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Editorial

Osteoporosis and Fragility Fractures in Type 2 Diabetes

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Type 2 diabetes mellitus (T2DM) and osteoporosis are associated with severe morbidity, increased mortality, and important social costs, mainly due to their chronic consequences [1]. Epidemiological data indicates that T2DM is associated with increased risk of fractures, suggesting that skeletal fragility should be considered among the chronic complications of T2DM [2, 3] and, in turn, T2DM should be considered among the causes of endocrine osteoporosis [4].

A common feature of the endocrine forms of osteoporosis is the reduced role of bone mineral density (BMD) in predicting fragility fractures [4]. In fact, T2DM does not represent an exception, since, even more than in the other endocrine related osteoporosis forms, it is generally characterised by normal or increased BMD [5]. As a consequence, in T2DM, the risk of fracture is largely independent of BMD, and the latter should not be considered a sensitive enough index of bone fragility [6]. Indeed, if in the presence of reduced BMD an increased risk of fracture has to be considered, then in the presence of normal BMD an increased risk of fractures could not be excluded [5]. Therefore, the fracture risk assessment algorithms, which are significantly based on BMD, are not accurate enough for identifying T2DM patients at risk for fractures [7, 8].

The simpler, but likely incomplete, explanation for the lack of association between BMD and fracture risk in T2DM is that in this endocrine form of osteoporosis reduced bone quality rather than bone density is the main cause of reduced bone strength [9]. This impaired bone quality can be attributed to different mechanisms, whose knowledge represents a challenge for researchers since this information could be used to identify possible targets for both predicting fractures and curing T2DM-related osteoporosis [10].

The reduced bone turnover and impairment of osteoblast activity have been advocated among the possible mechanisms underlying the reduction in bone quality in T2DM [11, 12].

For this special issue, we received different scientific contributions spanning from in vitro studies to animal and clinical research articles.

The paper by Zhang et al., entitled "FOXO1 Mediates Advanced Glycation End Products Induced Mouse Osteocyte-Like MLO-Y4 Cell Apoptosis and Dysfunctions," explored the capacity of advanced glycation end products (AGEs) to induce osteocyte apoptosis, thus impacting bone homeostasis. Using mouse osteocyte-like MLO-Y4 cells, the authors showed that FOXO1 plays a crucial role in AGE-induced osteocyte dysfunction and apoptosis through its regulation of caspase-3, sclerostin, and RANKL.

In the article by Mohsin et al., entitled "Type 2 Diabetes Mellitus Increases the Risk to Hip Fracture in Postmenopausal Osteoporosis by Deteriorating the Trabecular Bone Microarchitecture and Bone Mass," using a micro-CT, the authors analysed the changes in the trabecular bone microstructure due to T2DM at various time points in ovariectomised and nonovariectomised rats. Their data suggest that T2DM negatively affects the trabecular structure of the femoral heads of rats and that these changes are correlated with the T2DM duration, increasing the risk of hip fractures.

In the paper by Guo et al., entitled "Assessment of Risk Factors for Fractures in Patients with Type 2 Diabetes over 60 Years Old: A Cross-Sectional Study from Northeast China," the authors investigated the prevalence of bone fractures in elderly Chinese subjects (with and without T2DM) and evaluated the risk factors for fractures. In particular, when measuring the heel BMD and the timed "up and go" (TUG), the authors observed that low BMD and slow TUG times were independent risk factors for fractures in non-T2DM patients, while no associations were found in the T2DM population. Patients with T2DM had a higher risk for fractures, even when they had preserved BMD and a short TUG time. Therefore, the authors concluded that TUG and BMD underestimated the risk of fractures in the T2DM population.

The review by C. Eller-Vainicher et al., entitled "Pathophysiology and Management of Type 2 Diabetes Mellitus Bone Fragility," summarised the complex pathophysiological mechanisms underlying bone fragility in T2DM patients. In the first part of the review, the authors analysed the correct clinical approach for evaluating bone health in T2DM patients beyond dual X-ray densitometry, with particular attention to other imaging techniques that have been investigated in recent years, such as trabecular bone score, hip structural analysis, quantitative ultrasound, and peripheral quantitative computed tomography. Moreover, the authors examined the role of microindentation and bone turnover markers in the evaluation of bone fragility in T2DM patients. The second part of the review was dedicated to the factors that lead to bone fragility in T2DM, from disease duration, insulin use, glycometabolic control, and complications to the effects of the different antidiabetic drugs on bone and other metabolic aspects, such as obesity and cortisol secretion.

Finally, the article by Zhao et al., entitled "Association between Uric Acid and Bone Mineral Density in Postmenopausal Women with Type 2 Diabetes Mellitus in China-A cross-sectional Inpatients Study," retrospectively evaluated the association between uric acid levels and BMD in 262 postmenopausal women with T2DM. The authors concluded that uric acid levels were neither a protective factor nor a risk factor for osteoporosis in these subjects.

We think that the articles in this special issue contribute to increasing the knowledge of the pathogenesis of bone fragility in T2DM patients and may be the starting point for future research.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

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Iacopo Chiodini
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Luigi Gennari
Agostino Gaudio

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Research Article

Association between Uric Acid and Bone Mineral Density in Postmenopausal Women with Type 2 Diabetes Mellitus in China: A Cross-Sectional Inpatient Study

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Objective. To analyze the association between uric acid levels and bone mineral density in postmenopausal women with type 2 diabetes mellitus. *Methods*. We retrospectively analyzed 262 postmenopausal women with type 2 diabetes mellitus, to assess uric acid levels and bone mineral density using the T score of dual-energy X-ray absorptiometry. *Results*. (1) Women in the osteoporosis group demonstrated higher uric acid levels and lower estimated glomerular filtration rate ($p < 0.05$, respectively). (2) Uric acid levels were positively correlated with the hip and lumbar spine bone mineral density and T score ($r = 0.17$, $p < 0.05$; $r = 0.25$, $p < 0.05$; $r = 0.17$, $p < 0.05$; and $r = 0.28$, $p < 0.05$, respectively). Meanwhile, there was a positive relation between estimated glomerular filtration rate and hip bone mineral density ($r = 0.22$, $p < 0.05$). (3) Logistic regression analysis showed that age, body mass index, and diabetic duration are independent risk factors for osteoporosis in postmenopausal women with type 2 diabetes mellitus. The level of estimated glomerular filtration rate and uric acid levels were not independent effect factors for osteoporosis in menopausal women. *Conclusion*. Uric acid levels are neither a protective factor nor a risk factor for osteoporosis in women with type 2 diabetes mellitus.

1. Introduction

With continuously changing modern lifestyle and increase in the number of aging individuals, comorbidities such as type 2 diabetes mellitus (T2DM) and osteoporosis (OP), which gradually increase in incidence with age, have become common health problems [1, 2]. Higher blood glucose levels in T2DM patients increase the risk of diabetic complications, such as increased risk of brittle fracture [3]. Studies have shown that the changes in bone infrastructure in T2DM patients are due to multifactorial causes and manifest as decreased, increased, or normal bone mass. The bone mineral density (BMD) of T2DM patients is higher than that of nondiabetic people; however, the risk of fracture in T2DM is also significantly higher in T2DM patients [4–6]. In postmenopausal women with T2DM, there are disorders of glucose, lipid, and uric acid (UA) metabolism and bone metabolism, and the risk of osteoporosis is significantly increased. As an end product of purine metabolism, UA is an important endogenous antioxidant. A large number of studies have shown that UA has certain protective effects on a variety of diseases caused by high oxidative stress, including osteoporosis [7], so it is generally considered that UA is a protective factor of osteoporosis. However, hyperuricemia is a risk factor for insulin resistance and diabetes. Hyperuricemia can aggravate the progress of diabetes; hyperglycemia can also lead to bone fragility [8], so hyperuricemia can indirectly accelerate bone loss in T2DM patients. Therefore, whether the increase of UA is still related to the BMD is worth exploring. The purpose of this study is to explore the correlation between UA and BMD and bone metabolism indices in postmenopausal women with type 2 diabetes in China, so as to provide theoretical basis for clinical prevention and treatment of osteoporosis.

2. Methodology

2.1. Participants. In this retrospective study, 262 postmenopausal women with T2DM who were hospitalized in Peking University International Hospital endocrinology department from January 2017 to December 2019 were analyzed. The average age of the participants was 63.65 ± 7.90 years (50-80 years), and the average duration of T2DM was 11.61 ± 6.94 years. All subjects met the T2DM diagnostic criteria of the World Health Organization (WHO) in 1999 [9]. The exclusion criteria included (1) other type of diabetes mellitus; (2) nonphysiological menopausal women; (3) long-term use of drugs that affect bone metabolism; (4) patients with a history of primary or secondary bone cancer; (5) patients who have used OP drugs (estrogen, bisphosphonate, active vitamin D, etc.); and (6) patients who have previously been diagnosed with hyperuricemia and have taken hypouricemia drug (allopurinol, benzbromarone, etc.).

2.2. Methods

2.2.1. General Conditions

(1) Basic Information Collected. All participants' age, date of birth, diabetic duration, menopausal years, diabetes complications, including diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy, and types of antidiabetic drugs were collected and recorded.

(2) Height and Weight Measurement. All participants were asked to take off their shoes and socks and wear light and thin clothes, following which height (cm) and weight (kg) were measured with measuring instrument, and body mass index (BMI) was obtained according to the formula weight/height^2 (kg/m^2). Blood pressure including diastolic blood pressure (DBP) and systolic blood pressure (SBP) was measured in all participants.

2.2.2. Laboratory Measurement. All subjects were asked to fast for at least 8 hrs, and venous blood samples were collected in the morning. Chemiluminescence method was then used to test blood glucose and blood lipid profile. Other biochemical indices of the participants were then determined. High-pressure liquid chromatography (HPLC) was used to test glycosylated hemoglobin (HbAlc) level. The tests were carried out in the biochemical laboratory of Peking University International Hospital.

Laboratory measurements included fasting blood glucose (FBG), serum creatinine (sCr), glycosylated hemoglobin (HbA1c), calcium (Ca), uric acid (UA), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), parathyroid hormone (PTH), osteocalcin (OC), beta C-terminal telopeptide (β-CTX), procollagen 1 intact N-terminal (P1NP), and 25-hydroxyvitamin D (25(OH)D). The glomerular filtration rates (eGFRs) were estimated according to the sCr level.

sCr < 0.7 mg/dl: $\text{eGFRCKD-EPI-ASIA} = 141 \times (\text{sCr}/0.7)^{-0.329} \times 0.993^{\text{age}} \times 1.049$.

sCr > 0.7 mg/dl: $\text{eGFRCKD-EPI-ASIA} = 141 \times (\text{sCr}/0.7)^{-1.209} \times 0.993^{\text{age}} \times 1.049$.

2.2.3. BMD Measurement. Dual-energy X-ray absorptiometry (DXA) was used to detect the bone mineral density of the hip and lumbar spine of the participants. The T score was automatically generated by the computing system according to the BMD of each part by software (Hologic, USA) used in the laboratory of Peking University International Hospital. Participants were divided into 3 groups according to T score: the normal group (T score > 1.0): 41 women, the osteopenia group ($-1.0 \geq T$ score ≥ -2.5): 122 women, and the osteoporosis group (T score < −2.5): 99 women.

2.2.4. FRAX Score. According to the consensus of Chinese experts on fracture risk management of diabetic patients, FRAX score was determined for patients with T2DM, which was accessed at https://www.sheffield.ac.uk/frax/index. Under FRAX fracture risk assessment system, "Asia China mainland" mode was selected. The "calculate" button then provided the main OP fracture probability (PMOF) and hip fracture probability (PHF) within ten years.

2.3. Statistical Methods. All data were processed by SPSS 25.0. Normal distribution data were shown as mean standard deviation ($x \pm s$), and nonnormal distribution data were shown as mean median and quartile spacing. When data was normally distributed and variance was homogeneous, variance analysis was used for comparison among groups. When data was not normally distributed, variance analysis such as Kruskal Wallis test was used for comparison among multiple groups; Pearson correlation analysis and Spearman correlation analysis were used for correlation analysis; logistic regression method was used for analysis of the main influencing factors of OP in postmenopausal women with T2DM, and $p < 0.05$ was used for statistical significance.

3. Results

3.1. General Characteristics, Biochemical Indices, BMD, and Bone Metabolism Markers among the 3 Groups. Compared with the other two groups, the patients in the OP group were older, had lower BMI, and had been diabetic and menopausal for longer duration ($p < 0.05$). Compared with the other two groups, PMOF and PHF in the OP group were significantly different ($p < 0.05$). eGFR in the OP group was lower than that in the other two groups whereas the level of UA in the OP group was higher than that in the other two groups ($p < 0.05$, respectively). There was no significant difference in blood pressure and blood lipid levels among the normal group, the osteopenia group, and the osteoporosis group ($p > 0.05$, respectively). There was no significant difference among the three groups on incidence rate of diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy ($p > 0.05$, respectively). There was no significant difference on types of antidiabetic drugs among the three groups ($p > 0.05$, respectively). Upon comparison

Table 1: Comparison of general characteristics, diabetic complication, biochemical indices, BMD, and bone metabolism markers among the three groups.

Index	Normal group ($n = 41$)	Osteopenia group ($n = 122$)	Osteoporosis group ($n = 99$)	$F\left(X^2\right)$	p
Age (year)	59.71 ± 6.06	62.10 ± 7.85[a]	67.20 ± 7.27[a,b]	9.93	<0.05
BMI (kg/m^2)	26.23 ± 3.50	25.76 ± 3.63[a]	24.96 ± 3.77[a,b]	3.26	<0.05
Diabetes duration (year)	10.53 ± 6.83	12.03 ± 6.98	13.82 ± 6.96[a,b]	3.18	<0.05
Menopausal year (year)	11.15 ± 4.12	15.67 ± 5.28[a]	19.17 ± 6.93[a,b]	2.87	<0.05
SBP (mmHg)	139.34 ± 17.01	133.82 ± 16.82	137.12 ± 18.85	1.86	0.16
DBP (mmHg)	79.32 ± 7.49	77.09 ± 10.36	77.14 ± 11.91	0.75	0.47
PMOF (%)	2.5 (2.0-2.7)	3.6 (3.2-4.3)[a]	7.2 (5.8-9.7)[a,b]	2.96	<0.05
PHF (%)	0.3 (0.2-0.5)	1.0 (0.6-1.4)[a]	3.8 (1.8-5.3)[a,b]	2.45	<0.05
HbA1c (%)	8.45 ± 2.09	8.88 ± 1.91	8.29 ± 1.89	2.38	0.10
FBG (mmol/l)	8.53 ± 3.21	9.07 ± 3.83	8.07 ± 2.79	0.20	0.82
TC (mmol/l)	4.37 ± 1.14	4.44 ± 1.09	4.56 ± 1.36	0.42	0.66
TG (mmol/l)	1.97 ± 1.33	2.13 ± 1.62	1.83 ± 1.44	0.98	0.38
LDL-C (mmol/l)	2.51 ± 0.80	2.61 ± 1.06	2.78 ± 1.03	1.21	0.30
HDL-C (mmol/l)	1.04 ± 0.25	1.06 ± 0.27	1.10 ± 0.27	1.13	0.32
UA (μmol/l)	336.20 ± 92.41	324.84 ± 93.15[a]	312.67 ± 74.01[a,b]	3.26	<0.05
eGFR (ml/min/1.73^2)	97.25 ± 13.29	89.36 ± 19.85[a]	86.78 ± 20.25[a,b]	4.23	<0.05
Ca (mmol/l)	2.33 ± 0.09	2.32 ± 0.12	2.30 ± 0.08	1.34	0.27
PTH (pmol/l)	36.63 ± 13.60	38.63 ± 14.81	40.74 ± 15.78	1.16	0.32
Lumbar BMD (g/cm^2)	1.05 ± 0.17	0.89 ± 0.11[a]	0.72 ± 0.14[a,b]	100.71	<0.05
Hip BMD (g/cm^2)	0.82 ± 0.06	0.67 ± 0.07[a]	0.55 ± 0.09[a,b]	194.02	<0.05
OC (ng/ml)	17.14 ± 9.75	14.51 ± 6.89[a]	12.80 ± 5.05[a,b]	2.79	<0.05
β-CTX (ng/ml)	0.35 ± 0.23	0.43 ± 0.24	0.45 ± 0.27	2.24	0.11
P1NP (ng/ml)	42.56 ± 26.90	44.76 ± 24.10	50.62 ± 25.53	2.07	0.13
25(OH)D (ng/ml)	18.29 ± 4.99	16.10 ± 5.47[a]	13.89 ± 5.85[a,b]	2.99	<0.05
Complications					
Nephropathy (%)	7 (17.07%)	25 (20.49%)	17 (17.17%)	0.48	0.79
Neuropathy (%)	5 (12.20%)	13 (10.66%)	13 (13.13%)	0.33	0.85
Retinopathy (%)	6 (14.63%)	15 (12.30%)	10 (10.10%)	0.62	0.73
Types of antidiabetic drugs					
Metformin (%)	33 (80.49%)	89 (72.95%)	66 (66.67%)	2.89	0.24
SU (%)	10 (24.39%)	24 (19.67%)	22 (22.22%)	0.47	0.79
α-Glycosidase inhibitors (%)	10 (24.39%)	44 (36.07%)	37 (37.37%)	2.33	0.31
SGLT-2 inhibitor (%)	1 (2.44%)	2 (1.64%)	1 (1.01%)	0.41	0.81
TZD (%)	0	0	0	—	—
GLP-1 receptor agonist (%)	2 (4.88%)	4 (3.28%)	3 (3.03%)	0.32	0.85
DDP-4 inhibitor (%)	31 (75.61%)	78 (63.93%)	65 (65.66%)	1.92	0.38
Insulin (%)	12 (29.27%)	33 (27.05%)	19 (19.19%)	2.44	0.29

Note: [a]$p < 0.05$ compared with the normal group; [b]$p < 0.05$ compared with the osteopenia group. SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin; UA: uric acid; Ca: calcium; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; PTH: parathyroid hormone; eGFR: glomerular filtration rate; BMD: bone mineral density; PMOF: probability of a major osteoporotic fracture; PHF: probability of hip fracture; SU: sulfonylurea; SGLT-2: sodium-glucose cotransporter 2; TZD: thiazolidinedione; GLP-1: glucagon-like peptide 1; DDP-4; dipeptidyl peptidase 4.

of bone metabolism markers among the three groups, the OC level was the highest and 25(OH)D level was the lowest in the normal group, while the OC level was the lowest and 25(OH)D level was the highest in the osteoporosis group, and the difference was statistically significant ($p < 0.05$, respectively) (shown in Table 1).

Table 2: Correlation analysis between BMD and general conditions and biochemical indices in postmenopausal women with T2DM.

Index	Hip BMD		Lumbar spine BMD	
	r	*p*	*r*	*p*
Age (year)	-0.44	<0.05	-0.28	<0.05
BMI (kg/m^2)	0.16	<0.05	0.20	<0.05
Diabetes duration (year)	-0.18	<0.05	-0.25	<0.05
Menopausal year (year)	-0.28	<0.05	-0.33	<0.05
HbA1c (%)	-0.01	0.94	0.01	0.87
FBG (mmol/l)	-0.06	0.38	0.07	0.40
TC (mmol/l)	0.02	0.76	-0.01	0.95
TG (mmol/l)	0.06	0.41	0.01	0.89
LDL-C (mmol/l)	0.05	0.48	0.11	0.18
HDL-C (mmol/l)	0.02	0.74	-0.02	0.69
UA (μmol/l)	0.17	<0.05	0.25	<0.05
eGFR (ml/min/1.73^2)	0.22	<0.05	0.07	0.30
Ca (mmol/l)	0.14	0.06	0.08	0.23
PTH	0.12	0.08	0.18	0.24

Note: SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin; UA: uric acid; Ca: calcium; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; PTH: parathyroid hormone; eGFR: glomerular filtration rate; BMD: bone mineral density.

3.2. Correlation Analysis between BMD and Age, BMI, Diabetes Duration, Menopausal Year, HbA1c, Glucose and Blood Lipid Profile, UA, eGFR, and Other Biochemical Indices in Postmenopausal Women with T2DM. Among the three groups, age, diabetes duration, and menopausal year were negatively correlated with BMD (hip and lumbar spine) as well as the *T* score (hip and lumbar spine) ($p < 0.05$, respectively). On the other hand, BMI was positively correlated with BMD (hip and lumbar spine) as well as the *T* score (hip and lumbar spine) ($p < 0.05$, respectively). There was a positive correlation between the level of UA and BMD and *T* score ($p < 0.05$, respectively). Meanwhile, eGFR level was positively correlated with hip BMD ($r = 0.22$, $p < 0.05$) (shown in Table 2 and Figure 1).

3.3. Binary Logistic Regression Analysis of the Relationship between UA and Osteoporosis in Postmenopausal Women with T2DM. After adjusting the blood pressure, blood lipid profile, blood glucose, calcium, and PTH indices, eGFR and UA were not the independent factors for OP in postmenopausal women with T2DM; however, the age, lower BMI, and T2DM duration were independent risk factors (shown in Table 3).

