and increases bone resorption, with a worsening effect related to the duration of the disease and to the glycemic control.

6.2.2. Type 2 Diabetes. Increased fracture risk in T2D could be related, among the others, to alterations in skeletal structure. Dobnig et al. [211] have investigated the effect of T2D on bone turnover in 583 T2D patients and 1,081 control subjects, while hip and other nonvertebral fractures were monitored over 2 years. Diabetic patients had significantly higher age-, weight-, and mobility score-adjusted calcaneal stiffness ($P < 0.0001$), radial speed of sound ($P < 0.005$), and phalangeal speed of sound ($P < 0.05$) revelations

in comparison with controls. Serum PTH (−20.7%) and OCN levels (−22.3%) were significantly lower (both $P < 0.0001$) in T2D patients despite similar low serum 25OHD levels. However, a total of 110 hip fractures occurred during the observation period, with a similar hip fracture rate in controls and T2D (3.1 and 3.4 % per 100 patient years, resp.). Shu et al. [266] recruited 25 T2D women and 25 female control subjects and performed high-resolution peripheral quantitative computed tomography (HR-pQCT) and bone turnover markers. The results of the study showed that HR-pQCT did not differ among the two groups but both P1NP and OCN resulted lower in diabetic women than in controls ($P \leq 0.005$ and <0.001, resp.), suggesting that T2D could present a lower bone turnover regulation [266]. Okazaki et al. [176] performed a study on 78 T2D patients with a poor glycaemic control (HbA1c > 8%) and measured the serum bone markers at the baseline and after 3 weeks of glucose lowering treatment. Bone resorption markers were decreased at the beginning of the study while they were increased after the 3-week treatment, proving that a good glycaemic control influences bone turnover [176]. As stated above, COP cells have been recently identified and studied in osteoporotic patients. Rubin's group has correlated COP with bone histomorphometric structure and bone markers in T2D patients [216]. The results of the study showed reduced COP cells in T2D patients in comparison with control subjects. The bone formation markers P1NP and OCN were significantly lower in T2D (P1NP $P < 0.01$, OCN $P < 0.03$), as the bone resorption serum CTX ($P < 0.01$). Reduced histomorphometric indices of bone formation were observed in T2D subjects, including mineralizing surface (2.65 ± 1.9 versus $7.58 \pm 2.4\%$, $P < 0.02$), bone formation rate (0.01 ± 0.1 versus 0.05 ± 0.2 microm^3/um$^2 \times$ d, $P < 0.02$), and osteoblast surface (1.23 ± 0.9 versus $4.60 \pm 2.5\%$, $P < 0.03$) [216]. Although questions exist about the role of circulating OCN cells in bone formation, evidence that osteoblast progenitor cell maturation is inhibited in T2D could lead to interventions targeting improved bone formation to enhance bone strength in the diabetic skeleton.

Burghardt et al. [267], using HR pQCT, showed that T2D is associated with impaired microarchitecture and biomechanics at the peripheral skeleton of elderly women. Diabetic women had 10% higher trabecular volumetric BMD adjacent to the cortex and 13.8% higher trabecular thickness in the tibia. Cortical porosity was increased in the radius while pore volume tended to be higher in the tibia suggesting impaired resistance to bending loads and inefficient redistribution of bone mass in diabetes.

6.3. Bone Density and Strength

6.3.1. Type 1 Diabetes and BMD. It is not yet completely clear how BMD, osteoporosis, and the risk of fractures are related in T1D and T2D. Many, but not all, studies performed on T1D patients reported low BMD values at DEXA measurements [174] and focused their attention on the role of diabetic microvascular complications in the pathogenesis of osteoporosis. Insulin-dependent diabetic patients, in fact, show an approximately 10% decreased bone mineral content (BMC) a few years after clinical onset of diabetes [268]. However it seems that, in absence of diabetic microvascular complications, a further bone loss does not occur. Mathiassen et al. [268] studied 19 insulin-dependent diabetic patients and determined BMC with an interval of 11 years. At initial examination, no patient had diabetic microangiopathy, but at final examination 7 patients had developed diabetic microvascular complications. In comparison with gender- and age-matched controls, both subgroups showed significantly lower BMC at the initial examination, but, at final examination, BMC was significantly decreased in patients with microvascular complications than in patients without. Blood tests for bone metabolism showed a significantly increased fasting urinary excretion of calcium and hydroxyproline in patients with complications, but not in the group without complications, and there was a negative correlation between plasma OCN and HbA1C for all patients [268]. Forst et al. [269] found a 10% reduction of bone mineral density in the femoral neck ($P < 0.01$) and a 12% reduction in the distal radius ($P < 0.001$) compared with the control group. No significant difference was found in the lumbar spine. A link between decreased bone mineral density and diabetic neuropathy was observed for the femoral neck ($P < 0.001$), but not for the distal radius or axial skeleton, demonstrating that T1D microvascular complications may influence bone health [269]. Munoz-Torres et al. [270] performed a study on 94 patients affected by T1D and with disease duration ranging from 1 to 35 years. Diabetic patients showed reduced BMD in all sites and 19.1% met diagnostic criteria for osteoporosis. Diabetic complications were associated with lower BMD concluding that osteopenia and osteoporosis are a common complication of T1D and microvascular complications are a critical point in the progression of diabetic osteopenia [270].

6.3.2. Type 2 Diabetes and BMD. Barrett et al. [271] found that diabetic men had similar BMD compared to those with normal glucose tolerance, whereas diabetic women had higher BMD at all sites. The increased bone density in diabetic women was unexplained by age, obesity, cigarette smoking, alcohol intake, regular physical activity, and the use of diuretics and estrogen. Older women with T2D or hyperglycemia had better BMD than women with normal glucose tolerance, independent of differences in obesity and many other risk factors. No differences in bone density by diabetic status were observed in men. In conclusion, it is possible that the sex differences may be explained by the greater androgenicity reported in women with hyperglycemic and hyperinsulinemic conditions [271]. Similar findings were found by Stolk et al. [208] with higher bone mass associated with higher glucose and postload insulin levels at all bone sites. In men, the mean age-adjusted BMD at the lumbar spine increased by 4.64 per mmol/L serum glucose (95%CI 1.46–7.82) and 0.35 per mU/L postload insulin (0.17–0.53). In women, these values were 6.88 (4.37–9.39) for glucose and 0.25 (0.11–0.39) for insulin (for all analyses: $P < 0.01$) [208]. Following the same hypothesis, Barrett et al. found that hyperinsulinemia could play an osteogenic role showing that

each 10 microU/mL increase in fasting insulin was associated with a 0.57 g/cm^2 increase in lumbar spine [271].

6.3.3. Bone Strength. Bone strength may be reduced even in absence of changes in areal BMD because of geometric changes. In a case-control study, Petit et al. [272] examined the association between T2D and bone volumetric density, geometry, and estimates of bone strength at both tibia and radius. T2D patients had higher volumetric BMD (vBMD) and a smaller bone area, but no differences in estimated compressive bone strength at the distal trabecular bone regions. On the other hand, total bone area was smaller at the cortical bone midshaft sites resulting in lower bone bending strength despite a similar vBMD at these sites, suggesting that bone strength may be impaired in absence of vBMD changes.

Recently, Farr et al. [273] performed *in vivo* microindentation testing of the tibia to directly measure bone mineral strength in 60 postmenopausal women including 30 patients diagnosed with T2D for >10 yrs. Bone mineral strength was significantly lower in patients with T2D than controls and porosity tended to be increased in these patients despite no significant changes in other bone microarchitecture parameters. Glucose control was inversely related with strength and bone turnover markers were reduced [273]. Using HR pQCT, Patsch et al. [274] showed that fracturing diabetic patients had higher intracortical pore volume, relative porosity, and endocortical bone surface than diabetics without fractures. Relative porosity at the distal radius was 4.7-fold higher in fracturing diabetics compared with nonfracturing patients. Similarly, ultradistal tibia had more porosity and trabecular heterogeneity was higher in fractured diabetic patients [274]. On the other hand, nondiabetic fractured subjects and healthy controls only differed in a slight increase in pore volume. Similarly, using MRI at the distal radius, Pritchard et al. [275] found that women with T2D had larger holes within the trabecular bone network than women without T2D.

6.4. Predicting the Risk of Fractures. Even the FRAX (fractures risk assessment tool), an algorithm adopted by the WHO to assess the risk of fractures, does not seem useful in T2D patients [186]. In fact, Schwartz et al. have indicated that fracture risk was higher for a given T-score and age or for a given FRAX score [276]. Like BMD, FRAX score is only partially effective to predict hip and nonspine fracture risk in T2D patients.

A novel bone-state parameter is the trabecular bone score (TBS). It is a texture parameter that evaluates pixel gray-level variations in the spine DEXA image and is related to bone microarchitecture and fracture risk, independent of BMD. A positive correlation between lumbar spine TBS and skeletal deterioration in postmenopausal women with diabetes has been demonstrated, while in the same cases BMD is greater [277]. Data suggest that a TBS and BMD correlation could improve fractures prediction [278–281].

Therefore assessment of fracture risk in diabetics cannot be based only on traditional risk factors and commonly used algorithms and new predicting factors are needed.

6.5. Fractures in Type 1 Diabetes. Fractures risk is significantly higher in T1D when compared to the general population as well as to patients with T2D [282]. Most of the studies have focused on hip fractures finding a higher relative risk (RR) ranging between RR 1.7 and 12.3 [283]. Fractures at the spine and proximal humerus also were moderately increased [284, 285].

No gender differences were found although the small number of studies assessing this relationship does not allow a definite conclusion. A meta-analysis of 5 cohort studies showed that T1D was associated with an overall RR of 8.9 (95%CI: 7.1–11.2) [283]. In the large prospective Nurses' Health Study the incidence of hip fractures was reported as 383 per 100,000, a result 6-fold higher than the overall incidence of hip fracture in this population and 2.5-fold higher than in T2D [282].

Ivers et al. reported a higher risk of fractures in patients presenting retinopathy [286], while Strotmeyer et al. have shown that falls, lower performance state, neuropathy, and stroke were more frequent in fractured patients than in those without fractures [287]. Miao et al. reported a strong correlation between fracture risk and all types of complications, in particular with a lower BMD in patients with neuropathy and nephropathy than in patients without these complications [288].

6.6. Fractures in Type 2 Diabetes

6.6.1. Hip Fractures. Hip fractures contribute the most to the fracture risk seen in T2D [289–291]. This risk appears to be slightly higher in men compared with women [283, 292] and in black compared to white women [293]. The Nurses' Health Study showed that the incidence of hip fractures in women with T2D was 153 per 100,000 subjects compared with 63 per 100,000 [282]. The incidence was even higher in those women treated with insulin (209 per 100,000) [282]. Two large meta-analyses that assessed studies involving 1.3 million subjects confirmed that patients with T2D are at increased risk of hip fractures, with a RR of 1.7 (95%CI: 1.3–2.2) [283] and 1.38 (95%CI: 1.25–1.53) [294], respectively. The association increased further when the analysis was restricted to 4 cohorts with more than 10 years of follow-up, RR 2.7 (1.7–4.4) [283].

6.6.2. Vertebral Fractures. There is very little data available regarding vertebral fracture risk in T2D. Three studies have independently showed that risk for vertebral fractures is similar to nondiabetics [284, 287, 293]. However, a recent Japanese study found that diabetes was associated with increased risk in women (OR = 1.9; 95%CI: 1.11–3.12) and men (OR = 4.7; 95%CI: 2.19–10.20) [295]. Contrary to what shown in controls, age and BMD did not predict fractures in T2D patients.

6.6.3. Extremity Fractures. Fractures of wrist [296] and foot [289, 293] also seem to be more frequent in T2D. These data were confirmed in a recent meta-analysis and appeared to be

true only in those patients treated with oral hypoglycemic agents or insulin [283].

6.6.4. Atypical Femur Fractures. Atypical low-energy subtrochanteric and diaphyseal fractures have been reported as a possible adverse event associated with bisphosphonate therapy. A recent analysis of the Study of Osteoporotic Fractures (SOF) has shown that history of diabetes was the strongest independent predictor of this type of femur fracture (HR = 3.25; 95%CI: 1.55, 6.82) [297].

6.7. Relationship with Disease Duration and Complications. In some studies, T2D was not associated with fractures [211, 298, 299]; in other studies T2D even tended to be protective [300, 301] although the latter result was not significant either on initial report or when reanalyzed in meta-analyses. Interestingly, those studies which resulted only in minimal increase in fracture risk involved mainly diet controlled [291, 302] or early onset T2D. Liefde et al. that showed that people with impaired glucose tolerance tended to have lower fracture risk (HR 0.8; 0.63–1.00), which increased in treated type 2 diabetics (HR 1.69; 1.16–2.46) despite an equally increased BMD [296]. Consistent with these data, the association between diabetes and hip fractures in the meta-analysis conducted by Janghorbani et al. became stronger when the cohorts with more than 10 years of follow-up were evaluated separately [283]. It is possible that early on in the natural history of the disease higher BMD may protect from fractures. On the other hand, when diabetes progresses, factors such as hyperglycemia, chronic complications, and the need for multidrug treatments may impair bone quality and/or increase the risk of falls and subsequently fractures. For example, retinopathy reduces vision, polyneuropathy alters gait, and cardiovascular complications lead to heart failure and cardiac arrhythmias, all factors promoting falls [303–305]. Diabetic nephropathy increased hip fracture risk 12-fold in patients with T1D [306] and the fracture risk in the women's health study was related to the presence of diabetic complications such as neuropathy and use of TZD (in postmenopausal women) and insulin in patients with T2D [307].

6.8. Relationship with Blood Glucose Control. Although pathophysiological evidence suggests that treating hyperglycemia may revert mechanisms associated with diabetic bone loss, intensive blood glucose control may increase the rate of hypoglycemic episodes and therefore falls. However, the relationship between glucose control and fractures is not clear with observational studies reporting mixed results. Most of the studies did not show any association between HbA1c or fasting glucose and fracture risk [286, 287, 290, 307]. However, in a recent Japanese study on men with T2D, vertebral fractures identified with spine films were associated with HbA1c >9% among those who were obese or overweight [308]. In a clinical trial on 50 patients with T2D with high HbA1c (mean 11.6%) followed up to 1 year, improved blood glucose control was followed by an increase in bone density at the neck, and the bone formation marker OCN was reduced after the treatment [309]. Recently, additional data were provided by the ACCORD BONE ancillary study [310]. The ACCORD trial compared tight blood glucose control targeting normal HbA1c levels (i.e., <6%) with standard strategy in a population with long-standing T2D and history of cardiovascular disease or high cardiovascular risk [311]. The ancillary BONE study found that intensive glycemia did not increase or decrease fracture or fall risk in comparison with the standard strategy [310]. Despite an increased rate of hypoglycemic events no increased fractures or fall were showed in this study [310]. Moreover, the author suggested that achieving lower HbA1c for several years might not be sufficient to reduce these risks in diabetes patients.

6.9. Antidiabetic Drugs and Risk of Fractures. Antidiabetic drugs may influence bone turnover but the effective role of these medications is not always clear. The main evidence available comes from post hoc analysis of blood-glucose lowering trials, where the effective role of these medications is often difficult to discern. The increased risk of fall associated with hypoglycaemic events and diabetic complications may indirectly confound the bone-specific effect associated with hypoglycaemic drugs. For example, preclinical and some clinical evidence in T1D suggest that insulin is protective against bone loss while most of the trials indicated an increased risk of fractures in insulin-treated T2D patients.

6.9.1. Insulin. Most available studies report a higher incidence of bone fractures in insulin-treated patients, in comparison with non-insulin-treated T2D individuals. Monami et al. [312] published a case-controlled study in which the difference between control subjects and patients receiving long-term insulin treatment was analyzed. Within a cohort of 1,945 outpatients with diabetes with a follow-up of 4.1 ± 2.3 years, this study compared 83 cases of bone fractures and 249 controls matched for age, sex, duration of diabetes, BMI, levels of HbA1c, comorbidity, smoking, and alcohol abuse. Insulin-treated patients usually show a longer duration of diabetes and a higher prevalence of diabetes complications and it is possible that some studies, which did not provide adjustments for such confounders, could have overestimated the negative impact of insulin treatment. At the same time, treatment with insulin at the index date showed a significant association with bone fractures, maintained after adjusting for concomitant hypoglycemic medications. In fact the results showed that bone fractures in men were more frequent in insulin-treated patients (OR 3.20, 95%CI: 1.32–7.74), even if it was not confirmed in women (OR 1.41, 95%CI: 0.73–2.73). A study among older adults with diabetes showed that HbA1C <6% was related with greater risk of falls but only in those treated with insulin therapy [313]. These results are consistent with the hypothesis that insulin could increase the risk of fractures through falls caused by hypoglycemic episodes, without negative effects on bone metabolism. A recent analysis of the MrOS study evaluated the risk of nonvertebral fractures in 5,994 elderly men in relationship with diabetes status. In this study, diabetic men receiving insulin treatment had nearly double the risk of fractures

compared with those without diabetes after adjustment for multiple covariates. In diabetic men who were not using insulin or in subjects with prediabetes, the fracture rate was not increased during an average 9-year follow-up [314].

6.9.2. Thiazolidinediones. TZDs tackle insulin resistance via activation of PPARγ. Clinical studies suggest that TZDs reduce BMD and increase fracture risk. Most of the evidence comes from studies on rosiglitazone. A post hoc analysis of the ADOPT (A Diabetes Outcome Progression Trial) [315], which was designed to compare the efficacy of rosiglitazone versus metformin and glyburide to maintain durable normal blood glucose levels in 1,840 women and 2,511 men with prediabetes [316], showed an increased risk of fractures associated with rosiglitazone [315]. This effect was evident in women but not in men with hazard ratios of 1.81 and 2.13 for rosiglitazone compared with metformin and glyburide, respectively. Fractures were seen predominantly in the lower and upper limbs, but vertebral fractures were not assessed in this study [315]. Bone resorption was increased in women and, although no increased fracture risk was showed in men, the bone formation marker P1NP was reduced in both genders [317].

Evidence from other clinical studies substantiated this finding showing a twofold increased risk of fractures in women taking TZD, but still showing no effect in men [318]. Considering that TZDs are PPARγ agonists, a possible disruption in bone formation has been suggested. However, TZD are also known to lower RANKL activity and a recent study on ovariectomized rats indicates that bone impairment induced by rosiglitazone treatment is due to reduced bone strength coming from increased resorption mainly in sites rich in trabecular bone, which was reverted by treatment with alendronate [319]. Less evidence is available for pioglitazone. A recent study on 156 postmenopausal women with prediabetes showed that pioglitazone had no effect on BMD or bone turnover [320] with a similar result obtained by Grey et al. [321]. However, a meta-analysis of clinical studies has suggested an increased incidence of peripheral fractures in postmenopausal women with T2D taking pioglitazone [318].

6.9.3. Metformin. Metformin is an insulin sensitizer and the most widely used oral hypoglycemic drug. It has been shown that AMPK activation by metformin may decrease expression of SERBP-1, a transcription factor involved in adipocyte differentiation and increased the activity f Runx2 enhancing osteoblastogenesis [322]. On the other hand, it has a negative effect on osteoclast differentiation by decreasing RANKL and increasing OPG levels [323]. Consistent with this finding, an analysis of the Rochester cohort suggested that biguanides may have a beneficial effect on bone fractures (HR 0.7; 95%CI: 0.6–0.95) [307]; however, analysis of the ADOPT did not show any beneficial effect [315] and in another trial metformin did not prevent rosiglitazone induced bone loss [324]. The MrOS study showed no effect of metformin on nonvertebral fracture risk in elderly men with diabetes [314].

6.9.4. Sulphonylureas. Sulphonylureas work by stimulating insulin release by the β-cells. The post hoc analysis of the ADOPT study did not shown any effect of glyburide on fracture risk although treatment with this drug was associated with reduced bone formation marker P1NP [315]. In a recent analysis of the MrOS study, sulphonylurea use among elderly diabetic men was a risk factor for nonvertebral fractures (HR 1.66; 95%CI: 1.09–2.51) [314].

6.9.5. Incretins. The incretin pathway is attenuated in T2D and the therapeutic target of drugs used in T2D such as GLP-1 receptor analogues and inhibitors of DPP-4, which extend the half-life or the native incretins. As stated above there is some *in vitro* and *in vivo* evidence that this peptides exert positive effect on bone. However, the clinical data are still scant. A recent meta-analysis, taking into consideration twenty-eight trials enrolling 11,880 patients on DPP-4 inhibitors, showed a trend to reduced risk of fractures (odds ratio [MH-OR] 0.60, 95%CI 0.37–0.99, $P = 0.045$) in these patients compared to those on placebo [325]. Indeed, another meta-analysis on seven trials showed that GLP-1 receptor analogues do not modify the risk of bone fractures in diabetes compared with the use of other antidiabetic drugs [326].

6.9.6. Sodium-Glucose Cotransporters Inhibitors. Blockade of intestinal glucose uptake and renal glucose reabsorption via the sodium-glucose transporters (SGLT1, SGLT2) is a new approach to treat hyperglycemia in T2D [327]. Recently, the US Food and Drug Administration (FDA) approved two SGLT-2 inhibitors, canagliflozin and depagliflozin, as adjunctive therapy for T2D. These drugs inhibit selectively SGLT-2 in the kidney increasing urinary glucose excretion. Considering the mechanism of action, there is concern that renal tubular transportation of minerals and consequently bone health can be affected. In a 48-week trial assessing the efficacy of depagliflozin added on to pioglitazone in inadequately controlled T2D, Rosenstock et al. showed no clinically relevant changes in calcium, magnesium, phosphorous, or serum 25OHD levels [328]. A small increase in PTH and small mean changes in bone markers compared with placebo were shown. Two fractures occurred in the depagliflozin 5 mg group but all patients had received pioglitazone earlier [328]. In another study aimed to directly assess the effect of depagliflozin on bone turnover and BMD in patients inadequately controlled on metformin, Ljunggren et al. found no significant changes from baseline in P1NP, CTX, or BMD over 50 weeks of depagliflozin treatment [329]. Similar results were reported by Bolinder et al. [330]. In a pool analysis of eight clinical trials comprising 6,177 patients treated with canagliflozin, it was found that bone fracture incidence rates were 14.2, 18.7, and 17.6 per 1,000 patient years of exposure to comparator, canagliflozin 100 mg, and canagliflozin 300 mg, respectively. Patients treated with canagliflozin experienced more frequently extremity fractures than comparator [331]. On the other hand, a recent study on rats with a mixed SGLT1/2 inhibitor for 28 days resulted in marked changes in calcium and phosphorus homeostasis, suppression of PTH, $1.25(OH)_2D$, and bone turnover but had positive effects on

bone mass and strength [332]. However, available data on bone health are not conclusive and FDA has required a postmarketing bone safety study that the companies must conduct as a condition for approvals of both canagliflozin and depagliflozin.

7. Summary and Practical Hints

Diabetes increases the risk of osteoporotic fractures through several mechanisms. Hyperglycemia impairs osteoblasts function, generates abnormal modifications of bone protein matrix, induces a state of chronic inflammation, and fuels diabetic complications that are associated with an increased risk of falls and fractures. Specific pathophysiological elements linked to the type of diabetes, such as insulin deficiency in T1D or loss of incretin effect in T2D, are also involved in impaired bone health. Finally, increased release of adipokines by fat tissue further manipulates bone homeostasis. Taken together, these factors create, either directly or indirectly, a milieu that promotes MSC fate toward adipogenesis over osteoblastogenesis, describing a low bone turnover phenotype (Figure 1).

Fracture prediction may be challenging especially in patient with T2D. In these patients, BMD values and FRAX score should be carefully interpreted. Subjects with T2D have increased fragility despite normal or high T-score; similarly, the fracture risk is higher when compared with nondiabetic people for a given FRAX score. DEXA limitations might be overcome by techniques that take into account bone size and geometry such as pQCT while additional factors need to be studied in order to generate better predictive algorithms.

Although hyperglycemia may fuel several mechanisms associated with bone loss, a tight blood glucose control may increase the rate of hypoglycemia and therefore falls. Unfortunately, there is no consistent evidence from clinical trials that tight blood glucose control either positively or negatively influences bone fractures. However, it is well known that an HbA1c <7% prevents chronic complications [333–336], especially in younger and uncomplicated patients, thus possibly reducing the associated risk of falls and fractures. Diabetes treatment may impact bone health and treatment decisions should be individualized. TZD should be avoided in postmenopausal women if possible and weight loss in patients with T2D should be accompanied by increased physical activity to prevent bone loss.

Impaired vitamin D status is more prevalent in diabetes than in people without diabetes. Intervention studies assessing the effectiveness of vitamin D and calcium supplementation on diabetes-related fractures are not available and no consensus has been reached on the optimal vitamin D serum level [337]. However, 25OHD serum levels >20 ng/mL are advisable [338] and there is evidence from broad population trials that higher concentrations reduce hip fractures by 23% [339] and fall risk by 19% [340].

Evidences on osteoporosis drugs in diabetes are also scant and limited to observational or post hoc analysis of bisphosphonates studies. Alendronate has been shown to be as effective in diabetes as in postmenopausal osteoporosis in increasing BMD [341] and preventing hip fractures [342] with no differences between T1D and T2D [342]. Considering the increasing evidence that suggests low bone formation in diabetes, osteoanabolic therapies such as PTH-based drugs are attractive [343] but this hypothesis has not been substantiated by clinical studies yet. Ad hoc trials with antiresorptive and anabolic drugs investigating fracture outcomes in diabetes are needed.

Abbreviations

T2D:	Type 2 diabetes
MSC:	Mesenchymal stem cells
BMD:	Bone mineral density
T1D:	Type 1 diabetes
PPAR:	Peroxisome proliferator-activated receptors
LRP:	Low-density lipoprotein receptor-related protein
GSK:	Glycogen synthase kinase
Dkk:	Dickkoff
C/EBP:	CCAAT/enhancer binding protein
RANKL:	Receptor activator of nuclear factor-κB ligand
NF-κB:	Nuclear factor-κB
TNF:	Tumor necrosis factor
OPG:	Osteoprotegerin
BMI:	Body mass index
pQCT:	Peripheral quantitative computed tomography
IL-:	Interleukin-
TNFR1:	TNF receptor 1
ROS:	Reactive oxygen species
OCN:	Osteocalcin
11b-HSD:	11-β-hydroxysteroid dehydrogenase
DEXA:	Dual-energy X-ray absorptiometry
PTH:	Parathyroid hormone
$1,25(OH)_2D$:	$1,25(OH)_2$ vitamin D
25OHD:	25OH vitamin D
AGE:	Advanced glycation end-products
CTX:	Cross-linked C-telopeptide
IRS:	Insulin receptor substrates
NOD mouse:	Nonobese diabetic mouse
MRI:	Magnetic resonance imaging
GIP:	Glucose-dependent insulinotropic polypeptide
GLP-1:	Glucagon-like peptide 1
DPP-4:	Depeptidyl peptidase-4
ucOCN:	Undercarboxylated osteocalcin
P1NP:	Type 1 procollagen N-terminal peptides
COP:	Circulating osteogenic precursor cell
TRAP:	Tartrate-resistant acid phosphatase
BMC:	Bone mineral content
FRAX:	Fracture risk assessment tool
TBS:	Trabecular bone score
RR:	Relative risk
OR:	Odds ratio

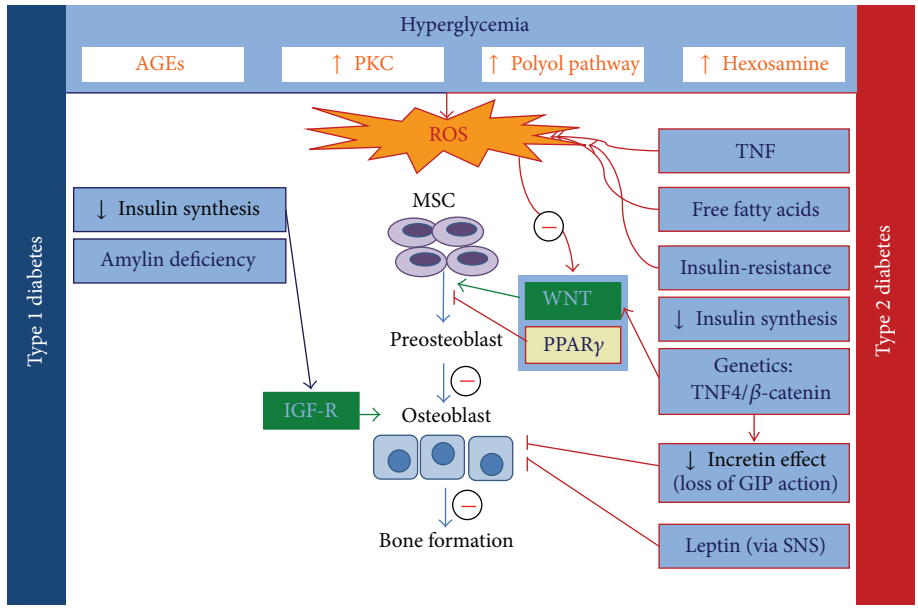

Figure 1: Diabetes-bone interaction. Several factors associated with diabetes may impair bone health. These factors may create, either directly or indirectly, a milieu that disrupts osteoblast differentiation and function, describing a low bone turnover phenotype. AGE: advanced glycation end-products; PKC: protein kinase C; ROS: reactive oxygen species; MSC: mesenchymal stem cells; TNF: tumor necrosis factor; GIP: gastric inhibitor peptide; IGF-R: insulin-like growth factor receptor; SNS: sympathetic nervous system.