4. Discussion

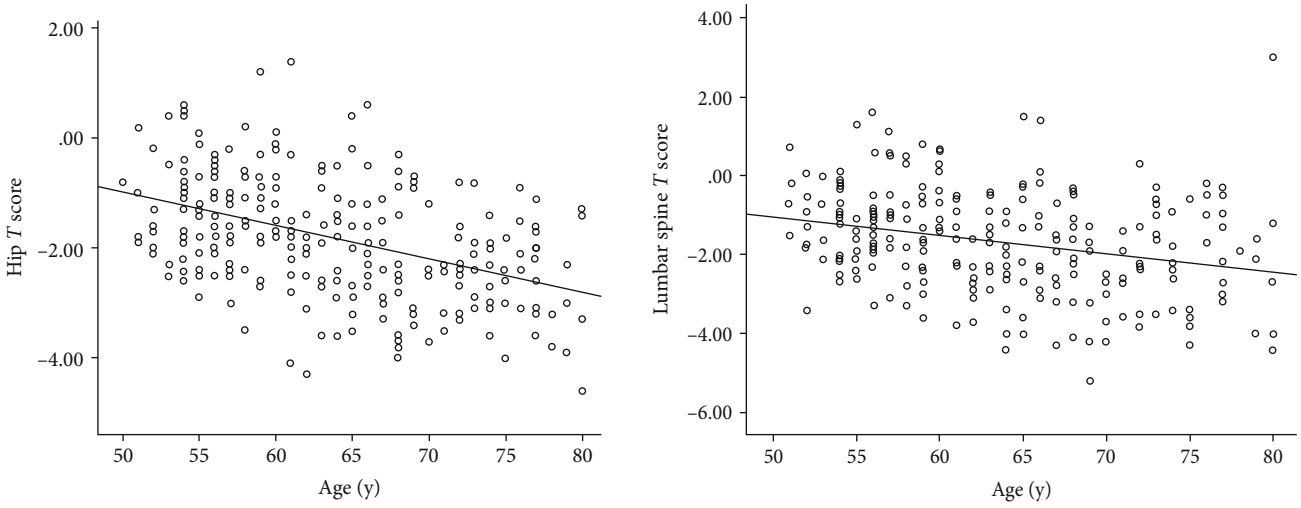
The relationship between T2DM and OP has been widely studied; however, the results are still controversial. Although T2DM patients have normal or even increased BMD, the risk of fracture is higher in T2DM patients than in nondiabetics. Because of this contradiction [10–12], screening for risk factors of OP in T2DM patients as early as possible is the key mechanism of OP prevention and treatment.

Currently, BMD is the gold standard used to evaluate bone mass and diagnose OP in the clinic. Although BMD is the most important factor to predict fracture risk, many brittle fractures in T2DM patients occur in individuals with *T* score higher than -2.5 as seen in clinical practice. Some studies [2] have proposed that increased fracture risk in T2DM patients results from various causes, including increased disease duration, poor blood glucose control, falls caused by hypoglycemia, decreased bone mass, impaired bone mass, and adverse drug reactions. In this study, T2DM duration is an independent risk factor for OP, suggesting that the incidence of OP in T2DM is complex and that the causes are multifactorial.

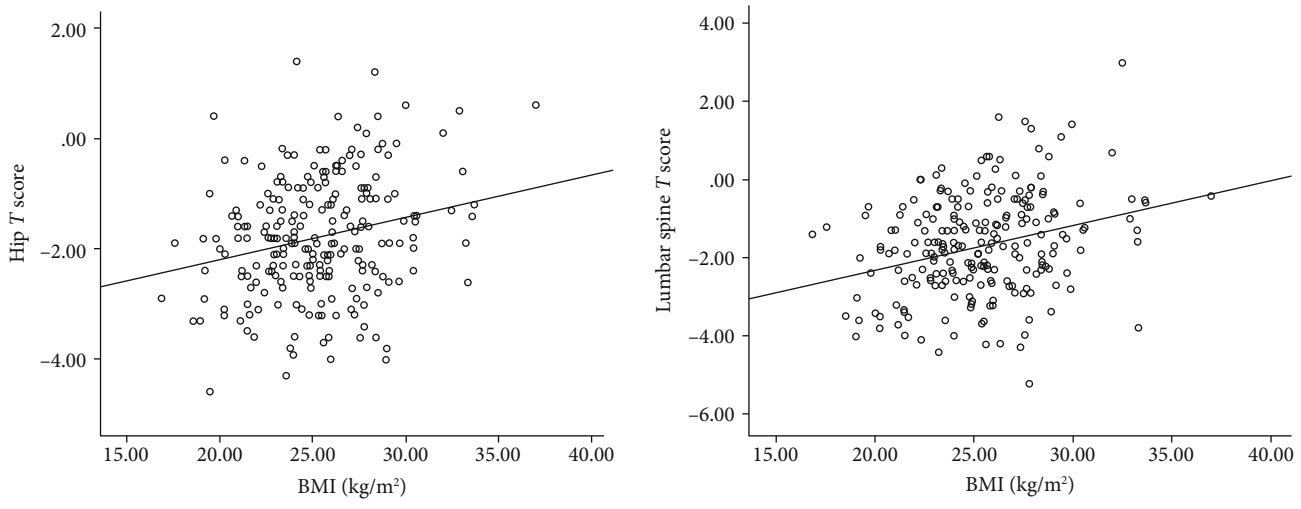
T2DM may affect bone health through a variety of complex ways. (1) Insulin resistance [13, 14]: insulin resistance is an important factor causing dysfunction of osteoblasts and osteoclasts activity. In addition, high blood glucose level can induce cell glycotoxicity, leading to osteoblast apoptosis. (2) Advanced glycation end products (AGEs): one of the inducers of brittle fracture in T2DM patients is age, with older age increasing the risk of brittle fracture in T2DM patients by inducing abnormal collagen arrangement [15, 16]. (3) Calcium loss in urine and vitamin D deficiency: diabetes caused by hyperglycemia results in an increase in calcium levels in the urine and the decrease in calcium level *in vivo*, leading to apoptosis of osteoblasts and the accelerated bone loss. (4) Diabetic complications: diabetic microvascular complications reduce blood supply to bone tissue, leading to bone loss [17]. (5) Use of some hypoglycemic drugs, such as insulin, thiazolidinediones, and sodium-glucose cotransporter 2 (SGLT-2), is related to bone loss and increased risk of fracture, especially in women [18].

This study screened the risk factors of OP in postmenopausal women with T2DM. Research shows that in the normal population, aging, menopause, and lower BMI are the independent risk factors of OP, which has been widely recognized [19]. This result was further confirmed in the postmenopausal women with T2DM in this study. As previously noted [20], this study also found that the increased T2DM duration is an independent factor for postmenopausal women. All of these findings indicate that T2DM patients who are older, with lower BMI, and with longer T2DM duration and menopausal year may have lower BMD and, therefore, higher incidence of OP and greater risk of fracture.

In recent years, studies [21] have shown that UA can promote the proliferation and osteogenic differentiation of human mesenchymal stem cells. UA is closely related to oxidative stress in the human body, and the increase of oxidative stress or the decrease of antioxidants will reduce the level of BMD. Whether UA is a protective factor or a risk factor of OP is controversial. At present, it is believed that UA has double effects on the body. The physiological concentration of UA has a protective effect on the stability of bone mass, while the excessive UA has the opposite effect. The mechanism of the increase of BMD induced by UA may be as follows: oxidative stress can inhibit the differentiation of osteoblasts and induce the death of osteoblasts. As a reducing



(a)



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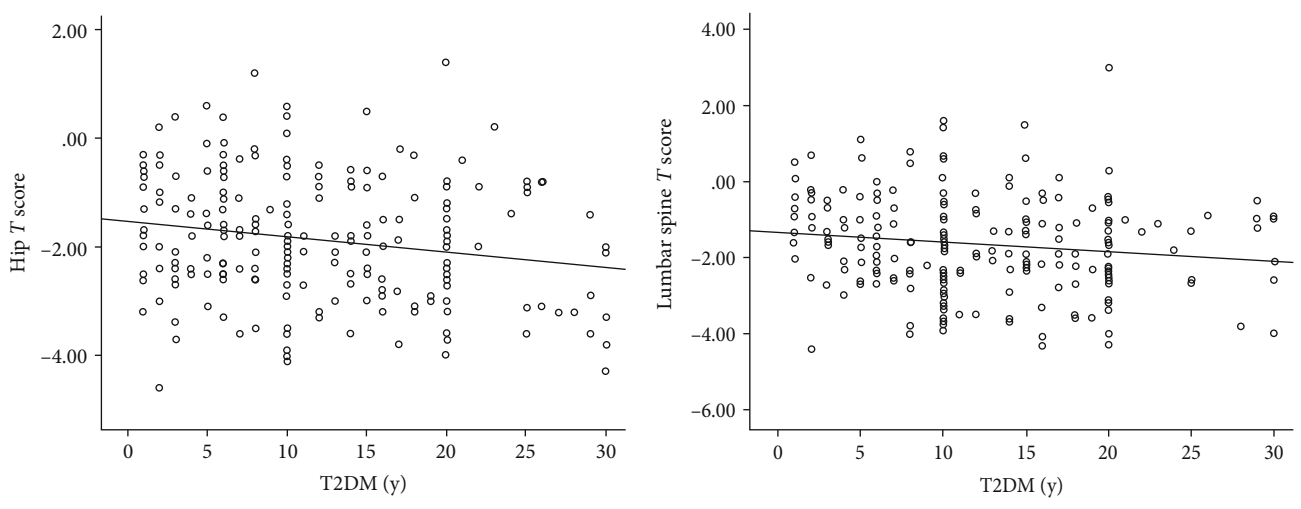

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Figure 1: Continued.

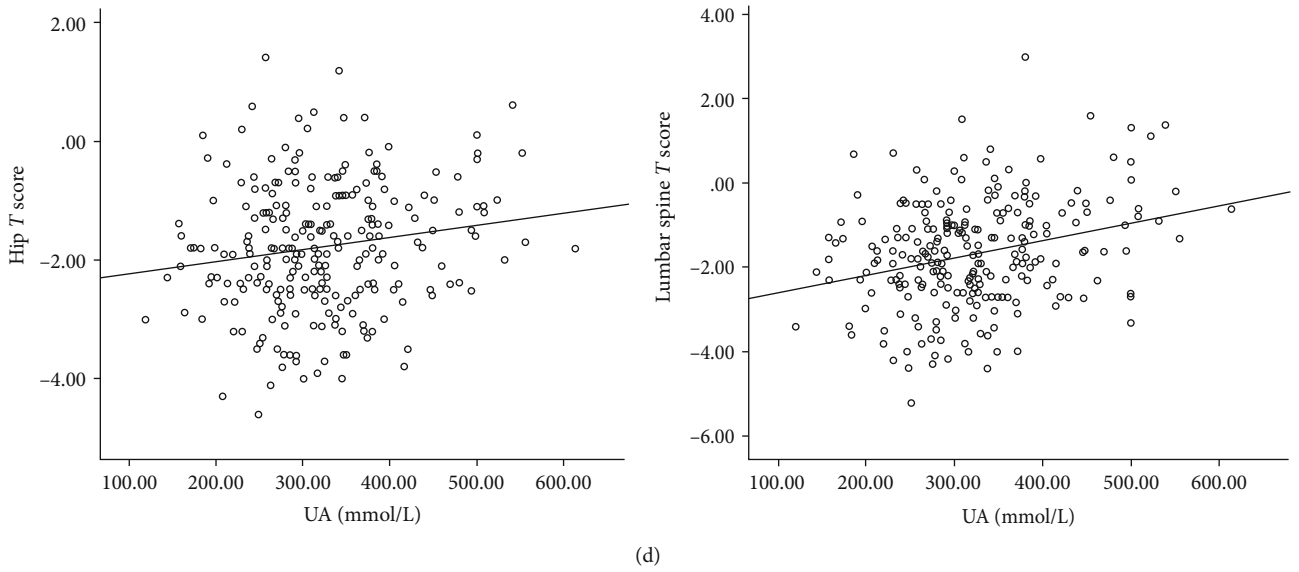

Figure 1: Comparison of the relation between age, BMI, and T2DM duration and hip and lumbar spine T score, respectively. (a) Correlation analysis revealed that age was negatively correlated with hip and lumbar spine T score ($r = -0.44$, $p < 0.05$; $r = -0.28$, $p < 0.05$, respectively). (b) Correlation analysis revealed that BMI was positively correlated with hip T score and lumbar spine T score ($r = 0.17$, $p < 0.05$; $r = 0.24$, $p < 0.05$, respectively). (c) Correlation analysis revealed that BMI was negatively correlated with hip T score and lumbar spine T score ($r = -0.21$, $p < 0.05$; $r = -0.19$, $p < 0.05$, respectively). (d) Correlation analysis revealed that UA was positively correlated with hip T score and lumbar spine T score ($r = 0.17$, $p < 0.05$; $r = 0.28$, $p < 0.05$, respectively).

Table 3: Binary logistic regression analysis of the relationship between UA and osteoporosis in postmenopausal women with T2DM.

Index	OP		
	βst	OR (95% CI)	p
Age (year)	0.14	1.15 (1.08, 1.22)	<0.05
BMI (kg/m^2)	-0.16	0.86 (0.77, 0.96)	<0.05
Diabetes duration (year)	0.05	1.05 (1.01, 1.11)	<0.05
eGFR (ml/min/1.73^2)	0.00	1.00 (0.98, 1.03)	0.78
UA (μmol/l)	-0.01	1.00 (0.99, 1.01)	0.81

Note: BMI: body mass index; UA: uric acid; eGFR: glomerular filtration rate.

substance, UA can prevent the production of reactive oxygen species in osteoblasts and stimulate the differentiation of osteoblasts, thus increasing bone formation [22]; UA can also inhibit the generation of osteoclasts, reduce the production of oxygen free radicals by osteoclast precursors, and reduce bone absorption. Foreign scholars [23] believe that there is a positive correlation between BMD and UA, and when UA is between 4 and 4.99 mg/dl, it reduces the risk of osteoporosis. Similarly, Ishii et al. [24] found that the level of hyperuricemia in the physiological range was linearly related to the increase of lumbar BMD in Japanese postmenopausal women, but whether there was still a positive correlation between the two indices in the hyperuricemia range is questioned. A larger population study believes that the increase of uric acid level is protective for bone density and bone strength [24, 25]. However, some studies have suggested that hyperuricemia is a risk factor for OP due to the role of inflammatory factors and the involvement of oxidative stress response [26, 27]. In this study, UA and eGFR were found to have positive correlation with bone mineral density and T score, which has previously been reported in patients without T2DM [28]. Due to the influence of metabolic indicators such as blood glucose, blood lipid, and blood pressure, the direct effect of UA on OP may not be found. In this study, after adjusting for BMI, age, blood pressure, blood glucose, blood lipid profile, and other factors, UA and eGFR were not found to be independent risk or protective factors of OP in postmenopausal women with T2DM. This finding suggests that the correlation between UA and eGFR and BMD might be due to the influence of BMI and metabolism index. After excluding the confounding factors, UA and eGFR were not found to be independent factors of OP in women with T2DM. In addition, in this study, the subjects were all inpatients. The level of blood glucose was higher than that of outpatients (the mean HbA1c level was 8.3-8.8%). Therefore, the risk of hypoglycemia was little, so the subjects are not fragile patients.

In this study, there was no significant difference among the three groups in the occurrence of complications and the application of hypoglycemic drugs, so the complications of diabetes and the interference of hypoglycemic drugs on the results were excluded as much as possible.

However, there are a few limitations in this study. The sample size needs to be larger to better assess the risk factors of OP in T2DM. Also, whether UA is a protective factor or a risk factor of OP in patients with T2DM could not be clarified due to the complexity of pathophysiological mechanism and the interference of multiple metabolic indicators. Therefore, further longitudinal research and large-sample epidemiological data is needed to confirm any finding.

More and more studies have shown that T2DM is a clinical risk factor that leads to increase in fracture. Many

commonly used clinical indices such as the effect of UA level on osteoporosis have not been confirmed. In postmenopausal patients with type 2 diabetes in our study, uric acid levels do not influence either positively or negatively bone mineral density. Therefore, it is of great clinical significance to find more risk or protective factors of OP for preventing the occurrence of fracture in such patients.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Review Article

Pathophysiology and Management of Type 2 Diabetes Mellitus Bone Fragility

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Individuals with type 2 diabetes mellitus (T2DM) have an increased risk of bone fragility fractures compared to nondiabetic subjects. This increased fracture risk may occur despite normal or even increased values of bone mineral density (BMD), and poor bone quality is suggested to contribute to skeletal fragility in this population. These concepts explain why the only evaluation of BMD could not be considered an adequate tool for evaluating the risk of fracture in the individual T2DM patient. Unfortunately, nowadays, the bone quality could not be reliably evaluated in the routine clinical practice. On the other hand, getting further insight on the pathogenesis of T2DM-related bone fragility could consent to ameliorate both the detection of the patients at risk for fracture and their appropriate treatment. The pathophysiological mechanisms underlying the increased risk of fragility fractures in a T2DM population are complex. Indeed, in T2DM, bone health is negatively affected by several factors, such as inflammatory cytokines, muscle-derived hormones, incretins, hydrogen sulfide (H2S) production and cortisol secretion, peripheral activation, and sensitivity. All these factors may alter bone formation and resorption, collagen formation, and bone marrow adiposity, ultimately leading to reduced bone strength. Additional factors such as hypoglycemia and the consequent increased propensity for falls and the direct effects on bone and mineral metabolism of certain antidiabetic medications may contribute to the increased fracture risk in this population. The purpose of this review is to summarize the literature evidence that faces the pathophysiological mechanisms underlying bone fragility in T2DM patients.

1. Introduction

Osteoporosis and type 2 diabetes mellitus (T2DM) are chronic disorders associated with severe morbidity and increased mortality. Their prevalence, due to the general population ageing, is rapidly increasing and will early become a global epidemic imposing an overwhelming burden on health-care systems [1–7].

Nowadays, skeletal fragility is considered a complication of T2DM [1]. These patients have an up to 3-fold increased hip fracture risk [3–5]. Fractures of the wrist and the foot also seem to be more frequent, while the evidences on vertebral fractures are more limited [2]. Anyhow, available data suggest a higher risk of vertebral fractures and in particular morphometric vertebral fractures [6, 7], which has been suggested to be present in a third of T2DM postmenopausal

women [8]. In T2DM patients, the fracture risk is increased for any given T-score with respect to the general population, so that fractures may occur despite a normal or even increased bone mineral density (BMD) [1, 5, 6], suggesting that the bone quality alterations rather than the BMD decrease may be the main factor influencing T2DM-related bone fragility [9]. Therefore, the assessment of BMD alone cannot represent a reliable tool to estimate fracture risk [9]. Similarly, fracture risk assessment algorithms, such as the WHO Fracture Risk Assessment Tool, underestimate fracture risk in these subjects [9–11].

Several pathophysiological mechanisms have been implicated in the deterioration of bone quality in T2DM. From a clinical point of view, the T2DM duration, the glycemic control, and the presence of the T2DM-related chronic complications (i.e., retinopathy, neuropathy, and macroangiopathy) are predictors of fragility fractures. Moreover, several T2DM therapies can have a direct negative role on bone metabolism. Chronic hyperglycemia and advanced glycation end product (AGE) accumulation, insulin resistance, altered bone marrow adiposity, inflammatory factors, and adipokines released by visceral fat and oxidative stress [2, 12] represent the principal mechanisms of T2DM-induced bone fragility.

Currently, the research in this field is getting rich by new evidences. Some data suggest that a decrease in hydrogen sulfide (H2S), which has a fundamental role for maintaining bone cell proliferation and differentiation, may be implicated in the pathogenesis of T2DM-related bone fragility [13]. Finally, starting from the similarities between the cortisol-related bone loss and T2DM-related bone fragility, the cortisol secretion, sensitivity, and peripheral activation (the so-called "cortisol milieu") have been suggested to play a role in T2DM-related bone fragility [8, 14, 15].

This review is aimed at exploring the current understanding of the pathophysiological mechanisms underlying T2DM-related bone fragility.

2. Methodology

According to PRISMA guidelines, PubMed and MEDLINE were searched from June 1968 to January 2020 for identifying published articles about bone metabolism and T2DM. In particular, we considered articles focused on the interactions between T2DM and bone fragility, such as hyperglycemia, insulin resistance, AGEs, bone marrow adiposity, inflammatory cytokines, H2S, and cortisol. Studies that analyzed how T2DM impacts on bone formation and resorption, collagen formation, skeletal muscle and the incretin system were evaluated. Only publications in English were included (Figure 1).

3. Evaluation of Bone Health in T2DM

3.1. Evaluation of Bone Fragility beyond Dual X-Ray Densitometry (DXA). In T2DM, individual fractures generally occur at higher BMD levels than in nondiabetic subjects, with T-score levels being often above the osteoporotic range. Thus, it has been estimated that in T2DM subjects, an increase in hip fracture risk, similar than in controls, occurs at 0.4 and 0.6 SD higher BMD levels in men and women, respectively [3, 15–17]. On the other hand, the T2DM duration (i.e., >10 years), insulin treatment, and the presence of the T2DM-related chronic complications are associated with fragility fractures regardless of BMD. These evidences justify the need of a spinal X-ray in patients with T2DM chronic complications or poorly controlled disease, in addition to the already fractured ones. Indeed, up to a third of postmenopausal T2DM women investigated by a lateral spine radiograph showed asymptomatic morphometric vertebral fractures [8] that represent a major risk factor for additional subsequent fractures [18].

Again, considering the inadequate reliability of BMD in a T2DM population, other imaging techniques have been investigated in the last years [19]. Different retrospective and cross-sectional studies have showed that Trabecular Bone Score (TBS), a textural index based on evaluating pixel grey-level variations in the lumbar DXA image, providing an indirect index of bone architecture, is often reduced in T2DM [19] and that it might predict fracture risk better than BMD [15, 19–21].

In some cohorts of T2DM patients, the hip structural analysis (HSA) that represents an additional tool that can be applied to DXA in order to obtain information on bone geometry and indirectly assess the bone resistance to axial compressive forces [22] showed a weaker geometry (e.g., a narrower neck width) and compromised estimates of skeletal load response (e.g., a lower buckling ratio) [22]. However, the additive role of HSA on the prediction of fractures in T2DM remains to be established.

Although quantitative ultrasound (QUS) devices of the calcaneus and the phalanxes are widely available and low-cost techniques, therefore potentially very useful for the screening of large populations such as the diabetic one, limited information has been released about their use in T2DM. Available data showed that QUS parameters may be useful [23], but data concerning their predictive role in discriminating patients with and without fragility fractures are conflicting [23, 24]. Moreover, a correlation between reduced QUS parameters and poor glycemic control or peripheral nerve dysfunction has been also described [24].

Recently, peripheral quantitative computed tomography (QCT) and high-resolution peripheral QCT of the distal radius and tibia have been employed to obtain a 3D assessment of bone size, volumetric BMD, and bone macro- and microarchitecture (e.g., cortical porosity and trabecular connectivity). Moreover, QCT images can also be employed for the estimation of the mechanical properties of bone by means of finite element analysis (FEA) [19]. However, the study results using these techniques have been quite inconsistent. Several studies, although not all, suggest that in T2DM women, the indices of trabecular microarchitecture are preserved but cortical porosity is increased and it is specifically associated with a deficit in biomechanical properties, particularly in those diabetic females with fragility fractures [25–28]. Data in T2DM men are even more scarce, but available ones indicate that the deficits in cortical bone affect both sexes, at least in older T2DM patients [29].

The use of magnetic resonance imaging to assess trabecular and cortical bone parameters at both peripheral and

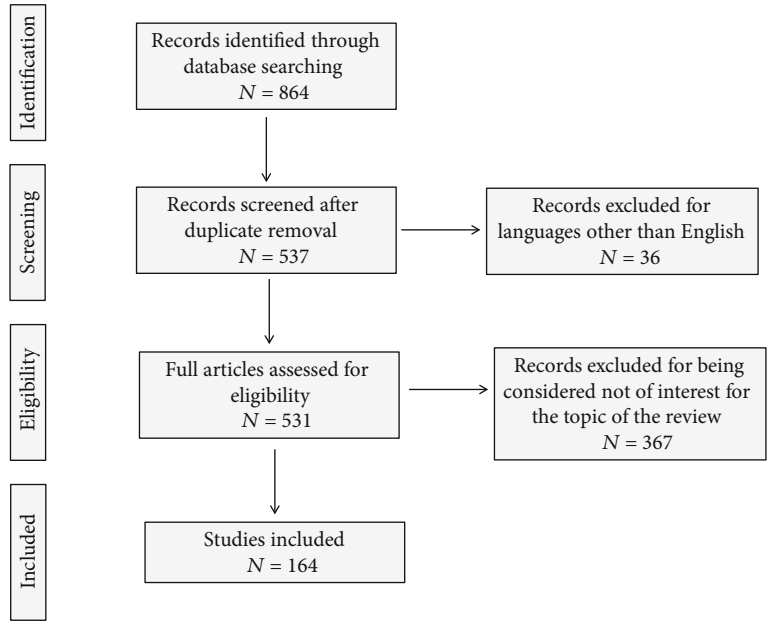

Figure 1: PRISMA flow diagram. According to PRISMA guidelines, PubMed and MEDLINE were searched from June 1968 to January 2020 for identifying published articles about bone metabolism and T2DM. In particular, we considered articles focused on the interactions between T2DM and bone fragility, such as hyperglycemia, insulin resistance, AGEs, bone marrow adiposity, inflammatory cytokines, H2S, and cortisol. Studies that analyzed how T2DM impacts on bone formation and resorption, collagen formation, skeletal muscle, and the incretin system were evaluated. Only publications in English were included.

axial skeleton could help in discriminating patients at higher fracture risk [25]. Remarkably, magnetic resonance spectroscopy of the vertebral bodies evidenced an altered bone marrow fat (BMF) composition (with lower unsaturation of bone marrow lipids) in T2DM postmenopausal women with fragility fractures [21]. This approach might represent a promising tool for fracture risk assessment in the future, given the negative role of BMF on bone health, as described later on [18, 25, 26].

Finally, an emerging invasive technique for direct measurement of mechanical characteristics of cortical bone in vivo is microindentation, which consists of the insertion of a specific probe into a cortical bone's surface at the anterior tibia to induce microscopic fractures. The impact microindentation, from which a ratio called bone material strength index (BMSi) can be derived as an index of fracture resistance, has been used for the assessment of bone properties in T2DM. Postmenopausal T2DM women showed significantly lower BMSi compared to controls, also after adjustment for BMI and despite similar BMD. Moreover, BMSi values were found to be negatively correlated with glycemic control and disease duration [19], thus confirming, using a direct in vivo measure, compromised bone quality in T2DM and the potential detrimental effects of prolonged hyperglycemia on bone.

Currently, the other available techniques beside DXA and vertebral morphometry, notwithstanding their potential role for investigating the mechanisms of the T2DM-related bone fragility, need to be tested in prospective studies and their scarce availability, high costs, microindentation and also the invasive nature of the procedure do not consent a routine use in clinical practice.

In addition to the indications in nondiabetic patients, a spinal radiograph for evidencing possible asymptomatic vertebral fractures should be performed in T2DM patients with clinical fragility fracture and/or with T2DM-related chronic complications, insulin use, and/or long T2DM duration (i.e., above 10 years).

3.2. Bone Turnover. In T2DM patients, histomorphometric studies have shown a reduction of the osteoblast number and of the osteoid amount [30] and a low bone formation rate [31]. Interestingly, this reduction in bone formation and mineralized surface has been found in the cancellous, intracortical, and endocortical surfaces of patients with T2DM but not of patients with type 1 diabetes [32]. However, some other data suggested that in addition to the reduction of the activation frequency of the bone remodeling units, in some patients, an increase in bone mineralization may be present. However, in T2DM, the nonenzymatic collagen crosslinking by pentosidine was found to be increased and directly associated with glycated hemoglobin (HbA1c) levels [33]. Overall, these data suggest a low bone turnover state in T2DM. Interestingly, the pentosidine level has been proposed as a bone fragility marker in T2DM [34, 35].

In keeping with these data, even clinical studies have shown a reduced bone turnover in T2DM. In particular, both bone apposition, as mirrored by osteocalcin levels, and bone resorption, as evaluated by the C-terminal telopeptide of type I collagen (CTX) levels, were found to be reduced and negatively associated with metabolic control [36–38]. In keeping with a reduction of bone turnover in T2DM, other markers of bone apposition and resorption, such as the procollagen type 1 amino-terminal propeptide and the N-terminal

telopeptide of type I collagen, respectively, were found to be reduced in patients with T2DM than in nondiabetic controls [39]. At variance, alkaline phosphatase total activity has been found to be increased in T2DM patients than in nondiabetic individuals. Even though both histomorphometrical and biochemical data point toward a low bone turnover osteoporosis, some data seem conflicting [37]. It must be considered, however, that the different studies are frequently not easily comparable, due to differences in disease duration, glycometabolic control, presence of chronic complications, age, ethnicity, and several other differences among study participants. Importantly, notwithstanding the potential role of a decrease of bone turnover in reducing the microcrack repairing in T2DM patients and, thus, in increasing the fragility fracture risk, we still do not know whether bone turnover markers can be used to predict fractures in T2DM patients.

Even a role of low levels of parathyroid hormone (PTH) has been hypothesized in T2DM-related bone fragility [40]. Indeed, some data suggested that a subtle hypoparathyroidism could contribute to low bone turnover in patients with diabetes mellitus. In keeping with this idea, PTH levels have been found to be directly associated with CTX, tartrate-resistant acid phosphatase 5b, and osteocalcin levels [40]. Interestingly, a chronic hypomagnesemia has been hypothesized to impaired PTH secretion in T2DM [41], and a renal calcium leak induced by glycosuria can determine a negative calcium balance, which seems to normalize after improving the glycometabolic control [42].

Summarizing, in the authors' opinion, the use of bone turnover markers and/or PTH level determination are not to be considered mandatory in the vast majority of T2DM patients. The determination of CTX and PTH levels should be reserved in doubtful cases (for example, if an additional secondary cause of osteoporosis is suspected) and on case-by-case basis.

4. Factors Leading to Bone Fragility in T2DM

4.1. Disease Duration, Insulin Use, Glycometabolic Control, and Complications. The difficulty in discriminating the independent effects of disease duration, metabolic control, and presence of T2DM complications is due to the existence of a strong link between these aspects of the T2DM condition. However, different studies found that a T2DM duration longer than 10 years significantly increases the fragility fracture risk, regardless of diabetes control [43–46]. Generally, the T2DM duration seems to negatively affect bone metabolism, even though it is important to keep in mind that T2DM may often remain undiagnosed for many years. Besides T2DM duration, a poor glycemic control (e.g., HbA1c levels ≥ 7.5 %) has been shown to be associated with increased fracture risk [47, 48]. A large (i.e., enrolling more than 4 thousand individuals) long-term prospective (i.e., about 12 years mean follow-up) study showed that the fracture risk is similar between nondiabetic subjects and adequately controlled T2DM patients [49], while the fracture risk was 1.6-fold increased in subjects with inadequately controlled T2DM. This relationship between T2DM control and fragility fracture risk was not confirmed in another study, in which, however, the median levels of HbA1c were only slightly elevated (~7.5%) [50]. Despite these evidences, it is important to keep in mind that the predictive value of a single HbA1c value in the determination of fracture risk is questionable.

Whether T2DM complications could represent independent risk factors for bone fragility is still a matter of debate. In a large case-control study, T2DM itself and all its complications were significantly associated with an increased overall risk of fractures [43, 51, 52], without a clear evidence of the independent contribution of each single factor. Interestingly, both neuropathy and insulin use may influence the risk of falls [53–55], which are of crucial importance in these patients, being associated with an increased risk of fracture, hospitalization, and death [56]. Indeed, as compared to nondiabetic subjects, the risk of falling more than once a year is known to be increased in older women with T2DM without insulin use and even higher in insulin users [54]. Sarcopenia, the age-related decline in skeletal muscle mass, quality, and function, may represent an additional contributing factor to the increased fall and fracture [57], and it is known to be associated with T2DM. Indeed, in T2DM patients, both muscle strength and function are decreased as compared to nondiabetic subjects [58, 59]. In addition, T2DM patients can present neuromuscular dysfunction, which may favor falls irrespective of sarcopenia [60]. However, the evidences of association between sarcopenia, fall risk, and bone fragility in T2DM cohorts are still limited.

Finally, some studies found an association between microvascular disease and bone microstructure as well as with fracture risk. This association might be explained by an altered vascular supply to the skeleton, in particular cortical bone, that may contribute in compromising bone formation [2].

Overall, in our opinion, great attention should be reserved to T2DM patients, who have a long-standing disease and/or chronic complications and/or are insulin treated.

4.2. Oral Antidiabetic Agents. The effect of oral antidiabetic agents on bone is summarized in Table 1. Among the possible mechanisms contributing to the increased risk of fracture in T2DM, the use of antidiabetic drugs with direct effects on bone cells or an indirect effect on bone metabolism has to be taken into consideration.

Metformin, the worldwide accepted first-line drug in the treatment of T2DM [61], exerts its effect decreasing liver glucose production, enhancing insulin sensitivity, and inhibiting fatty acid synthesis and promoting their oxidation [62]. In most animal studies, metformin seems to improve bone mass and strength [2, 63], by preventing the advanced glycation end product (AGE) accumulation, known to induce alterations in the osteoblastic cells. Moreover, metformin has been suggested to inhibit the formation of reactive oxygen species (ROS) and apoptosis in osteoblastic cultures exposed to high glucose concentrations [63]. Unfortunately, the evidence of a positive effect on bone of metformin administration in T2DM patients is less impressive and somewhat conflicting. However, overall metformin is reported having positive or neutral effects on fracture risk in T2DM patients [46, 64–66].

Table 1

Metformin			
Preclinical	Ref.	Effect	
	[2, 63]	↑ Bone mass and bone strength ↓ AGE accumulation ↓ ROS formation ↓ Osteoblast apoptosis	
Clinical	Ref.	Characteristics	Fracture risk
	[46]	Prospective cohort study among 1964 Rochester residents who first met glycemic criteria for diabetes in 1970-1994 (mean age, 61.7 ± 14.0 yr; 51% men)	The risk was decreased among users of biguanides (HR, 0.7; 95% CI, 0.6-0.96)
	[64]	Prospective cohort study, based on data from the Osteoporotic Fractures in Men (MrOS) study that enrolled 5,994 men (aged ≥65 years)	Metformin did not increase the risk of nonvertebral fracture
	[65]	Case-control study based on 498,617 subjects in Denmark	Decreased risk of fractures
	[66]	Population based study among 206,672 individuals	There was no association of hip fracture with cumulative exposure to metformin
			Overall: ↓ = fracture risk
Sulfonylureas			
Preclinical	Ref.	Effect	
	[2, 61, 63]	↑ Osteoblast proliferation and differentiation	
Clinical	Ref.	Characteristics	Fracture risk
	[46]	Prospective cohort study among 1964 Rochester residents who first met glycemic criteria for diabetes in 1970-1994 (mean age, 61.7 ± 14.0 yr; 51% men)	No significant influence on fracture risk was seen with sulfonylurea
	[65]	Case-control study based on 498,617 subjects in Denmark	Use of sulfonylureas was associated with a decreased risk of any fracture
	[66]	Population-based study among 206,672 individuals	There was no association of hip fracture with cumulative exposure to sulfonylureas
	[67]	Retrospective observational study on 361,210 patients with type 2 diabetes	ICD-9-CM-coded outpatient hypoglycemic events were independently associated with an increased risk of fall-related fractures
	[69]	Cross-sectional study on 838 Japanese patients with T2DM	Decreased risk of vertebral fractures in postmenopausal women (OR = 0.48, $P = 0.018$)
			Overall: ↓ = fracture risk, ↑ fall risk due to hypoglycemia
Thiazolidinediones			
Preclinical	Ref.	Effect	
	[2, 63]	↑ Osteoclastogenesis ↑ Osteocytes apoptosis	
	[70–72]	↑ Bone marrow adipogenesis ↓ Osteoblastogenesis	
Clinical	Ref.	Design	Fracture risk
	[73]	Longitudinal study on ADOPT data from 1,840 women and 2,511 men with T2DM	The increase in fractures with rosiglitazone representing hazard ratios (95% CI) of 1.81 (1.17-2.80) and 2.13 (1.30-3.51) for rosiglitazone compared with metformin and glyburide occurred in pre- and postmenopausal women, and fractures were seen predominantly in the lower and upper limbs
	[76]	Nested case-control study based on data of 32,466 T2DM from the Longitudinal Health Insurance Database 2000 (LHID2000) and the catastrophic illness patient registry (CIPR) in Taiwan	Increased risks for fracture in patients who used TDZs, especially in female patients younger than 64 years old, for whom the risk was elevated from a 1.74- to a 2.58-fold odds ratio
			Overall: ↑ fracture risk (peripheral fractures)

Table 1: Continued.

Incretins			
Preclinical	Ref.	Effect	
	[2, 63]	DPP-4 inhibitors ↓ Bone resorption; ↑ trabecular and cortical bone volume	
	[82, 83]	GLP1-RA ↑ Proliferation of bone marrow mesenchymal stem cells; ↓ differentiation adipocytes; ↓ sclerostin expression	
Clinical	Ref.	Design	Fracture risk
	[85]	Meta-analysis including 16 RCTs and a total of 11,206 patients to study the risk of bone fractures associated with liraglutide or exenatide, compared to placebo or other active drugs	Liraglutide treatment was associated with a significant reduced risk of incident bone fractures (MH − OR = 0.38, 95% CI 0.17-0.87); however, exenatide treatment was associated with an elevated risk of incident bone fractures (MH − OR = 2.09, 95% CI 1.03-4.21)
	[86]	Meta-analysis including 7 RCTs to assess GLP-1Ra-related fracture risk compared with other antidiabetic drugs	Use of GLP-1Ra does not modify the risk of bone fracture in T2DM compared with the use of other antidiabetic medications
	[88]	A case-control study nested within a cohort of 1,945 diabetic outpatients with a follow-up of 4.1 ± 2.3 yr	No significant association was observed between bone fractures and medications
	[89]	A retrospective analysis of real-world data that matched 4160 DPP4i ever users to never users in metformin-treated T2DM patients (mean age 61 ± 11 yr), in Germany	The use of DPP-4 inhibitors was associated with a significant decrease in the risk of developing bone fractures (all patients HR = 0.67, 95% CI 0.54-0.84; women HR = 0.72, 95% CI 0.54-0.97; men HR = 0.62, 95% CI 0.44-0.88)
	[90]	Meta-analysis based on 51 RCTs (N = 36,402; mean age 57 ± 5 yr), to assess fractures in T2DM, comparing DPP-4 inhibitors with either an active agent or a placebo	No association of fracture events with the use of DPP-4 inhibitor when compared with placebo (OR; 0.82, 95% CI 0.57-1.16; P = 0.9) or when DPP-4 inhibitor was compared against an active comparator (OR; 1.59, 95% CI 0.91-2.80, P = 0.9)
			Overall: ↓ fracture risk with liraglutide; =↓ fracture risk with DPP-4 inhibitors
SGLT-2 inhibitors			
Preclinical	Ref.	Effect	
	[94]	↑ Urinary calcium ↓ Serum PTH levels	
Clinical	Ref.	Design	Fracture risk
	[92]	Meta-analysis on 20 studies including 8,286 patients treated with SGLT-2 compared with placebo	Not increased fracture risk; pooled risk ratio of bone fracture in patients receiving SGLT2 inhibitors versus placebo was 0.67 (95% confidence interval, 0.42-1.07)
	[93]	Cumulative meta-analysis of 38 RCTs (10 canagliflozin, 15 dapagliflozin, and 13 empagliflozin) involving 30,384 patients	Compared with placebo, canagliflozin (OR 1.15; 95% CI 0.71-1.88), dapagliflozin (OR 0.68; 95% CI 0.37-1.25), and empagliflozin (OR 0.93; 95% CI 0.74-1.18) were not significantly associated with an increased risk of fracture
	[96]	Randomized phase 3 study on 10,194 T2DM patients to describe the effects of canagliflozin on bone fracture risk	Fracture risk was increased with canagliflozin treatment and may be mediated by falls
			Overall: = fracture rate or ↑ by canagliflozin
Insulin			
Preclinical	Ref.	Effect	
	[97–99]	↑ Bone anabolism; ↓ bone resorption ↑ BMD	
Clinical	Ref.	Design	Fracture risk
	[46]	Prospective cohort study among 1964 Rochester residents who first met glycemic criteria for diabetes in 1970-1994 (mean age, 61.7 ± 14.0 yr; 51% men)	Increased fracture risk in patients on insulin (HR, 1.3; 95% CI, 1.1–1.5)
	[64]	Prospective cohort study, based on data from the Osteoporotic Fractures in Men (MrOS) study that enrolled 5,994 men (aged ≥65 years)	The risk of nonvertebral fracture increased only among men with T2DM who were using insulin (HR 1.74, 95% CI 1.13, 2.69)

Table 1: Continued.