HR: Hazard ratio
HR-pQCT: High-resolution peripheral quantitative computed tomography
SGLT: Sodium-glucose transporters
FDA: Food and drug administration.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Nicola Napoli and Rocky Strollo contributed equally to this work.

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Clinical Study

Serum 25-OH Vitamin D in relation to Bone Mineral Density and Bone Turnover

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It is unclear which vitamin D status is optimal for bone health. In this study, we aimed to assess cutoffs of 25-hydroxyvitamin D (25OHD) derived by the literature (20, 25, or 30 ng/mL) in relation to bone turnover and bone mineral density (BMD). Serum 25OHD, PTH, osteocalcin, bone alkaline phosphatase, and C-telopeptide were measured in 274 consecutive postmenopausal women. BMD of the lumbar spine (L1–L4) and of femoral neck were also evaluated. 50 patients had normal BMD, while 124 had osteopenia and 100 had osteoporosis. 37.6%, 56.2%, and 70.8% subjects had serum 25OHD lower than 20, 25, or 30 ng/mL, respectively. No differences in bone turnover markers were found when comparing patients with low 25OHD defined according to the different cutoffs. However, a cutoff of 25 ng/mL appeared to differentiate better than a cutoff of 30 ng/mL in those subjects with reduced femoral neck BMD. The PTH plateau occurred at 25OHD levels of 26–30 ng/mL. In conclusion, vitamin D deficiency is common in Sicilian postmenopausal women and it may be associated with low BMD and increased bone turnover markers. Further studies are needed to better define the right cutoff for normal vitamin D levels in postmenopausal women.

1. Introduction

Vitamin D deficiency causes defects of bone mineralization and low vitamin D status has been detected in patients with hip fractures [1–3]. While in the past it was thought that vitamin D deficiency affects mostly northern countries [4, 5] and where there is a restricted exposure to sunlight or in elderly patients [6, 7], other studies have shown that vitamin D deficiency may be common also in subtropical countries [3, 8, 9] or southern Europe [10] including Italy [11]. In a large clinical trial on raloxifene, it was found that a vitamin D deficiency is common in southern Europe (8.3% of the patients) [10]. In the same study, 24.3% of the postmenopausal women had low-normal vitamin D status, in a range that could be considered partial vitamin D deficiency [10]. Several studies have shown a negative correlation between BMI and vitamin D at any ages and in different clinical conditions. Therefore, the increasing prevalence of obesity and metabolic syndrome, which are associated with decreased bioavailability of dietary and cutaneously synthesized vitamin D, is an additional factor contributing to the widespread of vitamin D deficiency [12]. It should be noted that vitamin D deficiency is associated with muscle impairment and it is one of the contributing factors of a clinical condition known as "sarcobesity."

However, there is no consensus on which levels of serum 25-hydroxyvitamin D (25OHD) should be considered abnormal [13–15]. In this study, we aimed to assess whether different cutoffs of 25OHD-deficiency are associated with altered bone turnover or bone mineral density (BMD) in a homogeneous population of postmenopausal women living in Sicily. Sicily is the most southern part of Italy; it is surrounded by the Mediterranean Sea and characterized by sun exposure for 2/3 of the year.

2. Experimental Subjects and Methods

2.1. Study Subjects. We enrolled 274 consecutive postmenopausal women, aged 48–65 years (mean age 57.7 ± 0.4),

Table 1: Clinical and biochemical features and T-scores of studied population. Data are mean ± standard error.

Age (years)	57.7 ± 0.8
BMI (Kg/m^2)	26.6 ± 0.4
25OHD (ng/mL)	26.04 ± 1.9
L1–L4 (SD)	−0.5 ± 0.01
Femoral neck (SD)	−0.4 ± 0.01
PTH (pg/mL)	27.6 ± 1.1
OC (ng/mL)	14.8 ± 0.9
BAP (μg/L)	18 ± 0.9
CTX (pmol/L)	2893 ± 154

who, from December to May, were referred to our outpatient clinic at University of Palermo, for osteoporosis assessment. Patients with hyperparathyroidism, Paget's bone disease, or secondary osteoporosis were not included in the study. We also excluded patients who were previously treated for osteoporosis or were taking calcium or vitamin D. In all postmenopausal women a fasting blood sample was taken in the morning for measurement of 25OHD, PTH, osteocalcin (OC), bone alkaline phosphatase (BAP), and C-telopeptides (CTX). All measurements were performed during winter-spring season (from December to May). Informed consent was obtained before enrollment and the protocol was approved by ethical committee of University of Palermo.

2.2. Bone Mineral Density Evaluation. BMD of the lumbar spine (L1–L4) and of femoral neck (F) was determined using dual X-ray absorptiometry (DEXA, Lunar DPX-Plus).

2.3. Biochemistry. 25OHD was measured using enzyme-linked immunosorbent assay (ELISA) using materials provided by Immunodiagnostic Systems (Boldon, United Kingdom). Intact PTH was measured by ELISA using materials provided by Biosource, Belgium. OC and CTX were measured by ELISA using materials provided by Biotech A/S (Herlev, Denmark). BAP was evaluated by ELISA using materials provided by Beckmann-Coulter (CA, USA). In all assays, the intra-assay coefficient of variation was 6% or less, and the interassay coefficient of variation was 15% or less.

2.4. Statistical Analysis. Analysis of variance and the Mann-Whitney U test were used for group comparisons. P less than 0.05 were considered statistically significant. Results were expressed as mean ± SD.

3. Results

Clinical and biochemical features of the studied population are shown in Table 1. 50 patients had normal BMD, while 124 patients had osteopenia (T-score between −1 and −2.5 SD) and 100 patients had osteoporosis (T-score ≤ −2.5 SD). In our population, mean 25OHD was 26.04 ± 10.14 ng/mL and 63 study subjects (23%) had serum 25OHD lower than 16 ng/mL (1 SD below 25OHD mean values). BMD and bone turnover were compared between subgroups delineated by different serum 25OHD levels. These cutoffs were based on literature data and were set to 20 ng/mL [13], 25 ng/mL [16], and 30 ng/mL [14].

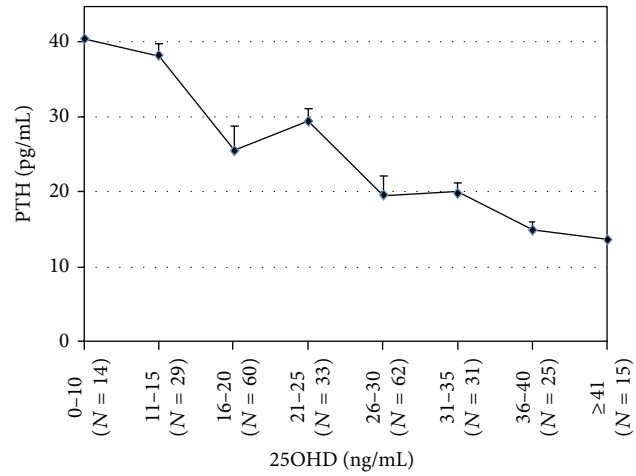

Figure 1: Mean (±SE) PTH by 25OHD subgroups. The graph shows subject PTH serum levels according to serum 25OHD subgroups defined by specific cutoffs. No clear inflection point was evident for the 25OHD cutoffs studies. However, there was a 34% increase in PTH levels (19.5 ± 1.53 versus 29.5 ± 3.3 pg/mL; $P = 0.002$) when comparing the two subgroups delineated by the 25OHD cutoff of 25 ng/mL. PTH levels did not change significantly differently when comparing the subgroups delineated by the 25OHD levels of 20 ng/mL (29.5 ± 3.3 versus 25.67 ± 1.51 pg/mL; $P = 0.228$), 30 ng/mL (19.5 ± 1.53 versus 20.06 ± 2.61 pg/mL; $P = 0.853$), 35 ng/mL (14.96 ± 1.28 versus 20.06 ± 2.61 pg/mL; $P = 0.110$), or 40 ng/mL (13.8 ± 1.16 versus 14.96 ± 1.28 pg/mL; $P = 0.543$), respectively.

Using a cutoff of 20 ng/mL, 103 study subjects (37.6%) had vitamin D deficiency. The prevalence of vitamin D deficiency increased to 56.2% (154 patients) using the 25 ng/mL cutoff and to 70.8% (174 patients) using the 30 ng/mL cutoff.

As shown in Table 2, study subjects with low serum 25OHD had higher serum PTH, BAP, and CTX, independently, on used cutoffs. In general, subjects with low serum 25OHD showed lower T-score independently of used cutoff, but this difference was lost on femoral neck when the cutoff at 30 ng/mL was used (−1.4 ± 1.0 versus −1.5 ± 1.0 SD). Therefore, a 25OHD level higher than 25 ng/mL appeared to differentiate better than a cutoff of 30 ng/mL in those subjects with reduced femoral neck BMD.

No significant differences in mean values of OC, BAP, and CTX were found when subjects considered as 25OHD-deficient, according to the different cutoffs, were compared. However, by plotting serum 25OHD to PTH levels, there was a significant 34% increase in PTH levels (19.5 ± 1.53 versus 29.5 ± 3.3; $P = 0.002$) when comparing the two subgroups delineated by the 25OHD cutoff of 25 ng/mL (Figure 1); on the other hand, PTH levels did not change significantly when comparing the subgroups delineated by the 25OHD levels of 20 ng/mL (29.5 ± 3.3 versus 25.67 ± 1.51 pg/mL; $P = 0.228$), 30 ng/mL (19.5 ± 1.53 versus 20.06 ± 2.61 pg/mL; $P = 0.853$), 35 ng/mL (14.96 ± 1.28 versus 20.06 ± 2.61 pg/mL; $P = 0.110$),

Table 2: Features, T-scores, and biochemical markers of subjects subdivided three times into two groups on the basis of the different 25OHD cutoff values (20, 25, and 30 ng/mL).

	Cutoff at 20 ng/mL		Cutoff at 25 ng/mL		Cutoff at 30 ng/mL	
	25OHD >20 ng/mL (n = 171)	25OHD <20 ng/mL (n = 103)	25OHD >25 ng/mL (n = 120)	25OHD <25 ng/mL (n = 154)	25OHD >30 ng/mL (n = 80)	25OHD <30 ng/mL (n = 194)
Age (years)	57.6 ± 6.4	57.1 ± 6.0	56.6 ± 5.5	57.8 ± 6.2	56.5 ± 6.0	57.7 ± 6.3
BMI (Kg/m^2)	26.6 ± 4.4	27.0 ± 3.5	25.9 ± 3.7	27.4 ± 4.1	25.9 ± 3.6	27.2 ± 4.2
Lumbar (L1–L4) T-score	−1.9 ± 1.3**	−2.2 ± 1.3	−1.7 ± 1.3**	−2.2 ± 1.6	−1.7 ± 1.3**	−2.1 ± 1.2
Femoral neck T-score	−1.2 ± 1.0**	−1.8 ± 1.0	−1.3 ± 1.2**	−1.5 ± 1.0	−1.5 ± 1.0	−1.4 ± 1.0
Osteocalcin (ng/mL)	18.8 ± 12.2	20.5 ± 12.1	18.2 ± 12.3	20.1 ± 11.6	18.2 ± 6.8	20.3 ± 13.5
BAP (μg/mL)	20.2 ± 7.4**	23.1 ± 8.4	19.3 ± 6.6**	23.0 ± 7.1	19.7 ± 6.5*	22.2 ± 9.0
CTX (pmol/L)	4426.2 ± 3546.9*	5439.5 ± 3143.0	4105.3 ± 2162.7*	5324 ± 3395	4002.9 ± 2484.6*	4909.3 ± 3112.0
PTH (pg/mL)	22.2 ± 15.6**	35.5 ± 18.5	20.0 ± 15.8**	33.3 ± 16.4	16.6 ± 13.0**	31.5 ± 19.0
25OHD (ng/mL)	30.2 ± 8.8**	14.4 ± 3.6	33.1 ± 8.6**	16.8 ± 7.8	37.2 ± 8.4**	18.8 ± 5.9

Values are mean ± SD. BMI = body mass index; BAP = bone alkaline phosphatase; CTX = C-telopeptides; PTH = parathormone; 25OHD = 25-hydroxyvitamin D. $^{**}P < 0.01$ versus subjects with 25OHD values lower than their respective cutoff value. $^{*}P < 0.05$ versus subjects with 25OHD values lower than their respective cutoff value.

or 40 ng/mL (13.8 ± 1.16 versus 14.96 ± 1.28 pg/mL; P = 0.543), respectively.

4. Discussion

There is no consensus on what levels of serum 25OHD should be considered abnormal [13–15], in part because vitamin D needs vary among different ethnic groups and geographical areas and also because there is limited data on which levels of 25OHD are associated with subtle abnormalities of bone metabolism, turnover, and neuromuscular function. The Institute of Medicine has set the optimal 25OHD level at 20 ng/mL (corresponding to 2 SD above the median needs) as it was suggested to meet the requirement of at least 97.5% of population in North America [13]. However, there is still some controversy about optimal levels [14] and the International Osteoporosis Foundation recommends a desirable 25OHD serum level of 30 ng/mL [15].

This issue is particularly difficult when studying populations with possible vitamin D deficiency. It is still an open question if the optimal cutoff should be obtained in the same population or should be derived from literature and obtained in populations with different genetic and environmental influences. We tried to answer this question studying a Sicilian population of postmenopausal women. While this cannot be considered an epidemiological study, it is representative of the women who come to an osteoporotic clinic for the assessment of their bone mass.

All cutoffs divided the population in two groups different for T-score, bone turnover, and PTH levels. For any analyzed cutoffs, BMD was generally lower in the vitamin D deficient groups with consequent significant increase in PTH. Both markers of bone resorption and formation resulted higher in the vitamin D deficient groups, indicating an increased bone turnover. These data confirm that, despite the chosen cutoff, lower vitamin D levels may always negatively affect bone health. Subjects with low serum 25OHD showed lower T-score independently of used cutoff, but this difference was lost on femoral neck when the cutoff at 30 ng/mL was used. Moreover, by plotting serum 25OHD to PTH levels, a significant change in PTH levels was evident when comparing the two subgroups delineated by the 25OHD cutoff of 25 ng/mL but not for higher or lower 25OHD cutoffs, suggesting that a plateau occurred at 26–30 ng/mL. This suggests that a status of vitamin D deficiency exists in women having vitamin D lower than 20 or 25 ng/mL while the level of 30 ng/mL may be too high. In fact, using this cutoff 2/3 of studied women could be considered as having a vitamin D deficiency. Our data are consistent with the finding of the National Health and Nutrition Survey (NHANES) III where the risk of hip fracture was significantly reduced among participants with 25OHD levels greater than 25 ng/mL compared with those who had lower concentrations, and the association resulted to be independent of bone density [16]. However, our data should be read with caution because the number of people included in this study, and particularly those with 25OHD higher than 30 ng/mL, is relatively small. Furthermore, although we found a significant change in PTH levels for 25OHD at 26–30 ng/mL, an inflection point was not clearly evident. In another study on a larger sample, Holick et al. found that an inflection point for PTH levels is evident for 25OHD less than 29.8 ng/mL [17]. On the other hand, our data corroborate recent findings showing that vitamin D deficiency is common in postmenopausal women living in Mediterranean countries [10] including Italy [18], despite the general belief that this condition is common only in elderly patients [6, 7] or in countries where exposure to sunlight is low and limited to short periods of the year [4, 5]. While the reasons for this are not clear, possibly a poorer intake or darker skin in Mediterranean population, our study supports

the idea that vitamin D status should be assessed in all postmenopausal women. A number of cross-sectional studies have found a positive association between 25OHD and BMD in postmenopausal women [5, 19–23], and the last NHANES in US [13] as well as a recent Italian study [18] showed that this relationship can be evident even in women before the onset of menopause. Interestingly, in the Italian study 25OHD levels were significantly lower in women from south sites compared with northern sites, despite a significantly higher sun exposure [18]. Moreover, impaired vitamin D status has been generally associated with an increased risk of fractures. A nested case control study from the Women's Health Initiative showed a near doubling of the odds ratio of risk for hip fracture in subjects with 25OHD lower than 20 ng/mL [24].

In conclusion, it is challenging to determine a precise cutoff for vitamin D deficiency in postmenopausal women, but, according to our study, a level of 25 ng/mL might be optimal. However, vitamin D deficiency causes bone loss and increased bone turnover and, therefore, vitamin D status should be assessed and corrected in populations at risk.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Nicola Napoli and Rocky Strollo contributed equally to this work.

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Research Article

Testosterone and Adipokines are Determinants of Physical Performance, Strength, and Aerobic Fitness in Frail, Obese, Older Adults

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In this study, we evaluated the independent and combined effects of baseline circulating gonadal, anabolic hormones and adipokines on physical function in 107 frail, obese (BMI $\geq$ 30 kg/m^2), and older ($\geq$65 yr) subjects. Our results showed significant positive correlations between circulating testosterone and insulin growth factor-1 (IGF-1) with knee flexion, knee extension, one-repetition maximum (1-RM), and peak oxygen consumption (VO_2 peak), while no correlation was observed with estradiol. Among the adipokines, high sensitivity C-reactive protein (Hs-CRP) and leptin negatively correlated with the modified physical performance testing (PPT), knee flexion, knee extension, 1-RM, and VO_2peak. Interleukin-6 (Il-6) negatively correlated with knee flexion and VO_2 peak and soluble tumor necrosis factors receptor-1 (sTNFr1) correlated with PPT, 1-RM, and VO_2 peak. Adiponectin correlated negatively with 1-RM. Multiple regression analysis revealed that, for PPT, sTNFr1 was the only independent predictor. Independent predictors included adiponectin, leptin, and testosterone for knee flexion; leptin and testosterone for knee extension; adiponectin, leptin, and testosterone for 1-RM; and IGF-1, IL-6, leptin, and testosterone for VO_2peak. In conclusion, in frail obese older adults, circulating levels of testosterone, adiponectin, and leptin appear to be important predictors of physical strength and fitness, while inflammation appears to be a major determinant of physical frailty.

1. Introduction

A high body mass index (BMI) is associated with impairment in activities of daily living, limitations in mobility, decreased physical performance, and increased risk for functional decline [1] leading to increased nursing home admissions [2]. The elderly obese are especially susceptible to the adverse effects of excess body fat on physical function, because of (1) decreased muscle mass and strength which occur with aging and (2) the need to carry greater weight due to obesity [3]. Investigators from our group first reported that 96% of community-living older adults with BMIs > 30 kg/m^2 were frail, as determined by physical performance test scores, peak oxygen consumption (VO_2 peak), and ability to perform activities of daily living [3].

Aging is associated with the decline in gonadal and anabolic hormones and increased in inflammatory cytokines. These changes are associated with loss of muscle mass and decline in strength and physical function. An increase in lean body mass, strength, and physical function has been demonstrated with testosterone [4, 5] and growth hormone (GH) [6] treatment among patients deficient of these hormones. On the other hand, aging is associated with increase in inflammatory cytokines, and high levels of these cytokines promote

muscle catabolism and reduced muscle volume [7]. Since loss of muscle volume leads to reduced muscle strength and function, it is possible that the age-related hormonal decline and increased inflammation contribute to deterioration in physical function leading to frailty. Furthermore, in the obese elderly patient, the added increase in inflammatory cytokine production from the excess adipose tissues would enhance inflammation perhaps leading to worse frailty syndrome than nonobese elderly patients. However, little information is available on the contribution of the interaction of circulating hormones and adipokines (some of them proinflammatory) on frailty and physical disability in obese elderly adults.

The objective of this study was to evaluate the independent and combined effects of circulating gonadal (testosterone and estradiol) and anabolic hormones (growth hormone/IGF-1) and adipose-tissue derived factors (adipokines) on physical function, strength and physical fitness in frail obese older adults.

2. Methods

2.1. Study Design and Study Population. This study is a cross-sectional analysis of baseline data from subjects who volunteered to participate in a previous lifestyle therapy trial of sedentary, frail, and elderly obese adults [8, 9]. This study was conducted at Washington University School of Medicine in accordance with the guidelines in the Declaration of Helsinki for the ethical treatment of human subjects. The protocol was approved by the Washington University Institutional Review Board and a written informed consent was obtained from each subject. Participants were recruited through newspaper and radio advertisements. Inclusion/exclusion criteria were as reported previously [8, 9]. Briefly, participants were ≥65 years of age, with BMI ≥ 30 kg/m^2, had sedentary lifestyle, (did not participate in regular exercise more than twice a week), had stable body weight (±2 kg) over the past year, and were on stable medications for 6 months before enrollment. Participants who were treated with bone-acting drugs (e.g., bisphosphonates, glucocorticoids, and sex-steroid compounds) during the previous year were excluded from participation. At enrollment, these subjects should not have cardiorespiratory or neuromuscular diseases that would limit their ability to exercise, diabetes mellitus, osteoporosis, hyperparathyroidism, chronic liver disease, uncontrolled or untreated hyperthyroidism, or significant renal impairment.

2.2. Physical Function. Physical function to determine frailty was assessed using the modified physical performance test (PPT) as previously described [8, 9]. The modified PPT includes seven standardized tasks (walking 50 ft, putting on and removing a coat, picking up a penny, standing up from a chair, lifting a book, climbing one flight of stairs, and performing a progressive Romberg test) plus two additional tasks (climbing up and down four flights of stairs and performing a 360-degree turn). The score for each task ranges from 0 to 4; a perfect score is 36.

2.3. Muscle Strength Testing

2.3.1. Knee Flexion and Knee Extension. Isokinetic knee extensor and flexor strength were evaluated by using a Biodex System 3 dynamometer (Shirley, NY) as previously described [10]. During the testing, the participants were seated with their backs supported and hips positioned at 120° of flexion and secured to the seat of the dynamometer with thigh and pelvic straps. All tests were performed on the right leg. Testing was performed at an angular velocity of 60° per second. The best of the 3 maximal voluntary efforts for each of the knee flexion and extension was used as the measure of absolute strength and reported as peak torque at 60° in Newton-meter (N.m) units. The test-retest reliability of this method based on follow-up isokinetic testing done one week following the initial tests showed an intraclass correlation coefficient of 0.99.

2.3.2. One Repetition Maximum. Total one-repetition maximum (1-RM) is the sum of the maximal weight a person can lift at one repetition for biceps curl, bench press, seated row, knee extension, knee flexion, and leg press [8, 10]. The test-retest reliability of this method based on follow-up 1-RM determinations one week following the initial tests showed an intraclass correlation coefficient of 0.96.

VO_2 peak, a measure of aerobic fitness, was assessed during graded treadmill walking by indirect calorimetry (True Max 2400, ParvoMedics Salt Lake City, UT), as previously described [3, 11]. Briefly, the incremental test started at a speed determined, during a warm-period, to elicit ~70% of age-predicted HR_{max}, and the speed remained constant throughout the test, while the grade was increased by 2% every 2 minutes. The test continued until the subject could no longer exercise because of exhaustion or until other conditions, such as ECG changes or development of symptoms, made it unsafe to continue [12, 13].

2.3.3. Body Weight. Body weight was measured in the morning after the subjects had fasted for 12 hours [8]. BMI was calculated as weight in kilograms/square of the height in meters (kg/m^2).

2.3.4. Biochemical Measurements. Blood samples were obtained in the morning after subjects fasted for at least 12 hours. Serum samples were extracted and stored at −80°C until analysis. Enzyme-linked immunosorbent assay kits were used to measure interleukin-6 (IL-6) (Quantikine, R&D Systems, Minneapolis, MN), soluble tumor necrosis factor receptor 1 (sTNFr1) (R&D, Minneapolis, MN, USA), and adiponectin (Quantikine, R&D Systems, Minneapolis, MN). Radioimmunoassay kits were used to measure serum estradiol (Ultra-sensitive estradiol DSL-4800; Diagnostic Systems Laboratories Inc., Webster, Tex), leptin (Leptin HL-81K; Linco Research Inc., St Charles, MO), and insulin-like growth factor 1 (IGF-1) (Diagnostic Products Group). High-sensitive C-reactive protein (Hs-CRP) was measured by immunoturbidimetric assay (Hitachi 917 analyzer), while serum total testosterone was measured by automated

Table 1: Clinical and biochemical characteristics of study participants.

Age (years)	69.4 ± 4.1
BMI (kg/m^2)	37.0 ± 4.9
Weight (kg)	116.7 ± 12.9
Testosterone (ng/dL)	119.3 ± 151.8
Estradiol (pg/mL)	52.5 ± 22.6
Adiponectin (ng/mL)	25.0 ± 15.3
Leptin (μg/mL)	36.6 ± 22.6
IL-6 (pg/mL)	2.3 ± 2.5
Hs-CRP (mg/L)	4.1 ± 4.5
sTNFrl (pg/mL)	167 ± 43.4
PPT (points)	27.6 ± 3.3
Knee flexion (Nm)	47.7 ± 16.4
Knee extension (Nm)	71.2 ± 25.5
Total 1-RM (lb.)	542.7 ± 194.0
VO_2 peak (mL/kg/min)	17.2 ± 3.3

BMI: Body mass index, IL-6: interleukin-6, Hs-CRP: high sensitivity C-reactive protein, sTNFr-1: soluble tumor necrosis factor receptor-1, PPT: physical performance test, Total 1-RM: total 1-repetition max, VO_2 peak: peak oxygen consumption, and Nm: Newton meter.

immunoassay (VITROS 5600). The CVs for these assays were <10%.

2.4. Statistical Analysis. Results are expressed as means ± SD. A P value of <0.05 was considered statistically significant. Normality for outcome variables was verified by Shapiro-Wilks test. Simple correlation analysis was performed to assess the individual associations between each hormone or adipokine with PPT, knee flexion, knee extension, total 1-RM, and VO_2 peak followed by multiple regression analysis to determine the independent contribution of each hormone or adipokine to the above tests.

3. Results

Our population consisted of 107 frail (PPT of ≤ 32) obese (BMI ≥ 30 kg/m^2) and elderly (≥65 years old) males (n = 41) and females (n = 66). The baseline characteristics of these patients have been reported previously [8] and summarized in Table 1.

Table 2 showed significant positive correlations between the testosterone with the PPT, all measures of strength (knee flexion, knee extension, and total 1-RM), and aerobic fitness (VO_2peak), while no correlation was observed between estradiol and any of these measures. Significant positive correlation was observed between IGF-1 and measures of strength and aerobic fitness but not with modified PPT. Among the different adipokines, there was a negative correlation between the proinflammatory cytokine Hs-CRP with PPT, strength, and aerobic fitness (VO_2peak). The other pro-inflammatory cytokine IL-6 also correlated negatively with knee flexion and VO_2peak. The last pro-inflammatory cytokine tested sTNFr1 also correlated negatively with PPT, total 1-RM, and VO_2peak.