[43]	Prospective study on 3,654 older Australians	Insulin treatment was associated with increased fracture risk (adjusted RR 5.9, 95% CI 2.6-13.5)
[101]	Prospective cohort study based on data from 9654 women, aged >65 yr in the Study of Osteoporotic Fractures	Insulin-treated diabetics had more than double the risk of foot (multivariate adjusted RR, 2.66; 95% CI, 1.18-6.02) fractures compared with nondiabetics
		Overall: ↑ fracture risk (especially nonvertebral fracture)

The role of sulfonylureas (insulin secretagogues, blocking ATP-regulated K+ channels, that enhance insulin release from pancreatic β-cells) on bone metabolism has been investigated only in few studies [61]. Available data evidence a potential stimulatory effect on osteoblast proliferation and differentiation and a protective role on osteoblasts against hyperglycemia [2, 63]. However, some studies reported an increased risk of falls and fractures that might be due to the increased risk of hypoglycemia associated with the use of these drugs [64, 67], while other studies reported a neutral or even positive effect on fracture risk [46, 65–69].

Pioglitazone and rosiglitazone, known as thiazolidinediones (TZDs), activating peroxisome proliferator-activated receptor gamma (PPARγ), reduce the extent of insulin resistance and improve β-cell response towards altered glucose levels. Despite the beneficial effect of TZDs on glycemic control, their prolonged use has been associated with negative effects on bone metabolism. Interestingly, in bone marrow stromal stem cells (BMSC), PPARγ activation increases adipogenesis and decreases osteoblastogenesis [70–72]. In keeping, TZDs have been shown to decrease bone formation, increase osteoclastogenesis, and promote osteocyte apoptosis [2, 63]. Several clinical studies have shown that in patients using TZDs, the bone formation markers decrease, while the bone resorption markers increase and BMD declines [2, 63]. Moreover, randomized controlled trials and prospective studies revealed an increased peripheral fracture risk in TZD-treated patients, especially in postmenopausal T2DM women [72–76]. Furthermore, BMD loss observed in TZD users seems to be not reversible after treatment discontinuation [77].

Gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are gut-derived hormones that stimulate insulin, suppress glucagon secretion, inhibit gastric emptying, and reduce appetite and food intake (so-called "incretin effect"). Patients with T2DM have a reduced incretin effect [78]. The therapeutic approaches for restoring the incretin action include degradation-resistant GLP-1 receptor agonists (GLP-1 mimetics) and inhibitors of dipeptidyl peptidase-4 (DPP-4) activity [79]. The presence of GLP-1 and GIP receptors in human osteoblastic cells at different stages of differentiation induced many authors to investigate the effect of these gut-derived hormones on bone metabolism [80]. Moreover, GLP-1 receptors are expressed even in BMSC and immature osteoblasts [81]. Several evidences suggest that GLP-1 stimulates proliferation of mesenchymal stem cells and inhibit their differentiation into adipocytes [82]. In vivo studies showed an osteogenic effect of GLP-1 that seems to be mediated through the inhibition of the expression of the sclerostin gene [83] and of the WNT pathway [81]. A study in rodents showed that the higher the doses of exendin-4 (a GLP-1 mimetic), the higher the increase in bone strength and bone formation [84].

From a clinical point of view, few meta-analyses or post hoc analyses of population-based studies have been performed on the relation between the incretin use and bone fragility in T2DM and showed conflicting results. A recent meta-analysis of 16 RCTs on the effect on fracture risk of the GLP-1 receptor agonists showed that, among the GLP-1 mimetics, while the exenatide use was associated with an increased fracture risk with respect to other antidiabetic agents, the liraglutide use was associated with a significantly reduced risk of fractures [85]. However, other studies did not report significant effects of GLP-1 receptor agonist fracture risk and BMD [86, 87]. Also, for DPP-4 inhibitors, available data are conflicting. In vitro studies show a neutral effect on osteoblast differentiation. However, in animal models, these agents have been found to increase trabecular and cortical bone volume, due to a suppression of bone resorption [2, 63]. As far as study in humans is concerned, although two clinical studies showed a positive effects on fracture prevention in patients treated with the DPP-4 inhibitors [88, 89], a recent meta-analysis reported a neutral role of these agents [90]. Overall, it should be underlined that none of these studies were specifically designed to assess the effect of DPP-4 inhibitors or of incretins on fracture prevention, and the information regarding fractures has been obtained only by analyzing the safety profile. This explains the small fracture number emerged from these studies.

A new class of blood glucose-lowering drug for T2DM is represented by the sodium-glucose cotransporter-2 (SGLT-2) inhibitors. These drugs inhibit SGLT2 in the proximal convoluted tubule preventing the reabsorption of glucose and inducing its excretion in urine. Importantly, even the tubular phosphate reabsorption is increased by using these agents. Available preclinical and clinical data suggest that the SGLT2 inhibitors might negatively affect bone health, but data on fracture risk are controversial [2]. Indeed, two pooled analyses of RCTs reported neutral effects of SGLT-2 inhibitors on fracture [91–93], while other studies found an increased fracture incidence, more evident with the use of canagliflozin, with fractures occurring already after 12 weeks of drug initiation and increasing over time [94–96]. At present, it is not clear whether the bone negative effects of SGLT-2 inhibitors are mechanism-based or compound-specific.

Even though no specific study regarding oral antidiabetic agents and fracture risk is available, in our opinion, a

particular attention at bone health should be paid in patients treated with TZDs and/or canagliflozin.

4.3. Insulin. The available data on the effect of insulin on bone are summarized in Table 1. In the presence of a treatment failure with the oral antidiabetic medications, insulin therapy represents the elective therapy for T2DM patients. In preclinical studies, insulin seems to play an important role in bone metabolism, in keeping with the presence of insulin growth factor receptors (IGFRs) on the surface of both osteoclasts and osteoblasts. *In vivo* and *in vitro* studies established that insulin exerts an anabolic effect on bone [97]. Mice with altered insulin signaling, due to the lack of IGFRs, have low bone turnover and reduced BMD [98]. On the other hand, insulin injection is able to induce bone formation, inhibit bone resorption, and lead to BMD improvement in adult mice [99].

At variance, in most clinical studies, the positive effect of the insulin treatment on both bone turnover markers and BMD [100] is not evident. Rather, its use has been associated with a higher risk of fractures (in particular, nonvertebral ones) [43, 46, 64, 101]. In a recent study on a large cohort of T2DM patients, insulin monotherapy was clearly associated with a 1.6-fold increased fracture risk in respect with metformin monotherapy [102]. However, recent data show that the use of long-acting insulins, less apt to induce hypoglycemia, was associated with a lower fracture risk as compared to other insulins [103], suggesting that, at least in part, the higher fracture risk associated with the insulin use might depend on a higher risk of hypoglycemia-related fall. Overall, it should be considered that insulin-treated T2DM patients have generally a longer disease duration and a higher number of comorbidities that could *per se* influence the fracture risk, regardless of the insulin use.

Eventually, is important to note that, although a relative insulin deficiency occurs in the later stages of T2DM, the predominant defect in this condition is the insulin resistance. We still do not know how insulin resistance affects bone and whether or not the skeletal loading might be compromised due to decreased muscle strength secondary to decreased glucose uptake by muscles.

As already mentioned, in the authors' opinion, insulin treatment has to be considered a risk factor for fragility fracture in T2DM patients.

4.4. Glucose Toxicity. As evidenced above, many evidences point toward a reduced bone turnover in T2DM, with a negative correlation between glycometabolic control and bone apposition and resorption markers. Hyperglycemia exerts troublesome effects on osteoblastogenesis since the early steps of differentiation, ultimately leading to low bone turnover. High blood glucose levels may reduce MSC viability and clonogenicity [104]. Several in vitro studies showed, in the presence of hyperglycemia, a downregulation of the BMSC proliferation, osteoblast gene expression, alkaline phosphatase (ALP) activity [105], and bone mineralization rate in BMSC isolated from streptozotocin- (STZ-) induced diabetic rats [105]. In addition, BMSC exposed to chronic high glucose exhibit enhanced adipogenic rather than osteogenic pathway, due to the PPARγ activation, and an enhanced expression of cyclin D3 [106] and decreased Runt-related transcription factor 2 (RUNX2) [107], ALP [108], and osteocalcin expression in osteoblasts. In keeping, studies in animal models confirmed a reduced mineralization and decreased trabecular bone volume in T2DM, probably due to the decreased RUNX2 gene expression and to reduction of osteocalcin, osteoprotegerin, bone morphogenetic protein-2 expression, and ALP [109–113] (Figure 2).

Recently, some evidences suggest that even the osteocytes, the most abundant bone cell type orchestrating bone remodeling, are affected by hyperglycemia. Indeed, in T2DM, sclerostin and Dickkopf-related protein 1 (Dkk1), two major contributors of bone formation via Wnt signaling inhibition, are increased and β-catenin is reduced [109]. Increased serum levels of sclerostin have been observed in T2DM patients [114] and have been shown to be associated with vertebral fractures [115]. In addition, in T2DM patients, the usual PTH-induced transcriptional suppression of sclerostin production is lost. In keeping, the treatment with sclerostin antibodies improves bone mass and strength in T2DM animal models.

Overall, these cellular and animal models indicate that in T2DM, a preferential differentiation of the BMSC toward adipocytes rather than osteoblast lineage is present. Interestingly, even clinical data are in line with this theory. Indeed, recent studies show that in T2DM, an inverse association exists between marrow adipose tissue (MAT) and glycemic control and T2DM women with poor glycemic control have significantly higher MAT levels than those with adequate glycemic control [116, 117]. The functional significance of MAT and its implications for bone quality remain to be clarified, as well as the relationship between MAT and other fat depots (i.e., visceral and subcutaneous fat stores) and possible hormonal determinants. Interestingly, another possible mechanism that may elucidate the prevalence of adipogenesis on osteoblastogenesis is the PI3K/Akt pathway, which is activated by the reactive oxygen species (ROS) production, which, in turn, is associated with hyperglycemia (Figure 2).

We know that AGE levels are increased in T2DM as a result of prolonged hyperglycemia and oxidative stress. The activation of AGE receptor, expressed in human-derived bone cells, enhances inflammatory cytokine production and ROS production, triggering a vicious cycle of inflammation and bone resorption [118]. Moreover, AGEs may reduce the expression of RUNX2, osteocalcin, and osterix [119], which are well-known important factors in osteoblast differentiation. Furthermore, on the one hand, AGEs suppress endoplasmic reticulum function, essential to osteoblast differentiation and activity [120] and, on the other hand, they increase osteoblast apoptotic death [119]. All these mechanisms induce a reduction of mineralization [31, 121] and a bone quality impairment. Finally, hyperglycemia may play a negative role also in osteoclastogenesis, inducing an impaired bone resorption. Hyperglycemia could especially impair embryonic stem cell differentiation in osteoclast, usually promoted by physiological glucose levels. In keeping with this, STZ-induced diabetic mice present impaired bone resorption due to reduced levels of dendritic cell-specific

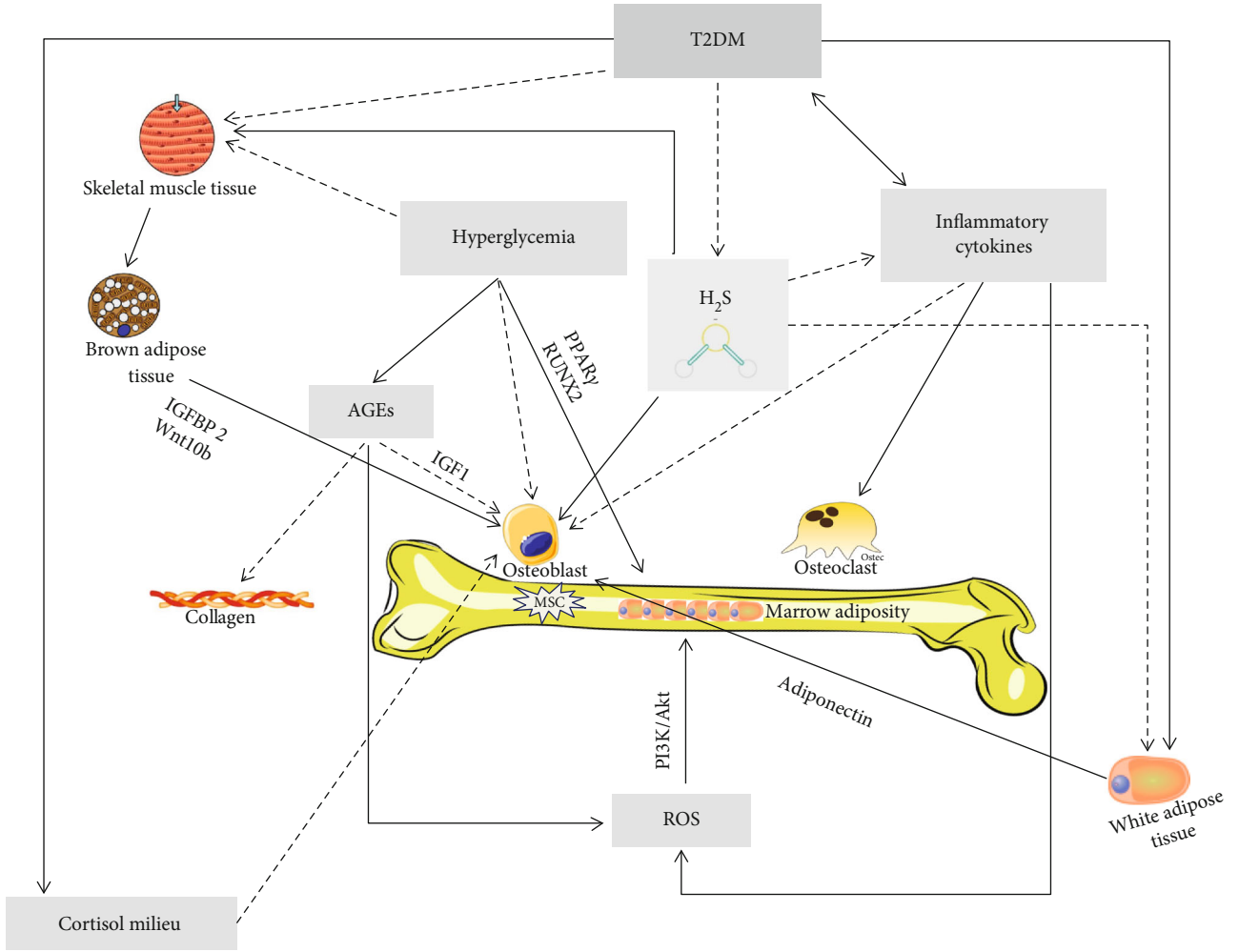

Figure 2: Mechanisms underlying bone fragility in type 2 diabetes mellitus (T2DM). In T2DM, the muscle tissue reduction, due to several factors including hyperglycemia per se, but probably also hydrogen sulfide (H2S) decrease, is thought to have a negative role on osteoblast lineage, via its crosstalk with the brown adipose tissue. Indeed, the muscle tissue is known to influence the brown adipose tissue, physiologically stimulating the secretion of factors (such as IGFBP2 and Wnt10b) thought to be important for osteoblast proliferation and activity. Osteoblast differentiation and activity, in T2DM, may be also impaired directly by the reduction of H2S levels that physiologically are thought to stimulate the osteoblast lineage. Hyperglycemia may directly reduce bone mesenchymal stem cell (MSC) viability and clonogenicity and also have an indirect negative effect on osteoblasts via the accumulation of advanced glycation end products (AGEs), which negatively affects osteoblasts through a reduction of the insulin-like growth factor-1 (IGF1) levels. The AGE accumulation impairs the normal collagen formation and leads to reactive oxygen species (ROS) increase that may augment marrow adiposity via the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/Akt) pathway. The inflammatory cytokine increase, directly and/or indirectly (due to the H2S reduction), may also impair osteoblastogenesis and increase osteoclast activity and ROS, ultimately leading to bone adiposity. Finally, in T2DM, osteoblasts may be also damaged by the low adiponectin levels due to the increase of white adipose tissue, which is a characteristic of T2DM itself but also a consequence of low H2S levels. Finally, even an altered cortisol secretion, peripheral activation, and sensitivity (i.e., "cortisol milieu") have been suggested to potentially impair osteoblast activity.

transmembrane proteins involved in osteoclast differentiation [122, 123]. However, other evidences show elevated bone resorption and osteoclast activity [111, 113] that may compromise the mineralization [124] (Figure 2).

Hyperglycemia exerts its negative effect on bone health also acting on extracellular matrix. It is well known that bone elasticity, toughness, and strength are dependent on the type of cross-links between the adjacent collagen molecules, while the mineral component of the bone matrix provides stiffness. Indeed, while enzymatic cross-links are essential to maintain bone strength, the formation of nonenzymatic AGE cross-links within collagen fibers negatively affects bone strength [125]. The low turnover of collagen leads to the accumulation of a huge quantity of altered type 1 collagen, which may induce, at both trabecular and cortical levels, biomechanical changes [126]. In addition, both in vitro and in vivo animal and human studies demonstrated that trabecular bone is susceptible to the accumulation of nonenzymatic glycation, which increases its propensity to fracture and decreased flexion strain and energy (Figure 2).

In an animal model, pentosidine levels and the pentosidine/total enzymatic cross-link ratio were negatively associated

with some mechanical bone properties such as energy absorption, stiffness, maximum load, and elastic modules [127]. Plasma and/or urinary pentosidine has been investigated as a potential clinical marker of bone damage in T2DM. In a T2DM Japanese cohort, pentosidine levels have been found significantly higher in postmenopausal women with vertebral fractures [34] than in nonfractured ones, regardless of the glycemic control, BMD, other osteoporosis risk factors, and renal function, all factors known to affect pentosidine levels.

Although these observations suggest that the impairment in collagen cross-links and AGE formation might explain the reduced bone quality in T2DM, larger and more robust studies are needed to confirm this hypothesis and to allow pentosidine being used as a marker for fracture prediction in T2DM patients.

4.5. Insulin Growth Factor 1 (IGF1), Inflammatory Cytokines, Brown/Beige Fat, and Adipokines. In T2DM, bone fragility is conceivably linked to an altered regulation of insulin growth factors (IGFs). Several *in vivo* studies have shown that high concentration of AGEs blunt the stimulatory IGF1 action on osteoblasts, probably through an osteoblast resistance to the IGF1 action [128, 129]. In postmenopausal women affected with T2DM, IGF1 were found to be inversely associated with the presence and the number of vertebral fractures, regardless of T2DM control, age, spinal BMD, renal function, and insulin secretion [130] (Figure 2).

Overall, T2DM is often described as a state of accelerated ageing. Inflammatory cytokines have been embroiled in the T2DM development as well as in its micro- and macrovascular complications. Inflammatory cytokines seem to have a role also in T2DM-related bone disease. Indeed, osteoclastogenesis can be activated by elevated cytokine levels, while osteoblast differentiation can be suppressed [131, 132]. Importantly, obese T2DM subjects show significantly higher levels of interleukin-6 and tumor necrosis factor-alfa that, at tissue level, may induce the ROS production, therefore affecting differentiation and survival of osteoblasts, osteoclasts, and osteocytes [133] (Figure 2).

Brown adipose tissue, which is typically thermogenically active, has been found to be reduced in T2DM and obesity [134]. Insulin-like growth factor-binding protein-2 and Wnt10b, factors secreted by brown adipose tissue, have an anabolic effect on bone metabolism and increase osteoblast activity [135]. In addition, the inactivation of TGFβ–SMAD3–myostatin signaling [136] promotes the browning of adipocytes. These recent data encourage the development of a novel class of TGFβ–myostatin antagonists that could be potentially used to treat both obesity and the T2DM-related bone disease (Figure 2).

Dysregulation of serum adipokine levels is also possibly linked to the T2DM-related low bone turnover. Indeed, T2DM patients present low adiponectin levels, an adipokine exclusively produced by the adipose tissue [137]. In vitro, adiponectin seems to have an inhibitory effect on osteoclasts and an anabolic effect on osteoblasts [138]. However, the studies aimed at investigating the link between adiponectin and BMD gave conflicting results, some data showing an inverse relationship [139, 140], while others showing a positive relationship between adiponectin and BMD at distal radius [141]. Furthermore, T2DM patients present low levels of leptin, another adipokine produced by white adipose tissue as well as by osteoblasts and bone marrow adipocytes. A Japanese study showed a significant negative correlation between leptin and bone resorption in T2DM subjects. Moreover, these authors showed that distal radius BMD was associated with leptin levels, but this association was not present for spine and hip BMD [141]. These results suggest that adipokines may exert a differential effect on cortical versus trabecular bone [142].

Further research is needed to confirm if the adipokine levels may be associated with bone disease in T2DM and if their determination may be useful in the clinical practice (Figure 2).

4.6. Obesity, T2DM, and Bone Fragility: A Concept of "Circular Health" in Body Energy Control. Human health can be regarded as a system of communicating vessels, particularly true when abnormalities in the management of the energy balance exist. The concept of "circular health" would suggest an interdisciplinary approach to identify and treat the multifactorial determinants of chronic diseases. The ability to adapt and adjust to different environmental conditions has been enabling the humans in survival. In a period of famine or hunger, the following conditions occur: (a) decrease in basal metabolic rate, leptin production, muscle mass, and lipolysis and (b) increase in cortisol secretion and lipogenesis. The opposite occurs when abundant food is available. Currently, in industrialized countries, rarely radical fluctuations in diet and metabolism occur, and, consequently, unfavorable health conditions such as obesity and diabetes mellitus develop. Over the past 20 years, it has been suggested that human skeleton may exert an important role also in energy metabolism through "local" hormonal connection, such as adipokines, mainly released by adipose tissue, but not exclusively, insulin/insulin-like growth factor 1 (IGF1), and osteocalcin/undercarboxylated osteocalcin pathways [143, 144], together with organs known to be involved in metabolic control. Such molecular pathways may be fundamental in maintaining energy homeostasis by controlling and coordinating both "fuel" uptake and energy expenditure in the human body, probably within a more complex network in which also central nervous system neurons and peripheral energy centers, sensing and regulating the energy needs, cooperate. The common cellular origin of osteoblasts, myocytes, and adipocytes makes not surprising the hypothesis that the skeleton either has a role in energy metabolism or may suffer in both skeletal muscle and adipose cell diseases, even if the underlying molecular mechanisms involved are still to be understood. Diabetic animal models and in vivo human studies suggested a strict interaction between whole body metabolism and skeletal health. It is well known that obesity and T2DM have a negative impact on fracture risk, but the knowledge on possible interactions of obesity, T2DM, and fracture still needs to be elucidated. Several studies suggested that alterations of adipose tissue-released hormones, such as adipokines, may exert harmful effects on bone cells. In

particular, an in vitro study revealed that adiponectin, produced by adipose tissue, may exert either an anabolic effect on osteoblasts or an inhibitory effect on osteoclasts [138], and low levels of adiponectin are found in patients with T2DM [137]. However, conflicting results concerning a clinical evidence on the link between adiponectin and BMD exist [139–141]. Impaired leptin production, produced by white adipose tissue, bone marrow adipocytes, and osteoblasts, has been observed in diabetic patients, and a significant negative correlation between its serum levels and the bone resorption marker urinary NTX has been reported in T2DM Japanese subjects, who showed a significant positive correlation between serum levels of leptin and Z-scores at the distal radius but neither at the lumbar spine nor at femoral neck levels, as if a differential effect on cancellous versus cortical bone existed [141, 142]. A fracture-related morbidity seems to be a higher in obese than in nonobese women [145]. Higher fat depots negatively act on bone, and the cytokines produced by visceral fat exhibit a proresorptive effect while an increased intramuscular fat accumulation associates with a reduced and less effective skeletal muscle function, powering both the attenuation of loading effects and the increase of risk for falls typically observed also in T2DM [146]. Metabolic syndrome, a cluster of cooccurring conditions highly increasing the risk for cardiovascular heart diseases, T2DM, excess body fat around the waist, and abnormal cholesterol or triglyceride levels, and dysmobility syndrome, a cluster of coexisting conditions such as osteoporosis, sarcopenia, obesity, ultimately increasing the risk for falls and fractures in affected subjects [147], may coexist in obese-T2DM patients. However, a common denominator in both syndromes, represented by higher individual fragility and impairment of the energy balance of the body, either as its generation or its dissipation/transformation, can be hypothesized. The importance of these metabolic pathways is underlined by common metabolic diseases, such as osteoporosis, diabetes, and obesity, caused by genetic or environmental disturbances in endocrine control mechanisms. The impact of coexisting obesity and diabetes determines rising health costs and disability, other than a poor health status.

In a circular health model, a common multitasking diagnostic-clinical-therapeutic management of these patients is to be recommended [148, 149].

4.7. Cortisol Secretion, Peripheral Activation, and Sensitivity. The low bone turnover with a decreased osteoblastic function typical of the T2DM-related bone damage is also a feature of the glucocorticoid-induced osteoporosis. Interestingly, in T2DM patients, the cortisol secretion and/or sensitivity have been suggested to influence the diabetic disease. Indeed, in T2DM patients, an increased (even though still within the normal range) cortisol secretion is present, particularly in those affected with the diabetic complications [13] and the different glucocorticoid receptor (GR) gene polymorphisms have been found to potentially influence the disease control [150]. Interestingly, the cortisol secretion and sensitivity (as represented by the N3S3S sensitizing variant of GR gene) have been suggested to be associated with the presence of asymptomatic vertebral fractures in postmenopausal T2DM patients [8, 14]. These clinical data, suggesting a potential role of the degree of cortisol secretion and sensitivity in the T2DM-related bone osteoporosis, are in line with recent in vitro data showing that the shift in the balance between osteoblastogenesis and adipogenesis of MSC may be mediated by the GR genetic variants [151]. In addition, even the degree of the interconversion of cortisone in cortisol at the peripheral tissue levels (including bone), due to the activity of the 11βhydroxysteroidodehydrogenase type 1 (11HSD1), may influence bone in T2DM. Indeed, the selective inhibition of 11HSD1, which has been suggested as potential treatment for T2DM in humans [152], has been also demonstrated to improve diabesity and osteoblast differentiation in a mouse model [153]. Finally, in T2DM, a vicious circle could be hypothesized between cortisol "milieu," bone, and glycometabolic control. Indeed, the low bone turnover induced by the increased cortisol secretion, peripheral activation, and sensitivity could contribute in reducing the undercarboxylated osteocalcin levels [154], which, in turn, could worsen the glycometabolic control, eventually leading to a perpetuation of low bone turnover (Figure 3).

These data may have an important clinical application. Indeed, if bone damage in T2D were related, at least partially, to the degree cortisol secretion and/or sensitivity, the treatment with an 11HSD1 inhibitor could improve glycometabolic control and reduce the fracture risk at the same time.

At present, however, the clinical usefulness of the cortisol "milieu" assessment for individuating T2DM patients at risk of fracture is still to be determined.

4.8. Hydrogen Sulfide. Hydrogen sulfide (H_2S) is a new gaseous signaling molecule which acts as a key messenger in many physiological and pathological conditions. Endogenously, H_2S is produced within cells by the catabolic pathway of sulfurated amino acids, also known as the transsulfuration pathway, by means of the two enzymes cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE) [155]. Physiologically, H_2S freely diffuses through cell membranes and is released in the circulation, where it can be present in the form of free H_2S or bound sulfane sulfur. Decreased nitric oxide (NO) bioavailability and deficiency of H_2S are considered to be involved in the pathophysiology of both T2DM [155] and osteoporosis [156]. Cystathionine beta-synthase is abundantly expressed in several tissues and in particular in BMSC, in insulin-secreting pancreatic β-cells, and several studies showed a role of H_2S in both inhibition of insulin secretion mediated by ATP-sensitive K+ channels and a pro- or antiapoptotic effects on β-cells [157] and in skeletal muscles. Most studies indicate that in both animal models of diabetes and T2DM patients, H_2S blood levels are decreased.

In BMSC, H_2S has a fundamental role for maintaining cell proliferation and differentiation [158]. Indeed, H_2S deficiency in BMSC attenuates both osteogenesis and proliferation. In keeping with this, CBS-deficient mice have decreased serum and intracellular levels of H_2S and a severe osteoporotic phenotype [158, 159]. The H_2S administration to CBS deficient mice of can restore normal bone homeostasis [158]. One of the supposed mechanisms is the increase of

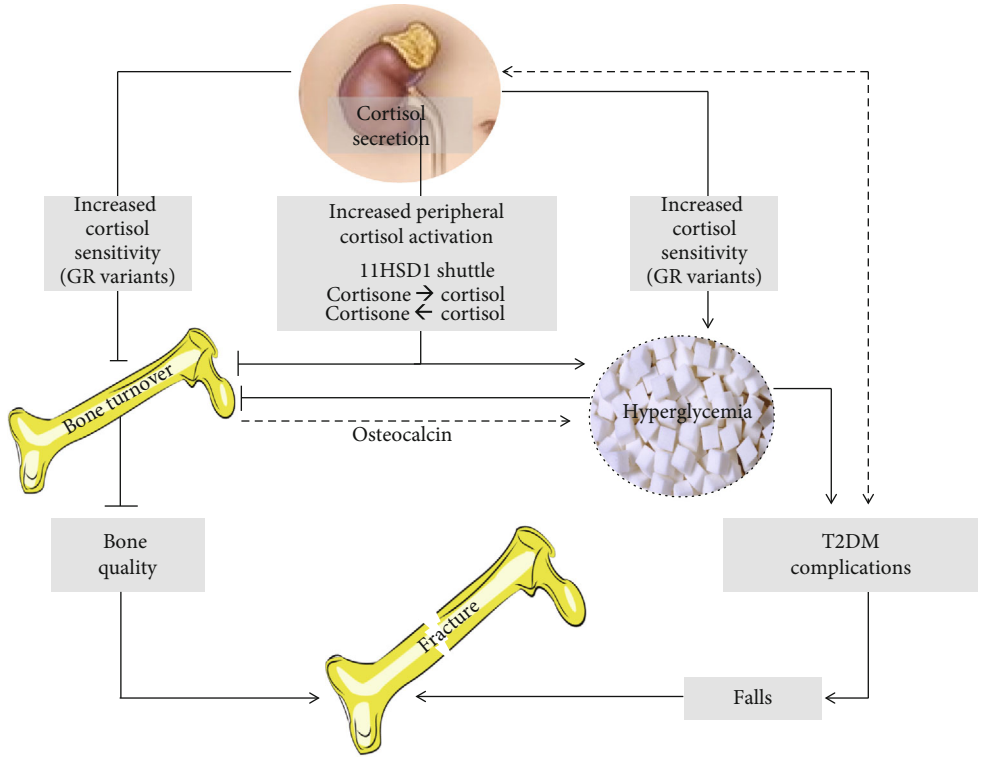

Figure 3: Cortisol milieu and bone fragility in type 2 diabetes mellitus (T2DM). In T2DM patients, an increased (even though still within the normal range) cortisol secretion is present, particularly in those affected with the diabetic complications, which in turn is hypothesized to be a trigger for the increased cortisol secretion itself. The sensitizing variants of the glucocorticoid receptor (GR) may increase the negative effect of cortisol on both T2DM control and bone metabolism, contributing to the shift in the balance between osteoblastogenesis and adipogenesis of mesenchymal stem cells in bone. The degree of the interconversion of cortisone in cortisol, due to the activity of the 11βhydroxysteroidodehydrogenase type 1 (11HSD1), may influence bone metabolism in T2DM. Indeed, in humans, the selective inhibition of 11HSD1, which has been even suggested as potential treatment for T2DM, has been also demonstrated to improve diabesity and osteoblast differentiation in a mouse model. Finally, in T2DM, a vicious circle could be hypothesized between the increased cortisol secretion, peripheral activation, and sensitivity (i.e., "cortisol milieu") and bone and glycometabolic control. Indeed, the low bone turnover induced by this activated cortisol milieu could contribute in reducing the undercarboxylated osteocalcin levels, which decrease and, in turn, may worsen the glycometabolic control, therefore perpetuating the mechanisms leading to reduced bone turnover. The final effects of these alterations of the cortisol milieu in T2DM may be on one side of the reduction of bone quality, since the low bone turnover reduces the possibility of the microcrack repairing, and, on the other side, the worsening of the T2DM complications that ultimately could lead to an increased risk of falls. The reduction of bone quality together with the increased risk of falls is among the most important factors associated with bone fragility in T2DM.

Hcy that leads to and oxidative damage and dysfunction of the BMMSCs. Moreover, several studies showed that osteoporosis derived from estrogen deficiency is associated to a defective H2S biosynthesis [160] and the treatment with an H2S donor prevents the bone loss induced by stimulating bone formation through the activation of the Wnt signaling cascade by increased production of the Wnt ligands.

H2S regulated insulin sensitivity, gluconeogenesis, and glycogenolysis and inhibits glucose utilization and glycogen storage. It seems also to regulate adipose tissue lipolysis, adipokine production, and inflammation, processes important for local and systemic insulin sensitivity [161] (Figure 2).

Recently, a study showed, in a rat model of diabetes, a reduced expression of CBS and other enzymes involved in H2S production in skeletal muscles and suggested a possible relationship between sarcopenia and H2S deficiency. Indeed, in this animal model, the treatment with H2S donor showed to lead to an improvement in muscle mass and functionality (Figure 3).

Nowadays, although being an extremely promising research field, H2S cannot be considered among the drugs possibly available in the very next future.

5. Conclusions

Nowadays, reduced bone quality and an increased fracture risk should be considered among the possible complications of T2DM. In T2DM individuals, the risk of fractures is increased for a given BMD and bone turnover markers are relatively low in these patients. These features explain the difficulty in identifying patients at high fracture risk, since physicians could not rely on the BMD measurement and/or on bone turnover assessment.

Notwithstanding the current limitations, the increasing knowledge regarding the pathophysiology of the T2DM-related bone damage gives us some information regarding which T2DM individual may be at higher risk for bone fragility. Indeed, T2DM patients with longer (≥10 years) disease duration, insulin use, poor glycometabolic control, and diabetic complications are predisposed to fracture and, in these subjects, beside the BMD determination, looking for vertebral morphometric fracture is advisable. Therefore, in the authors' opinion, a spinal and femur BMD determination by DXA spinal and femur BMD evaluation should be done in T2DM patients in the presence of clinical fragility fractures and/or a morphometric vertebral fracture and/or with a long T2DM duration (i.e., >10 years) and/or insulin use and/or T2DM-related chronic complication(s).

In the future, evaluating the ROS and AGE levels and the degree of cortisol secretion, peripheral activation, and sensitivity could increase our ability in predicting the fracture risk in the single T2D patient. In addition, a better understanding of the mechanisms leading to bone fragility in T2DM, such as the bone marrow fat, adipokine production, and cortisol milieu could consent to both the development of drugs able to reduce the fracture risk in T2DM and individuate those antidiabetic drugs more prone to damage the skeletal tissue. In this regard, the clinical similarities between bone damage in glucocorticoid-induced osteoporosis and T2D-related bone involvement seem to find some biological confirmation. Indeed, very recent data show that in rats, dexamethasone decreases serum H2S and two key H2S-generating enzymes in the bone marrow and the H2S treatment significantly relieved the inhibitory effect of dexamethasone on bone formation [162]. Therefore, it is possible to hypothesize that the reduced H2S levels in T2DM may depend on the increased cortisol levels at least in some diabetic patients.

In general, it is conceivable that the mechanisms underlying bone fragility are different among patients with T2DM. Therefore, the identification of the main cause of bone fragility in the single patient may consent to personalize the diagnostic approach and treatment of choice in T2DM patients at risk for fracture.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

All authors contributed equally to the literature review and drafting of the manuscript. All authors approved the last version of the manuscript.

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Research Article

Assessment of Risk Factors for Fractures in Patients with Type 2 Diabetes over 60 Years Old: A Cross-Sectional Study from Northeast China

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Aims. Previous evidence has demonstrated an increased fracture risk among the population with type 2 diabetes mellitus (T2DM). This study investigated the prevalence of bone fractures in elderly subjects (with and without type 2 diabetes) and identified any fracture risk factors, especially the risk factors for common known fractures in particular diabetic populations. *Methods*. This cross-sectional study was conducted with community-dwelling people over 60 years old in nine communities from the city of Shenyang, which is the capital of Northeast China's Liaoning Province. A total of 3430 elderly adults (2201 females, mean ± standard deviation age 68.16 ± 6.1 years; 1229 males, 69.16 ± 6.7 years) were included. Our study measured the heel bone mineral density (BMD) and used the timed "up and go" (TUG) test and other indicators. In addition, we performed logistic regression analysis to explore the risk factors for fractures in the general population and the diabetic population and to analyze the differences. *Results*. The results revealed that a total of 201 elderly persons (5.8%), with an average age of 70.05 ± 6.54 years, suffered from a history of fragility fractures, which affected more females (74.6%) than males ($p = 0.001$). The prevalence of fractures in the T2DM population was 7.3%, which was much higher than the 5.2% in non-T2DM population ($p < 0.05$). In the non-T2DM population, the BMD was lower and the TUG time was longer in the fracture group than in the nonfracture group ($p < 0.001$). However, in the T2DM population, the BMD and TUG values were similar between the fracture group and the nonfracture group ($p > 0.05$). Logistic regression analysis showed that the female sex (OR 1.835), TUG time > 10.2 s (OR 1.602), and T-score≤−2.5 (OR 1.750) were independent risk factors for fragility fractures in the non-T2DM population, but they were not risk factors in the T2DM population. *Conclusions*. This study found that low BMD and slow TUG time were independent risk factors for fractures in non-T2DM patients, while no associations were found in the T2DM population. Patients with T2DM have a higher risk for fractures even when they have sufficient BMD and a short TUG time. TUG and BMD underestimated the risk for fractures in the T2DM population.

1. Introduction

The prevalence rate of type 2 diabetes mellitus (T2DM) has increased to 18% (aged 65-99) around the world in 2017 [1], and the prevalence of T2DM in China's elderly population has increased along with the aging of the Chinese population and the rise in unhealthy lifestyle habits, and environmental pollution [2]. Individuals with T2DM have a higher risk for fractures than those without T2DM, but epidemiological data are limited [3–5]. Fractures seriously affect the quality of life, and many more prediction methods that are simple and practicable should be explored. Bone strength includes not only bone density but also bone quality, and it is typically used as a measure of skeletal disorders that are associated with fractures [6]. Most studies have revealed that bone mineral density (BMD) is not lower in patients with T2DM, and in fact, it is higher than that in non-T2DM persons [6–8], but the cause of this phenomenon is not yet clear. The timed "up and go" (TUG) test was originally described by Podsiadlo as a mobility test for frail older persons [9]. Some studies have shown that the TUG test is a sensitive and specific measure for community-dwelling adults in

predicting falls and an independent risk factor for fracture [10, 11], but the relationship between TUG and fracture risk in T2DM-specific populations is unclear. Thus, it is of great interest to us to evaluate whether the TUG results could predict fractures in diabetic populations. We performed a cross-sectional study to investigate the prevalence of fractures in a population of 3430 elderly subjects (with and without type 2 diabetes) in order to identify fracture risk factors and, in particular, to assess the risk factors for common fractures in particular diabetic populations.

2. Materials and Methods

2.1. Study Population. We conducted a cross-sectional study of permanent residents over 60 in nine communities in Shenyang, Northeast China, from May to October 2017. The exclusion criteria were as follows: secondary osteoporosis; cancer; glomerular nephritis, or creatinine clearance (Ccr) < 30 mL/min; hyperthyroidism or hypothyroidism; and previous diagnosis of osteoporosis and treatment. A total of 3430 seniors took part in our survey after removing incomplete samples, including 1073 suffering from T2DM and 201 samples suffering from fragility fractures. We used a stricter standard for fragility fractures in the population who had fractures caused by a minor crash or fall and obtained a definite diagnosis from a clinician without distinguishing the fracture location.

This study was approved by the ethics committee of the First Affiliated Hospital of China Medical University and was conducted in accordance with the principles described in the Declaration of Helsinki. All subjects provided written informed consent prior to participation. The research has been registered on the Chinese Clinical Trials Registry (ChiCTR-ERC-17011100).

2.2. Clinical Data Collection. All subjects were assessed with a standardized questionnaire based on the Community Health Questionnaire administered by trained doctors, including basic demographics, history of present illness, past medical history, lifestyle risk factors such as smoking and alcohol consumption, and medication used. Each subject was examined for height, weight, waist circumference (WC), and hip circumference (HC); each measurement was evaluated twice and then averaged (accurate to 0.1 cm). Body mass index (BMI) was calculated with the following equation: weight (kg)/(height (m^2)). The waist to hip ratio (WHR) was the ratio of WC (cm) to HC (cm). Systolic pressure (SBP) and diastolic pressure (DBP) were also measured twice and averaged.

2.3. Biochemical Measurements. Blood samples were collected following overnight fasting. Serum fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), uric acid (UA), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), creatinine (Cr), serum, and calcium (Ca) were measured by an automatic biochemical analyzer, and 25-hydroxyvitamin D_3 (25 $(OH)D_3$) was measured by mass spectrometry. And Ccr was calculated using the formula from Cockcroft and Gault [12].

2.4. TUG. The timed "up and go" (TUG) test records the time it takes to rise from an armed chair, walk 3 meters, and return to sit in the chair. In this study, a TUG result > 10.2 s was defined as poor mobility, according to a previous study [9].

2.5. BMD. The heel BMD was measured by an ultrasonic bone densitometer (Hologic Sahara ultrasound bone density densitometer, software: version3.1, American Hologic Corporation), which has good correlations with dual-energy X-ray absorption (DXA) measurement. In this study, we defined normal density, osteopenia, and osteoporosis (OP) as a *T*-score≥−1.0, between -1.0 and -2.5, and ≤-2.5, respectively, following the World Health Organization definitions [13].

2.6. Statistical Analysis. All statistical analyses were performed with Statistical Package for the Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA), and significant differences were assumed to be present at $p < 0.05$ (two tailed). Data are expressed as the mean ± SD for continuous variables or percentages (%) for categorical variables. *t*-tests for continuous variables or chi-square test for categorical variables was used to compare parameters between two groups. Logistic regression analysis was performed to identify risk factors.