The adipokine leptin negatively correlated with PPT, all measures of strength, and VO_2 peak. The adipokine adiponectin negatively correlated with total 1-RM.

Multiple regression analysis (Table 3) showed that, for PPT, sTNFr1 was the only independent predictor. Independent predictors for knee flexion included adiponectin, leptin and testosterone. Independent predictors for knee extension included: leptin and testosterone. Adiponectin, leptin and testosterone were independent predictors of total 1-RM while independent predictors of peak oxygen consumption (VO_2 peak) included: IGF-1, IL-6, leptin and testosterone.

4. Discussion

Our results demonstrated that although there were correlations between gonadal and anabolic hormones and several adipokines with physical function, different measures of strength, and aerobic fitness (VO_2 peak), sTNFr1 was the only independent predictor of overall physical performance (PPT), while testosterone seemed to be a consistent predictor of all measures of physical strength and fitness. Leptin and adiponectin also appeared to be independent determinants of the different measures of strength, while leptin additionally predicted VO_2peak. On the other hand, IGF-1 only independently predicted physical fitness, while estradiol appeared to have no role in physical function, strength, or fitness. Thus, our results suggest that circulating hormones and adipokines alone or in combination may contribute to frailty and physical disability in obese older adults.

Advancing age is associated with a decline in fat-free mass (FFM; primarily skeletal muscle) and function, known as sarcopenia [14]. Obesity is associated with higher absolute volume of FFM, but there is a disproportionately higher volume of fat mass relative to FFM resulting in relative muscle mass deficit exacerbating age-related muscle loss in the elderly obese [3]. Thus, obesity does not protect against sarcopenia and in fact it acts synergistically with sarcopenia (sarcopenic obesity) resulting in worst disability. Indeed, our group reported that 96% of obese elderly patients meet criteria for frailty [3].

Both obesity and aging have additive effects on chronic inflammation [15, 16] putting the elderly obese in a continuous state of heightened inflammation. Sarcopenic obesity is associated with increased levels of inflammatory cytokines [15] which have direct catabolic effects on skeletal muscle such as: TNF-α which suppresses muscle protein synthesis and interleukin-6 (IL-6) which inhibits the anabolic effects of IGF-1 [7]. Aside from adipose tissue production, skeletal muscles also express cytokines that have direct autocrine and paracrine effects within skeletal muscles [16]. Increased circulating concentrations of IL-6 are associated with lower muscle mass or strength and impaired mobility and in conjunction with low IGF-1 levels contribute synergistically to produce disability [17]. Our results showed that high levels of proinflammatory cytokines were independently associated with impaired strength and physical function and in fact, sTNFr1 was the only independent predictor of physical performance as assessed by the PPT score.

Table 2: Simple correlation analysis between physical function, strength, and aerobic fitness with different hormones and adipokines.

	Testosterone	Estradiol	IGF-1	IL-6	sTNFr1	Hs-CRP	Leptin	Adiponectin
Modified PPT	0.23*	−0.03	0.10	−0.11	−0.30**	−0.22*	−0.22*	−0.01
Knee flexion	0.49**	0.14	0.26*	−0.25*	−0.15	−0.22*	−0.53**	−0.11
Knee extension	0.51**	0.18	0.26**	−0.16	−0.14	−0.26**	−0.44**	−0.10
Total 1-RM	0.66**	0.14	0.23*	−0.13	−0.20*	−0.26**	−0.52**	−0.22*
VO_2 peak	0.56**	0.04	0.20*	−0.28**	−0.23*	−0.28**	−0.61**	−0.09

Data are mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$. VO_2 peak: peak oxygen consumption, Nm: Newton meter, BMI: body mass index, IL-6: interleukin-6, Hs-CRP: high sensitivity C-reactive protein, sTNFr-1: soluble tumor necrosis factor receptor-1, PPT: modified physical performance test, and Total 1-RM: total 1-repetition max.

Table 3: Final model of the hormonal and adipokine predictors of physical performance test, strength measures, and aerobic fitness (VO_2peak) in frail obese older adults.

	R^2 (%)	Beta estimate	SE	P
PPT	10			
sTNFr1		−0.025	0.008	0.003
Knee flexion	37.9			
Adiponectin		−0.37	0.16	0.02
Leptin		−0.29	0.09	0.002
Testosterone		0.034	0.013	0.01
Knee extension	27.4			
Leptin		−0.28	0.13	0.04
Testosterone		0.06	0.02	0.003
Total 1-RM	54.3			
Adiponectin		−3.22	1.07	0.004
Leptin		−2.23	0.81	0.006
Testosterone		0.71	0.12	<0.001
VO_2 peak	54.3			
IGF-1		0.02	0.01	0.02
IL-6		−0.31	0.11	0.009
Leptin		−0.05	0.01	<0.001
Testosterone		0.007	0.002	0.001

Variables entered in the model: sTNFr1, adiponectin, leptin, testosterone, estradiol, IGF-1, IL-6, leptin, age, and sex. PPT: modified physical performance test, Total 1-RM: total 1-repetition max, VO_2 peak: peak oxygen consumption, sTNFr1: soluble tumor necrosis factor receptor-1, IGF-1: insulin growth factor-1, and IL-6: interleukin-6.

Our results also demonstrated a significant contribution of testosterone to physical strength and fitness, while estradiol appeared to have no role in physical function, strength, or fitness in our study population. Although part of decline in physical function and strength with aging is attributed to abnormalities in GH/IGF-1 production and signaling, our study showed that IGF-1 had very little role in these parameters in frail obese older men and women. Aging is associated with decrease in gonadal hormones in both sexes. In women, the drastic drop in estrogen levels with menopause is associated with reduction in muscle mass [18]. In men after age of 40, testosterone production gradually decreases at a rate of 1.6% per year for total and to 2-3% per year for bioavailable testosterone [19]. Because of the age-related increase in sex hormone binding globulin, the magnitude of the decrease in bioavailable testosterone in men is even greater than the decline in total testosterone levels [20]. This reduction in testosterone production in men parallels the (1) age-associated loss of muscle mass that leads to sarcopenia and impairment of function and (2) age-associated loss of bone mass that leads to osteopenia and fracture risk [21, 22].

In men, several randomized controlled trials (RCTs) have demonstrated that testosterone significantly increases FFM (1.1 to 3.7 kg) and decreases fat mass (1.1 to 4.5 kg) [23–25]. In a review of seven trials, testosterone therapy was associated with a significantly greater increase in lean body mass (2.7 kg; 95% CI, 1.6–3.7) and a greater reduction in fat mass (−2.0 kg; 95% CI 3.1–0.8) than placebo [12]. The body weight change did not differ significantly between groups (−0.6 kg; 95% CI −2.0–0.8). In several studies, the increase in lean mass was accompanied by increase in muscle strength as assessed by hand grip strength, [25] maximum voluntary strength in a leg-press and chest-press exercise, [26] and isokinetic knee extension peak torque [27]. Furthermore, in a meta-analyses of 11 RCTs in elderly men, it was concluded that testosterone increased muscle strength particularly in the lower extremity (effect size: 0.63, 95% CI = 0.03–1.28) [28]. Moreover, testosterone replacement has been shown to improve physical function in healthy and mostly frail elderly men, as assessed by physical performance test [27], timed physical function test [25], aggregate locomotor function [27], and loaded stair-climbing [26].

On the other hand, although it is well-established that testosterone administration in postmenopausal women resulted in improvement in sexual function not only in surgically menopausal women but also in women with natural menopause [29–31], very few studies have investigated the effects of testosterone administration on body composition, muscle performance, and physical function in women. Huang et al. reported dose-related increases in lean body mass and improvement in strength [32] in postmenopausal women with low gender-adjusted testosterone suggesting the potential for testosterone to improve body composition and physical function in women. Their results were supported by data from another recent study showing that muscle protein synthesis in postmenopausal women is actually stimulated by testosterone and progesterone but not by estradiol [33].

Leptin and adiponectin share similarities in that both are produced by adipose tissues, have receptors in skeletal muscle, and have effects on muscle metabolism [34]. Leptin

stimulates fatty acid oxidation in skeletal muscles and inhibits fat storage while promoting intramuscular lipolysis [35, 36]. Nevertheless, despite this positive effect of leptin on muscle metabolism, epidemiologic studies demonstrate a negative association between leptin with muscle mass and function [13, 37]. This inconsistency is believed to result from leptin resistance that develops with high-fat feeding and obesity [38, 39]. Similarly for adiponectin, aside from stimulating glucose utilization, it stimulates fatty acid oxidation and inhibits intramuscular fat lipid deposition [40, 41]. However, resistance to adiponectin in peripheral tissues similarly develops with high fat feeding and obesity [42] and may explain the inverse association found between adiponectin, with muscle strength and function [43, 44]. Thus, these adipokines may be useful as biomarkers for frailty.

Our study has limitations. It is cross-sectional study and therefore does not provide a direct evidence of the actual physiologic role of each factor on physical function, strength, and frailty. In addition, our sample size was relatively small which thus needs confirmation in a larger population of obese older adults.

In summary, our results indicate that circulating testosterone levels and adipokines alone or in combination influence physical function, strength, and aerobic fitness in the frail obese older adults. Our group previously demonstrated that lifestyle intervention by diet and exercise was safe and improves physical function and ameliorates frailty in this population [8]. However, we also showed that although weight loss-associated muscle and bone loss can be attenuated by exercise, it did not totally prevent these complications. Given testosterone's effect on increasing bone density, muscle mass and strength, whether adding testosterone to lifestyle therapy, would be able to prevent muscle and bone loss from weight loss, while at the same time improving strength needs to be examined in future studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Hip Osteoarthritis and Osteoporosis: Clinical and Histomorphometric Considerations

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Although an inverse relationship between osteoarthritis (OA) and osteoporosis (OP) has been shown by some studies, other reports supported their coexistence. To clarify this relationship, we analyzed the interplay between clinical and histomorphometric features. Bone mineral density (BMD) and histomorphometric structure were assessed in 80 patients of four different age-matched groups undergoing hip arthroplasty for severe OA or OP-related femoral fracture. Harris Hip Score was also performed. Surgical double osteotomy of the femoral head was performed and microscopic bone slice samples analysis was performed by using a BioQuant Osteo software. Bone volume fraction (BV/TV) was lower ($P < 0.01$) in subjects with femoral neck fracture ($20.77 \pm 4.34\%$) than in subjects with nonosteopenic OA ($36.49 \pm 7.73\%$) or osteopenic OA ($32.93 \pm 6.83\%$), whereas no difference was detected between subjects with femoral neck fractures and those with combined OA and OP ($20.71 \pm 5.23\%$). Worse Harris Hip Score was found in those patients with the lowest BMD and BV/TV values. Our data support recent evidences indicating the possibility of impaired bone volume fraction in OA patients, with a high risk of developing OP, likely for their decreased mobility. Further studies are needed in order to investigate biomolecular pathway and/or growth factors involved in bone volume impairment in OA patients.

1. Introduction

The improvement of living conditions and advances in medicine in the last 50 years increased life expectancy, allowing ageing-related diseases to become a common cause of death and disability. Osteoarthritis (OA) and osteoporosis (OP) are extremely frequent among elderly people and their impact on life quality makes them of high sociohealth relevance [1–3]. Several observations reported an inverse association between OA and OP and large longitudinal studies suggested a protective effect of one disease on the other one [4–7]. This belief was partly supported from the evidence of opposite mechanisms driving the development of bone changes associated with OA and OP. Reduction of the bone mass and quality are key features of OP and determine a high risk of fractures [8, 9]. Instead, OA is characterized by increased bone density [10–14] and cartilage remodelling opposite to those of OP [10, 14–16]. However, other studies failed to show an inverse relationship between OA and OP and reported impaired bone quality and increased risk of fracture in patients with OA [17–20]. Histomorphometry is a recently developed method aimed at evaluating microscopic structure of bone that reflects changes and turnover activities of absorption and formation [21]. Histomorphometric assessment allows for a comprehensive semiquantitative analysis of microscopic organization and structure of bone by using specific grids and software. This allows a computer-assisted analysis of images and obtaining detailed information on volumes and surfaces occupied by different bone component, with particular reference to the distribution of the bone volume compared to the total area. In order to better clarify the relationship between OA and OP, we compared clinical and microscopic bone features in patients with OA or fracture undergoing hip arthroplasty. Dual energy X-ray

absorptiometry (DXA) and histomorphometry were used in different subgroups of patients to evaluate bone mass and microarchitectural bone parameters, respectively. The comparative analysis gave better comprehensive information on the relationship between hip OA and OP and the hypothetical mechanisms underlying this association.

2. Materials and Methods

2.1. Selection of Patients. From June 2011 to September 2012, 119 patients underwent hip arthroplasty in the Orthopaedic Department of Tor Vergata University; patients' written consensus was obtained. Before surgery, each patient with OA underwent DXA examination of the lumbar spine and femoral neck on the same limb on which the operation was planned to estimate the bone mineral density (BMD) and the possible condition of OP according to WHO criteria [22]. Hip X-rays were taken to establish the grade of OA; spine X-rays were also performed in patients with femoral fracture or back pain to evaluate the presence of a vertebral compression fracture (VCF). Lumbar spine and nonfractured femur BMD were also evaluated few days after the surgery. To evaluate hip function, Harris Hip Score (HHS) was also calculated. It gives a maximum of 100 points; the higher the HHS, the less the dysfunction. Exclusion criteria were as follows: history of primary or secondary malignant bone tumors, smoking habit, alcohol abuse, diabetes, hypercholesterolemia and use of glucocorticoids, and a previous fracture on the same or contralateral femur. Patients did not take antiosteoporotic drugs. Among fractured group, 7 patients received a supplementation of calcium and vitamin D.

Four different groups were made according to BMD results and principal diseases (i.e., femoral neck fracture and OA) by enrolling 20 consecutive age-matched patients responding to the following criteria: (1) hip OA and T-score greater than −1 DS (group OA-norm); (2) hip OA and T-score between −1 and −2.5 DS, a condition indicative of osteopenia (group OA-op); (3) hip OA and T-score less than −2.5 DS, a condition indicative of osteoporosis (group OA-OP); (4) femoral neck fracture and T-score less than −2.5 DS, a condition indicative of OP (group FX-OP). Differences among the data of the four groups were analyzed, and their significance was evaluated by Student's t-test. In general, P values less than 0.05 were considered statistically significant.

2.2. Evaluation of Bone Mineral Density. BMD was evaluated by iDXA (Lunar, GE Healthcare, Diegem, Belgium). Lumbar spine (L1–L4) and femoral (neck and total) scans were performed, and BMD was measured (in grams per square centimeter) and analyzed as just described. The coefficient of variation percentage (CV%) of lumbar spine (L1–L4) and proximal femur was 1.1% and 0.7%. Additional quality controls were done every morning for the DXA equipment according to the manufacturer's guidelines, to verify the stability of the system, and did not show any shift during the entire study period. In all groups, measurements were performed while participants lay supine on an examination table with their limbs abducted away from the trunk.

2.3. Preparation and Analysis of Specimens. During surgery, a 5 mm thick sagittal slice was obtained from the femoral head. Samples were fixed in 10% buffered formalin and subsequently decalcified in Decalcifier II (Surgipath, Leica Microsystems Srl., Milan Italy) [23]. Successively, after accurate sampling of all slices, samples were dehydrated in increasing concentrations of ethanol and embedded in paraffin. Serial 5 μm thick sections were cut, placed on positively charged slides, and stained with haematoxylin and eosin for microscopic examination [24].

2.4. Histomorphometric Analysis. For each femoral head, we evaluated at least 15 microscopic images randomly selected from at least three bone slides, for a total of 15 acquisitions per patient. Images were selected at 40x magnification by using a Nikon Eclipse E600 light microscope connected to a Nikon digital camera and saved at a resolution of 1280×1024 pixels. Successively, the images were analyzed by using a BioQuant Osteo software (version 7.20.10; BIOQUANT Image Analysis Corporation, Nashville, USA), specific for histomorphometric bone analysis, according to the manufacture's suggestions. Among the results contained in the BioQuant Osteo software report, we considered the bone volume fraction as percentage of bone volume/total volume ratio (BV/TV), corresponding to the percentage of the bone in the examined surface/field.

3. Results and Discussion

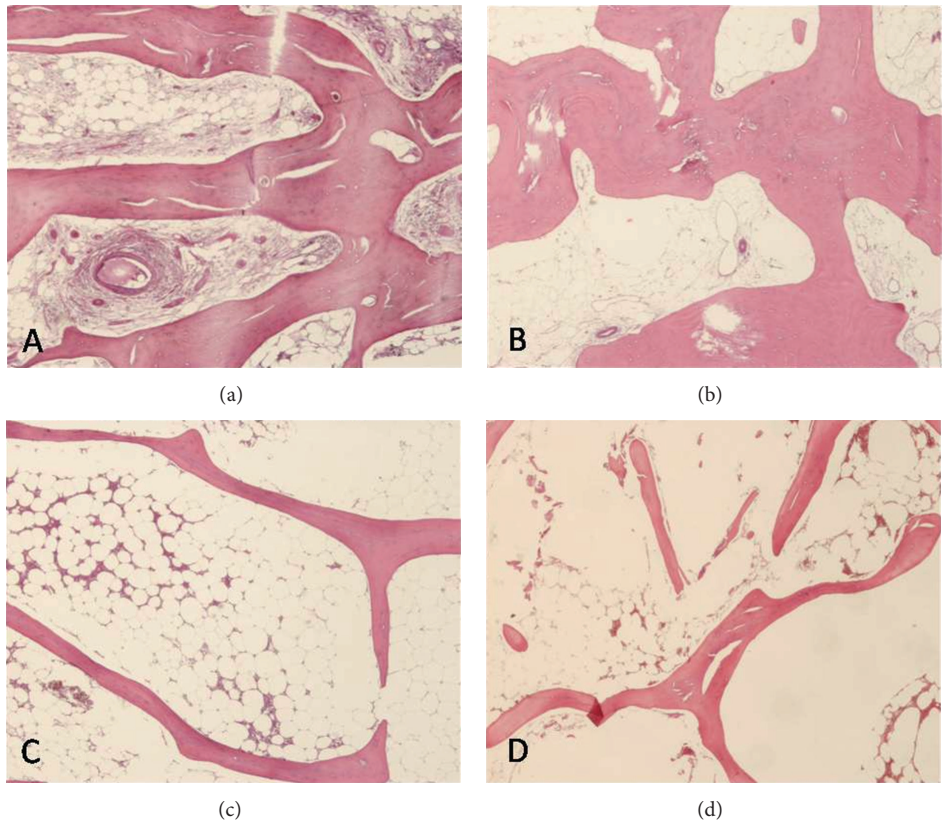
Examples of acquired fields of microscopic bone structure of four age-matched different groups are reported in Figure 1 and clinical features are summarized in Table 1. Regarding the hip functional assessment, OA patients with normal or osteopenic BMD values displayed a higher HHS score (mean value 41.2 ± 8.6 and 33.4 ± 7.2, resp.) compared with osteoporotic OA patients (mean score 25.5 ± 7.6). Spine X-ray examination documented a VCF in 8 patients (40%) with femoral neck fracture, in line with the literature [25]; 4 patients with OA complained of back pain, but only one patient with osteoporotic BMD displayed a VCF.

Histomorphometric analysis of femoral head samples (Table 1) highlighted significant differences in BV/TV between fractured patients and OA patients with normal BMD ($P < 0.0001$) and between fractured patients and OA patients with osteopenic BMD ($P < 0.0001$), while there was no significant difference between fractured and osteoporotic OA patients ($P = 0.975$), neither between OA patients with normal or osteopenic BMD ($P = 0.192$). The identification of a subset of OA patients with osteoporotic or osteopenic BMD suggested that OA and OP can coexist in some cases, with no evident protective role of one disease on the other one. Our results also highlighted a good correlation between clinical score and histomorphometric features. The reduced BV/TV in OA patients suggests a potential risk for the development of OP. Decreased mobility of patients due to severe OA could be able to impair bone quality and probably increase the risk of fracture. Nevertheless, biomolecular pathways involved in the reduction of BV/TV in OA patients are largely unknown. Many factors or biomarkers have been evocated to mediate

Table 1: Comparison of clinical and histomorphometric parameters in the 4 different age-matched groups of patients with hip osteoarthritis and osteoporosis.

	Osteoarthritis	Osteoarthritis + osteopenia	Osteoarthritis + osteoporosis	Femoral neck fracture
Number of patients (male/female)	11/9	11/9	9/11	8/12
Age (years)	71.7 ± 5.2	71.6 ± 7.2	73.0 ± 4.5	71.9 ± 9.0
Harris Hip Score	41.2 ± 8.6	33.4 ± 7.2	25.5 ± 7.6	73.9 ± 13.2*
Bone mineral density (T-score)	0.89 ± 0.85	−1.32 ± 0.84	−2.57 ± 0.51	−2.67 ± 0.51
Bone volume fraction (BV/TV) %	36.49 ± 7.73	32.93 ± 6.83	20.71 ± 5.23	20.77 ± 4.34

Values are ±SD; *calculated on the contralateral femur. P values are reported in the text.



(a) (b) (c) (d)

Figure 1: Example of microscopic images acquired from each group of patients. (a) Patients with osteoarthritis (OA) and normal bone mineral density (BMD), (b) OA patients with osteopenic BMD, (c) OA patients with osteoporotic BMD, and (d) fractured patients with osteoporotic BMD. The latter shows a greater separation among bone trabeculae that appear thinner in patients with osteoporotic BMD than in those with normal or osteopenic BMD. Haematoxylin and eosin staining, original magnification ×40.

bone tissue remodelling. Upregulation of VEGF and its receptors has been shown to be expressed in OA cartilage [26]. Deep chondrocytes normally express antiangiogenic protease inhibitors and their failure can facilitate angiogenic-mediated cartilage remodelling and bone deposition [27]. Insulin growth factor-1 (IGF-1) is the most abundant growth factor in the bone matrix and maintains bone mass in adults. Recently, it has been reported that IGF-1 is markedly decreased in osteoporotic bone in old subjects [28], suggesting a main role of mesenchymal stem cells in the Akt/mTOR pathway mediated bone homeostasis, as also documented for other tissues [29]. During osteogenesis, mesenchymal stem cells also overexpress sortilin [30], and its age-related reduction may cause bone volume loss associated with OP and also pathological vascular remodelling [30, 31]. A better knowledge of mechanisms regulating growth factor expression may suggest new therapeutic opportunities [32, 33] in selected subgroups of OA patients.

The main limit of the present study was the relatively small cohort of patients, which deserves the investigation of additional cases to reinforce the present conclusions. Another limiting aspect was the mainly observational nature

of the study, which should be integrated from the analysis of involved tissue growth factor expression to explain the different behaviour of OA patients also developing OP.

4. Conclusions

It remains an unsolved question whether OA and OP are related or not. In this study, we addressed this problem combining clinical and structural features from OA or fractured patients. Our preliminary data support the hypothesis that hip OA and OP can coexist, for the presence of a specific subgroup of OA patients with reduced BV/TV and high risk of developing OP.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

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Clinical Study

Role of Serum Fibrinogen Levels in Patients with Rotator Cuff Tears

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Although rotator cuff (RC) tendinopathy is a frequent pathology of the shoulder, the real understanding of its aetiopathogenesis is still unclear. Several studies showed that RC tendinopathy is more frequent in patients with hyperglycemia, diabetes, obesity, or metabolic syndrome. This paper aims to evaluate the serum concentration of fibrinogen in patients with RC tears. Metabolic disorders have been related to high concentration of serum fibrinogen and the activity of fibrinogen has been proven to be crucial in the development of microvascular damage. Thus, it may produce progression of RC degeneration by reducing the vascular supply of tendons. We report the results of a cross-sectional frequency-matched case-control study comparing the serum concentration of fibrinogen of patients with RC tears with that of a control group of patients without history of RC tears who underwent arthroscopic meniscectomy. We choose to enrol in the control group patients with pathology of the lower limb with a likely mechanic, not metabolic, cause, different from tendon pathology. We found no statistically significant differences in serum concentration of fibrinogen when comparing patients with RC tears and patients who underwent arthroscopic meniscectomy ($P = 0.5$). Further studies are necessary to clarify the role of fibrinogen in RC disease.

1. Introduction

Rotator cuff (RC) tendinopathy is a frequent disorder of the shoulder, producing great healthcare costs in the industrialized countries and representing the most costly problem in Workers' Compensation Systems after low-back pain [1–4]. The incidence of RC tears ranges from 5% [5] to 39% [6], being the third cause of musculoskeletal disease (16%), after the spine (23%) and the knee (19%) [7].

The clear understanding of the aetiology and aetiopathogenesis of RC tendinopathy remains a challenge [8–11]. Combinations of intrinsic factors (age, gender), extrinsic factors (such as load, sport, and work), and metabolic factors have been described in the development of RC tears [12, 13].

The role of hyperglycemia as a risk factor for RC tear has been investigated [14]. Preliminary reports focused on the analysis of the nonenzymatic glycosylation process, which changes collagen cross-links causing tendon degeneration [15–17]. The presence of statistically significant higher level of fasting plasma glucose in nondiabetic patients undergoing arthroscopic RC repair has been already demonstrated [14]. Other authors showed an association between both type 1 and type 2 diabetes mellitus and chronic RC tendinopathy in men but not in women [18]. These findings confirm the fact that metabolic syndrome may play a role in RC tendinopathy and it may be relevant to predict which patients may have an increased risk of developing RC tendinopathy.

Some authors focused their attention on the correlation between serum levels of lipids and RC tears [19, 20]. The interest in this relationship arises from the potential role of high serum lipid concentration in complete rupture of the Achilles tendon [21, 22]. Fatty degeneration, or

tendolipomatosis, was found in the histopathological examination of specimens harvested during surgery for tendinopathy in the lower limb [23]. However, similar results were not obtained from tendon samples of the RC [24] and the long head of the biceps [25]. In a previous study [26], no statistically significant difference in serum triglyceride and total cholesterol concentrations between patients undergoing arthroscopic RC repair and patients of a similar age undergoing arthroscopic meniscectomy has been reported. On the other hand, Abboud and Kim [19] observed higher levels of total cholesterol, triglycerides, low-density lipoprotein cholesterol, and lower levels of high-density lipoprotein cholesterol in patients with RC tears compared to patients with shoulder pain but without RC tears. Nevertheless, this data was not confirmed by histological/pathological evidence of cholesterol deposition. Consequently, no definitive conclusion on the role of serum cholesterol and triglyceride concentration in the pathogenesis of RC tears can be formulated.

Obesity can be considered another important risk factor for the development of RC tendinopathy. Overweight patients have elevated cholesterol, atherosclerosis, diabetes, hypertension, metabolic syndrome, and decreased physical activity. Since vascular supply is essential for the metabolic processes of the tendons, all these conditions associated with obesity or with increased body mass index (BMI) may represent a cause of decreased vascularity, interplaying in the onset and progression of RC tears. In a recent cross-sectional study [18], abdominal obesity was associated with chronic RC tendinopathy. Furthermore, both obesity and metabolic syndrome are associated with increased concentration of proinflammatory cytokines including IL-1, IL-6, and TNFα [27–32], as well as reactive oxygen species (ROS). Proinflammatory cytokines have been proved to be upregulated inrat and human models of RC tendinopathy [33]. In this respect, raised circulating IL-1, IL-6, and TNFα may aggravate shoulder complaints, for example, by maintaining inflammation. Moreover, they play a crucial role in the apoptosis process, particularly in that induced by oxidative stress, leading to tendon degeneration [34].

The aim of this cross-sectional frequency-matched case-control study was to compare the serum levels of fibrinogen in patients with RC tears and those in patients without history of RC tears who underwent arthroscopic meniscectomy and who were used as control group.

2. Materials and Methods

The study included 164 subjects (72 male and 92 female) who underwent arthroscopic RC repair or meniscectomy at our institution.

Group 1 (study group) included 82 patients (36 men and 46 women; mean age: 57.7 ± 10.2 years, range 29 to 76) who underwent arthroscopic repair of RC tears. The dominant arm was affected in 68 patients. The rotator cuff tears were classified as small (≤3 cm) in 32 patients, medium (3 ≥ 5 cm) in 36 patients, and large (>5 cm) in 14 patients. The tear involved the supraspinatus tendon in 37 patients; both supraspinatus and infraspinatus tendons in 31 patients; and both supraspinatus and subscapularis tendons in 14 patients (Table 1).

Table 1: High fibrinogen serum levels.

	Rotator cuff damage	Controls	P
Patients with elevated FNG	15 (5 male; 10 female)	10 (1 male; 9 female)	0.5

Group 2 (control group) included 82 patients (36 men and 46 women; mean age: 55.9 ± 9 years, range 30 to 73) who underwent arthroscopic meniscectomy for a meniscal tear with no history of RC symptoms. These patients were frequency-matched by age (within 3 years) and gender with patients of Group 1.

2.1. Inclusion Criteria. Patients in Group 1 were included in the study if they had RC tear diagnosed on clinical and imaging grounds and confirmed at the time of surgery. Conservative management, including nonsteroidal anti-inflammatory drugs, physiotherapy, and rest, failed in all patients, and they continued to experience unacceptable pain and weakness in the affected shoulder. None of the patients had undergone prior surgery on the affected shoulder. All patients fulfilled the following criteria: (1) positive rotator cuff lag signs on preoperative examination (at least one among Jobe test, Napoleon test, lift-off test, and Patte test), (2) no episodes of shoulder instability, (3) no radiographic sign of fracture of the glenoid or the tuberosities, (4) magnetic resonance imaging (MRI) evidence of cuff tear, (5) RC tear of 1 or more tendons at arthroscopic examination, and (6) no lesion of the glenoid labrum or of the capsule at arthroscopic examination.

Patients in Group 2 were included in the study if they had a meniscal tear diagnosed on clinical and imaging grounds and confirmed at the time of surgery.

2.2. Exclusion Criteria for All Participants. Patients were excluded in case of primary osteoarthritis of the operated or contralateral shoulder, previous operations on the shoulder or knee, and inflammatory joint disease.

Patients in Group 2 were also excluded from the study if they have had history of shoulder pain or rotator cuff pathology diagnosed by imaging or on clinical grounds.

2.3. Measurement of Serum Fibrinogen Levels. All blood samples were collected in an identical manner between 07.00 and 07.30 after an overnight fast started at 12.00 midnight. Samples were collected into a plastic or siliconized glass tube, 9 parts of freshly drawn venous blood and 1 part of trisodium citrate 3.8%. The plasma was separated after centrifugation of the mixture for 10 minutes at 1500 ×g. The determination of fibrinogen with thrombin clotting time was performed using the method originally described by Clauss (in the presence of an excess of thrombin, fibrinogen is transformed into fibrin and clot formation time is inversely proportional to the concentration of fibrinogen in the sample plasma).

2.4. Statistics. Data were entered in a commercially available database. Descriptive statistics were calculated, and analytical

Table 2: Comparison of fibrinogen serum levels.

Serum fibrinogen values	Group 1 (study group)				Group 2 (control group)			
	Male		Female		Male		Female	
	mg per decilitre	Millimoles per litre	mg per decilitre	Millimoles per litre	mg per decilitre	Millimoles per litre	mg per decilitre	Millimoles per litre
Mean	317.6	0.093	350.3	0.103	311.2	0.091	343.9	0.016
Median	320.5	0.094	353.5	0.104	307	0.09	329.5	0.017
SD	70	0.02	67	0.019	57	0.016	79.6	0.002
Range	171–512	0.05–0.1	238–498	0.019–0.14	211–435	0.062–0.127	205–607	0.009–0.021

statistics were performed with the unpaired sample t-test using Statistical Programs for the Social Sciences (SPSS). P values lower than 0.05 were considered statistically significant.

3. Results

The serum levels of fibrinogen were measurable in all patients. When comparing the two groups, no statistically significant differences in serum concentration of fibrinogen were present ($P = 0.5$) (Table 1). Besides, we were not able to determine any statistically significant differences in serum concentration of fibrinogen in patients with small, medium, or large RC tears. Equally, there were no statistically significant differences in serum concentration of fibrinogen in patients with a supraspinatus tendon tear or supraspinatus and infraspinatus tendons' tear or supraspinatus and subscapularis tendons' tear.

Therefore, for the purposes of this study, all tears were grouped together.

3.1. Group 1. In Group 1 (study group), the mean fibrinogen serum concentration was 335.9 ± 171 mg/dL (range 70–512; median 328.5) (Table 2). Fibrinogen concentration was higher than 400 mg/dL in 15 (18%) patients (5 male; 10 female) (Table 1).

Of these patients, 6 (40%) had a small or medium tear, while 3 (20%) patients had a large tear. In 7 (47%) patients, the RC tear involved the supraspinatus tendon or both supraspinatus and infraspinatus tendons, while in 1 (6%) patient the RC tear involved both supraspinatus and subscapularis tendons (Table 3).

3.2. Group 2. In Group 2 (control group), the mean fibrinogen serum concentration was 329.6 ± 205 mg/dL (range 72–607; median 322.5) (Table 2). Fibrinogen concentration was higher than 400 mg/dL in 10 (12%) patients (1 male; 9 female) (Table 1).

Table 3: Extension of RC tear: RC tendon involved and fibrinogen serum levels.

	High FNG	Normal FNG
Extent of RC damage <3 cm	6 (40%)	26 (39%)
Extent of RC damage 3–5 cm	6 (40%)	30 (45%)
Extent of RC damage >5 cm	3 (20%)	11 (16%)
Supraspinatus	7 (47%)	24 (35%)
Supraspinatus + infraspinatus	7 (47%)	30 (45%)
Supraspinatus + subscapularis	1 (6%)	13 (20%)

4. Discussion

Following the evidences on the relationship between metabolic disorders and RC tendinopathy and taking into account that high serum levels of fibrinogen have been described in different metabolic disorders in which has been demonstrated also an increased incidence of RC tears [34], such as obesity [35], metabolic syndrome [36], hyperglycemia, and diabetes [37], we hypothesized that the serum concentration of fibrinogen could be a predictor factor for the onset of RC tendinopathy.

In the present study, which is a cross-sectional frequency-matched case-control study, the serum levels of fibrinogen obtained from patients who underwent arthroscopic RC repair were compared with the serum levels of fibrinogen obtained from a control group of patients of a similar age who underwent arthroscopic meniscectomy. To our knowledge, this is the first study on this topic.

Patients with RC tear showed no statistically significant difference in serum fibrinogen concentrations compared to subjects of the same age and sex undergoing arthroscopic meniscectomy who had no history of RC injury. However, the serum concentration of fibrinogen was higher in patients with RC tears compared to patients of the control group. Moreover, no statistical significant relationship has been found between extension of the RC tear, as well as RC tendon involved, and serum concentration of fibrinogen.

The activity of fibrinogen has been proven to be crucial in the development of microvascular damage. High concentration of serum fibrinogen determines an increased viscosity of the plasma producing erythrocyte and platelet aggregation, impairing vascular contractility and endothelial integrity with a consequent damage of the microcirculation. High levels of fibrinogen may ultimately produce progression of RC degeneration reducing the vascular supply of tendons.

However, despite several studies [34] showing that RC tears are more frequent in patients with obesity, diabetes, metabolic syndrome, and hyperglycemia and all these pathologies are related to high concentration of serum fibrinogen, the serum concentration of fibrinogen does not correlate with an increased incidence of RC tears.

Although several advances have been made in the surgical management of RC tears, the aetiology of RC tendinopathy is still unclear, and its understanding is fundamental because RC tears represent an important healthcare problem producing elevated costs in the industrialized countries. Combinations of intrinsic (age, gender) and extrinsic factors (such as load, sport, and work), as well as biological factors, were described in the development of injury of the RC.

Intrinsic factors include injuries to the RC via tensile overload, aging, or microvascular supply through traumatic, reactive, or degenerative insults to the tendons [24, 25, 38]. Extrinsic factors include injuries to the RC through compression of the tendons by bony impingement or direct pressure from the surrounding soft tissue [39] or microtrauma [24]. Anyhow, genetics and biological factors play a role in RC pathology. Siblings of patients diagnosed with full thickness RC tears had more than twice the relative risk for developing a lesion and nearly five times the risk of experiencing symptoms than spousal controls [40, 41]. Furthermore, the correlation between tendinopathy and serum levels of some substances was largely demonstrated. The role of hyperglycemia as a risk factor for RC tear has been investigated [14]. We have already showed that statistically significant higher fasting plasma glucose levels within the normoglycemic range have been found in nondiabetic patients undergoing arthroscopic RC repair, compared with patients of a similar age undergoing arthroscopic meniscectomy [14]. Patients with RC tear are likely to have hypercholesterolemia when compared with a control group [42]. We could not find similar results in our population of patients with RC tears and our data suggest no role of the serum cholesterol and triglyceride concentration in RC tears.

Strengths of the present study include the systematic collection of blood samples, the use of preoperative imaging and arthroscopy to diagnose RC and meniscal tears, and the relatively large sample size of our study group. Nevertheless, we acknowledge the cross-sectional nature of the present investigation, which cannot completely resolve issues concerning temporality or rule out other factors that may influence RC tendinopathy. Another limitation of our study was that the control group did not include healthy people. Among the various diseases of the lower limb, we choose to enroll in the control group patients with pathology of the lower limb with a likely mechanic, not metabolic, cause, different from tendon pathology.

On the basis of our study, we think that fibrinogen serum levels do not correlate with RC tears, even though further research is necessary to reach definitive conclusion.

5. Conclusions

Patients with metabolic disorders such as metabolic syndrome, diabetes, obesity, hyperglycemia, and dyslipidemia have an increased incidence of RC tears or tendinopathy when compared with healthy population. However, the molecular processes underlying the onset of RC tendinopathy are still unclear.

The role of serum lipids in RC tendinopathy remains unclear since studies on the topic are discordant. Actually, no definitive conclusion on the role of serum cholesterol and triglyceride concentration in the pathogenesis of RC tears can be made, and further studies are necessary to clarify the issue.

We hypothesized that the serum concentration of fibrinogen may be the link between metabolic disorders and RC tears. In the present study, the serum concentration of fibrinogen was higher in patients with RC tears when compared with patients in the control group. However, this difference was not statistically significant. Moreover, no statistical significant relationship has been found between extension of the RC tear, as well as RC tendon involved, and serum concentration of fibrinogen. On the basis of our study, we doubt that fibrinogen serum levels have a causative role in the pathogenesis of RC tears, even though we advocate more research to reach a definitive conclusion.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Irisin Enhances Osteoblast Differentiation *In Vitro*

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It has been recently demonstrated that exercise activity increases the expression of the myokine Irisin in skeletal muscle, which is able to drive the transition of white to brown adipocytes, likely following a phenomenon of transdifferentiation. This new evidence supports the idea that muscle can be considered an endocrine organ, given its ability to target adipose tissue by promoting energy expenditure. In accordance with these new findings, we hypothesized that Irisin is directly involved in bone metabolism, demonstrating its ability to increase the differentiation of bone marrow stromal cells into mature osteoblasts. Firstly, we confirmed that myoblasts from mice subjected to 3 weeks of free wheel running increased Irisin expression compared to nonexercised state. The conditioned media (CM) collected from myoblasts of exercised mice induced osteoblast differentiation *in vitro* to a greater extent than those of mice housed in resting conditions. Furthermore, the differentiated osteoblasts increased alkaline phosphatase and collagen I expression by an Irisin-dependent mechanism. Our results show, for the first time, that Irisin directly targets osteoblasts, enhancing their differentiation. This finding advances notable perspectives in future studies which could satisfy the ongoing research of exercise-mimetic therapies with anabolic action on the skeleton.

1. Introduction

The benefits of exercise have been widely recognized, indeed the physical activity is reported as the better nonpharmacological treatment for cardiovascular, metabolic, and bone diseases [1, 2]. However, for long time, the molecular mechanisms by which exercise exerts its healthful effects remained mostly unknown. A successful deal for researchers and clinicians should be to reveal these mechanisms, encouraging practicing physical activity and promoting the development of exercise-mimetic drugs.

Recently, several lines of evidence are suggesting that skeletal muscle is crucial in the regulation of energy homeostasis. Therefore, the skeletal muscle is now considered an endocrine organ that secretes a number of myokines including the newly identified Irisin [3]. In this work, Boström and colleagues have reported that physical exercise activity induces an increase of the transcriptional regulator Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC-1α) in the skeletal muscle, which in turn drives the production of the membrane protein Fibronectin type III domain-containing protein 5 (FNDC5). This is subsequently cleaved as the myokine Irisin, which acts on white adipose tissue (WAT), stimulating uncoupling protein 1 (UCP1) expression, one of the master genes of brown adipose tissue (BAT), and activating the browning response [3]. The authors showed that, after 3 weeks of free wheel running, plasma Irisin levels in mice were increased by 65% and, in healthy humans, plasma Irisin levels were found to double after 10 weeks of endurance exercise [3]. These results opened new frontiers for searching the involvement of Irisin in a broader network, suggesting the intriguing feasibility that this myokine might represent an endocrine molecule that could target other organs besides the adipose tissue.

Notably, muscle is important for bone healing and activity. Indeed, the hypothesis that muscle supports bone mass is

confirmed by studies of microgravity and bed rest [4], as well as results showing the associated development of sarcopenia and osteoporosis at the same time [5].

Physical exercise is fundamental for the development of an efficient weight-bearing skeleton. For instance, the exercise strengthens bones, given the evidence that tennis players develop high bone mass in the playing arm compared to the nonplaying arm [6]. On the other hand, the absence of physical exercise or, even worse, the complete disuse of muscles, ensuing by severe pathological conditions, leads to a likewise severe bone loss. For instance, child growing with congenital neuromuscular diseases develop fragile long bones with reduced periosteal circumference [7]. Furthermore, long bones in a paralyzed limb do not achieve their normal ossification and show delay of mineralization in newly laid-down bone matrix [8]. Consequently, this paralytic phenotype, associated with decreased bone mass, is prone to severe fractures as frequently occurs in progressive disease such as spina bifida [8].

Moreover, the propensity of sarcopenic patients to falls can often degenerate into an osteoporotic hip fracture. This event may reduce the life expectancy of up to two years and this corresponds to increased mortality of 20–25% in the first year after the fracture [2]. For this reason, the onset of simultaneous sarcopenia and osteoporosis, also described as "the hazardous duet," has been defined as one of the most devastating threats during old age [2].

Although this tight relationship between skeletal muscle and bone is well recognized, it has hitherto been mainly explained concerning mechanical loading, as overall effect [9]. Therefore, the description of bone response to mechanical load is currently defined as the ability of bone cells to perceive paracrine signals produced by mechanical stimulus [10]. This mechanotransduction effect is highly anabolic in bone. Hence, it could be extremely useful to deepen the understanding of the molecular mechanism involved, revealing the identity of all these signalling-paracrine molecules able to affect bone metabolism. For this reason, we propose a model where exercise induces muscle to release myokines, which regulate mechanotransduction in bone, basing on the physical proximity of these two tissues. Noteworthy, we postulated that the protective effect of muscles on bone could be dependent on the paracrine action of the myokine Irisin. To validate the potential role of Irisin on bone metabolism, we investigated whether Irisin targets bone cells directly, demonstrating its ability to increase the differentiation of bone marrow stromal cells into mature osteoblasts.

The relevance of these findings opens new frontiers in searching the Irisin mechanism of action on bone metabolism. The *in vivo* data, confidently obtained in future, could further correlate the well-known beneficial effects of physical exercise with bone recovery and improvements.

2. Materials and Methods

2.1. Materials. Antibody anti-FNDC5 (Irisin cleaved form) was from Abcam; Antibody anti-Collagen I and β-Actin were from Santa Cruz, Antibody anti-β-Tubulin was from Ori-Gene Technologies. Ascorbic acid, b-Glicerophosphate and Alkaline Phosphatase (ALP) staining kit were from Sigma Aldrich. Primers for qPCR are ALP/S-aaacccagacacaagcattcc; ALP/AS-tccaccagcaagaagaagcc; Coll I/S-ggctcctgctcctcttag; Coll I/AS-acagtccagttcttcattgc.

2.2. Exercise Protocol. 2-month-old C57BL/6 male mice were subjected to 3 weeks of rest activity or free wheel running activity, as described previously [3]. The rest activity was performed isolating each mouse in one cage, in order to avoid their tendency to fight with cage mates, preventing any exercise-mimetic activity. The wheel mice were individually housed, in order to avoid that the dominant mouse in the cage inhibited other mice in the free use of wheel. Animals were euthanized by cervical dislocation and their tissues were surgically excised.

2.3. Primary Cell Cultures. Primary myoblasts were obtained from digestion of vastus lateralis specimens with a solution of trypsin, collagenase, and $CaCl_2$. The isolated cells were preplated on an uncoated petri dish for 1 hour to remove fibroblasts and then transferred on tissue culture plate and cultured with α-MEM/10% FCS. Cells were then cultured for 14 days until multinucleated, spontaneously contracting myotubes were formed. After 3 days, the conditioned media (CM) were collected. Firstly, CM were centrifuged at 1300 rpm to eliminate floating cells. Then, CM were purified by centrifugation at 13 K rmp to eliminate debris.

Bone marrow stromal cells, obtained by flushing bone marrow of 2-month-old C57BL/6 mice, were cultured to induce osteoblast differentiation with α-MEM/5% FCS in the presence of 50 μg/mL ascorbic acid and 10^{-2} M β-glycerophosphate or with 1/2 CM from primary myoblast +1/2α-MEM/10% FCS in the presence of 50 μg/mL ascorbic acid and 10^{-2} M β-glycerophosphate. Thereafter cells were subjected to alkaline phosphatase staining and mRNA and protein analysis.

2.4. RT-PCR. qPCR was carried out after RNA extraction using spin columns (RNasy, Qiagen) according to the manufacturer's instruction. By using SuperScript First-Strand Synthesis System kit (Invitrogen), the resulting cDNA (20 ng) was subjected to quantitative PCR and, thereafter, to ITAQ SYBR Green Supermix with ROX kit (Bio-Rad) on an iCycler iQ5 Cromo4 (BioRad). Each transcript was assayed 3 times, and cDNA was normalized to murine Gapdh, 18S or β-Actin and quantitative measures were obtained using the $\Delta\Delta C_T$ method. Analyses were performed using unpaired Student's t-tests (Excel) for significant differences at $P < 0.05$.

2.5. Western Blot. Protein amounts from all samples were assessed using the BCA-kit (Biorad) followed by protein concentration normalization before all western blot experiments. 30 μg of cell proteins was subjected to SDS-PAGE. Subsequently proteins were transferred to nitrocellulose membranes (Hybond, Amersham). The blots were probed using primary antibodies, described in Materials section, and IRDye-labeled secondary antibodies (680/800 CW) (LI-COR Biosciences). For immunodetection, the Odyssey infrared

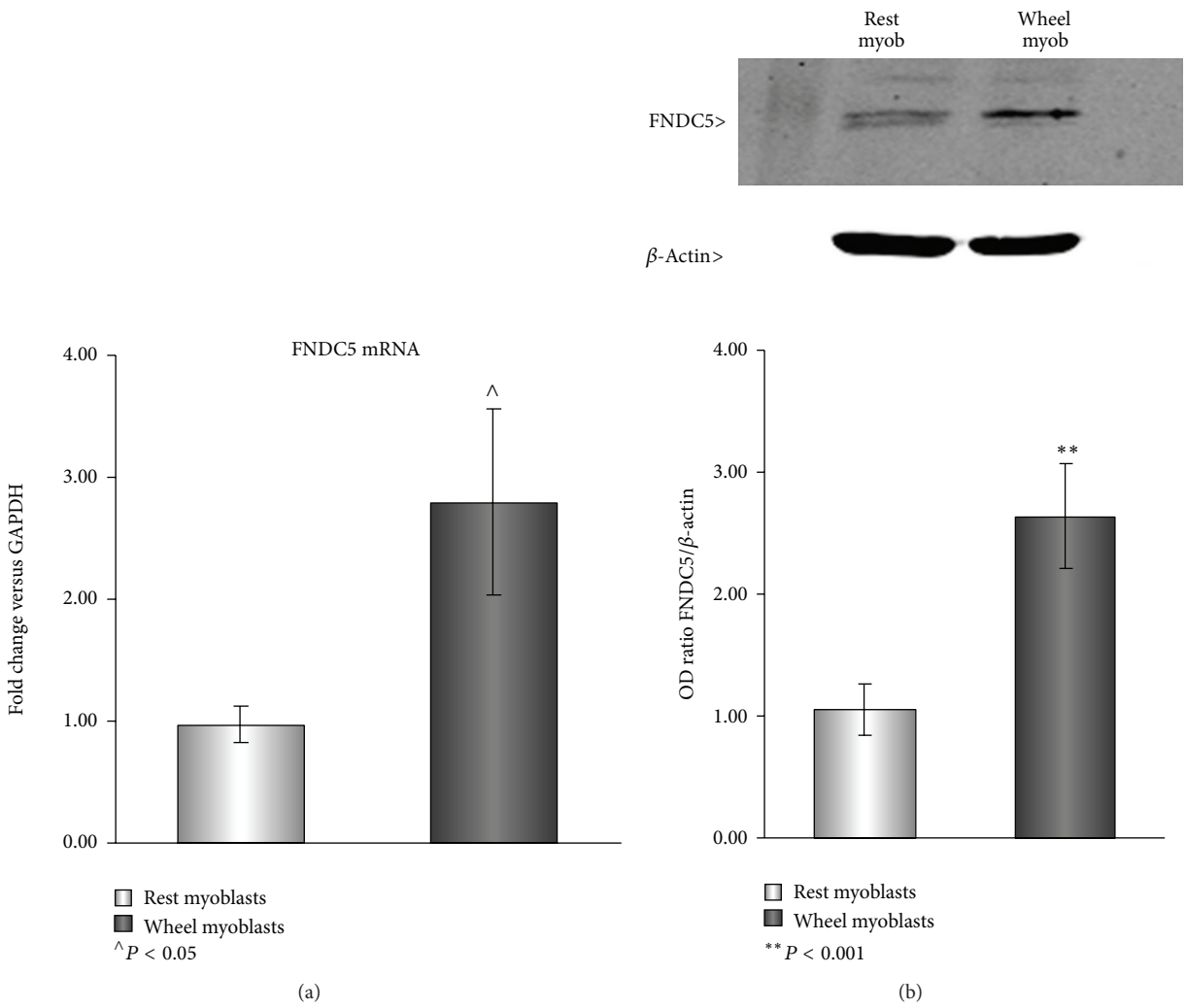

Figure 1: qPCR analysis of FNDC5 in mRNA extracts (a) and western blot analysis of Irisin/FNDC5 in total cell lysates (b) from primary culture of myoblasts obtained from mice subjected to 3 weeks of rest activity or free wheel running activity. $N = 8$ for each group, repeated in 3 separate experiments. Data is presented as mean ± SEM. $^{**}P < 0.001$ and $^{\wedge}P < 0.05$ compared to rest group. Student's t-test was used for single comparison.

imaging system was utilized (LI-COR Corp., Lincoln, NE). All data were normalized to background and loading controls.

3. Results

3.1. Myoblast from Exercised Wheel Mice Express Higher FNDC5/Irisin Than Rest Mice. FNDC5 is highly expressed in skeletal muscle [3, 15]. Therefore, we confirmed the effects of exercise on FNDC5 expression in our exercise regimen, based on 3 weeks of voluntary free wheel running. By qPCR analysis, we detected a 2-fold increase in FNDC5 mRNA of wheel myoblasts (Figure 1(a)). This result was confirmed by the analysis of FNDC5/Irisin protein expression (Figure 1(b)). Indeed, we were able to detect a stronger band, corresponding to FNDC5/Irisin, in cell lysates of myoblasts from wheel mice compared with those from rest mice (Figure 1(b)). These data are, according to previous observations, showing an increase of about 65% in muscle of mice subjected to three weeks of voluntary exercise [3]. It should be further noted that FNDC5/Irisin is slightly detectable also in rest myoblasts, suggesting a constitutive expression of this myokine even in nonexercised state that might be related to a basal metabolism.

3.2. Conditioned Medium from Wheel Myoblasts Enhances Osteoblast Differentiation. In the last years, accumulating evidences have shown that skeletal muscle release hormone-like substances. These secreted proteins are largely myokines and play important regulatory role in intercellular communication [16]. Our model of primary murine skeletal muscle cells allowed us to recapitulate *in vitro* what occurs *in vivo* when muscle is subjected to exercise and releases these circulating myokines. The conditioned medium (CM) collected

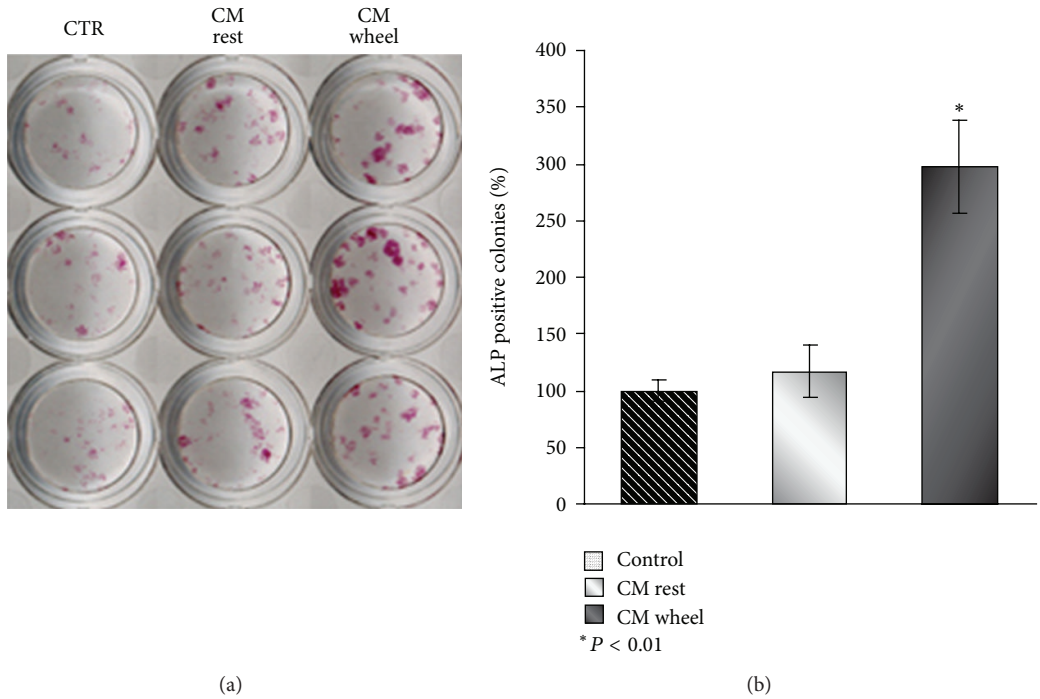

Figure 2: Histochemical staining for ALP in osteoblasts primary culture obtained from mouse bone marrow stromal cells treated with α-MEM/5% FCS in the presence of 50 μg/mL ascorbic acid and 10^{-2} M β-glycerophosphate (CTR) or with 1/2 CM from primary myoblast (rest or wheel) +1/2α-MEM/10% FCS in the presence of 50 μg/mL ascorbic acid and 10^{-2} M β-glycerophosphate. The graph shows quantification of ALP positive colonies as percentage ($^{*}P < 0.01$) compared to control and is representative for 3 independent experiments. Data is presented as mean ± SEM. Student's t-test was used for single comparisons.

from these myoblasts, which likely contained several released myokines, was used to evaluate its ability in regulating maturation of undifferentiated bone marrow stromal cells toward osteoblast differentiation. Our result shows that the CM obtained from wheel myoblasts increased by 2.5-fold the number of alkaline phosphatase (ALP) positive colonies compared to control medium (Figure 2). This assay, based on a histochemical staining for ALP, is the first evidence of osteoblast differentiation, since the ALP enzyme is established as the osteoblastogenesis relevant marker. The result suggests, for the first time, that muscles could exert a direct anabolic effect on osteoblasts through the paracrine action of released myokines, rather than the solely mechanotransduction action on osteocytes, the mechanosensor cells of bone.