3. Results

3.1. Baseline Characteristics and Laboratory Parameters. The baseline characteristics and laboratory parameters of this study population were stratified by a history of fragility fractures into fracture and nonfracture groups (Table 1). There were 3430 people in this study, of which 2201 (64.2%) were female and 1229 (35.8%) were male, with ages ranging from 60 to 92 years old. A total of 201 subjects (5.8%) suffered from a history of fragility fractures. Compared with the nonfracture group, the fracture group was older (70.05 ± 6.54 vs. 68.43 ± 6.30, $p < 0.001$) and included more females (74.6% vs. 63.5%, $p = 0.001$). TUG and BMD were measured in the study population. The BMD was found to be much lower and the TUG time much longer in the fracture group than in the nonfracture group ($p < 0.001$). In the fracture group, the prevalence rate of T2DM was 38.8%, which was much higher than that in the nonfracture group ($p < 0.05$).

3.2. The Risk for Fracture. We performed a logistic regression analysis for the total population and found that diabetes was a risk factor for fracture (OR 1.357). Moreover, the female sex (OR 1.663), older age (OR 1.026), slow TUG time (OR 1.454), and osteoporosis (OR 1.799) were risk factors for fractures after adjusting for the confounding factors in this study (Table 2).

3.3. The Baseline Characteristics and Risk Factors Stratified by T2DM and Fracture. We divided all subjects into a diabetes population and a nondiabetic population and analyzed the differences in sex, age, uric acid, Ccr, FBG, HbA1c, $25(OH)D_3$, Ca, WHR, BMI, BMD, and TUG between two populations (Table 3). In our study, the prevalence rate of T2DM was 31.3%, and the prevalence of hypertension rate was 56.5% (not shown). The BMD was similar between the

Table 1: Baseline characteristics and laboratory parameters of total population stratified by fracture.

Variables	Fracture ($n = 201$, 5.8%)	Nonfracture ($n = 3229$, 94.2%)	p value
Sex, female (%)	150 (74.6)	2051 (63.5)	0.001*
Age (years)	70.05 ± 6.54	68.43 ± 6.30	<0.001**
Height (cm)	159.87 ± 8.06	160.99 ± 8.15	0.058
Weight (kg)	63.13 ± 9.76	64.50 ± 10.44	0.07
BMI (kg/m^2)	24.71 ± 3.37	24.84 ± 3.27	0.572
WC (cm)	88.66 ± 8.96	87.33 ± 9.36	0.17
HC (cm)	97.94 ± 7.16	97.94 ± 7.16	0.873
SBP (mmHg)	140.01 ± 19.62	138.63 ± 20.42	0.826
DBP (mmHg)	81.37 ± 11.59	80.13 ± 11.79	0.878
WHR	0.9 ± 0.06	0.89 ± 0.06	0.015*
BMD (g/cm^2)	0.33 ± 0.08	0.36 ± 0.08	<0.001**
TUG (seconds)	10.14 ± 4.55	9.18 ± 3.09	<0.001**
HbA1c (%)	6.11 ± 1.47	5.9 ± 1.17	0.075
FBG (mmol/L)	6.44 ± 2.23	6.09 ± 1.76	0.023*
UA (μmol/L)	292.23 ± 93.75	303.58 ± 102.49	0.126
Ccr (mL/min)	73.10 ± 19.65	75.56 ± 21.40	0.114
TC (mmol/L)	5.06 ± 0.95	5.12 ± 1.02	0.388
TG (mmol/L)	1.69 ± 1.03	1.80 ± 5.33	0.776
HDL-C (mmol/L)	1.39 ± 0.38	1.37 ± 0.42	0.4
LDL-C (mmol/L)	3.07 ± 0.79	3.16 ± 0.91	0.165
Ca (mmol/L)	2.41 ± 0.1	2.42 ± 0.09	0.258
25 (OH)D_3 (ng/mL)	22.38 ± 7.93	22.19 ± 7.62	0.735
History of hypertension (%)	101 (50.2)	1391 (43.1)	0.047*
History of T2DM (%)	78 (38.8)	995 (30.8)	0.018*
History of smoking (%)			0.126
Never	173 (86)	2591 (80.2)	
Current	18 (9)	422 (13.1)	
Quit	10 (5)	216 (6.7)	
Current drinking (%)			0.24
Never	165 (82.1)	2498 (77.4)	
Current	30 (14.9)	639 (19.8)	
Quit	6 (3)	92 (2.8)	
Therapy of T2DM			0.49
Oral	10 (12.8)	198 (19.9)	
Insulin	7 (9)	87 (8.7)	
Oral and insulin	3 (3.8)	30 (3.1)	
Without medicine	58 (74.4)	680 (68.3)	

Abbreviations: BMI: body mass index; WC: waist circumference; HC: hip circumference; WHR: waist hip ratio; SBP: systolic pressure; DBP: diastolic pressure; HbA1c: glycosylated hemoglobin; FBG: fasting blood glucose; UA: serum uric acid; Ccr: creatinine clearance; TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; 25 (OH)D_3: 25-hydroxyvitamin D3; BMD: bone mineral density; TUG: timed "up and go." **$p < 0.001$, *$p < 0.05$.

two populations, while the TUG time was longer in the T2DM population than in the non-T2DM population.

We divided all subjects into a diabetic population and a nondiabetic population and analyzed the differences in sex, age, UA, Ccr, FBG, HbA1c, 25 (OH)D_3, Ca, WHR, BMD, and TUG time between the fracture group and nonfracture group for the two populations. We found that sex, age, UA, BMD, and TUG time were different between the fracture group and the nonfracture group in the nondiabetic population. However, we did not find significant differences in TUG time and BMD between the fracture group and the nonfracture group in the diabetic population (Table 4). The

Table 2: Analysis of risk factors for fracture in all subjects.

Variables	OR	95% CI	p value
Sex, female	1.663	1.192-2.312	0.003*
Age (years)	1.026	1.002-1.050	0.032*
TUG > 10.2 s	1.454	1.040-2.032	0.029*
History of T2DM	1.357	1.008-1.826	0.044*
T-scores			
Normal	1		
Osteopenia	1.261	0.744-2.137	0.389
Osteoporosis	1.799	1.072-3.02	0.026*

Abbreviations: TUG: timed "up and go." *$p < 0.05$.

Table 3: Baseline characteristics and laboratory parameters stratified by T2DM.

Variables	T2DM (n = 1073, 31.3%)	Non-T2DM (n = 2357, 68.7%)	p value
Sex, female (%)	691 (64.4)	1510 (64.1)	0.85
Age (years)	69.09 ± 6.53	68.26 ± 6.21	<0.001**
UA (μmol/L)	321.37 ± 86.6	311.49 ± 83.13	0.002*
Ccr (mL/min)	76.46 ± 21.51	74.94 ± 21.2	0.054
FBG (mmol/L)	7.65 ± 2.43	5.4 ± 0.6	<0.001**
HbA1c (%)	6.96 ± 1.47	5.44 ± 0.58	<0.001**
25 (OH)D_3 (ng/mL)	22.19 ± 7.62	22.21 ± 7.65	0.95
Ca (mmol/L)	2.42 ± 0.09	2.41 ± 0.09	0.001*
WHR	0.91 ± 0.06	0.89 ± 0.06	<0.001**
BMI (kg/m^2)	25.29 ± 3.31	24.63 ± 3.25	<0.001**
BMD (g/cm^2)	0.36 ± 0.09	0.35 ± 0.08	0.131
TUG (s)	9.82 ± 4.16	8.97 ± 2.61	<0.001**

Abbreviations: HUA: serum uric acid > 420 μmol/L; Ccr: creatinine clearance; TUG: timed "up and go." **$p < 0.001$, *$p < 0.05$.

differences in TUG and BMD values between the fracture and nonfracture groups in diabetic and nondiabetic populations were stratified by sex, and the differences in TUG and BMD values in nondiabetic women were found to be statistically significant (Figures 1(a)–1(d)). We used logistic regression analysis to assess the relationship between fractures and multiple risk factors in the T2DM and non-T2DM populations, and the results are shown in Table 5. The female sex (OR 1.835), TUG time > 10.2 s (OR 1.602), and T-score≤−2.5 (OR 1.750) were found to be independent risk factors for fragility fractures in the nondiabetic population but not in the T2DM population after adjusting for confounding factors (Table 5).

3.4. Multiple Metabolic Parameters May Affect TUG Time and BMD in the Diabetic Population. We stratified the diabetic population into groups according to TUG time > 10.2 s and T-score≤−2.5. We analyzed the relationships among TUG time, BMD, and multiple metabolic parameters that are usually combined with diabetes, including age, BMI, SBP, DBP, HbA1c, Ccr, UA, TC, TG, HDL-C, LDL-C, Ca, 25 (OH)D_3, history of HTN, and T2DM therapy. TUG time and T-score were found to be related to multiple factors (Table 6) that may influence the results showing that TUG time and BMD are not independent risk factors for fragility fractures in the diabetic population.

4. Discussion

This study population was from a high latitude region in China with cold weather, low levels of sunlight, and high risk for slips and falls in the winter. In addition, the age of the population was over 60 years old, which is considered retirement age and is defined as geriatric in China. The sample size and demographic characteristics of the population in this study differ from those in other studies.

Previous studies have researched multiple risk factors for fragility fractures, such as vitamin D intake or serum concentration [14], level of UA [15], obesity or low weight [16, 17], body composition [18], osteoporosis, and falls. Individuals with T2DM and type 1 diabetes mellitus (T1DM) have a higher risk for fractures, particularly hip fractures, than nondiabetic subjects, including both men and women [5, 19]. Previous studies have shown that the risks of fractures in those diagnosed with diabetes were higher than those in non-Hispanic black (HR 1.86 (95% CI 1.05–3.30)) and Mexican American (HR 2.29 (95% CI 1.41–3.73)) adults without diabetes [20]. T2DM factors such as a longer disease duration [21], diabetic complications, poor glycemic control [22], insulin resistance (IR) [23], and the use of insulin or oral antidiabetic medication [19, 24] have a complex pathophysiological interaction with fractures. And an increased risk for falls were also reported to increase the fracture risk [25].

Our study found that the prevalence rate of fragility fractures in the diabetic population was 7.3%, which is much higher than the 5.2% in the non-T2DM population ($p = 0.018$, Table 1), and analyzed the association between fractures and diabetes, hypertension (HTN), HbA1c, blood lipids, BMI, level of serum 25 (OH)D_3, insulin use, oral antidiabetic medication, Ccr, etc. We found that T2DM, female sex, older age, slower TUG time, and osteoporosis are risk factors for fractures.

BMD is measured to assess osteoporosis in many medical institutions. Although previous studies have reported that the BMD of patients with T2DM was normal or even higher than that of nondiabetic controls, the fracture risk was higher in patients with T2DM [4, 26]. Our study also showed that the BMD levels between T2DM and non-T2DM patients were similar ($p = 0.131$), although the T2DM population had a higher risk for fractures (OR 1.357).

The TUG test is usually performed to reflect the risk for falls and fractures [6, 11, 27]. D.C.C. de Abreu et al.'s [28] research reported that the TUG times did not present correlations with fall's history, which reminds us that a slow TUG time may be related to a higher risk for fractures but did not totally account for the higher fall risk; some other aspects may also be involved. The TUG test could reflect muscle strength, impaired gait and balance, and increased fall risk,

Table 4: Baseline characteristics and laboratory parameters of the study population stratified by T2DM and fracture.

Variables	T2DM (n = 1073, 31.3%)		p value	Non-T2DM (n = 2357, 68.7%)		p value
	Fracture (n = 78, 7.3%)	Nonfracture (n = 995, 92.7%)		Fracture (n = 123, 5.2%)	Nonfracture (n = 2234, 94.8%)	
Sex, female (%)	56 (71.8)	635 (63.8)	0.157	94 (76.4)	1416 (63.4)	0.003*
Age (years)	70.83 + 6.4	68.95 + 6.53	0.014*	69.55 ± 6.61	68.19 ± 6.18	0.018*
UA (μmol/L)	325 + 83.00	321.08 ± 86.90	0.691	288.65 ± 69.33	312.77 ± 83.66	<0.001**
Ccr (mL/min)	70.11 ± 18.94	76.96 ± 21.63	0.007*	74.98 ± 1.8	74.98 ± 19.93	0.983**
FBG (mmol/L)	8.03 ± 2.88	7.62 ± 2.39	0.154	5.44 ± 0.56	5.40 ± 0.60	0.449
HbA1c (%)	7.17 ± 1.79	6.95 ± 1.44	0.28	5.43 ± 0.59	5.44 ± 0.58	0.848
25 (OH)D_3 (ng/mL)	22.68 ± 6.96	22.15 ± 7.68	0.559	22.19 ± 8.51	22.20 ± 7.60	0.98
Ca (mmol/L)	2.42 ± 0.11	2.45 ± 0.09	0.46	2.41 ± 0.09	2.41 ± 0.09	0.298
WHR	0.91 ± 0.06	0.91 ± 0.06	0.374	0.90 ± 0.07	0.88 ± 0.06	0.05
BMD (g/cm^2)	0.344 ± 0.08	0.36 ± 0.09	0.182	0.32 ± 0.08	0.35 ± 0.08	<0.001**
TUG (s)	10.58 ± 5.77	9.76 ± 4.0	0.09	9.86 ± 3.56	8.92 ± 2.54	<0.001**

Abbreviations: HUA: serum uric acid > 420 μmol/L; Ccr: creatinine clearance; TUG: timed "up and go." $^{**}p < 0.001$, $^{*}p < 0.05$.

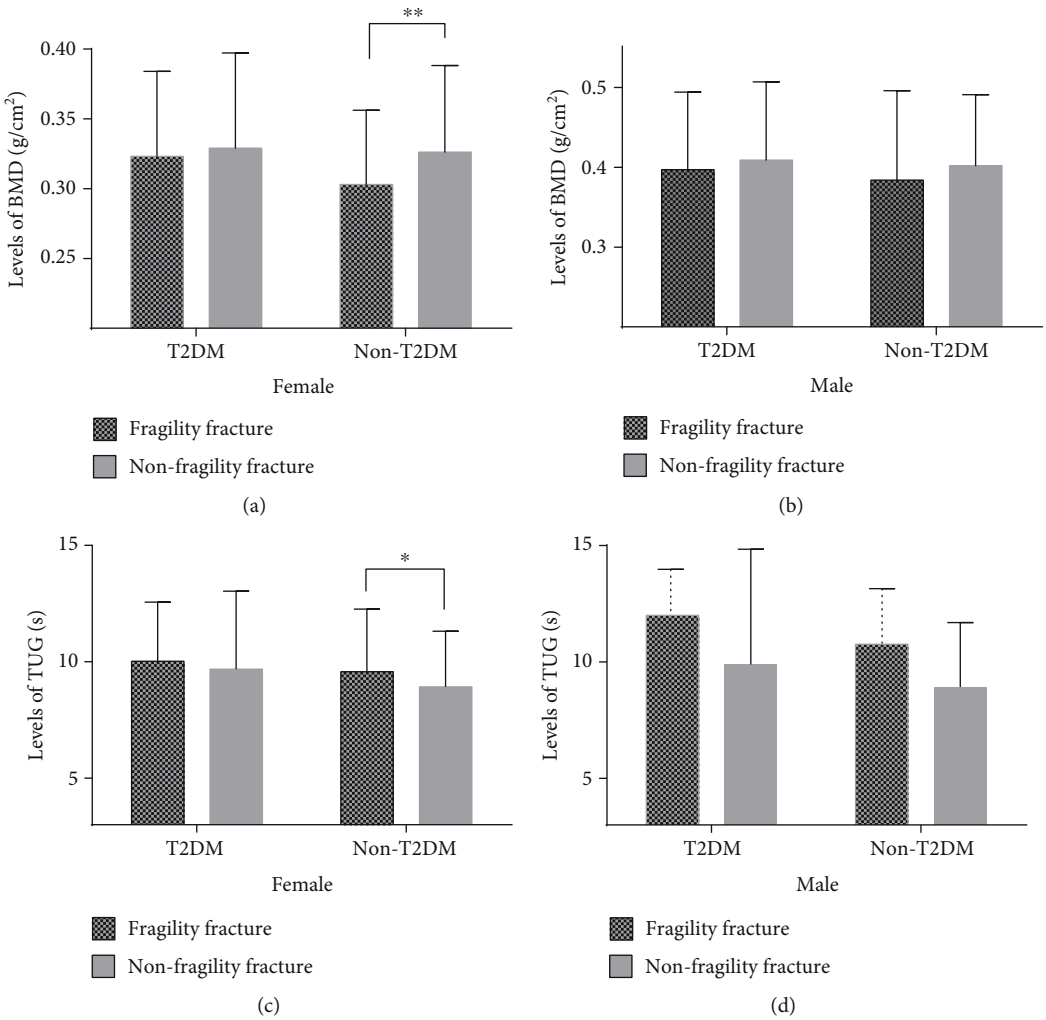

Figure 1: (a) Levels of BMD in different groups for female. (b) Levels of BMD in different groups for male. (c) Levels of TUG in different groups for female. (d) Levels of TUG in different groups for male. $^{**}p < 0.001$, $^{*}p < 0.05$.

Table 5: Logistic regression analysis for the risk for fracture in T2DM and non-T2DM subjects, respectively.

Variables	T2DM $N = 1073$ (31.3%) OR (95% CI)	p value	Non-T2DM $N = 2357$ (68.7%) OR (95% CI)	p value
Sex, female	1.538 (0.886-2.669)	0.126	1.835 (1.178-2.860)	0.007*
Age	0.987 (0.944-1.031)	0.552	0.969 (0.937-1.003)	0.07
HUA	1.109 (0.507-2.427)	0.795	1.160 (0.547-2.460)	0.698
Ccr (mL/min)	1.010 (0.996-1.025)	0.172	0.993 (0.984-1.001)	0.101
TUG > 10.2 s	1.349 (0.790-2.305)	0.273	1.602 (1.031-2.491)	0.036*
T-score≤−2.5	1.204 (0.744-1.949)	0.449	1.750 (1.196-2.562)	0.004*

Abbreviations: HUA: serum uric acid > 420 μmol/L; Ccr: creatinine clearance; TUG: timed "up and go"; OP: osteoporosis; $^*p < 0.05$.

Table 6: The relationship between diabetic parameters and TUG and T-score.

Variables	TUG >10.2 s ($n = 306$)	TUG ≤10.2 s ($n = 767$)	p value	T-score ≤-2.5 ($n = 434$)	T-score >-2.5 ($n = 639$)	p value
Sex, female (%)	200 (65.4)	391 (64.0)	0.678	295 (68)	396 (62)	0.044*
Age (years)	72.82 ± 6.963	67.6 ± 5.714	<0.001**	69.82 ± 6.48	68.59 ± 6.53	0.002*
BMI (kg/m^2)	26.11 ± 3.54	24.96 ± 3.15	<0.001**	25.32 ± 3.47	25.26 ± 3.19	0.762
SBP (mmHg)	145.42 ± 21.34	139.59 ± 19.94	<0.001**	141.63 ± 20.44	140.99 ± 20.56	0.614
DBP (mmHg)	79.72 ± 13.79	79.94 ± 11.32	0.783	79.28 ± 12.24	80.29 ± 11.95	0.178
HbA1c (%)	7.05 ± 1.43	6.93 ± 1.48	0.243	6.96 ± 1.41	6.97 ± 1.50	0.92
Ccr (mL/min)	71.17 ± 22.22	78.57 ± 20.87	<0.001**	74.45 ± 21.82	77.84 ± 21.20	0.012*
UA (μmol/L)	324.96 ± 89.95	319.94 ± 85.24	0.395	317.31 ± 83.09	324.15 ± 88.88	0.207
TC (mmol/L)	5.05 ± 1.15	5.15 ± 1.07	0.173	5.13 ± 1.08	5.11 ± 1.10	0.785
TG (mmol/L)	1.80 ± 1.01	1.94 ± 1.28	0.085	1.91 ± 1.28	1.88 ± 1.17	0.696
HDL-C (mmol/L)	1.27 ± 0.36	1.32 ± 0.47	0.082	1.32 ± 0.46	1.30 ± 0.43	0.585
LDL-C (mmol/L)	3.15 ± 0.98	3.16 ± 0.91	0.87	3.17 ± 0.93	3.15 ± 0.92	0.782
Ca (mmol/L)	2.42 ± 0.09	2.43 ± 0.09	0.62	2.42 ± 0.09	2.42 ± 0.09	0.961
25 (OH)D_3 (ng/mL)	21.05 ± 7.73	22.65 ± 7.54	0.002*	22.21 ± 8.04	22.17 ± 7.33	0.93
Hypertension (%)	215 (70.3)	382 (49.8)	<0.001**	239 (55.1)	358 (56)	0.757
Therapy of T2DM			0.139			0.681
Oral	65 (21.2)	143 (18.6)		77 (17.7)	131 (20.5)	
Insulin	35 (11.4)	59 (7.7)		41 (9.4)	53 (8.3)	
Oral and insulin	9 (2.9)	24 (3.1)		15 (3.5)	18 (2.8)	
Without medicine	197 (64.4)	541 (70.5)		301 (69.4)	437 (68.4)	

Abbreviations: BMI: body mass index; SBP: systolic pressure; DBP: diastolic pressure; HbA1c: glycosylated hemoglobin; UA: serum uric acid; Ccr: creatinine clearance; TC: total cholesterol; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; 25 (OH)D_3: 25-hydroxyvitamin D3; BMD: bone mineral density; TUG: timed "up and go." $^{**}p < 0.001$, $^*p < 0.05$.

which are associated with the risk for fractures, especially in geriatric patients [27].