3.3. Irisin Secreted from Wheel Myoblasts Increases Alkaline Phosphatase and Collagen I Expression. Given the ability of CM from wheel myoblasts to enhance osteoblast differentiation, we investigated which bone proteins were upregulated. By qPCR analysis, we demonstrated that osteoblasts treated for 3 days with CM from wheel myoblasts have an increased expression of ALP and Collagen I mRNA (Figure 3). These data, further confirming the previously shown increased number of ALP positive colonies (Figure 2), is supported by an enhanced expression of ALP mRNA, the marker gene of osteoblasts. Moreover, the upregulation of Collagen I, the most abundant bone protein, greatly corroborates the beneficial effect on osteoblasts exerted by molecules released from the exercised muscle. Subsequently, our effort has been to obtain evidence about the involved myokine, present in CM, responsible for such a great effect on osteoblast differentiation. Given the increased expression of FNDC5/Irisin seen in myoblasts from wheel mice (Figures 1(a) and 1(b)), we choose Irisin as candidate myokine. For this challenge, we cultured osteoblasts with CM from myoblasts in presence of a neutralizing antibody direct against Irisin. We showed that the increase in Collagen I and ALP was completely reversed by neutralizing Irisin in wheel CM used to treat osteoblasts (Figures 4(a) and 4(b)). This finding demonstrated that the enhanced osteoblastogenesis, induced by exercised muscle, is Irisin-dependent.

It should be noted that the molecular weight of the secreted form of FNDC5/Irisin has remained for long time controversial. Now it is well ascertained that the sequence of mouse FNDC5 is cleaved from aa 29 to aa 151 to give its released form, as Irisin. Being aware of this, we used an anti-FNDC5/Irisin antibody (amino acids 50–150 from Abcam) directed against the predicted Irisin cleaved form.

4. Discussion

Boström and colleagues have recently reported that physical exercise activity induces the release, from skeletal muscle to bloodstream, of the myokine Irisin which was so called (from Iris, the messenger goddess) to highlight its role as positive messenger which targets an endocrine signal from skeletal

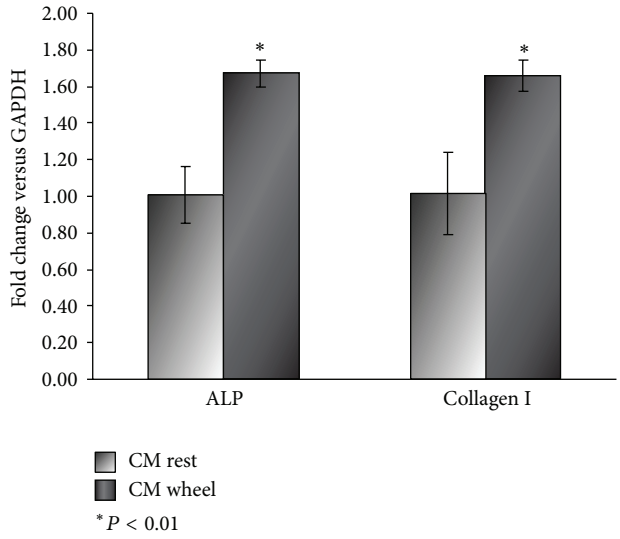

Figure 3: qPCRanalysis of ALP and Collagen I in mRNA extracts from osteoblasts treated with conditioned medium (CM) of myoblasts from rest and wheel mice. $N = 8$ for each group, repeated in 3 separate experiments. Data is presented as mean ± SEM. $^{*}P < 0.01$ compared to rest CM. Student's t-test was used for single comparisons.

muscle to adipose tissue (WAT). Irisin induces browning of WAT (i.e., conversion from WAT to BAT), that is a well-known new avenue for its great therapeutic potential in diabetes and obesity [3].

In the present study, we show that mature exercised primary myoblasts and myotubes secrete factor(s), which increase osteoblast differentiation *in vitro*. We also establish that this enhanced osteoblastogenesis, induced by exercised muscle, is mediated through Irisin. These results presented here add new insights in the complex relationship between muscle and bone tissue, indeed the mechanical influence of skeletal muscle on bone has long been documented but the molecular mechanisms involved remain still poorly understood [2]. Skeletal muscle and bone are tightly connected: they have a common origin, share the same integrated system that provides shape and physical function, and display significant changes across the lifespan. In elderly, the severe decline of skeletal muscle function, known as Sarcopenia, is associated with impaired function of bone (osteopenia). These two simultaneous losses of function lead to increased risk of falls and bone fractures. Therefore, developing a better understanding of the complex relationship between these important components of the musculoskeletal system may reveal new strategies for early identification, prevention, and treatment of sarcopenia and osteopenia, as well as their consequences [2, 8, 17, 18].

Currently, the most effective measure to counteract both diseases is exercise [1], but not all patients can perform a physical exercise program; thus, our evidence that exercise-induced Irisin could account for this effect greatly improves the chances of achieving this goal.

We show here that Irisin directly targets osteoblasts, enhancing their differentiation *in vitro*, proving that myokines, produced by exercised muscle, might be among the molecules regulating mechanotransduction in bone. In our system, mice subjected to three weeks of voluntary exercise showed an increased expression of Irisin/FNDC5 in skeletal muscles.

This result confirms previously published data demonstrating that endurance exercise training for 10 weeks increased plasma Irisin levels in healthy adult [3]. Conversely, Timmons et al. were not able to confirm FNDC5 gene activation by aerobic exercise in younger subjects [19]. These discrepancies have been explained by another study, which demonstrated that Irisin levels increase only when more energy is needed, such as in circumstances where ATP concentration in muscle is strongly decreased [20].

Moreover, we achieved evidence that conditioned medium from primary culture of myoblasts and myotubes, obtained from exercised muscles, were able to enhance the number of alkaline phosphatase (ALP) positive colonies in culture of undifferentiated bone marrow stromal cells.

Noteworthy, this is the first study showing the osteogenic potential of Irisin released from exercised skeletal muscle. From a physiological point of view, this result adds another explanation of the tightly connection between muscle and bone, which share a common fate even in positive scenarios, like in this concomitant gain of mass.

The effects of physical exercise are systemic and, obviously, cannot be solely related to the energy expenditure in muscle [21]. The study of Böstrom et al. [3] reported a new mechanism that explains how the total body energy expenditure is increased by muscle activity, elucidating the molecular circuit triggered by exercised muscle. Analysis of subcutaneous fat tissue depots, in mice overexpressing the muscle-specific PGC-1α, revealed that white adipocytes displayed signatures of brown fat cells [3]. Delineating muscle genes activated by PGC-1α, authors identified the myokine Irisin, able to drive the white-to-brown adipocyte transdifferentiation [3, 22].

By considering the tight relationship between skeletal metabolism and energy homeostasis, clinical and experimental results are giving great importance to the role of BAT on bone metabolism. Due to the evidence that an inducible form of BAT exists during adulthood and given the importance of its ability to dissipate the stored energy with thermogenesis [23–26], BAT induction is becoming a significant promise for the treatment of obesity and metabolic syndrome [13]. A cross-sectional study, carried on 15 young women, has shown a positive correlation between the amount of BAT and bone mineral density (BMD) [27]. Data derived from experimental mice model showed that, FoxC2(AD)(+/Tg) mice, overexpressing FoxC2 as well-established model for induction of BAT have high bone mass due to increased bone formation associated with high bone turnover [28]. On the contrary, mice named Misty (m/m), carrying a very low amount of BAT, albeit partially functional, have accelerated age-related trabecular bone loss and impaired brown fat function, such as reduced temperature and lower expression of PGC-1α [29].

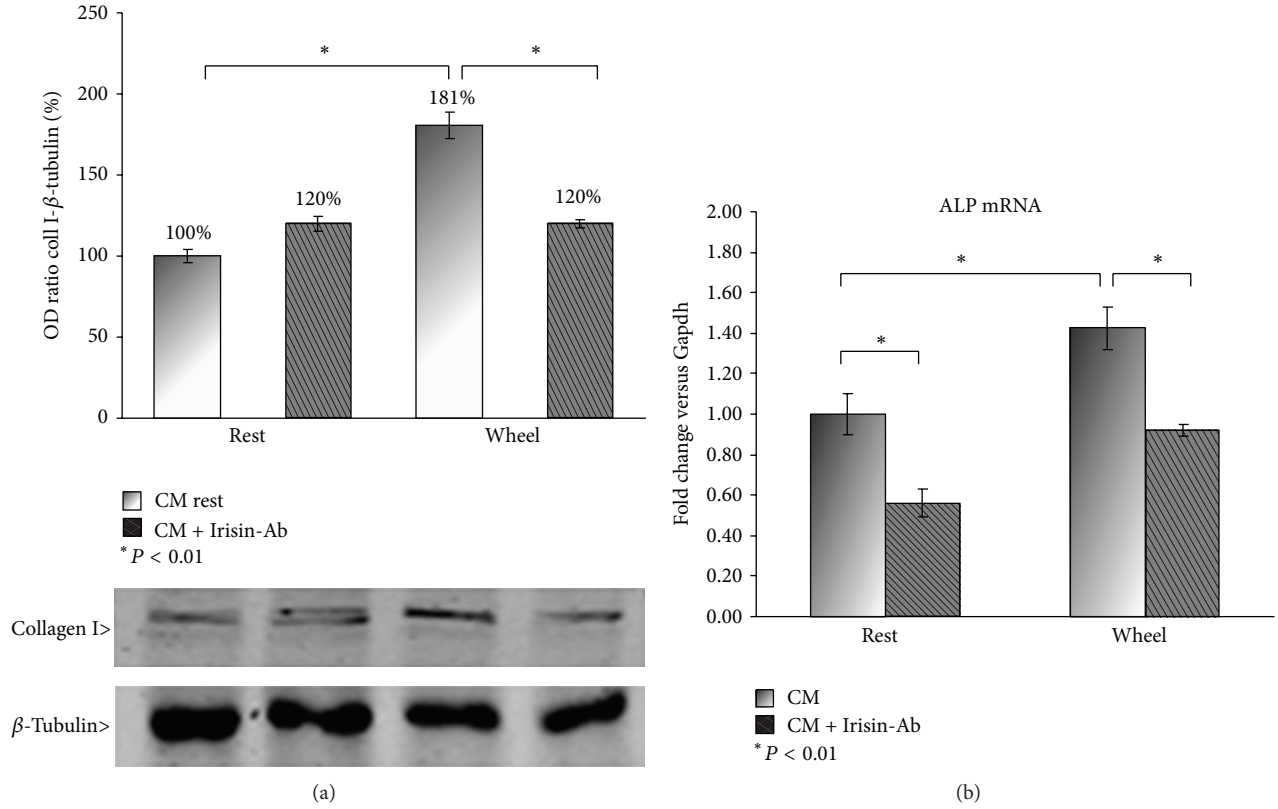

Figure 4: Western blot analysis of Collagen I in total cells lysates (a) and qPCR analysis of ALP in mRNA extracts (b) from osteoblasts treated with conditioned medium (CM) of myoblasts from rest and wheel mice ± Irisin/FNDC5 neutralizing antibody. The graph (a) shows quantification of OD ratio Collagen I/β-Tubulin as percentage ($^{*}P < 0,01$) compared to rest CM and is representative for 3 independent experiments. Data is presented as mean ± SEM. Student's t-test was used for single comparisons.

This growing body of evidences suggests a functional fat-bone axis [30], but the discovery of Irisin, together with our new findings, add a new protagonist to this axis, allowing enlarging it as the muscle-fat-bone axis (Figure 5).

Based on the scenario described, in which both muscle and BAT affect bone metabolism, it might be questioning whether the effect of physical activity on the skeleton could also be mediated by BAT, which in turn has been affected by Irisin. This would imply double, direct and indirect, Irisin action on bone *in vivo*. In our hands the Irisin-dependent action on osteoblasts is further supported by the increase of ALP and Collagen I expression, observed in osteoblasts cultured in the presence of CM from muscle cells of wheel mice. The Irisin involvement was proved by the fact that the CM-induced upregulation of ALP and Collagen I was abolished when cells were treated with CM containing anti-Irisin antibody. This suggests that Irisin does not target only adipocytes but also other body compartments, according to recent data demonstrating that Irisin could play a role in the central nervous system. In this context, recent studies revealed that cerebellar Purkinje cells of rat and mice express Irisin [31], which is also required for the proper neural differentiation of mouse embryonic stem cells [32]. Hence, given that physical activity improves neurogenesis, by reducing risk of neurodegenerative diseases such as Alzheimer and Parkinson [33, 34], Irisin could represent the molecular link between exercise and healthy brain.

5. Conclusions

In conclusion, we showed a novel role of Irisin, adding new evidence to the complex muscle-fat-bone axis (Figure 5). This seems remarkably promising, considering the aforementioned tight relationships between skeletal muscle and bone. Our future efforts will focus on a deepen analysis of the molecular signalling triggered by its action and, in particular, on the overall effect of Irisin in the bone context. Moreover, the characterization of its receptor will allow a more clear understanding of Irisin-induced signalling. In this respect, we would also elucidate whether this myokine affects osteoclasts, the bone resorbing cells. This might better explain the global regulation of skeletal homeostasis exerted by physical exercise or, conversely, by lack of mechanical loading.

Future studies could reveal whether expectations on the potential role of Irisin as pharmacological treatment will be confirmed. Hopefully, with regard to the skeleton, Irisin could represent a new anabolic therapy to gain bone mass in osteopenia caused by muscle-disabling diseases, such as sarcopenia, tumor-associated cachexia, neuromuscular

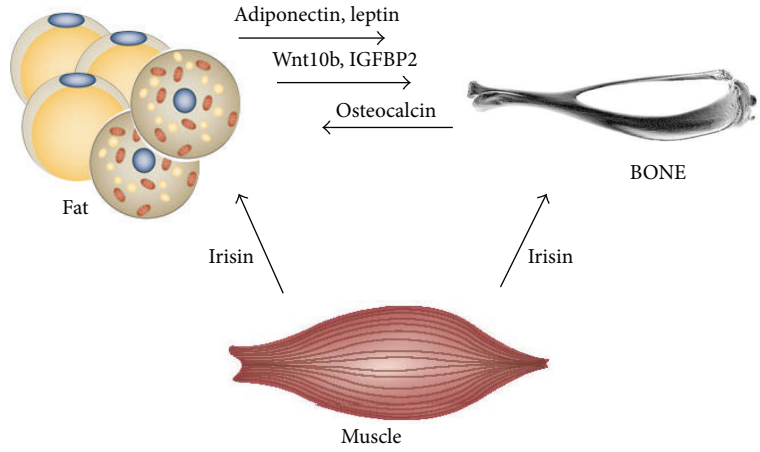

Figure 5: The muscle-fat-bone axis. It is been ascertained that a local network exists between the adipose and the bone tissue, which creates what has been defined as the fat-bone axis. In this paracrine circuit, fat influences bone both positively and negatively by secreting Leptin [11] and Adiponectin [12], respectively. Recently, it has been emphasized the function of brown adipocytes which also affect bone tissue byproducing factors that may be secreted to circulation or act directly in the bone marrow environment to induce osteoblast differentiation and osteocyte support for bone formation and bone turnover. Two of these factors, insulin-like growth factor binding protein 2 (IGFBP2) and wingless related MMTV integration site 10b (WNT10b), gather considerable interest because they regulate both bone remodelling and energy metabolism [13]. Moreover, beside its classical functions, bone acts in turn as endocrine organ secreting Osteocalcin, a hormone active on glucose and fat metabolism, stimulating insulin secretion and β-cell proliferation [14]. Of further significance, the discovering of Irisin, which is released from muscle, acts as endocrine molecule targeting adipose tissue by increasing energy expenditure [3] and bone by enhancing osteoblast differentiation. As shown in this work, Irisin is a new protagonist of the axis, which now could be considered as the muscle-fat-bone axis.

disease, or situations with forced lack of mechanical loading, such as absence of gravity which astronauts undergo.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Mitochondrial DNA Copy Number in Peripheral Blood Is Independently Associated with Visceral Fat Accumulation in Healthy Young Adults

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Aims. Visceral obesity is associated with an increased risk of cardiometabolic diseases and it is important to identify the underlying mechanisms. There is growing evidence that mitochondrial dysfunction is associated with metabolic disturbances related to visceral obesity. In addition, maintaining mitochondrial DNA (mtDNA) copy number is important for preserving mitochondrial function. Therefore, we investigated the relationship between mtDNA copy number and visceral fat in healthy young adults. *Methods*. A total of 94 healthy young subjects were studied. Biomarkers of metabolic risk factors were assessed along with body composition by computed tomography. mtDNA copy number was measured in peripheral leukocytes using real-time polymerase chain reaction (PCR) methods. *Results*. The mtDNA copy number correlated with BMI ($r = -0.22$, $P = 0.04$), waist circumference ($r = -0.23$, $P = 0.03$), visceral fat area ($r = -0.28$, $P = -0.01$), HDL-cholesterol levels ($r = 0.25$, $P = 0.02$), and hs-CRP ($r = 0.32$, $P = 0.02$) after adjusting for age and sex. Both stepwise and nonstepwise multiple regression analyses confirmed that visceral fat area was independently associated with mtDNA copy number ($\beta = -0.33$, $P < 0.01$, $\beta = 0.32$, and $P = 0.03$, resp.). *Conclusions*. An independent association between mtDNA content and visceral adiposity was identified. These data suggest that mtDNA copy number is a potential predictive marker for metabolic disturbances. Further studies are required to understand the causality and clinical significance of our findings.

1. Introduction

The prevalence of obesity is increasing worldwide. Obesity is a well-known risk factor for numerous health problems, including cardiovascular disease (CVD), diabetes mellitus (DM), and cancer [1, 2]. Recent data shows that the regional distribution of body fat, rather than overall obesity, contributes to disease processes [3]. Visceral fat is more metabolically active than subcutaneous fat [4] and affects the development of metabolic disturbances, including insulin resistance [5] and dyslipidemia [6]. The precise role of visceral adiposity in metabolic disturbance is still unknown but proinflammatory cytokines and adipokines secreted by visceral adipocytes are believed to be involved [7].

Mitochondria are organelles that play an important role in the energy synthesis of the cells. Mitochondria synthesize the molecules essential for the body metabolism and influence metabolic homeostasis of the entire body [8]. Mitochondrial function decreases with aging and mitochondrial dysfunction is related to various age-related conditions including type 2 DM and CVD [9]. Mitochondria are highly vulnerable to oxidative damage [10, 11] and mitochondrial dysfunction induced by oxidative damage is considered to contribute to the development of cardiometabolic diseases [9].

Mitochondrial DNA copy number, which reflects the content of mtDNA, is associated with mitochondrial gene stability and mitochondrial biogenesis [12]. Mitochondrial

dysfunction reduces the contents of mitochondria, which is expressed as a decreased mtDNA copy number [12]. Furthermore, reduced mitochondrial DNA content of peripheral blood as well as specific organs was associated with the development of IR, type 2 DM [13], cognitive function [14], and metabolic syndrome [15]. Adipose tissue is the main source of cytokines and adipokines that increase systemic oxidative stress. Thus, obesity may decrease mitochondrial function. Previous results show that human obesity is associated with mitochondrial dysfunction. However, few studies have investigated the quantitative changes in mitochondria according to increased adiposity. The studies that have been performed have yielded mixed results. Furthermore, the potential differences in mitochondrial content according to the regional distribution of adiposity were not fully evaluated.

Adipose tissue is the main source of cytokines and adipokines that increase systemic oxidative stress [16]. Thus, obesity may decrease mitochondrial function. Previous results show that human obesity is associated with mitochondrial dysfunction [17]. However, few studies have investigated the quantitative changes in mitochondria according to increased adiposity. The studies that have been performed have yielded mixed results [18, 19]. Furthermore, the potential differences in mitochondrial content according to the regional distribution of adiposity were not fully evaluated.

Therefore, we investigated the association between peripheral blood mtDNA copy number and visceral fat accumulation among 94 healthy young-aged people.

2. Materials and Methods

2.1. Study Sample. This was a secondary data analysis from the Yonsei Aging Cohort, which was designed to investigate health-related markers among people of various ages [20]. Participants visited the Department of Family Medicine at Severance Hospital for routine health checkups and not for investigations or treatments of specific symptoms or diseases. All subjects participated in the study voluntarily, and written informed consent was obtained from each participant. Questionnaires about lifestyles and underlying medical conditions, overnight-fasting blood tests, and fat measurements with computed tomography were performed as baseline tests. Two additional samples of blood were collected from participants who agreed to store their blood samples for 10 years for future analysis. An additional separate written informed consent was obtained from each participant before performing the additional laboratory test with the stored blood samples.

Because the aim of our study was to investigate the association between visceral obesity and mtDNA copy number in healthy young participants, we selected 203 people aged from 20 to 40 years. Mitochondrial DNA copy numbers were measured with the stored blood samples. Thus we excluded 75 participants who did not agree to store their blood samples. Fifteen additional participants were excluded, because data for their abdominal visceral fat areas were missing. To select a healthy population, participants with histories of hypertension, diabetes mellitus, coronary artery occlusive disease, chronic liver disease, chronic renal disease, or cancer were not included. Subjects who participated in regular exercise were also excluded from the data analysis. Regular exercise was defined as physical exercise or physical work that was performed for more than 30 minutes, three times per week. We also excluded participants who used medications, including antihypertensive agents, lipid-reducing drugs, oral hypoglycemic agents, and nutrient supplements, which could affect cardiometabolic functions. Ninety-four patients were included in our analyses. The study complied with the Declaration of Helsinki, and the institutional review board of Yonsei University College of Medicine approved this study.

2.2. Measurements. All participants were questioned about lifestyle factors, including alcohol consumption and smoking. Alcohol consumption was defined as drinking alcohol more frequently than once per week. Smoking was defined as current cigarette smoking.

Anthropometric measurements were made by a single examiner. After a 10-minute resting period, blood pressure was measured in the sitting position. Body mass index was calculated as weight (kg) divided by height squared (cm^2).

Abdominal fat tissue area was calculated using computed tomography (Tomoscan 350; Philips, Mahwah, NJ, USA) as described previously [21].

Blood samples were collected after at least an 8-hour overnight fasting period. Fasting glucose, high sensitive C-reactive protein (hs-CRP), total cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol levels were measured using an ADVIA 1650 chemistry system (Siemens Medical Solution, Tarrytown, NY, USA). Fasting insulin levels were determined using electrochemiluminescence immunoassays with an Elecsys 2010 (Roche, Indianapolis, IN, USA). Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) index: (insulin [μIU/mL] × fasting blood glucose [mg/dL]/18)/22.5.

2.3. Measurement of Mitochondrial DNA Copy Numbers in Peripheral Blood. To reduce variations in measurements, one examiner measured all parameters throughout the study. mtDNA in peripheral leukocytes was extracted from 1 mL of whole blood using the QIAamp Tissue Kit 250 (Qiagen Inc., Valencia, CA, USA). The relative mtDNA copy number was measured by a real-time polymerase chain reaction (QPCR) and corrected by simultaneous measurement of the nuclear DNA according to the method of Wong and Cortopassi [22] and Liu et al. [11]. Reactions were performed using a Light Cycler-Fast Start DNA Master SYBR Green I kit, purchased from Roche Applied Science (Pleasanton, CA, USA). The forward and reverse primers of β-globin (used to amplify a 268 bp product) were 5′-GAAGAGCCAAGGACAGGTAC-3′ and 5′-CAACTTCATCCACGTTCACC-3′, respectively. The forward and reverse primers of the mitochondrial gene (ND1 gene) used to amplify a 153 bp product were 5′-AACATACCCATGGCCAACCT-3′ and 5′-AGCGAAGGG-TTGTAGTAGCCC-3′, respectively. After denaturation at 95°C for 300 seconds, DNA samples were treated at 95°C for

0.1 seconds, 58°C for 6 seconds, and 72°C for 18 seconds for 40 cycles. A total of 20 ng of DNA was used and the number of PCR cycles to reach this amount of DNA was defined as the threshold cycle (Ct). The following equation was used to quantify the mtDNA copy number relative to β-globin: relative copy number = $2^{\Delta Ct}$ ($\Delta Ct = Ct_{\beta\text{-globin}} - Ct_{ND1}$) [23]. The intra-assay and interassay coefficients of variation of Ct values for the ND1 gene were 4.5% and 5.8%, respectively.

2.4. Statistical Analyses. Normally distributed data are expressed as the mean ± standard deviation (SD). Nonnormally distributed data are expressed as median and interquartile range. mtDNA, fasting insulin, HOMA-IR, total cholesterol, triglyceride, and hs-CRP were log transformed to improve the skewness of the distribution. Pearson correlation analyses were performed to evaluate relationships between mtDNA and other metabolic variables. Stepwise multiple linear regression analysis was performed to identify factors that contributed to mtDNA copy number. If there was a significant correlation ($r > 0.7$) between two variables, only one variable was selected and entered into the model to avoid multicollinearity. In addition, nonstepwise multiple linear regression analysis was performed. Variables with $P < 0.05$ in the univariate analysis and clinically important variables, including age, BMI, and HOMA-IR, were entered into the nonstepwise analysis.

We performed all statistical analyses using the Statistical Package for the Social Sciences, version 18.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

3. Results

The clinical characteristics of the study subjects are shown in Table 1. The mean age of the study subjects was 32.26 ± 9.14 years, and the median (25–75th percentile) mtDNA copy number was 302.08 (48.98–891.25). After adjusting for age and sex, mtDNA copy numbers positively correlated with HDL-cholesterol levels ($r = 0.25$, $P = 0.02$) and negatively correlated with BMI ($r = -0.22$, $P = 0.04$), waist circumference ($r = -0.23$, $P = 0.03$), visceral fat area ($r = -0.28$, $P = -0.01$), and hs-CRP ($r = 0.32$, $P = 0.02$) (Table 2). The mean mtDNA level in the nonsmoking group (2.50 ± 0.81) was significantly higher than that of the smoking group (1.80 ± 0.74, $P < 0.001$). In addition, the mean mtDNA level of female subjects (2.57 ± 0.80) was significantly higher than that of male subjects (2.15 ± 0.83, $P = 0.02$). There were no significant differences in mean mtDNA levels between subjects that consumed alcohol (2.17 ± 0.76) and subjects that did not (2.42 ± 0.88, $P = 0.17$). Figure 1 shows the different relationships between mtDNA copy number and abdominal adiposity according to the regional fat distribution. The mtDNA copy numbers negatively correlated with visceral fat area. However, there was no significant correlation with subcutaneous fat.

In stepwise multiple linear regression analyses, visceral fat area, hs-CRP, HDL-cholesterol, and smoking accounted for 35% of the variance in mtDNA copy number. Thus, these variables were considered to be explanatory variables for mtDNA copy number. In addition, nonstepwise multiple regression analyses indicated that visceral fat, smoking, and hs-CRP levels were independently associated with mtDNA copy numbers, as these variables accounted for 58% of the variance (Table 3).

Table 1: Clinical characteristics of study subjects ($n = 94$).

Variables	Total ($n = 94$)
Age (years)	29.57 ± 0.95
mtDNA copy number[#]	302.08 (48.98–891.25)
Male (n, %)	54 (57.4)
Adiposity index	
BMI (kg/m^2)	27.70 ± 4.36
Waist (cm)	93.06 ± 11.19
Visceral fat area (cm^2)	95.22 ± 45.45
Subcutaneous fat area (cm^2)	245.93 ± 100.14
Blood pressure (mmHg)	
Systolic	125.21 ± 15.59
Diastolic	76.96 ± 12.87
Fasting glucose (mg/dL)	87.77 ± 11.40
Fasting insulin (μIU/mL)[#]	7.51 (4.53–11.84)
HOMA-IR[#]	1.57 (0.97–2.68)
Lipid profile (mg/dL)	
Total cholesterol[#]	181.00 (164.00–215.25)
Triglyceride[#]	89.00 (63.00–131.25)
HDL-cholesterol	52.25 ± 11.92
Hs-CRP[#] (mg/L)	0.46 (0.10–1.42)
Smoking (n, %)	23 (24.5)
Alcohol consumption (n, %)	34 (36.2)

Note: BMI: body mass index; HOMA-IR: Homeostasis Model of Assessment of Insulin Resistance; HDL: high-density lipoprotein; LDL: low-density lipoprotein; hsCRP: high sensitive C reactive protein.
Alcohol consumption was defined as drinking alcohol more frequently than once per week.
Normally distributed data are shown as the mean (±SD).
[#]Non-normally distributed data are presented as medians (25–75 percentiles) and analyzed after log-transformation to correct for skewed distribution.