Mousa et al. [27] conducted a case-control study of 138 elderly individuals aged over 60 years from a hospital who showed abnormal TUG times; a TUG time > 20 s was defined as poor mobility and was strongly associated with a reduced BMD and increased fracture risk, but the study did not analyze the T2DM population in particular.

In this study, we defined TUG times > 10.2 s as poor mobility according to Podsiadlo and Richardson [9]. The populations were from communities and were in better physical condition than populations from medical institutions, and the mean TUG times were lower than 10 s. Thus, the cut-off point of <10.2 s was highly suitable for study inclusion.

The risk for fractures in the diabetic population is much higher than that in the nondiabetic population, so we wondered whether other risk factors of fractures differ between T2DM and non-T2DM populations. We divided all subjects into T2DM and non-T2DM populations and analyzed the differences in risk factors for fracture, including but not

limited to TUG times and BMD. We found that, after adjusting for confounding factors, a slow TUG time and lower BMD were risk factors for fracture in the nondiabetic population but not the diabetic population, which is quite interesting. What influenced the difference between the two population types? Diabetic patients often have multiple metabolic diseases and take multiple medications. Were there some confounding factors of diabetes that influence the specificity? We therefore explored the factors that may affect TUG time and BMD in the T2DM population that are usually associated with diabetes. As shown in Table 5, we found several confounding factors that are involved, such as hypertension, Ccr, BMI, and 25 $(OH)D_3$. Since the data we collected could not represent all the abnormalities in people with diabetes, we did not perform further analyses on what factors exactly affect TUG time and BMD.

At present, many specialists have recognized that BMD does not predict the risk for fractures in diabetic patients very well, which is similar to our results, but there is a lack of understanding of the predictive value of the TUG time. Clinically, many doctors use the TUG test to predict the risk for fractures in diabetes. However, our study showed that individuals with T2DM and fractures may have a good TUG test time but a high risk for fractures. We still accept that the TUG test can reflect a person's physical state, but it is not appropriate for screening the risk for fractures in T2DM patients.

There were some limitations in this study. First, the study design was cross-sectional. But this was not a cohort study and could not follow the outcomes of the patients. Therefore, more prospective studies with intervention strategies are needed to verify our results. Second, our study did not distinguish the fracture location because the data were incomplete. Third, we roughly collected the therapy methods for diabetes, but not in detail. The ages of the participants were all above 60 years, but not all ages may have the same conclusion.

5. Conclusion

This study found that low BMD and slow TUG time were independent risk factors for fractures in non-T2DM patients, while no associations were found in the T2DM population. Patients with T2DM have a higher risk for fractures even when they have sufficient BMD and a short TUG time. TUG and BMD underestimated the risk for fractures in the T2DM population.

Data Availability

The cross-sectional data used to support the findings of this study have not been made available because it involves patient privacy.

Conflicts of Interest

All the authors declare that there is no conflict of interest.

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Research Article

FOXO1 Mediates Advanced Glycation End Products Induced Mouse Osteocyte-Like MLO-Y4 Cell Apoptosis and Dysfunctions

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Osteocyte plays an essential role in bone metabolism by regulating osteoblast and osteoclast activities. Dysfunction or apoptosis of osteocyte will severely endanger the bone homeostasis and result in bone diseases such as osteoporosis. Osteoporosis has been considered as one of the diabetes complications; however, the mechanism is still to be discovered. Advanced glycation end products (AGEs), as the main pathogenic factor of diabetes mellitus, have the capacity to induce osteocyte apoptosis thus sabotaging bone homeostasis. Here, we examined the role of AGE during osteocyte apoptosis and how this effect would affect osteocyte's regulation of osteoblast and osteoclast. Mouse osteocyte-like MLO-Y4 cells were used to study the properties of osteocyte and to examine its biological and pathological function. MTT assay and Annexin V assay showed that AGE significantly induce MLO-Y4 cell apoptosis. qPCR and Western blot results have shown that AGE upregulates proapoptotic gene p53 and its downstream target gene Bax, which leads to enhanced activation of caspase-3, thus inducing apoptosis in MLO-Y4 cells. Increased expression of sclerostin and RANKL in osteocytes has shown that AGE induces osteocyte dysfunction thus severely damaging the bone homeostasis by decreasing osteoblast and increasing osteoclast activities. Furthermore, the role of the transcription factor FOXO1, which is intensely associated with apoptosis, has been determined. Western blot has shown that AGE significantly decreases Akt activities. Immunofluorescence has shown that AGE promotes FOXO1 nuclei localization and enhances FOXO1 expression. Silencing of FOXO1 suppressed AGE-enhanced apoptosis; mRNA and protein expressions of cleaved caspase-3, sclerostin, and RANKL were downregulated as well. Moreover, exogenous FOXO1 increased caspase-3 mRNA levels and caspase-3 transcriptional activity. Lastly, ChIP assay has established the capacity of FOXO1 binding directly on the caspase-3, sclerostin, and RANKL promoter region in AGE environment, providing the mechanism of the AGE-induced osteocyte apoptosis and dysfunction. Our results have shown that FOXO1 plays a crucial role in AGE-induced osteocyte dysfunction and apoptosis through its regulation of caspase-3, sclerostin, and RANKL. This study provides new insight into diabetes-enhanced risk of osteoporosis given the critical role of AGE in the pathogenesis of diabetes and the essential part of osteocyte in bone metabolism.

1. Introduction

Osteoporosis, increasing dramatically with population aging, has been considered as a systemic skeletal disease characterized by decreased in bone mineral density (BMD) and increased risk of fracture. Fractures induced by osteoporosis severely affect daily life; vertebral and hip fractures may even lead to increased morbidity and mortality [1]. However, the risk is not limited to weight-bearing bone fracture. Osteoporosis also affects oral health by deteriorating alveolar bone quality which leads to tooth loss [2, 3]. It has been acknowledged that osteoporosis promotes the occurrence and development of periodontal disease and affects the integration and stability of dental implants [4]. Diabetes mellitus (DM) is an exceedingly chronic metabolic disorder which affects bone metabolism deleteriously, which frequently coexists with osteoporosis in the elderly [5, 6]. Recent studies have shown that patients with DM have an elevated risk of

osteoporotic fractures [7–9]. Nowadays, osteoporosis has been recognized as one of the diabetic complications.

Under physiological conditions, the progression of bone resorption by osteoclast and bone formation by osteoblast are dynamically balanced. Bone tissue is continuously under remodeling for adaptation to mechanical use and calcium homeostasis [10]. Osteocyte, the most abundant cell in the bone tissue, plays a critical role during the bone remodeling process by regulating osteoclast and osteoblast activities [11, 12]. Osteocyte regulates osteoclastogenesis through its secretion of receptor activator of nuclear factor-κB ligand (RANKL), which is the critical regulator for osteoclast differentiation and activation. Other than osteoblasts and bone marrow stromal cells, osteocytes are the primary source of RANKL secretion in the bone [10, 13]. Sclerostin, which is a protein expressed explicitly by osteocyte, inhibits osteoblast activities and decreases bone formation [14]. Thus, it is safe to say that osteocyte acts as an orchestrator of the bone remodeling process. Therefore, dysfunction or apoptosis of osteocyte will harm the bone homeostasis and result in bone diseases. Osteoporosis is the consequence of an imbalanced bone metabolism which is the excessive bone resorption without the corresponding amount of neoformed bone. It has been recently demonstrated that osteocytes are even involved in the occurrence of osteoporosis directly besides its regulation role in osteoblast and osteoclast behavior.

Accelerated accumulation of advanced glycation end products (AGEs) has been considered as a characteristic feature of DM and osteoporosis [15, 16]. Under hyperglycemia and oxidative stress condition, nonenzymatic chemically modified proteins produce AGEs. Recent studies have demonstrated that AGEs mediate the pathogenesis of osteoporosis by impairing osteoblastic development and function [17, 18]. Several works of literature have argued that AGEs induced osteocyte apoptosis directly, increased sclerostin expression, and decreased RANKL expression in osteocyte-like cells [12, 19, 20]. However, the underlying mechanism of AGE-induced osteocyte dysfunction is still under discovery. It has been reported that AGE stimulates fibroblast apoptosis through transcription factor FOXO1, and FOXO1 silencing can rescue this effect [21]. Other shreds of evidence have shown that FOXO1 is highly involved in the pathogenesis and development of DM and mediates bone metabolism by the regulation of RANKL secretion [22–25]. FOXO1 is a member of forkhead box-O (FOXO) transcription factor, which typically regulates gene expression by binding to DNA and modulating transcription [26]. However, it is difficult to predict the impact of FOXO1 since its function is regulated by both epigenetic and posttranslation protein modifications and its downstream targets are modified by the microenvironment [23, 27–29].

Taken together, we hypothesized that AGEs induce osteocyte dysfunction and apoptosis through a FOXO1-dependent mechanism. Results demonstrate that AGEs lead to a significantly increased osteocyte apoptosis through upregulating p53, Bax, and cleaved caspase-3 expressions. Besides inducing osteocyte apoptosis directly, increased expression of sclerostin and RANKL in osteocyte has shown that AGE-induced osteocyte dysfunction leads to decreased osteoblast activities and increased osteoclast activities thus severely damaging the bone homeostasis. AGE significantly suppresses p-Akt expression, translocates FOXO1 from the cytoplasm into the nucleus, and promotes its function as a transcription factor. FOXO1 has the capacity to direct interaction with the caspase-3, sclerostin, and RANKL promoter regions in osteocytes and enhances their transcriptional activity. Conversely, knockdown of FOXO1 rescued the AGE-enhanced apoptosis and dysfunction of osteocyte. Our results have shown for the first time that FOXO1 mediates the AGE-induced osteocyte apoptosis and its dysregulation of osteoblast and osteoclast. Thus, it is possible that FOXO1 plays a crucial role in the pathogenesis development of DM-induced osteoporosis.

2. Materials and Methods

2.1. Preparation of AGEs. AGEs were prepared as previously described by Okazaki et al. [30]. Briefly, 50 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) was incubated with 0.1 M DL-glyceraldehyde (Sigma-Aldrich) in 0.2 M phosphate buffer (pH 7.4) at a 37°C sterilized incubator for 1 week, then exhaustively dialyzed against phosphate-buffered saline (pH 7.4) for three days. Nonglycated BSA was prepared at the same time, except that no DL-glyceraldehyde was added. Fluorescence strength of AGE solution was detected at an excitation/emission wavelength of 370/440 nm, which is fortyfold higher than the BSA control.

2.2. Cell Culture and Treatment. Experiments were performed with mouse osteocyte-like MLO-Y4 cells from Cell Bank (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai). This cell line enables us to study the properties of osteocytes and to examine its biological and pathological function [31]. MLO-Y4 cells were cultured on type I collagen-coated plates in a-MEM (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 1% penicillin-streptomycin (Solarbio, Beijing, China). Cells were kept in an incubator with 5% CO_2 at 37 degrees Celsius, and the culture medium was changed twice per week. MLO-Y4 cells were incubated with (200 μg/ml) AGE for up to three days after seeding; the control group was incubated with the same concentration of unmodified BSA.

2.3. MTT Assay. MLO-Y4 cells were seeded in 96-well plates at 1×10^3 cells per well and treated with 200 μg/ml BSA or AGE for up to three days. The effect of AGE treatment on cell apoptosis and viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Sigma, Ronkonkoma, NY, USA). Data were detected by a spectrophotometer (Bio-Rad) at 0, 24, 48, and 72 hours.

2.4. Annexin V-FITC Apoptosis Detection Assay. MLO-Y4 cells were incubated with 200 μg/ml BSA or AGE separately for three days and collected 24 hours after the transfection of siFOXO1 or its relative control siRNA. Cell apoptosis was assessed with Annexin V-FITC apoptosis detection kit

I (BD Biosciences) according to the manufacturer's instructions. Subsequently, 10,000 cells per treatment were counted to assess the number of positively stained cells. Cells with both Annexin V and PI positively stained were recognized as apoptotic cells. Data were exhibited as fold change compared with the control group.

2.5. Immunofluorescence. Cells were plated in 96-well plates at 1×10^3 cells per well and incubated with 200 μg/ml BSA or AGE separately for three days before experiments. 4% formaldehyde supplemented with 0.5% Triton X-100 were used for fixation. 2% BSA was used for nonspecific blocking followed by the procedure of incubation with primary anti-FOXO1 antibody (Abcam ab39670, Cambridge, MA, US) or nonspecific IgG (I-1000, Vector Laboratories). The biotinylated secondary antibody was used to associate with primary antibody, and the nucleus was stained with DAPI (S2110; Solarbio, Beijing, China). Exposure time was first set up using the threshold of the highest exposure time with no signal in the nonspecific IgG control. Then, all images were captured at 200 × the original magnifications using the same exposure time by a fluorescence microscope (Olympus, Japan). Analysis was performed using an Olympus AX-70 analysis system. Percentage of FOXO1 nuclei-positive cells was set by the number of FOXO1 nuclei-positive cells divided by the total number of FOXO1-positive cells.

2.6. RNA Isolation, Reverse Transcription, and qPCR. RNA isolation was performed using the TRIzol (Invitrogen) method. PrimeScript RT reagent kit (RR047, TaKaRa Bio, Toyobo Osaka, Japan) was used for reverse transcription. qPCR was performed using TB Green Advantage kit (#639676, Clontech, Toyobo Osaka, Japan) according to the manufacturer's instructions. Specific primers were as follows: p53: 5′-AGTAAAGGCTCTAAAGCTCACCC-3′ (forward) and 5′-GTAAGAGGTCGGCATTGGAAG-3′ (reverse); Bax: 5′-TGAAGACAGGGGCCTTTTTG-3′ (forward) and 5′-AATTCGCCGGAGACACTCG-3′ (reverse); caspase-3: 5′-TGGTGATGAAGGGGTCATTTATG-3′ (forward) and 5′-AATTCGCCGGAGACACTCG-3′ (reverse); sclerostin: 5′-CGGAGAATGGAGGCAGAC-3′ (forward) and 5′-GTCAGGAAGCGGGTGTAGTG-3′ (reverse); and RANKL: 5′-AGGCTGGGCCAAGATCTCTA-3′ (forward) and 5′-GTCTGTAGGTACGCTTCCCG-3′ (reverse). GAPDH was used as a reference gene and its PCR primers included 5′-GAAGGTGAAGGTCGGAGTC-3′ (forward) and 5′-TTCGGCTTTCCAGTCAGACTC-3′ (reverse). qPCR calculation was performed using ΔΔCT method.

2.7. Western Blot. MLO-Y4 cells were harvested and lysed after three days of incubation of AGE or BSA control. Proteins were separated on an SDS/polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Followed by nonspecific blocking, membranes were then incubated with the primary antibody (GAPDH: sc-32233, Santa Cruz; p53: ab32389, Abcam; Bax: #5023, Cell Signaling Technology; FOXO1: Abcam ab39670; sclerostin: bs-10200R, Bioss; RANKL: bs-0747R, Bioss; caspase-3: #9662, Cell Signaling Technology; p-Akt: #4058, Cell Signaling Technology; and Akt: #9272, Cell Signaling Technology). The membranes were extensively washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). The antigen-antibody complexes were visualized by West-Q-Chemiluminescent Sub Kit Plus (BioTang, Waltham, MA).

2.8. Transfections and Dual Luciferase Reporter Assay. MLO-Y4 cells were plated into 48-well plates with 70% confluence for siRNA transfections which were performed with 10 nM siFOXO1 (GenePharma, Suzhou, China) or scramble (GenePharma, Suzhou, China) control using siRNAFect (Cwbio, Beijing, China) following the manufacturer's instructions. Full culture medium was replaced by OptiMEM (Gibco) medium one hour prior to transfection. Lipofectamine 3000 was used to perform the transfection according to the manufacturer's instruction. Transfection medium was replaced back to full culture medium after 6 hours. Luciferase reporter assay kit from Promega (cat#: E1960) was used for detection. Cotransfection was performed with empty vector and wild-type FOXO1 vector along with luciferase reporter. Vector and reporter were added at a 1 : 1 ratio according to the transfection efficiency test. For normalizing the verification caused by transfection efficiency, relative luciferase expression values were calculated using firefly luciferase activities divided by Renilla activities as the company instructed.

2.9. Chromatin Immunoprecipitation. Approximately 1.5×10^8 MLO-Y4 cells were collected after three days of incubation of BSA and AGE. ChIP-IT Express Enzymatic from Active Motif (#53009) was used to perform chromatin immunoprecipitation (ChIP) according to the manufacturer's instructions. FOXO1 (FKHR) antibody (Abcam ab39670) or nonspecific IgG (I-1000, Vector Laboratories) was used for pulldown. DNA was purified using chromatin IP DNA purification kit (#58002, Active Motif) following the company's instructions before endpoint analysis. The caspase-3 promoter region of 560-745 which contains several consensus FOXO1 elements was detected using the following primers: forward: 5′-GTGTACGTCAGTCCCTTACATC-3′ and reverse: 5′-AGACTCTGACTCTGGGAAGT-3′. The RANKL promoter region of 1046-1245 which contains several consensus FOXO1 elements was detected using the following primers: forward: 5′-GATCTCTGAGTTTGAGGTCAGC-3′ and reverse: 5′-GGACCTGAATTTGACCAGAAGA-3′. The sclerostin promoter region of 562-707 which contains several consensus FOXO1 elements was detected using the following primers: forward: 5′-CTGGATTCCGCCTTCTGTAG-3′ and reverse: 5′-GCAGTCAGGCTGTGGTT-3′. Results were quantified as a percentage of input.

2.10. Statistical Analysis. Two-way ANOVA with Tukey's post hoc test was performed for experiments with multiple comparisons. Student's t test was performed for comparisons between two groups. All error bars are standard error of the mean. Significance was set at $p < 0.05$. Triplicate samples were examined per group, and experiments were repeated with similar results.

3. Results

3.1. AGE Induces MLO-Y4 Cell Apoptosis and Dysfunction. We examined the effect of AGE treatment on MLO-Y4 cells. MTT assay was performed to detect cell viability. Cells were incubated with AGE or its negative control BSA for three days. Percentages of cell viability were analyzed at 0, 24, 48, and 72 hours. Results have shown that MLO-Y4 cells' viability gradually reduced by AGE treatment and reached the bottom at 72 hours (Figure 1(a)). This result indicated 72 hours of incubation time as a perfect time point to detect the effect of AGE on osteocyte apoptosis. Annexin V/PI staining was performed to detect AGE's role on apoptosis of MLO-Y4 cells. Result has shown that cell apoptosis exhibits a 1.6-fold increase after incubation of AGE compared with the BSA control group (Figure 1(b)). Furthermore, osteocyte-derived protein sclerostin and RANKL were detected by qPCR and western blot to determine how AGE would affect osteocytes' regulation of osteoblasts and osteoclasts (Figure 1(c)). Western blot results show that AGE leads to a 4.7-fold change in sclerostin expression and a 2.5-fold change in RANKL expression compared with BSA group (Figures 1(d) and 1(e)). qPCR results show that AGE enhances sclerostin expression to 3.8-fold and increases RANKL expression to 3.2-fold compared with BSA group (Figures 1(f) and 1(g)).

3.2. AGE Enhances MLO-Y4 Cell Apoptosis by Upregulating p53, Bax, and Caspase-3. mRNA expression of proapoptotic gene p53 and Bax was detected by qPCR after 72 hours of AGE treatment. Quantifications have shown that after AGE treatment, both p53 and Bax mRNA-relative expressions have surged to more than tenfold compared with those in the BSA control group (Figures 2(a) and 2(b)). To verify the mRNA results, Western blot was performed to detect the protein levels of p53 and Bax. Consistent with mRNA results, the protein levels of p53 and Bax were significantly upregulated by AGE (Figure 2(c)). Quantifications have shown that Bax was remarkably increased to elevenfold, while p53 was strikingly increased to sixteenfold when compared with the BSA control group (Figures 2(d) and 2(e)). Cleaved caspase-3 was then detected by Western blot to further determine the apoptosis in MLO-Y4 cells. Result shows that AGE significantly increases cleaved caspase-3 expression (Figure 2(c)). Quantifications have shown that cleaved caspase-3 in the AGE group was twice as much as that in the BSA control group which confirmed AGE-enhanced osteocyte apoptosis (Figure 2(f)).