4. Discussion

Our cross-sectional study revealed a relationship between peripheral blood mtDNA copy number and visceral obesity in a healthy Korean young-aged population. This association remained significant after adjusting for BMI and other confounding factors. In addition, our study showed a significant relationship between mtDNA copy number with smoking, the components of metabolic syndrome (waist circumference, blood pressure, and HDL-cholesterol), and cardiovascular risk factors (systolic and diastolic BP), which is consistent with the findings of previous studies [15, 24].

The mitochondrion is an organelle with diverse functions, including energy synthesis, cellular remodeling, and regulation of cell metabolism. Mitochondrial dysfunction induces various metabolic diseases, including insulin resistance,

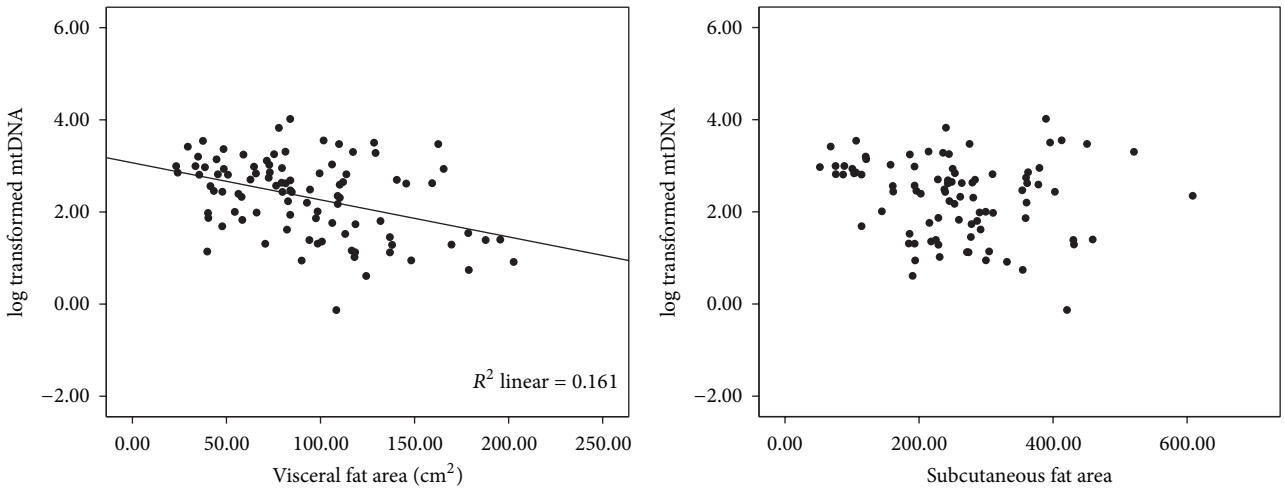

Figure 1: The relationship between abdominal visceral fat area, abdominal subcutaneous fat area, and mtDNA copy numbers. Coefficients (r) and P values were calculated using the Pearson correlation model.

Table 2: The correlation between mtDNA copy numbers[#] and various parameters.

Variables	Unadjusted		Age, sex adjusted	
	r	P-value	r	P-value
Age (years)	−0.28	0.01		
Adiposity index	−0.25	0.01	−0.22	0.04
BMI (kg/m^2)	−0.33	<0.01	−0.23	0.03
Waist (cm)	−0.40	<0.01	−0.28	0.01
Visceral fat area (cm^2)[#]	−0.16	0.14	−0.21	0.09
Subcutaneous fat area (cm^2)				
Blood pressure (mmHg)				
Systolic	−0.22	0.04	−0.11	0.32
Diastolic	−0.29	0.01	−0.15	0.17
Fasting glucose (mg/dL)	0.19	0.09	0.14	0.21
Fasting insulin (μIU/mL)[#]	−0.12	0.23	−0.17	0.12
HOMA-IR[#]	−0.07	0.15	−0.12	0.27
Lipid profile (mg/dL)				
Total cholesterol[#]	−0.09	0.42	−0.03	0.80
Triglyceride[#]	−0.37	<0.01	−0.18	0.09
HDL-cholesterol[#]	0.37	<0.01	0.25	0.02
Hs-CRP (mg/L)	−0.42	<0.01	0.32	0.02

[#]Values analyzed after log-transformation to correct for skewed distribution. Coefficients (r) and P values were calculated using the Pearson correlation model.

Table 3: Multiple regression analyses for mtDNA copy number[#].

(a) Stepwise model

	β coefficient	SE	P-value
Visceral fat area	−0.33	0.00	<0.01
Hs-CRP (mg/L)	−0.32	0.05	<0.01
Smoking[#] (%)	−0.21	0.18	0.01
HDL-cholesterol (mg/dL)	0.21	0.39	0.04

r^2 = 0.35. Variables included in the stepwise model for mtDNA were age, sex, BMI, alcohol consumption, smoking, systolic BP, total cholesterol, HDL-cholesterol, fasting glucose, HOMA-IR and visceral fat area.
To avoid multi-collinearlity, diastolic BP, triglycerides and subcutaneous fat area were not included in the stepwise model.
[#]Values analyzed after log-transformation to correct for skewed distribution.

(b) Non-stepwise model

Variables	β coefficient	SE	P-value
Age (years)	−0.05	0.02	0.30
Male (%)	0.03	0.20	0.89
BMI (kg/m^2)	−0.12	0.03	0.43
Smoking (%)	−0.42	0.20	0.03
Systolic BP (mm Hg)	0.00	0.01	0.69
Visceral fat area (cm^2)	−0.32	0.00	0.03
HDL-cholesterol (mg/dL)	0.21	0.42	0.07
Fasting glucose (mg/dL)	−0.04	0.00	0.66
HOMA-IR	−0.04	0.16	0.41
hs-CRP (mg/L)	−0.19	0.06	0.02

r^2 = 0.58. Variables included in the non-stepwise model for mtDNA were age, sex, BMI, smoking, systolic BP, HDL-cholesterol, fasting glucose, HOMA-IR and hs-CRP.

type-2 diabetes mellitus, and CVD [9]. Multiple biochemical mechanisms, including impaired fatty-acid oxidation, and mitochondrial reactive oxygen stress, explain the link between mitochondrial dysfunction and pathologic conditions [25]. Although mitochondria are present in all types of cells, increasing evidence indicates that mitochondrial function in adipocytes is important for metabolic regulation. In an experimental animal model, rats with visceral obesity showed defective oxidative metabolism and reduced mitochondrial gene expression [26]. Kraunsøe et al. reported that mitochondrial respiration was reduced in the visceral adipose tissues of obese humans compared to that in subcutaneous fat tissues [27]. Furthermore, obese people have been shown to have defective mitochondrial ATP formation compared with that of nonobese people [28]. However, there are mixed

results from human clinical studies regarding the quantitative aspects of mtDNA and regional distribution of adiposity. Yin et al. reported a modest decrease in mtDNA content of omental adipocytes from obese men compared with that of nonobese men [29]. In a Korean study, an inverse relationship was reported between peripheral mitochondrial DNA copy number and visceral fat mass [18]. However, some studies showed no correlation between mtDNA copy number and regional distribution of adiposity. One study showed no correlation between mtDNA copy number and waist-hip ratio which reflects visceral obesity [30]. Furthermore, results that are opposite to those of our study have also been reported [19, 31, 32]. The investigator of those studies suggested that mtDNA content may increase secondary to mitochondrial dysfunction. Therefore, the association between visceral obesity and mtDNA copy number remains unclear. Our study participants were apparently healthy, young subjects from 20–40 years of age without chronic metabolic diseases. Therefore, although we could not determine causality, our results suggest that visceral fat accumulation may affect mitochondrial DNA content in apparently healthy population without metabolic disturbances.

The precise mechanism that explains the association between mitochondrial DNA copy number and visceral fat mass remains unknown. We could not find the causal factor through our cross-sectional study. However, the results suggest possible mechanisms.

First, increased chronic systemic inflammation according to the secretion of proinflammatory cytokines and adipokines may play important roles in the relationship. Visceral adipose tissue is the main site of secretion of proinflammatory cytokines, which induce mitochondrial dysfunction by affecting signaling pathways associated with mitochondrial biogenesis. Visceral adipose tissue is the main site of secretion of proinflammatory cytokines, which induce mitochondrial dysfunction by affecting signaling pathways associated with mitochondrial biogenesis. For example, in cultured fat and muscle tissue, TNF-α depleted endothelial nitric oxide synthase expression along with mitochondrial biogenesis defects and adipocytes with defective TNF-α signaling showed partial recovery of mitochondrial function in obese mice [33]. In our study, increased mtDNA copy number was significantly associated with decreased hs-CRP level which reflects the total inflammatory status of human body. Furthermore hs-CRP level was also positively associated with visceral fat accumulation after it was adjusted for age and sex ($r = 0.5$, $P < 0.05$) (data not shown). Because both visceral adiposity and mitochondrial DNA copy number were associated with systemic inflammatory status, increased inflammation level may explain the observed link between visceral obesity and decreased mitochondrial contents. However, the association between visceral fat accumulation and mitochondrial copy number remained statistically significant after adjustment for hs-CRP, suggesting that the association was, at least in part, independent of systemic inflammation. Furthermore, it is impossible to find out the specific roles of proinflammatory cytokines and adipokines in the observed association in our study. Therefore, measurement of proinflammatory cytokines and adipokines should be performed in the future.

Second, free fatty acids that accumulated in the visceral adipose tissue may affect the decreased mtDNA copy number. Increased levels of free fatty acids promote increased synthesis of toxic fatty-acid-delivered metabolites. These metabolites elevate the level of oxidative stress, driving mitochondrial dysfunction [34]. Therefore increased free fatty acids according to the visceral fat accumulation may induce the decreased mitochondrial contents.

This study has several limitations. First, the cross-sectional design of our study cannot determine a causal relationship between mtDNA copy number and visceral obesity and the small sample size is another limitation of the current study. Although the correlation was statistically significant, the low power was another limitation. Therefore, we cannot generalize the results to the population at large. In addition, we did not perform fat biopsy, which is the gold standard for investigation of mitochondrial function. However, it is easier to obtain peripheral blood leukocytes than muscle tissue. And decreased mtDNA copy number in peripheral blood leukocytes correlated well with mitochondrial dysfunction in skeletal muscle [35, 36]. Finally, this study did not measure levels of proinflammatory cytokines and adipokines. Therefore our study cannot directly investigate the role of cytokines and adipokines as mediators between visceral adiposity and reduced mitochondrial DNA copy number. We agree that assessing inflammatory cytokine and adipokine levels will provide additional important information in future studies.

In conclusion, our study shows that peripheral blood mtDNA copy number is associated with abdominal visceral fat area in 94 healthy young-aged subjects. Although the causal direction of the relationship between mtDNA copy number and visceral obesity cannot be determined, our study collectively suggests that decreased mitochondrial contents may be a mediator that links visceral obesity and metabolic disturbances. Further studies are required to better understand the pathophysiological and clinical significance of our findings.

Conflict of Interests

The authors declare there is no conflict of interests.

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Research Article

Negative Influence of a Long-Term High-Fat Diet on Murine Bone Architecture

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A correlation between obesity and bone metabolism is strongly assumed because adipocytes and osteoblasts originate from the same precursor cells and their differentiation is conversely regulated by the same factors. It is controversially discussed if obesity protects bone or leads to loss of bone mass. Thus, the aim of the present study was to investigate the influence of diet-induced mild obesity (11% increased body weight compared to control) on bone microstructure in mice. Four-week-old male C57BL/6J mice received a high-fat diet (HFD, 60% kcal from fat) and were analyzed by means of dual X-ray absorptiometry, histological methods, real-time RT-PCR, and transmission electron microscopy in comparison to control animals (10% kcal from fat). The cancellous bone mass, collagen 1α1 expression, amount of osteoid, and cohesion of cells via cell-to-cell contacts decreased in HFD mice whereas the bone mineral density and the amount of osteoblasts and osteoclasts were not modified. The amount of apoptotic osteocytes was increased in HFD mice in comparison to controls. We conclude that moderately increased body weight does not protect bone architecture from age-dependent degeneration. By contrast, bone microstructure is negatively affected and reduced maintenance of cell-cell contacts may be one of the underlying mechanisms.

1. Introduction

Obesity is characterized by a body-mass index of $\geq 30\,kg/m^2$ and excessive body fat accumulation [1]. Globally, estimated 502 million adults and 170 million children were classified as overweight or obese in 2008 and the rates of obesity are still increasing [2]. Obesity is associated with many chronic disorders such as type 2 diabetes mellitus, coronary heart disease, sleep-breathing disorders, certain cancers, and osteoarthritis [1]. A correlation between obesity and bone metabolism is strongly assumed. Bone forming osteoblasts are derived from stem cells that can also give rise to adipocytes and chondrocytes [3]. Stem cells proliferate and differentiate into preosteoblasts and finally into mature osteoblasts that are characterized by cessation of cell division, production of bone matrix, and synthesis of several essential marker enzymes and proteins for bone formation, for example, collagen type 1, osteocalcin, and alkaline phosphatase (ALP). Collagen type 1 is with approximately 90% of the main component of the bone matrix. In lamellar bone, its fiber organization allows the highest density per unit volume of tissue. In the biomechanically weaker woven bone collagen fibers are not so tightly packed and found in randomly oriented bundles. Crystals of hydroxyapatite are situated on the collagen fibers, within them, and in the matrix around them. They tend to be oriented in the same direction as the collagen fibers. ALP is the main enzyme for formation of hydroxyapatite crystals and is therefore important for mineralization of bone and bone mineral density (BMD). Besides the collagens several non-collagenous proteins are present in the bone matrix. Osteocalcin is the major non-collagenous protein. It is produced by osteoblasts, makes up 1% of the matrix, and plays a role in calcium binding and stabilization of hydroxyapatite in the matrix [4]. Factors stimulating formation of bone

are inhibiting adipogenesis and vice versa [3]. For example, mechanical loading promotes osteoblastogenesis and inhibits differentiation of stem cells into adipocytes by increasing stable beta-catenin and reducing peroxisome proliferator-activated receptor-gamma (PPARγ) whereas stimulation of PPARγ decreases osteoblast differentiation and enhances adipogenesis [5–10]. However, the loss of bone mass determined in aging, osteoporosis, and after administration of glucocorticoids is associated with an increase in bone marrow adipogenesis (reviewed in [10]). The underling mechanisms for these effects are still unknown. The involvement of adipocyte-derived proinflammatory cytokines and hormones is discussed [10–12]. Since several studies focused on obesity associated with low-grade chronic inflammation, there is an increasing amount of reports describing detrimental effects of excessive body fat on bone [13–15]. There is a higher incidence of clinical fractures in obese postmenopausal women [16] and in overweight adolescents [17–19]. Several animal studies supported this negative effect on bone strength [20, 21], bone mineral density [22], and bone formation [15]. However, the traditional view of obesity is that overweight is beneficial to bone [11, 23–25] since the femoral neck of obese women especially with osteoporotic bones showed a reduction in fracture risk [25] and an increase in BMD [23]. The enhanced BMD on the weight-bearing site implies that the mechanical effect of overweight stimulates bone mineralization [24] in addition to upregulation of bone formation enhancing molecules, for example, adipocytic estrogens [26]. However, fat and bone are linked by multiple pathways. Thus, we analyzed in the present study the bone density, the cellular structures, and the molecular bone markers. Since Cao et al., 2010, [27] reported detrimental effects on bone in a murine obesity model with high-fat diet (HFD) determined by means of μCT experiments, we hypothesized that our investigation will also point out negative effects of obesity on bone properties using several cell biological methods for investigation of in vivo bone samples. The expected results will gain new insights into the mechanism underling alterations of bone structure by obesity.

2. Materials and Methods

2.1. Animals and Experimental Model. All animal procedures were approved by the local ethics committee (GI20/11-Nr. A17/2010) and conducted in accordance to the Declaration of Helsinki. Seven 4-week-old male C57BL/6J mice (Janvier, France) were fed for 23 weeks with a high-fat diet (Altromin, Lage, Germany, 60% kcal from fat) and six with normal diet (Altromin, 10% kcal from fat). The animals were kept under a 12-hour (h) light and dark cycle and had free access to chow and water. The body weight was measured every week. At the age of 27 weeks the animals were euthanized by inhalation of CO_2 and directly scanned via Dual-X-Ray Absorptiometry (DXA, lunar prodigy, GE Healthcare, Munich, Germany) for determination of bone mineral density (BMD). Afterwards bones were extracted and used for conduction of cell and molecular-biological methods.

2.2. Histology. Samples of femur and vertebrae L2 were fixed in 4% phosphate buffered paraformaldehyde (Carl Roth, Karlsruhe, Germany) and demineralized in 10% ethylene diamine tetra acetic acid (Merck, Darmstadt, Germany) in 0.281 M Tris-buffer. The samples were dehydrated using increasing ethanol concentrations (70%, 80%, and 96% each for 2 h, 3x 100% for 3 h) and incubated in xylol (Carl Roth, 3x 1 h) and afterwards in liquid paraffin. After blocking out, paraffin sections were cut with a thickness of 4-5 μm at the rotation microtome (RM 2155, Leica, Bensheim, Germany). Sections were stained with hematoxylin and eosin (HE, Merck) or used for enzyme- or immunohistochemistry (IHC).

2.3. Tartrate-Resistant Acidic Phosphatase (TRAP). To detect macrophages and osteoclasts the tartrate-resistant acidic phosphatase (TRAP) enzyme histochemistry was performed. Therefore paraffin sections were deparaffinized with xylol and a decreasing series of ethanols. After washing in 0.1 M acetate buffer (pH 5.2) sections were incubated in a solution of naphthol-AS-TR-phosphate (Sigma-Aldrich, Steinheim, Germany), di-sodium-tartrate-dihydrid (Merck), and fast red TR salt (Sigma-Aldrich) in acetate buffer at 37°C for 30 minutes (min). After washing in aqua dest., sections were counterstained with hematoxylin and coverslipped with Kaisers Glyceringelatine (Merck).

2.4. Alkaline Phosphatase (ALP). The rehydrated paraffin sections were incubated in a solution of 5-bromo-4-chloro-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT, KPL, Gaithersburg, MD, USA) salt for 45 min in a moist chamber at 37°C. After thoroughly washing in aqua dest., sections were counterstained with nuclear fast red, dehydrated, and coverslipped with DePex (Serva, Heidelberg, Germany).

2.5. Immunohistochemistry. Immunohistochemical incubation with an antibody for detection of collagen-1 was conducted in the present study to evaluate bone architecture. Therefore rehydrated paraffin sections were treated with a Tris-NaCl buffer (TBS, pH 7.4) containing 0.025% Triton-X-100 (Merck). Afterwards the endogenous peroxidase was blocked by incubation with 3% H_2O_2. After washing in TBS, the sections were incubated with the primary collagen-1 antibody (Biomex, Heidelberg, Germany) in a dilution of 1 : 50 in diluting buffer (Dako, Hamburg, Germany) overnight at 4°C. After washing in TBS, sections were incubated for 30 min with a biotinylated goat anti-rabbit secondary antibody (dilution 1 : 500; Vector Laboratories Inc., Burlingame, CA, USA) and subsequently with the streptavidin-biotin-peroxidase complex (Vector Laboratories Inc.). To visualize the peroxidase a Nova-Red substrate kit (Vector Laboratories Inc.) was used and according to the manufacturer's protocol with an incubation time of 5 min. The nuclei were counterstained with hematoxylin and the sections were coverslipped with DePex. As negative control the procedure was performed omitting the first antibody.

All labeled paraffin sections were evaluated light microscopically with a photomicroscope (Axiophot-2, Zeiss, Jena,

Table 1: Primers used for RT-PCR.

Targets	Sequence	Length (bp)	Annealing (°C)	Accession
β-actin				
fwd[1]	TGTTACCAACTGGGACGACA	165	58	NM_007393.3
rev[2]	GGGGTGTTGAAGGTCTCAAA			
Bglap[3]				
fwd	TTCTGCTCACTCTGCTGACC	111	58	NM_007541.2
rev	TATTGCCCTCCTGCTTGGAC			
ctsK[4]				
fwd	GAGGCGGCTATATGACCACT	119	58	NM_007802.3
rev	CTTTGCCGTGGCGTTATACA			
coll[5]				
fwd	TGGCATCCCTGGACAGCCTG	144	62	NM_007742.3
rev	ATGGGGCCAGGCACGGAAAC			

[1]Forward; [2]reverse; [3]bglap: gene name of osteocalcin; [4]cathepsin K; [5]collagen 1α1.

Germany) equipped with a digital camera (DC 500, Leica, Bensheim, Germany).

2.6. Bone Histomorphometry. For quantification of two dimensional trabecular regions in relation to the whole tissue histomorphometrical analyses were performed according to the methods described by Dempster et al., 2013, [28]. In brief, an area of interest from the tissue was defined (in mm^2) in which the trabeculae were marked and calculated in mm^2 with the Image-Pro Plus Software (Media Cybernetic, Maryland, USA). Afterwards the relation was calculated in percentage. As area of interest the metaphyseal region of the proximal and distal femur and the spongious part of the corpus vertebrae were used.

2.7. Real-Time RT-PCR. For expression analyses humeri and vertebrae L3 were collected in RNA-later (Ambion Applied Biosystems, Foster City, CA, USA), homogenized with a mortar, and transferred into 1 mL Trizol (Invitrogen, Darmstadt, Germany). After 5 min 200 μL chloroform (Sigma-Aldrich) was added, the samples were centrifuged (12,000 g, 15 min, 4°C), and the upper phase containing the RNA was transferred into a new cup. Isopropanol (0.5 mL) was added and total RNA precipitated by centrifugation (12,000 g, 10 min, 4°C).

For reverse transcription of total RNA the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) was used. In brief, 1 μg RNA was cleaned from genomic DNA by incubation with 2 μL DNA Wipeout buffer for 2 min at 42°C. Afterwards RNA was transcribed to cDNA with 1 μL Quantiscript reverse transcriptase, 4 μL buffer, and 1 μL primer mix containing Oligo(dT)s and random-primers at 42°C for 30 min. Reverse transcriptase was inactivated at 95°C for 3 min. Subsequent real-time RT-PCR was performed in the Lightcycler (Roche, Rotkreuz, Schweiz). Therefore 2 μL of cDNA was added to 2 μL Roche reagent (LightCyclerFastStart DNA Maser SYBR Green I, Roche, Mannheim, Germany), 0.2 μL forward and reverse primer (Eurofins MWG Operon, Ebersberg, Germany, Table 1), and 6.8 μL RNase free water. The samples were incubated for 10 min at 95°C, followed by 40 cycles of 5 seconds (sec) heating at 95°C, annealing for 5 sec at 58–62°C, and elongating for 5 sec at 72°C. Subsequently the PCR product was controlled by melting curve and gel electrophoresis. As controls RT negative controls were performed where the reverse transcription was conducted without the enzyme. In addition negative controls were made where the template was omitted and water was used instead (water control). Using the Lightcycler software Cp-values were measured, normalized to the reference gene β-actin and the $\Delta\Delta$Cp, and relative expression was calculated according the $\Delta\Delta$Cp method.

2.8. Transmission Electron Microscopy (TEM). Vertebrae L4 were fixed in yellow fix (2% paraformaldehyde, 2% glutardialdehyde and 0.02% picrinic acid in 0.01 M phosphate buffer, pH 7.2) for 6 h. After washing in 0.1 M cacodylate buffer samples were incubated for 2 h in 1% osmium tetroxide solution. Afterwards they were dehydrated through an increasing ethanol series, washed 3x in xylol, and incubated in a solution of xylol and Epon (Serva). Finally the samples were polymerized in Epon and cut into semi-thin (500 nm) and ultra-thin sections (60–80 nm). The semi-thin sections were stained with toluidine blue and safranin-O and evaluated with a light microscope. The ultra-thin sections were contrasted with uranyl acetate and lead citrate and analyzed with a TEM (LEO EM 912, Zeiss, Oberkochen, Germany) equipped with a CCD-Camera (Olympus, Münster, Germany).

2.9. Statistical Analysis. The SPSS software (version 21.0; SPSS Institute Inc, Chicago, IL, USA) was used for statistical analysis. Comparisons were performed by the Mann-Whitney test. A P value of less than 0.05 indicates a significant difference.

3. Results

3.1. Body Composition. Body weight was recorded weekly. During the experimental period of 23 weeks the weight of

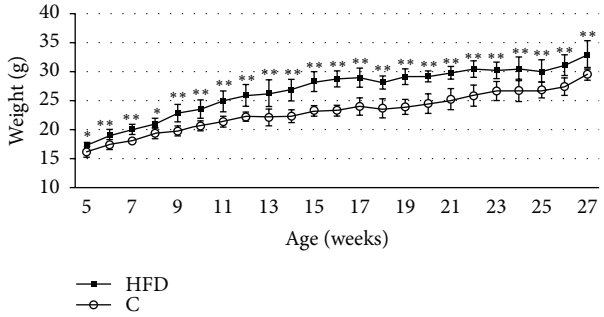


Figure 1: Body composition. The body weight was significantly upregulated in the high-fat diet (HFD, 60% kcal from fat) mice ($n = 7$) compared to control (C) mice ($n = 6$). Significant data were labeled with $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$. Data are shown as mean ± SD.

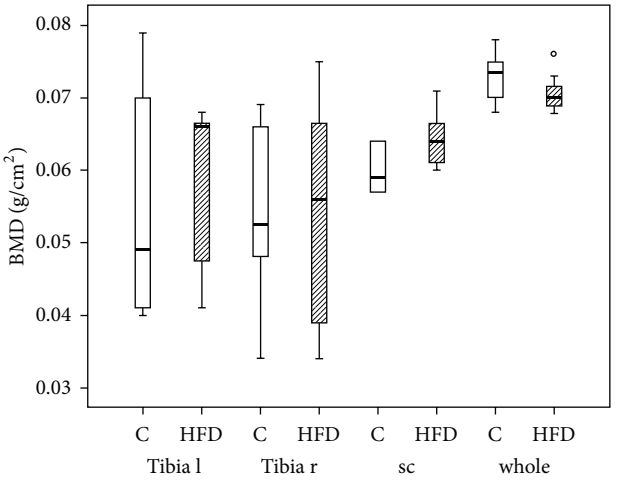


Figure 2: Bone mineral density. DXA measurement revealed no significant differences in bone mineral density (BMD) between the mice receiving high-fat diet (HFD; $n = 7$) and the controls (C; $n = 6$). Data are presented as box plot with the median indicated by solid line within the box. The circle represents data beyond 1.5x the interquartile range of the median. l = left, r = right, sc = spinal column, and whole = whole body.

mice receiving a HFD (60% kcal of energy from fat) was 11% higher ($P = 0.001$) than that of control animals (10% kcal of energy from fat) (Figure 1).

3.2. Bone Mineral Density. DXA-scan did not reveal significant differences in bone mineral density between both experimental groups (Figure 2).

3.3. Light Microscopic Observations. The trabecular structure was evaluated using Epon embedded calcified semithin sections (Figure 3) and demineralized paraffin sections (Figure 4) that were stained routinely with hematoxylin and eosin (HE) (Figures 4(a), 4(b), 4(e), and 4(f)) or immunohistochemically labeled with an antibody against collagen-1 (Figures 4(c) and 4(g)). Semi-thin and HE sections demonstrated that the trabeculae of animals fed with HFD were smaller (Figures 3(a) and 3(b)), peaked at the ends (rod-like shape, Figures 4(b) and 4(f)), and less linked with other trabeculae (Figures 3(a) and 3(b)). The lamellar structure was often dissolved inside the trabeculae so that there was space in between the different lamellae (Figure 4(f)). In the HFD group the trabeculae contained more woven bone and less lamellar bone than in the control group as shown by collagen-1 IHC (Figures 4(c) and 4(g)). Furthermore the animals with high-fat diet exhibited less osteoid and more megakaryocytes in the bone marrow of the same samples (Figure 3(b)). However, no changes could be observed in the amount, distribution, and size of osteoclasts identified with the TRAP enzyme histochemical staining (Figure 4(h)). The ALP staining could not point out differences between both animal groups. No alterations were shown in the labeling intensity, amount, and distribution of ALP (Figure 4(c)).

Table 2: Histomorphometry.

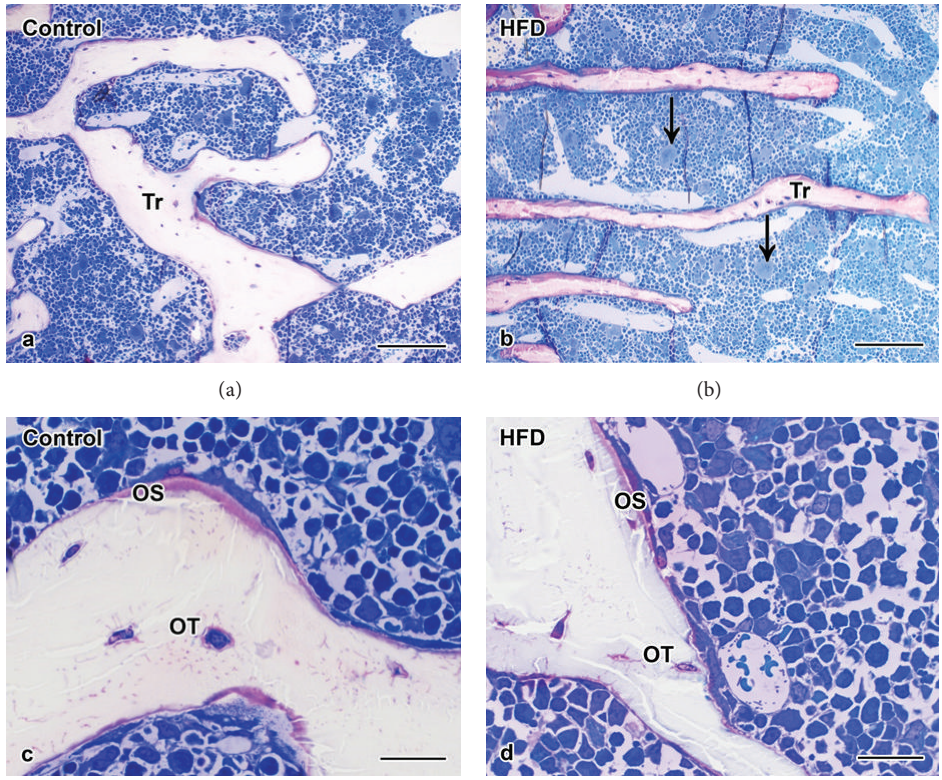
Region	Group	Bone area (%)	SD	P value
Proximal femur	C	21.0009	±2.0087	$P = 0.065$
	HFD	16.8641	±4.2989	
Distal femur	C	22.0639	±5.0217	$P = 0.026$
	HFD	16.7619	±1.7276	
Vertebra L2	C	23.5852	±2.657	$P = 0.101$
	HFD	19.854	±3.961	

HFD: high-fat diet, $n = 7$; C: control mice, $n = 6$.

3.4. Histomorphometry. The ratio of bone area in correlation with tissue area was measured using histomorphometry. The bone area was calculated as percentage (%) of the whole tissue. Comparing the average of the values a distinct decrease in bone was observed for all measured areas (proximal, distal femur, and vertebrae, Table 2). However, because of the high standard deviation (SD) only the values of the distal femur showed significant changes ($P = 0.026$, Table 2).

3.5. Real-Time RT-PCR. The expression of collagen 1α1 was significantly downregulated in humeri ($P = 0.002$, relative expression 0.34 ± 0.19) and vertebrae ($P = 0.004$, relative expression 0.4 ± 0.14) whereas osteocalcin and cathepsin K were not significantly changed due to HFD (Figure 5).

3.6. Ultrastructure. TEM analysis further confirmed the presence of less osteoid in HFD mice compared to controls (Figure 6). Even if collagen fibrillae were found the striation was less distinct than in control mice. Besides, we observed a dissolving of the cell-cell and cell-matrix connections (Figure 6(d)). Spaces were formed between the single osteoblasts. In addition, osteoblasts included less rough endoplasmatic reticulum (Figure 6(d)). The membrane of osteocytes and osteoblasts was sometimes leaky and folded (Figures 6(e) and 6(f)) and more apoptotic cells were found in the HFD group.



(a) (b)

(c) (d)

Figure 3: Microstructure of cancellous bone in mineralized semi-thin sections. The trabeculae of (a) control mice were wider and more connected than the trabeculae of (b) HFD mice. Higher magnification revealed that (c) control mice dispose more osteoid than (d) HFD mice. Tr = trabculae, OS = osteoid, OT = osteocyte, C = control mice, and arrow = megakaryocytes. Scale bar: 100 μm in ((a)-(b)) and 20 μm in ((c)-(d)).

4. Discussion

The presented study was conducted to analyze the bones of male mice (strain C57BL/6J) after feeding with a HFD (60% kcal from fat) for 23 weeks. The diet started at an age of 4 weeks. This animal procedure is a well-known model for induction of obesity that is used in a number of studies [15, 29–31]. Obesity is one of the most important risk factors for several musculoskeletal disorders [32]. It remains still unclear through which mechanism the adipose tissue affects the musculoskeletal system. It is known that additional load leads to functional and structural limitation of the soft structures such as tendons [33]. Furthermore overweight results in less physical activity [34] and that causes a loss of bone mass [35]. On the other hand adipose tissue secretes adipose-derived hormones and cytokines (e.g, TNFα, IL-1, and IL-6) leading to a low-grade systemic inflammation [12, 13, 15]. These factors are also involved in the cross talk of bone cells [14]. However, relation between obesity and bone is still controversially discussed, and therefore the aim of the present study was to investigate the bone structure of obese mice in comparison to normal animals by means of DXA, histological methods, histomorphometrical measurement, real-time RT-PCR, and transmission electron microscopy.

DXA-scan allows the calculation of BMD which in our study showed no differences between obese mice and controls getting normal chow (10% kcal from fat). A close correlation of body weight and BMD, however, was found in humans where an approximate increase in 10 kg body weight causes a 1% increase in BMD [36]. This positive correlation is stronger in women than in men and in postmenopausal than in premenopausal women. This effect could be explained by the conversion of androgens into estrogen in adipocytes and by the protective role of estrogen against osteoporosis and bone loss (reviewed in [10]). Besides, Ehrlich and Lanyon described that the biomechanical loading of the additive weight stimulates bone formation and therefore increases bone mass and BMD [37]. Contrasting studies demonstrated that the bone strengthening effects of heavy bodies were not only due to adipose tissue but also due to elevated muscle mass [38]. However, in the present study no significant alterations were measured regarding the BMD.

Changes in bone mineral density are often induced by an imbalance in the amount and activity of bone forming osteoblasts and bone resorbing osteoclasts. Enzyme histochemistry of alkaline phosphatase is a common method for testing the activity of osteoblasts [39]. ALP activity showed no alteration in bone sections from mice receiving HFD

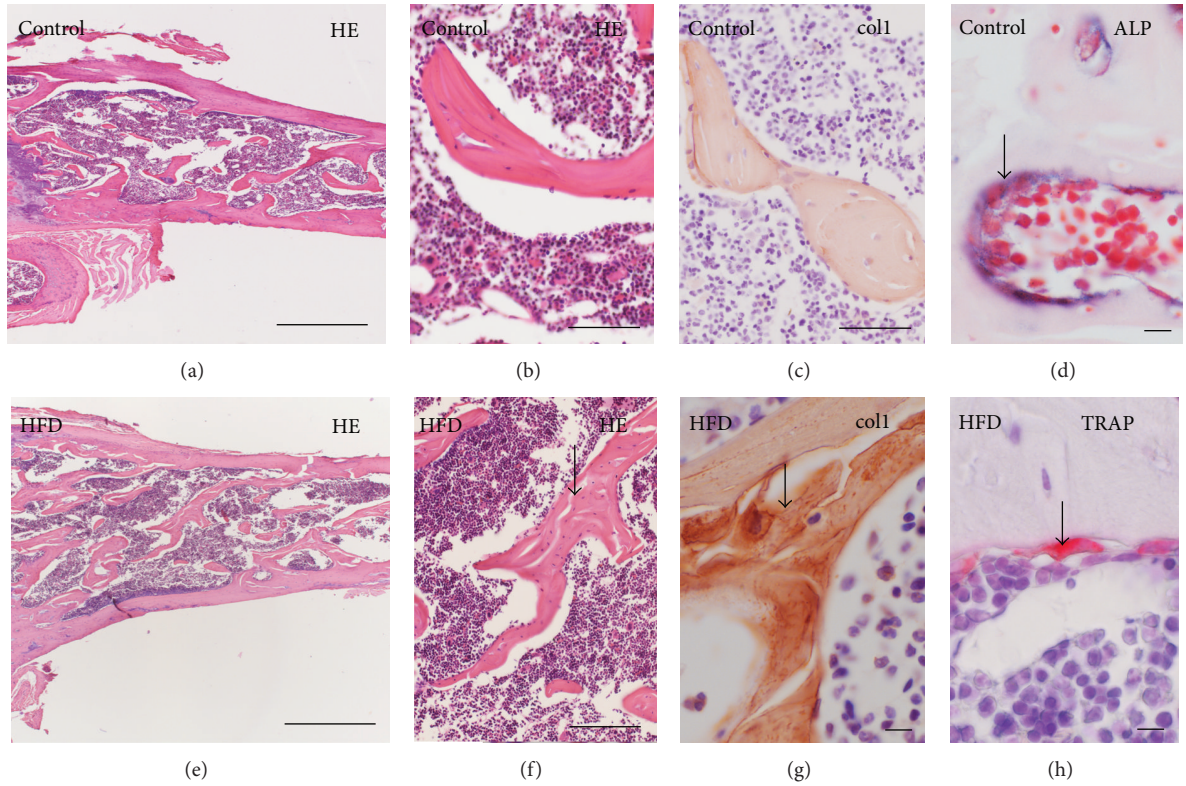


Figure 4: Microstructure of cancellous bone in demineralized paraffin sections. Trabeculae of control mice ((a)–(d)) exhibit a plate-like shape ((a)-(b)) whereas trabeculae of HFD mice ((e)-(f)) show a more rod-like form ((e)-(f)). The trabecular lamellae were interrupted by spaces in HFD mice (arrow, (f)). Collagen-1 immunohistochemistry (col1, (c), (g)) brought out an increase in woven bone (arrow) in (g) HFD mice in comparison to the lamellar trabeculae in (c) control animals. Changes were neither demonstrated in enzyme histochemistry of alkaline phosphatase (arrow, ALP, (d)) nor in tartrate-resistant acidic phosphatase (arrow, TRAP, (h)). Scale bar: 500 μm in (a), (e); 50 μm in (b), (c), (f); 20 μm in (d), (g), (h).

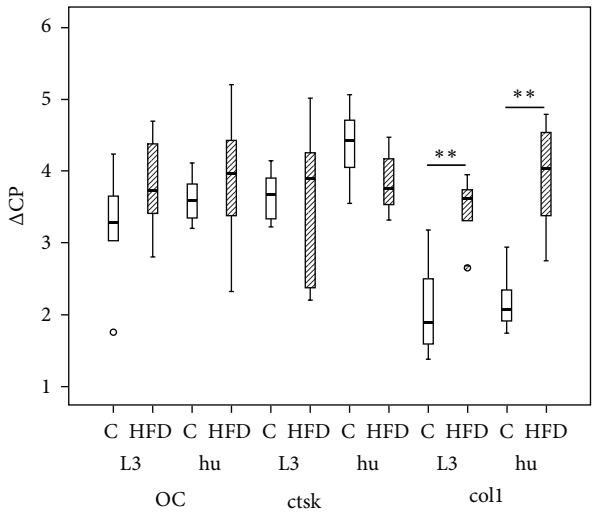


Figure 5: Real-time RT-PCR. The expression of collagen 1α1 (col1) was decreased in humerus (hu; HFD $n = 7$, C $n = 6$) and vertebrae L3 (L3; HFD $n = 6$, C $n = 6$) in HFD mice in comparison to controls (C). No regulation was found for osteocalcin (OC) and cathepsin K (ctsK) expression. Data presented as box plots with the median indicated by solid line within the box. Small circles represent data beyond 1.5x the interquartile range of the median. $^{**}P \leq 0.01$.

compared to control diet. Cao et al., 2009, found an upregulation of ALP positive colony forming units after culturing bone marrow stromal/osteoblastic cells of mice fed with HFD [40]. The direct comparison between these results is not possible because of the different methods and the different HFDs. In the present study we used a HFD where 60 kcal% energy as fat was given whereas the mice in Cao et al.'s [40] study got a HFD with 45 kcal% energy as fat. Hence, there could be a metabolic window were an increase in fat has positive effects on bone formation. Such a window would also explain the controversial discussion in the literature and this would be in line with the report of Núñez et al., 2007, who described that extreme obesity reduces BMD in animals and humans [41]. Furthermore, bone stimulating effects are only found when the energy upregulation is not correlated with an increase of insulin. In patients with type II diabetes mellitus osteoblasts increased their cell division and proliferation in presence of insulin (1.2- to 1.7-fold) but the ALP activity and the production of mineralized matrix was reduced to 55% in comparison to control [39]. However, in the present study no alteration was observed in ALP activity. ALP is an enzyme that is necessary for the mineralization of the bone matrix. It is linked to the membrane of matrix vesicles via a glycosylphosphatidylinositol (GPI) anchor by means

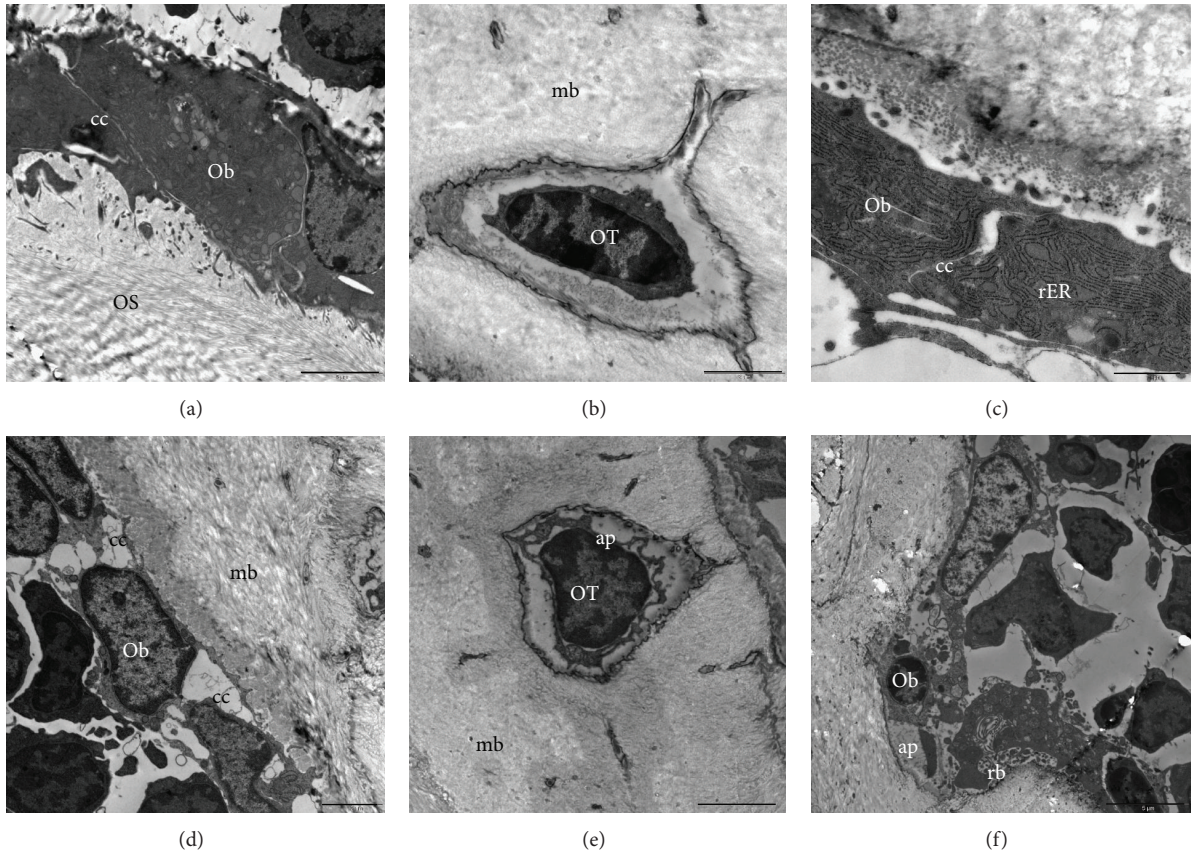


Figure 6: Transmission electron microscopy. In comparison to controls (a)–(c) the HFD mice (d)–(f) showed less cell-to-cell (cc) and cell-to-matrix contacts ((a), (d)). More apoptotic formations (ap) were found in osteocytes (OT, (e)) and osteoblasts (Ob, (f)) of HFD mice that also contained less rough endoplasmatic reticulum (rER) in comparison to control animals (c). mb = mineralized bone; rb = ruffled border of a osteoclast. Scale bar: 5 μm in (a), (f), 2 μm in (b), (d)-(e), and 1 μm in (c).

of posttranslational modification. ALP is involved in the formation of hydroxyapatite crystals within the vesicles by hydrolyzing monophosphate esters at a pH of 8–10. After the matrix vesicles are budding from the osteoblast, the hydroxyapatite crystals penetrate the vesicle membrane and fill the space between the collagen fibrils (reviewed in [42]).

Besides ALP, osteocalcin and collagen-1 are very prominent in bone. With an amount of 1% of the bone matrix, osteocalcin is the most important member of the group of noncollagenous matrix proteins. In our study we analyzed the possible regulation of osteocalcin on mRNA level where we did not find any alteration. On the protein level Cao et al. [40] could measure a significant down-regulation and therefore supposed a delay in bone formation. Besides, collagen-1 is the main component of the non-mineralized bone matrix. Using real-time RT-PCR we observed a significant down-regulation of collagen 1α1 mRNA in both bone types, long bone (tibia) as well as irregular bone (vertebrae) of HFD mice. Thus, on mRNA level, the formation of non-mineralized matrix is changed in our obesity model. Since the collagen builds up the skeletal structure of bone, we measured the cancellous area in relation to the whole tissue by means of histomorphometry. Our results showed a distinct downregulation in the relative trabecular area of HFD mice in comparison to the control mice. A decrease in cancellous bone mass has also been reported by other studies using HFD in mice as model for obesity [29, 30, 40]. In addition to histomorphometry used in our study Patsch et al. also used microcomputed tomography (μCT) [29] and Fujita et al. and Cao et al. focused on μCT and serum levels of bone markers [30, 40]. μCT analysis possesses the advantage of analyzing the bone structures 3-dimensionally whereas histomorphometry is restricted to 2 dimensions. All three reports [29, 30, 40] measured a decrease in the ratio of bone volume to tissue volume and trabecular number. In addition, Patsch et al. and Cao et al. also described an increase in trabecular separation, connectivity density, and structure model index (SMI) [29, 40]. In contrast to our study the reports of Fujita et al. [30] and Patsch et al. [29] correlated these effects with the duration of the HFD. Fujita et al. analyzed animals after 4, 8, and 12 weeks of HFD. The effects of HFD on the bone structure increased with time [30]. In our study we used only a long-term HFD (23 weeks) where we could confirm the results of Patsch et al. [29] and Fujita et al. [30]. Fujita et al. [30] correlated structure of trabecular and cortical bone. Although cortical bone formation was slower in obese mice compared to controls, the periostal bone formation increased with age. Thus, they assumed that the underlying mechanism of bone

loss was different at these two sites of bone [30]. Ionova-Martin et al. confirmed these results in a study with young animals receiving a HFD. When the HFD was given to adults they measured a decrease in femoral diameter, bone strength, fracture toughness, and alignment of osteocytes [31]. Our results also depict a lamellar disorganization that affects the osteocyte microarchitecture.

Beyond the bone forming osteoblast lineage, osteoclasts are accountable for a loss of bone mass. Osteoclasts are multinucleated cells with a specific endowment of enzymes and transporters that facilitate them to resorb bone [43]. In the present study osteoclasts were analyzed on mRNA level by measuring cathepsin K expression and histologically by TRAP enzyme histochemistry. No differences were observed in the osteoclast population between HFD mice and controls using both methods. Multinucleated osteoclasts with a ruffled border, sealing zone, and several vesicles in their cytoplasm were found to be located at the surface of the mineralized bone. No structural aberration or obvious difference in the amount was found in the HFD mice in comparison to the controls. Despite this, striking alterations were found for the osteoblasts. Osteoblasts are usually situated on the surface of growing bone as a closely packed layer of cells [4] that are connected to each other, to osteocytes, and to osteoid via gap junctions, connexons, and hemi-channels [44]. An unusual big space was observed between the osteoblasts among themselves and between osteoblasts and the bone surface. The TEM observations did not give information about the connections from osteoblast to the osteocytes. However the cell-to-cell and cell-to-matrix communications are important for maintaining bone homeostasis. The expression of connexin43, which is the main component of gap junctions and hemi-channels is needed for functioning of mature osteoblasts and osteocytes [45]. This report indicates that gap junctions and hemi-channels play an important role for bone cell survival. In HFD mice several apoptotic formations were observed for osteocytes and osteoblasts, and additionally, TEM analysis showed a reduced osteoid production and striation and a decrease in osteoblast endoplasmatic reticulum in HFD mice. Thus, we suspect that the reduction of cell-to-cell contacts is followed by an increase in apoptosis of bone forming cells and therefore the assembling of new bone is delayed.

In summary, the presented study demonstrated a missarrangement of cell-cell and cell-matrix contacts, osteoblast and trabecular structure, and collagen-1 and osteoid synthesis that altogether outlines a negative effect of obesity on bone microstructure.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Intermuscular Fat: A Review of the Consequences and Causes

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Muscle's structural composition is an important factor underlying muscle strength and physical function in older adults. There is an increasing amount of research to support the clear disassociation between the loss of muscle lean tissue mass and strength with aging. This disassociation implies that factors in addition to lean muscle mass are responsible for the decreases in strength and function seen with aging. Intermuscular adipose tissue (IMAT) is a significant predictor of both muscle function and mobility function in older adults and across a wide variety of comorbid conditions such as stroke, spinal cord injury, diabetes, and COPD. IMAT is also implicated in metabolic dysfunction such as insulin resistance. The purpose of this narrative review is to provide a review of the implications of increased IMAT levels in metabolic, muscle, and mobility function. Potential treatment options to mitigate increasing levels of IMAT will also be discussed.

1. Introduction

The unique ability of adipose tissue to expand throughout life and release a host of chemical messengers makes adipose not only a distinctive tissue but also the largest endocrine organ in the body [1]. In the last twenty years, a rapid expansion of our understanding of this unique organ has occurred. Once thought to be an inert storage depot for excess calories, important only to energy homeostasis, we now know that adipose tissue expresses and secretes a multitude of hormones and proinflammatory cytokines thereby acting in an autocrine, paracrine, and endocrine manner signaling the heart, musculoskeletal, central nervous, and metabolic systems [1–3]. Not all adipose depots are alike. Recent studies have suggested that the location [4–8] and type [9] of excess adipose tissue, rather than simply total body adiposity, may be important in the systemic increase of circulating cytokines and the rise in metabolic diseases such as diabetes [9–14] (for a more complete review of the types and roles of adipose tissue, see Wronska 2012 and Stehno-Bittel 2008) [1, 9]. Adipose tissue stored in subcutaneous depots, particularly in the gluteal-femoral region, is a negative predictor of metabolic syndrome and is cardioprotective [4–7, 15, 16]. However, adipose tissue stored in ectopic locations outside of the subcutaneous tissue such as in the muscle, liver, and abdominal cavity is linked with chronic inflammation [10, 17–19], impaired glucose tolerance [4–6, 20, 21], increased total cholesterol [8, 16, 22], and decreased strength and mobility in older adults [23–31]. Advancing age results in a redistribution of fat depots, despite stable or decreasing overall fat, with adipose storage sites changing from subcutaneous locations to the more harmful ectopic locations [3, 32, 33]. In particular, intermuscular adipose tissue (IMAT), an ectopic fat depot found beneath the fascia and within the muscles, may be of specific interest to rehabilitation professionals.

IMAT has been studied in a variety of individuals with metabolic [5, 6, 8, 14, 28, 34–36], orthopedic [37, 38], and neurologic [39, 40] conditions commonly seen in rehabilitative settings. High levels of IMAT are associated with insulin resistance [5, 6, 8, 14, 28, 34–36], a loss of strength [23–31],

and mobility dysfunction [23, 41–43]. High levels of IMAT can be found in many patient populations, including, but not restricted to, the paraspinal muscles of individuals with chronic back pain [37, 38] and the locomotor muscles of individuals diagnosed with HIV [44], spinal cord injury [39], CVA [40], diabetes [6], and COPD [45]. Furthermore, older adults with increased IMAT levels in the locomotor muscles are known to experience increased levels of muscle weakness, decreased mobility function [23, 41–43], and an increased risk of future mobility limitation [42, 43]. IMAT has potential clinical implications that rehabilitation professionals should recognize and attempt to manage in rehabilitation settings when working with older adults and those with diseases and disabilities associated with IMAT.

The purpose of this narrative review is to inform rehabilitation professionals about the potential metabolic, muscle, and mobility associations of increased IMAT in the locomotor muscles of adults. This review will focus on three areas. First, the definition and measurement of IMAT will be presented; second, the implications of increased locomotor muscle IMAT in metabolism, muscle strength, and mobility will be reviewed; and third, recommendations for future research and treatment for adults with increased levels of IMAT will be made. Literature targeted for this review included peer reviewed cross-sectional, longitudinal, epidemiologic, and clinical studies in adult humans.

2. Definitions and Measurements of IMAT

IMAT has been referred to in the literature by a variety of names and definitions including myostasis, intermuscular fat, intramuscular fat, and low density lean tissue. Intermuscular fat is typically the broadest definition of fatty infiltration in the muscle referring to storage of lipids in adipocytes underneath the deep fascia of muscle. This includes the visible storage of lipids in adipocytes located between the muscle fibers (also termed intramuscular fat) and also between muscle groups (literally intermuscular) [46] (See Figure 1). While not frequently isolated as a separate fat depot by itself, there also exists a smaller group of lipids stored within the muscle cells themselves known as intramyocellular lipids or IMCL; IMCL has been reviewed extensively elsewhere [47]. Increased levels of IMCL are found both in obese insulin resistant individuals and in highly trained endurance athletes; these paradoxical findings have led to the conclusion that lipids stored within muscle cells are not always harmful to the cell [47]. For the remainder of this review, the term IMAT will refer to any measure of fat beneath the deep fascia of the thigh, not including studies that have used methods that independently quantify IMCLs (i.e., histochemical or spectroscopic methods).

IMAT is most commonly measured via computed tomography (CT) or magnetic resonance imaging (MRI). While IMAT has been quantified in numerous studies, it is not yet routinely measured or quantified in clinical imaging studies. CT scans have been extensively used to quantify IMAT in numerous studies [5, 6, 10, 14, 20, 23, 28, 40, 42, 43, 48–52] and were first described by Kelley et al. in 1991 [53]. CT is a fast imaging method that utilizes X-rays for an indirect measurement of IMAT based on the tissue density of an area. On a continuum of density where bone is the most dense and fat is the least dense, lean muscle mass falls between these two extremes. Lean tissue seen on a CT scan can be further divided into areas of high-density lean tissue and areas of low-density lean tissue. High-density lean is an area where little fatty infiltration occurs, and low-density lean tissue is an area where increased levels of adipocytes are found between and within muscle fibers and result in decreased density on CT scan. An individual with a higher proportion of low-density lean is assumed to have increased levels of both IMCL and IMAT. If the density of a muscle increases, or the area of low-density lean decreases after an exercise program, it is presumed that the exercise program has resulted in a loss of both IMCL and IMAT.

With MRI, direct measurements of IMAT [46] can occur without the use of harmful radiation; therefore, MRI is increasingly used to quantify IMAT [25–27, 29–31, 35, 36, 39, 46, 54–65]. MRIs utilize the chemical properties of fat and muscle to directly measure the amount of IMAT within a region of interest [46]. However, while MRI studies of IMAT avoid the use of harmful radiation, they do typically require time-consuming manual segmentation for a region of interest. This process can be difficult and less reliable for small, irregularly shaped areas. Comparative studies of MRI and CT have demonstrated that MRI has a higher sensitivity than CT for identifying early fatty replacement in muscle and that MRI, because it is not density based, provides better anatomical details of soft tissue than CT [46, 66, 67]. Studies comparing CT and MRI measurements have generally shown good agreement and both methods are acceptable precise measures of IMAT [68, 69]. The same definition and method for measuring IMAT should be used in pre- and poststudies. Both CT and MRI appear to be appropriate and advanced techniques for measuring IMAT; however, drawing conclusions concerning absolute amounts of IMAT across studies may be difficult if different methods of measurement are employed. Many studies have used slightly different definitions of IMAT (i.e., adipose tissue in a muscle, adipose tissue between muscles, or adipose tissue under the fascia of the thigh), and conclusions drawn across studies should be interpreted within this context.

3. IMAT and Metabolism

IMAT is positively associated with insulin resistance and an increased risk of developing type 2 diabetes [5, 6, 8, 14, 28, 34–36] (Figure 2). The link between IMAT and insulin resistance could be theoretically attributed to the relationship of IMAT and BMI. Generally, as BMI increases so does IMAT [7, 21, 23]. However, even when BMI is statistically accounted for, IMAT remains a strong predictor of fasting glucose and insulin levels in both younger [5] and older adults [6, 22, 54], suggesting that these metabolic impairments are not simply due to obesity alone. Compared to subcutaneous fat, IMAT is a much smaller fat depot, accounting for as little as 8% of the adipose tissue in the thigh [5]. Despite its small

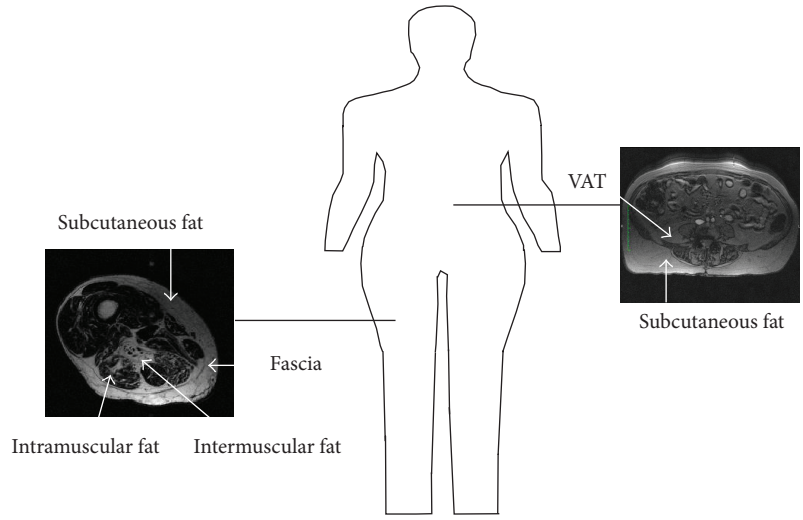


Figure 1: Intermuscular fat is generally considered to be any fat (including the fat between muscle groups and within a muscle) found beneath the fascia of a muscle and is the widest definition for fat beneath the fascia of a muscle. Intramuscular fat is the visible fat found within a muscle. Intermuscular is considered to be an ectopic fat depot similar to visceral adipose tissue (VAT) found in the abdomen.

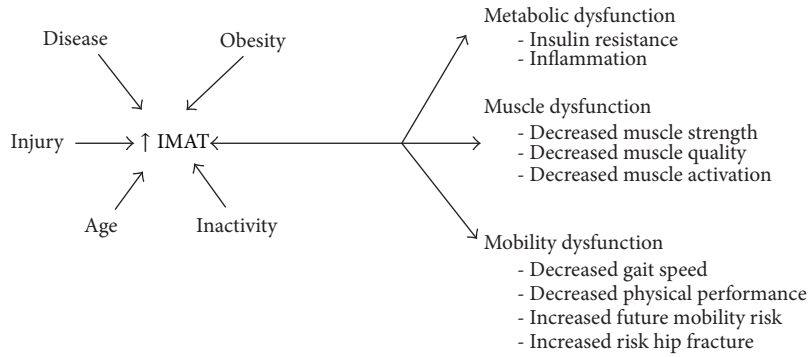


Figure 2: Muscle injury, obesity, age, disease status, and inactivity are all factors that are associated with increased levels of IMAT. Increased levels of IMAT may also lead to a myriad of metabolic, muscle, and mobility dysfunctions.

size, IMAT is strongly associated with insulin sensitivity in obese individuals [5]. It is currently unknown if IMAT acts merely as a marker of metabolic dysfunction or if it may have an intermediary or modifying role in insulin resistance. Since IMAT sits in close proximity to the muscle fibers, it is possible that IMAT may interact with muscle fibers through a yet unknown pathway leading to muscle dysfunction and insulin resistance [10, 26]. Muscle dysfunction may lead to further inactivity and increased levels of IMAT precipitating a cycle of increased IMAT, insulin resistance, and muscle dysfunction. This close relationship between the muscle fibers and IMAT becomes particularly important in populations that are known to have increased IMAT, muscle dysfunction, and insulin resistance including individuals with diabetes [70] and survivors of stroke [28, 40] and spinal cord injury [39, 57, 58].

After a stroke (CVA), muscle volume decreases and both subcutaneous adipose tissue and IMAT increase in the paretic limb [28, 40]. We noted that, in the paretic limb, the subcutaneous adipose depot was 6% higher and IMAT was increased 4% compared to the nonparetic limb in older stroke survivors [28]. Similar to the findings in older adults with type 2 diabetes, a positive relationship also exists between IMAT and fasting insulin levels in those post-CVA [28]. In this study of 70 adult stroke survivors, we found that decreased muscle attenuation (indicating increased IMAT levels) was associated with increased fasting insulin levels [28]. Similar results are found in those who have suffered a spinal cord injury. One study found that thigh IMAT increased on average 26% in just three months after a complete spinal cord injury [39]. This large increase in IMAT accounted for a 70% reduction in glucose tolerance in these same individuals [39]. The strong relationship observed between decreased glucose tolerance and increased IMAT postspinal cord injury suggests that accumulation of IMAT may have a deleterious effect on glucose homeostasis particularly in those who are mobility limited. Further studies are necessary to determine if IMAT plays a direct role in decreased glucose tolerance or if it is only a marker of metabolic dysfunction.

Despite not knowing the specific mechanism behind IMAT's potentially harmful influence on muscle metabolism, there are several lines of evidence that support this relationship. Multiple authors have suggested that IMAT, an ectopic fat depot similar to visceral adipose tissue, may release a host of proinflammatory cytokines resulting in local inflammation within the muscle [10, 26, 48, 65, 71]. Other ectopic fat depots, such as those found in the liver or the abdomen, are known to have increased systemic levels of proinflammatory cytokines [72]. Beasley et al. also reported a relationship between the amount of IMAT within the thigh and systemic measures of proinflammatory cytokines, as measured in the serum suggesting that IMAT may in fact be related to increased whole body inflammation [10]. We reported for the 1st time increased IMAT in the paretic leg of stroke survivors [40], which we followed with our examination of skeletal muscle TNF-a [73]. We found that both IMAT [40] and inflammation [73] are increased in the paretic leg of stroke survivors [28, 73]. However, to date, we are unaware of any published examinations of the direct relationship between IMAT and the local inflammatory environment within the muscle. Skeletal muscle is the primary site for glucose metabolism in the body. While it is currently unknown by which mechanism IMAT may act on metabolism, it does appear that a relationship exists between increased levels of IMAT and decreased whole body glucose metabolism particularly in those who have suffered an injury that reduces muscle function. It is theorized that the close proximity of IMAT to the muscle fiber may impair the local muscle environment through aforementioned increase in local proinflammatory cytokines [10, 59], impaired blood flow [5, 8], or increasing the rate of lipolysis within skeletal muscle resulting in an increased concentration of glucose within the skeletal muscle itself, leading to insulin resistance [5, 8].

4. IMAT and Muscle Function

The structural composition of muscle is an important factor in its function [23]. It is now well established that a loss of lean muscle mass in older adults does not directly translate into a loss of strength [41, 74]. The Baltimore Longitudinal Study of Aging found that while grip strength and muscle mass both declined with age, older adults were weaker than the loss of muscle mass alone would predict [74]. Similar results were found in a 3-year longitudinal study of 1800 healthy older adults. In this finding from the Health ABC Study, muscle strength declined even in those individuals who gained lean muscle mass. While lean mass decreased by approximately 1% a year, strength decreased up to 4% during the same time period [41]. This clear dissociation between lean mass and strength advocates for factors other than lean muscle mass being responsible for the declines in muscle function seen with aging. IMAT is one such factor that may impact the muscle function losses that are associated with aging.

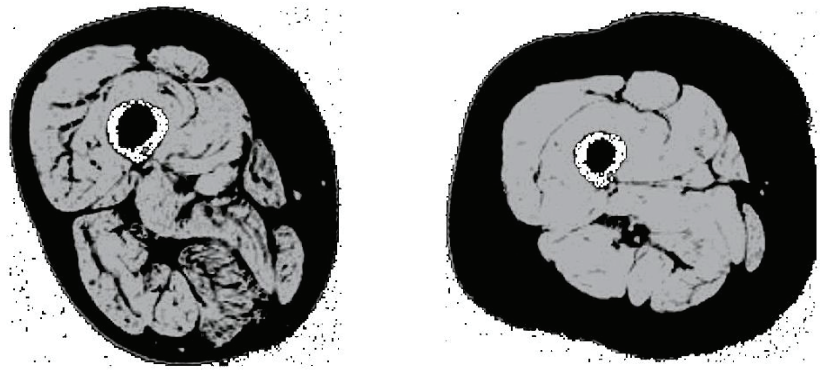
An emerging body of literature supports IMAT as a significant predictor of both muscle and mobility function in older adults suggesting that increased IMAT may at least partially explain a loss of strength and mobility seen with aging [23–31] (Figure 2). Older adults with higher levels of IMAT in the legs have lower muscle strength [23, 30] as well as muscle quality [23] or the force produced per unit of cross-sectional area of muscle, as demonstrated by the two women whose thigh images are presented in Figure 3. Decreases in muscle quality may lead to difficulties in functional activities [75] and several studies have also demonstrated that adults with comorbid conditions such as COPD [45], stroke [28], osteoarthritis [76], kidney disease [77], and cognitive decline [78] demonstrate decreases in muscle quality. The relationship of increased levels of IMAT and decreased strength and muscle quality is reported in multiple studies in the thigh [23] and calf muscles [30], in healthy elders [23], and in adults with comorbid conditions including diabetes and peripheral neuropathy [30]. It is intriguing that this relationship does not appear to be confined to older adults [26]. After 30 days of single limb suspension, Manini et al. found that young (~20 years) healthy individuals experienced an increase of 15–20% in IMAT of both the calf and thigh muscles. This increase in IMAT also exceeded the loss of lean tissue suggesting that IMAT was not just merely "filling" the space left by lean tissue atrophy [26]. The increase in IMAT also accounted for a 4–6% of loss of strength, again emphasizing that IMAT is more than an inert storage depot, but may also play a role in inactivity related strength loss.

High levels of IMAT are also associated with decreased activation of the quadriceps muscles in older adults [31]. We found a moderate significant negative relationship between IMAT and quadriceps muscle activation in a small sample of older adults. Muscle activation, in this study, was quantified by the central activation ratio, a measure of a muscle's ability to fully activate during a maximal effort voluntary isometric contraction. It appears that not only may IMAT impair a muscle's ability to produce force but also it may actually hinder the improvement in muscle quality typically seen with resistance training [59]. We examined changes in muscle quality after 12 weeks (3x/week) of resistance training in 70 older adults with a history of falls and found that only individuals with low amounts of IMAT in the thigh at the start of training were able to significantly improve muscle quality. Similar to the loss of muscle quality with high levels of IMAT, a decrease in muscle activation in the presence of high amounts of IMAT suggests that IMAT may be partially responsible for inhibiting muscle force production and improvements with strength training.

5. IMAT and Mobility Function

Perhaps even more important than the association between IMAT and muscle function is the relationship between IMAT and mobility. There is an increasing amount of evidence linking IMAT with mobility impairment in older adults [25, 27, 29, 30, 42, 43]. Increased levels of IMAT are associated with decreased six-minute walk distance [27, 30, 79], decreased gait speed [43], decreased physical performance [25, 30], difficulty with repeated chair stands [43], and slower stair descent and timed up and go tests [27]. This relationship has consistently been reported in a variety of populations of older

	Timed up and go (s)	Stair up (s)	Stair down (s)	Lower extremity power (W)	Knee extension strength (N)
Subject 07	8.4	6.6	7.0	88.2	194.8
Subject 44	6.5	4.9	4.4	139.5	248.3
Difference	25%	29%	45%	45%	24%



Subject 07
Lean: 99.5 cm^2
IMAT: 18.8 cm^2

Subject 44
Lean: 100.7 cm^2
IMAT: 9.8 cm^2

Figure 3: Two women with similar age, BMI, and levels of lean muscle mass but with differing levels of IMAT in a cross-sectional MRI image of the thigh. Subject 7 has double the level of IMAT (black within the muscle) in her thigh as subject 44. While both women have similar levels of lean tissue (seen in grey), they have different levels of mobility and muscle function. The increased levels of IMAT and decreased muscle and mobility function of subject 7 are consistent with literature that reports that increased levels of IMAT are associated with decreased muscle and mobility function.

adults including healthy elders [43], those with a history of diabetes [25, 30], COPD [45], falls [27], and cancer [27].

IMAT is frequently associated with mobility function even when lean tissue is not suggesting that IMAT may in fact be an important variable when referring to mobility function in older adults [80]. IMAT is also predictive of future mobility limitations [42]. A large study of over 3000 older adults aged 70–79 followed up for two and one half years revealed that individuals with the greatest amounts of baseline IMAT were 50 to 80% more likely to develop mobility limitations over the following two and one half years when compared with those with the lowest levels of baseline IMAT [42]. This finding was consistent even after adjusting for baseline total body fat and muscle strength.

High levels of IMAT may not only impair mobility but also increase the risk for developing disability. Increased levels of IMAT correlate with low bone mineral density and an increased risk of hip fracture [81, 82]. IMAT levels of the mid-thigh are noted to be a strong and independent determinant of bone mineral density [82]. Additionally, the Health ABC Study, a large longitudinal investigation of over 2500 individuals between the ages of 70 and 79 years, reported a large increase in the risk for hip fracture with increased IMAT [81]. A decrease of one standard deviation of muscle density of the thigh as measured with CT conferred a 50% increase in hip fracture risk [81]. Even after adjusting for bone mineral density, an increase in IMAT raised the risk of a hip fracture by 40% [81].

It is clear that increased levels of IMAT are associated with decreased muscle and mobility function in older adults but whether IMAT is a marker of muscle dysfunction or whether it has a direct effect on muscle dysfunction is not currently known. IMAT may act as an intermediary modifying preexisting pathological process as IMAT's harmful relationship with muscle and mobility function has been theoretically attributed to an increase in proinflammatory cytokines [10, 26, 48, 65, 71] similar to the attributed effects of proinflammatory cytokines on metabolic function. Interestingly, several authors have reported relationships between increased proinflammatory cytokines and decreased muscle [83, 84] and mobility function [85–87] that are strikingly similar to those reported between IMAT and muscle and mobility function [19].

IMAT may also be harmful to muscle and mobility function due to mechanical changes in muscle that occur in the presence of IMAT that can lead to changes in muscle fiber orientation [56]. Studies of rotator cuff injuries suggest that the loss of force in a muscle may be related to increased levels of IMAT [56]. After a supraspinatus tear, elasticity of the muscle decreases and passive tension of the supraspinatus is increased. This decreased elasticity leads to a poorer ability to actively generate force, resulting in a loss of maximal tension of the muscle [88]. In addition to the loss of elasticity in rotator cuff muscles, it has been hypothesized that excess IMAT leads to an alteration in contractile fiber pennation angle, hence resulting in an unfavorable mechanical angle

and a concomitant reduction in force production [56, 89]. We are unaware of studies that have examined the effect of IMAT on elasticity or of pennation angle in locomotor muscles. While the impact of IMAT relative to elasticity or pennation angle might be expected to be similar in other muscles, the results from rotator cuff studies should be interpreted cautiously due to differences in the muscle's architecture and function. Additionally, fatty infiltrate in rotator cuff muscles follows a known musculotendinous injury, that is, a rotator cuff tear. The cause of the increased fatty infiltration associated with many metabolic or systemic diseases is not as easy to pinpoint as there is no direct muscular injury. Future research should elucidate the mechanisms behind increased IMAT and decreased muscle and mobility function in older adults and importantly should determine if minimizing IMAT is accompanied by improved muscle and mobility function.

6. Aging, Weight Loss, Activity, and IMAT

Several authors have implied that IMAT is an unwanted but inevitable consequence of aging as epidemiological, longitudinal, and cross-sectional studies have reported significant positive relationships between aging and IMAT [7, 48, 63, 90]. Some have theorized that whole body IMAT increases as little as 9 grams/year [7] to as much as 70 grams/year [63]. The majority of studies examining the effects of aging on increases in IMAT have been small and cross-sectional and have failed to account for activity levels and disease status or have investigated only a narrow age range. These caveats call into question the definitive assertion that IMAT is an inevitable consequence of aging [7, 63, 90]. In the largest longitudinal study to date, Delmonico et al. followed up over 1600 older adults between the ages of 70 and 79 for 5-years [48]. After accounting for race, weight changes, health status, and activity levels, they found decreased thigh muscle density even in those who lost weight or were weight stable over a 5-year period. However, it should be noted that increases in IMAT were clearly influenced by increases in body weight as those who gained the most body weight over five years also gained the most IMAT. Furthermore, the study did not report the reasons for loss of body weight (i.e., illness). Weight loss due to intentional caloric restriction and exercise may have a different influence on IMAT than weight loss due to illness as numerous intervention studies have found that intentional weight loss leads to decreases in IMAT [52, 62, 91, 92].

More recent work suggests that increases in IMAT may be more a product of illness, disuse, or inactivity than aging per se [24, 29, 64]. This is a clinically important finding as it suggests that IMAT may be amenable to change via a physical activity intervention (Figure 4). Longitudinal twin studies have demonstrated that after 32 years of difference in activity habits, inactive twins had 54% higher IMAT in their mid-thigh compared to their more active twin [35]. High levels of spasticity after spinal cord injury have also been shown to protect against the accumulation of IMAT [57]. Further support for the assertion that physical activity has a strong influence on IMAT is found in studies of young, healthy adults following periods of inactivity [26], when comparing younger to older athletes [14, 64] and when comparing obese active to inactive individuals [29]. After 30 days of single limb suspension, a method of immobilizing one leg, young, healthy adults demonstrate an increase of 15% IMAT in the immobilized thigh and 20% in the calf [26]. In a cross-sectional study examining master athletes from age 40 to 81 who consistently participated in high levels of physical activity it was found that younger and older adults did not differ in IMAT levels [26]. Even in a population of obese adults with diabetes and peripheral neuropathy, conditions known to be associated with increased IMAT, there still exists a significant relationship between the number of steps taken in a day and the volume of IMAT in the calf [29]. Tuttle et al. reported that the average daily step count was able to explain up to 19% of the variance in IMAT in the calf of older adults with diabetes and peripheral neuropathy [29]. Based on these studies, it appears that IMAT may be amenable to change via increasing physical activity levels. However, the magnitude of changes reported questions the clinical significance of these changes. It may be that significant weight loss, via physical activity or diet, may be necessary to achieve meaningful changes in IMAT.

Multiple studies have examined the effects of diet, exercise, or a combination of diet and exercise on IMAT [20, 22, 24, 51, 52, 55, 59, 61, 62, 91–100]. Most have reported decreased IMAT following intervention [20, 22, 51, 52, 55, 61, 62, 91–94, 96, 97, 99]. The current general consensus among studies examining changes in IMAT with weight loss alone or with exercise is that weight loss is necessary to see significant changes in IMAT [20, 51, 52, 55, 62, 91–93, 97]. However, it is possible that exercise, when performed at a sufficient intensity and duration to induce weight loss, is actually superior at decreasing IMAT levels compared to weight loss induced by reduced calorie intake [55, 62, 97]. Murphy et al. compared the effects of exercise induced weight loss to weight loss induced by calorie restriction alone in overweight adults aged 50–60 [62]. They found that when exercise resulted in weight loss, the loss of IMAT was two times greater than calorie restriction alone. This finding is in agreement with Christiansen et al. who found that the combination of calorie restriction and exercise resulted in an 11% decrease in IMAT while calorie restriction alone resulted in a 7% decrease in IMAT [55]. While weight loss may be necessary to decrease IMAT, this may not be a desirable option for some older adults. Weight loss in frail, older adults with already low body mass indexes may be accompanied by loss of muscle mass and function and therefore may not result in a positive outcome. There is currently a paucity of literature that examines the effects of any intervention on IMAT in frail, older adults. Most studies of IMAT to date have examined younger [22, 55, 92, 95, 96], obese, [22, 52, 55, 91–93], or overweight [20, 22, 51, 52, 62, 91, 94–96, 101] populations, making generalization to frail, older adults difficult. Goodpaster et al. reported that physical activity nearly ameliorated the increase in IMAT that occurs with sedentary behavior in older adults with a mean age of 76 years [24]. A modest walking program of 1-2 times per week for as little as 30 minutes per session stabilized IMAT accumulation in these individuals. In contrast, in this same study, the control group that did not participate in any

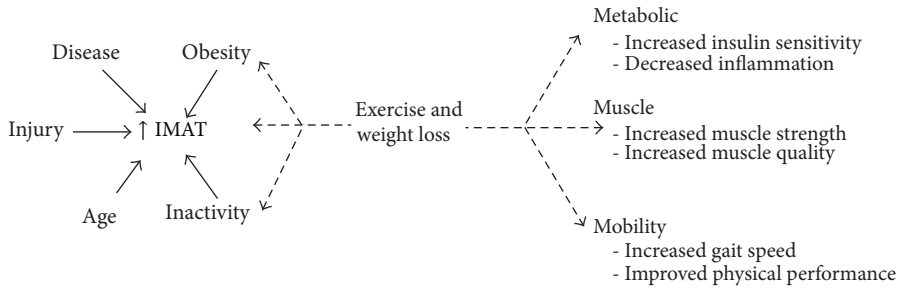


Figure 4: Exercise and weight loss may act to directly decrease IMAT, improve factors associated with increased IMAT such as obesity and inactivity, and improve metabolic, muscle, and mobility dysfunction.

formal exercise program experienced an 18% gain in thigh IMAT over 12 months [24]. This suggests that physical activity might mitigate the accumulation of IMAT in older adults. However, only two small studies have found that increasing physical activity, one through walking and the other through resistance training, decreased IMAT in this population [93, 94]. We also found that resistance training decreased IMAT in the thigh muscles of older adults (~65 years) who had a CVA [101]. This is a promising finding as it suggests that IMAT may respond to physical activity interventions, even in older adults with comorbid health conditions. However, more research is needed to (1) verify these findings, (2) determine the most effective method of reducing IMAT, and (3) assess the clinical impact of doing so in older adults.

7. Future Directions and Rehabilitation Considerations

More work is necessary to determine the role of increased IMAT on metabolic, mobility, and muscle dysfunction. It has not yet been determined if IMAT is merely a marker of dysfunction or if it has some direct or indirect role in modifying metabolic, muscle, and mobility function. If IMAT does impair muscle activation, then using exercise as a method to reduce IMAT may have a limited effect particularly in frail, older adults. Impaired muscle contraction may minimize the muscles ability to mobilize and utilize IMAT as a fuel source, and it is possible that a combination of therapies will be necessary to reduce IMAT. This may be particularly true in frail, older adults with limited ability or need to change their body mass. The addition of electrical stimulation to exercise may be one method to reduce IMAT and improve muscle function. In a small study of nine individuals with complete spinal cord injury which compared the use of electrical stimulation on the quadriceps muscles twice a week for 12 weeks combined with calorie restriction to calorie restriction alone, the addition of electrical stimulation was shown to significantly decrease IMAT [58]. While the decrease in IMAT was still relatively small (approximately 3%); particularly noteworthy is the observation that the calorie restriction group increased IMAT by 3% during this same period of time [58]. The use of electrical stimulation may result in increased muscle contraction and perhaps an increased ability to use IMAT as a fuel source thus decreasing IMAT within the muscle. This has yet to be explored and is currently only speculative.

Another promising direction that may yield new therapeutic targets is research into the origins of IMAT. Studies investigating the cellular origins of IMAT [102, 103] are attempting to determine the cellular processes that precipitate increased IMAT. While these origins are currently unknown, if found to be similar to other ectopic fat depots such as those found in the liver, pharmacological interventions used in combination with exercise may be a treatment option worth future exploration [72]. Current recommendations for the treatment of nonalcoholic fatty liver disease that results in the accumulation of fat within the liver, similar to IMAT accumulation in the muscle, include the combination of diet, exercise, and in some cases medication [72]. While we are unaware of any trials examining the effects of medication on IMAT, the use of anti-inflammatory or other medications that have been effective at treating other ectopic fat depots such as thiazolidinediones may be useful in the treatment of IMAT, particularly in older frail adults [72].

Large randomized control trials examining the effect of exercise on decreasing IMAT are limited, though it does appear that physical activity, at a minimum, may serve as a preventive strategy to halt the infiltration of IMAT into muscle [24] and may even decrease IMAT in muscles that have already undergone this abnormal adaptation [20, 22, 51, 52, 55, 61, 62, 91–94, 96, 97, 99]. The majority of studies that have demonstrated a decrease in IMAT have been studies that employed a combination of calorie restriction and aerobic exercise for at least 6 months [20, 51, 52, 62, 91, 93, 96]. It also appears that resistive exercise alone [94, 101] or in combination with weight loss [97] or aerobic exercise [55, 61] may decrease IMAT.

It is theorized that exercise training may access IMAT as a fuel source during times of increased activity of the muscle [20, 30]. While speculative, IMAT may be preferentially metabolized as a fuel source to support the increased demands of the muscle thus resulting in a decrease of IMAT with long-term activity [20, 30]. While exercise should be a lifelong activity, to decrease IMAT levels a minimum of 12 weeks of intervention appears to be required to decrease IMAT, though 6 months may be superior. It is important to note that exercise interventions have multiple effects on physiology and the improvements found in these studies may not be due to a reduction in IMAT. Further research is

needed to elucidate the role of decreased IMAT on muscle and metabolic function as well as the most effective exercise prescription to target a reduction in IMAT in older adults.

As our population ages and larger number of individuals with metabolic, muscle, and mobility dysfunction require effective interventions, there is an increase in the need for understanding and treating the multiple negative metabolic and muscle adaptations that may occur. IMAT is now recognized as an important predictor of muscle metabolism and function and also appears to be a modifiable muscle risk factor. Exercise and physical activity appear to be effective countermeasures against increases in IMAT. Future research should focus not only on the causes and mechanisms of increased fatty infiltration but also on establishing whether and how IMAT is involved in the development of the pathologies discussed as well as effective intervention regimes to decrease IMAT.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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