3.3. AGE Enhances FOXO1 Expression and Promotes FOXO1 Nuclei Localization. Akt has been well established to be one of the most important manipulative factors of FOXO1 which induces its phosphorylation and degradation. Akt activities were detected by Western blot using both Akt- and p-Akt-(Ser-473-) specific antibodies. Result has shown that AGE suppresses 70% of p-Akt expression when compared with the BSA control (Figures 3(a) and 3(b)). Western blot was then performed to examine FOXO1 at the protein level. Result has shown that AGE increases FOXO1's expression, and quantification has shown FOXO1 expression was up to fourfold as much as compared with the BSA control group (Figures 3(c) and 3(d)). Immunofluorescence was performed to visualize FOXO1's location after AGE treatment. Images have shown that FOXO1 resides in the cytoplasm in the BSA control group while translocated into the nucleus in the AGE group (Figure 3(e)). Total FOXO1-positive cells and the nucleus FOXO1-positive cells were counted to quantify the percentage of FOXO1 nuclei localization. Quantification has shown that more than 27% of FOXO1 is nuclei positive in the AGE group compared with only 2% in the BSA control group (Figure 3(f)). This result showed that AGE promotes about 25% of FOXO1 translocate from the cytoplasm into the nucleus and functions as a transcription factor. Nuclei localization will clearly lead to an enhanced FOXO1 transcription efficiency and increased the yield of FOXO1 target gene expression.

3.4. FOXO1 Deletion Rescued the AGE Enhanced MLO-Y4 Apoptosis and Dysfunction. For a better understanding of FOXO1's function during this event, scramble siRNA or siFOXO1 was transfected into MLO-Y4 cells briefly after AGE incubation. Cell apoptosis, mRNA, and protein expression of cleaved caspase-3, sclerostin, and RANKL were detected after transfection. Annexin V/PI staining result has shown that silencing of FOXO1 reduced about 50% of the AGE-enhanced apoptosis (Figure 4(a)). qPCR result demonstrated that 60% of caspase-3 and sclerostin and 70% of RANKL mRNA expressions were reduced by FOXO1 silencing (Figures 4(b), 4(c), and 4(d)). Western blot result demonstrates that the decreased mRNA expression caused by FOXO1 silencing leads to a reduction in protein level (Figure 4(e)). Quantification has shown that siFOXO1 leads to a 30% decrease in cleaved caspase-3, 60% decrease in sclerostin, and 65% in RANKL expression when compared with scramble siRNA in AGE environment (Figures 4(f), 4(g), and 4(h)).

3.5. FOXO1 Mediates AGE-Induced MLO-Y4 Cell Apoptosis by Direct Binding to Caspase-3 Promoter Region. To further illustrate the underlying mechanism of this regulation, ChIP assay was performed to determine if FOXO1 is bound directly to the caspase-3, sclerostin, and RANKL promoter regions. Quantifications of caspase-3 results show that in the AGE group, FOXO1 pulldown is eightfold stronger compared with the nonspecific IgG, while no significant difference was found between FOXO1 and IgG in the BSA group (Figure 5(a)). Sclerostin results show that FOXO1 pulldown in AGE group exhibits a 5.5-fold higher compared with the IgG control and RANKL results show a 4.8-fold change (Figures 5(b) and 5(c)). Luciferase assay was then performed to verify the ChIP assay results. Results have shown that exogenous FOXO1 increases caspase-3 twice compared with empty vector in the AGE group but no significant difference was found in the BSA control group, and AGE alone without FOXO1 vector leads to a twofold change in caspase-3 expression (Figure 5(d)). Quantifications show that FOXO1 vector enhances sclerostin expression 2.7-fold and increases RANKL expression twofold compared with empty vector in the AGE group. AGE leads to a 2.9-fold change in sclerostin

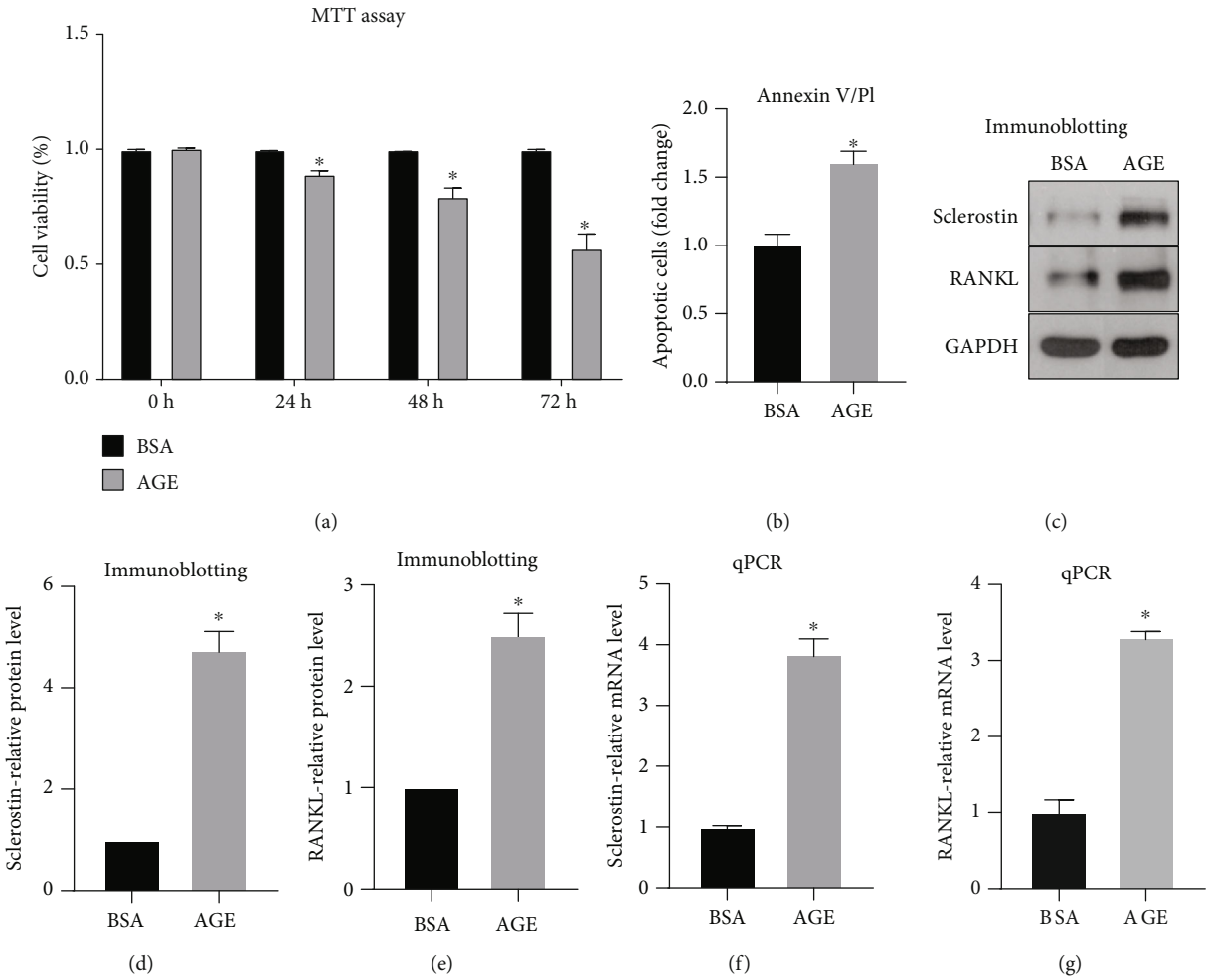

Figure 1: AGE treatment induces MLO-Y4 cell apoptosis and dysfunction. (a) MLO-Y4 cells were incubated for BSA or AGE for up to three days and cell viability was detected by MTT assay at 0 h, 24 h, 48 h, and 72 h. (b) Annexin V/PI assays were performed to detect AGE's role on MLO-Y4 cell apoptosis. Western blot was performed to examine the protein expression of sclerostin and RANKL. (c) Western blot representative images. (d) Quantifications of sclerostin-relative protein expression. (e) Quantifications of RANKL-relative protein expression. qPCR was performed to examine the mRNA expression of sclerostin and RANKL. (f) Quantifications of sclerostin-relative mRNA expression. (g) Quantifications of RANKL-relative mRNA expression. All error bars are standard deviation of the mean. $^{*}p < .05$ compared to the BSA control group.

and a 2.3-fold in RANKL when compared with BSA group (Figures 5(e) and 5(f)). These results are consistent with the ChIP assay results, which FOXO1 only binds to caspase-3, sclerostin, and RANKL promoter regions in the AGE group. Besides that, AGE alone is capable of increases in caspase-3, sclerostin, and RANKL expressions which is consistent with our previous result.

4. Discussion

Our study revealed that AGE significantly enhances the expression of p53 and Bax, which results in an increased caspase-3-dependent apoptosis in MLO-Y4 cells. Besides inducing osteocyte apoptosis directly, AGE significantly upregulates osteocyte-derived sclerostin and RANKL indicating that AGE impairs the biological function of osteocytes and leads to inhibited osteoblast activities and enhanced osteoclast activities. These results offered a critical insight that AGE not only induces osteocyte apoptosis but also caused its dysfunction. Furthermore, we provide the foundations for a mechanism as to how these effects were regulated. Akt is an essential manipulative factor of FOXO1 which induces its phosphorylation and degradation. Immunoblotting results have shown that AGE significantly suppresses the activities of Akt, which will lead to upregulated FOXO1 activity. Western blot and immunofluorescence assay verified that AGE upregulates FOXO1 and strengthens its regulation of downstream target genes by translocating FOXO1 into the nucleus. Moreover, silencing of FOXO1 rescued the AGE-enhanced osteocyte apoptosis, sclerostin, and RANKL expressions thus confirming FOXO1's role as a critical regulator during this event. ChIP assay was performed to establish FOXO1's ability to regulate caspase-3, sclerostin, and RANKL expressions through direct promoter binding, and

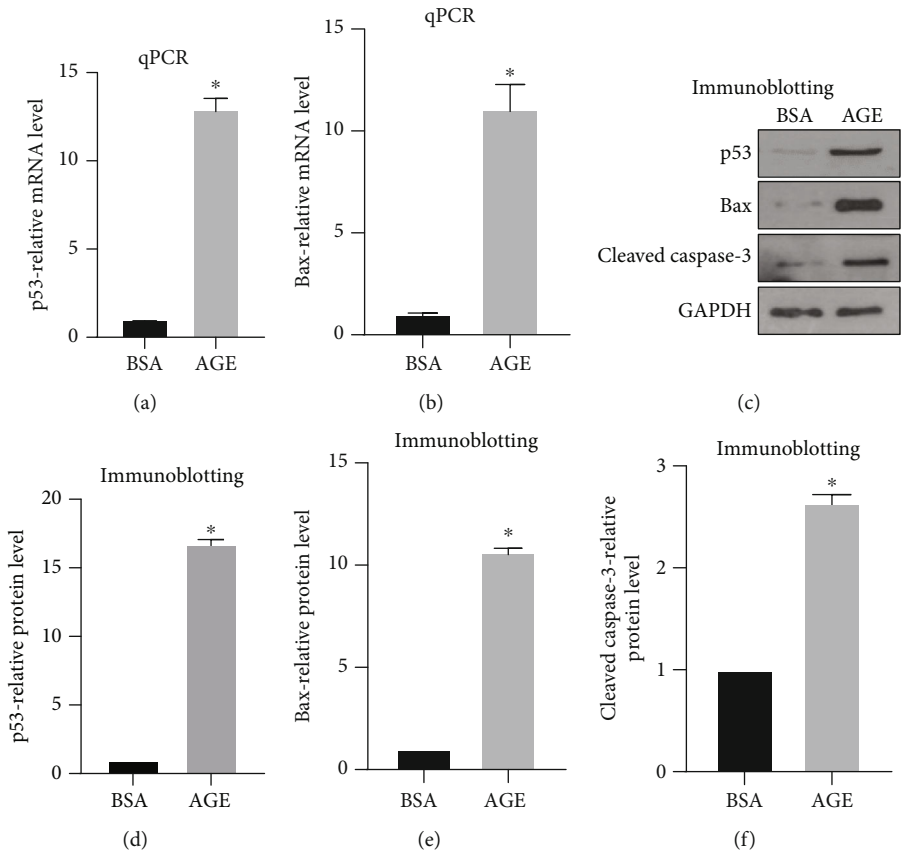

Figure 2: AGE enhances the expression of proapoptotic genes and proteins in MLO-Y4 cells. The mRNA expression of proapoptotic genes p53 and Bax was measured via qPCR. (a) Quantifications of p53-relative mRNA expression. (b) Quantifications of Bax-relative mRNA expression. Western blot was performed to detect p53, Bax, and cleaved caspase-3 expression in protein level. (c) Western blot representative images. (d) Quantifications of cleaved caspase-3-relative expression. (e) Quantifications of p53-relative expression. (f) Quantifications of Bax-relative expression. All error bars are standard deviation of the mean. $^*p < 0.05$ compared to the BSA control group.

this regulation is verified by luciferase assay. Our results clarified that AGE induces osteocyte apoptosis and dysfunction in a FOXO1-dependent method. These results are critical as they proposed the explanation and possible mechanism for why we are likely seeing increased osteoporosis risk in diabetic patients.

Accumulation of AGE seriously endangers the balance of bone metabolism and results in osteoporosis by increasing osteocyte apoptosis and decreasing osteoblast and osteoclast activities [12, 19, 32]. Our study is aiming to detect the underlying mechanism of how AGE induces osteoporosis and the role of transcription factor FOXO1. Annexin V-FITC apoptosis detection assay results have shown that AGE significantly induces osteocyte apoptosis. Cleaved caspase-3 expression in MLO-Y4 cells in the AGE group was twice as much as that of the control group. These results verified that AGE causes osteocyte apoptosis in a caspase-3-dependent pathway. Furthermore, proapoptotic genes p53 and Bax, which are upper stream signaling of caspase-3, were detected after AGE treatment. Results have shown elevated expressions of p53 and Bax in both mRNA and protein levels as we anticipated. p53-mediated mitochondria intrinsic apoptosis has been intensely studied. It can rapidly translocate from the cytoplasm to the mitochondrial surface upon activation by oxidative stress, leading to apoptosis through interacting with BCL-2 family members, such as inhibiting antiapoptotic gene Bcl-2 and activating proapoptotic gene Bax functions [33–35]. These results suggest that AGE induces osteocyte apoptosis through p53-mediated mitochondria intrinsic pathway which is executed by caspase-3. Other than inducing osteocyte apoptosis, AGE also affects osteocytes' regulation of osteoblast and osteoclast activities. Sclerostin and RANKL expressions were detected, and the results have shown that both cytokines secreted by osteocytes were elevated. The previous study [12] found that AGE2 and AGE3 reduce RANKL expression in the MLO-Y4 cell lysate but upregulate its expression in the supernatant. The main difference between the two studies is that they were using 100 μg/ml of AGEs while we are using 200 μg/ml as a working concentration. This working concentration was decided based on our previous study [36]. 200 μg/ml of AGEs would represent approximately 4.8 nmol/ml of carboxymethyllysine, which approximates the levels of carboxymethyllysine reported in serum (approximately 2.6 nmol/ml). It has been reported that 200 μg/ml of AGEs significantly induce fibroblast apoptosis and increase RANKL expression in

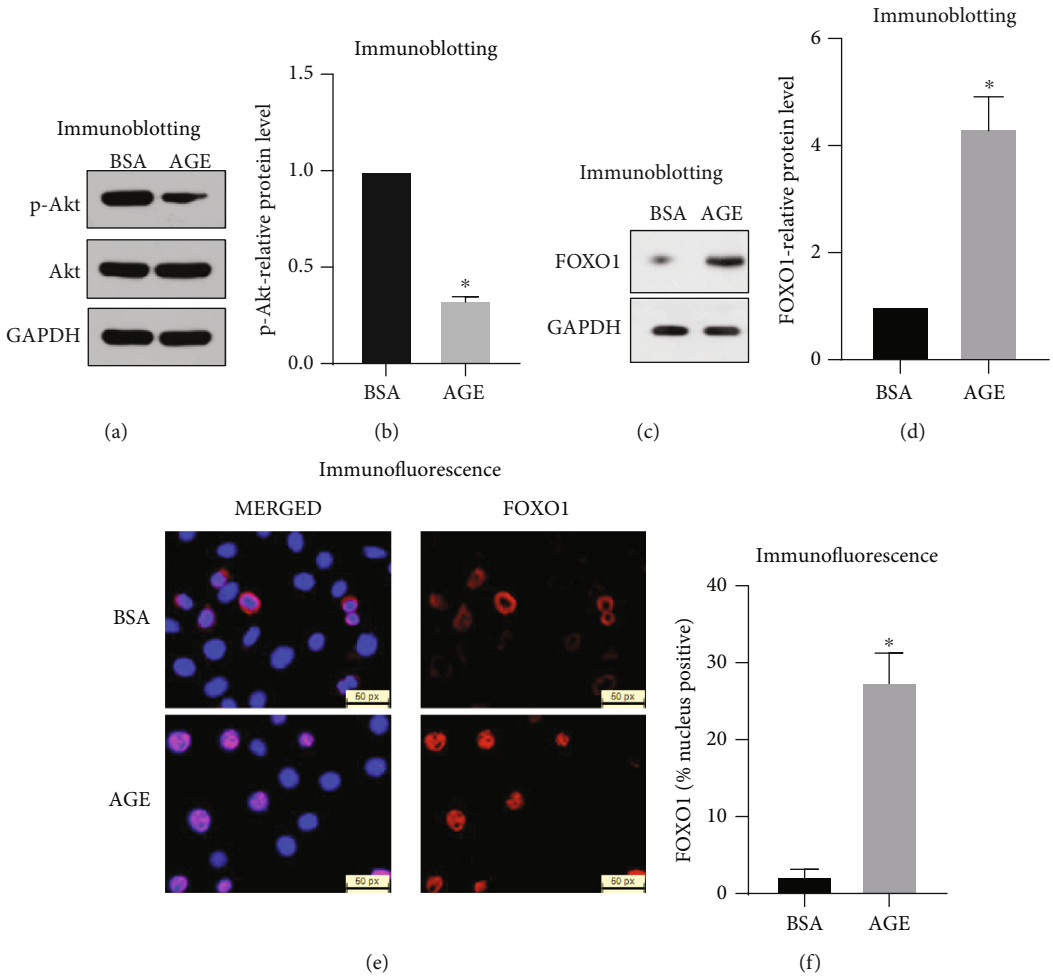

Figure 3: AGE enhances FOXO1 expression and promotes FOXO1 nuclei localization. Western blot was performed to examine the expression of Akt and p-Akt. (a) Akt and p-Akt representative images. (b) Quantifications of p-Akt expression. Western blot was performed to examine the expression of FOXO1. (c) FOXO1 representative images. (d) Quantifications of FOXO1-relative expression. Immunofluorescence pictures taken with FOXO1 antibody to detect FOXO1 nuclei localization, images were taken at 40× for FOXO1-positive cell counting and 100× for exhibiting nuclei localization. (e) Immunofluorescence representative images for FOXO1 nuclei translocation. (f) Percentage of FOXO1 nuclei-positive cells was set by the number of FOXO1 nuclei-positive cells divided by the total number of FOXO1-positive cells. All error bars are standard deviation of the mean. $^*p < 0.05$ compared to the BSA control group.

chondrocytes [21, 22]. However, it is possible that different levels of AGEs have various abilities to induce RANKL expression. Taken together, our results indicate that AGE induces osteocyte apoptosis and has the capacity to cause osteocyte dysfunction, which would suppress osteoblast activities and enhance osteoclast activities, thus sabotaging bone homeostasis and leads to osteoporosis.

Several studies have revealed that FOXO1 is closely associated with apoptosis. Tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) is regulated by FOXO1 and responsible for FOXO1-induced apoptosis [37]. In addition to that, FOXO1 is capable of transactivation of Bim which is a proapoptotic member of the Bcl-2 family that functions in the intrinsic mitochondrial apoptotic pathway [38, 39]. Our study established that FOXO1 mediates AGE-induced osteocyte apoptosis through caspase-3 and promotes AGE-caused osteocyte dysfunction by upregulating sclerostin and RANKL. Silencing of FOXO1 decreases osteocyte apoptosis and cleaved caspase-3, sclerostin, and RANKL expressions indicating that FOXO1 is a key regulator in the AGE-aggravated osteocyte dysfunction and apoptosis, and these reduced expressions are likely due to the direct interaction of FOXO1 with the caspase-3, sclerostin, and RANKL promoter regions that all contain a FOXO1 consensus response element. ChIP assay results have proved that FOXO1 binds to caspase-3, sclerostin, and RANKL promoter regions in the AGE group but not in the BSA control group. This is likely due to AGE significantly suppressing Akt activities, which promotes FOXO1 translocate into the nucleus and increases its transcriptional activities. Indeed, luciferase assay has provided that without exogenous FOXO1, AGE alone induces caspase-3 expression, which could be explained by

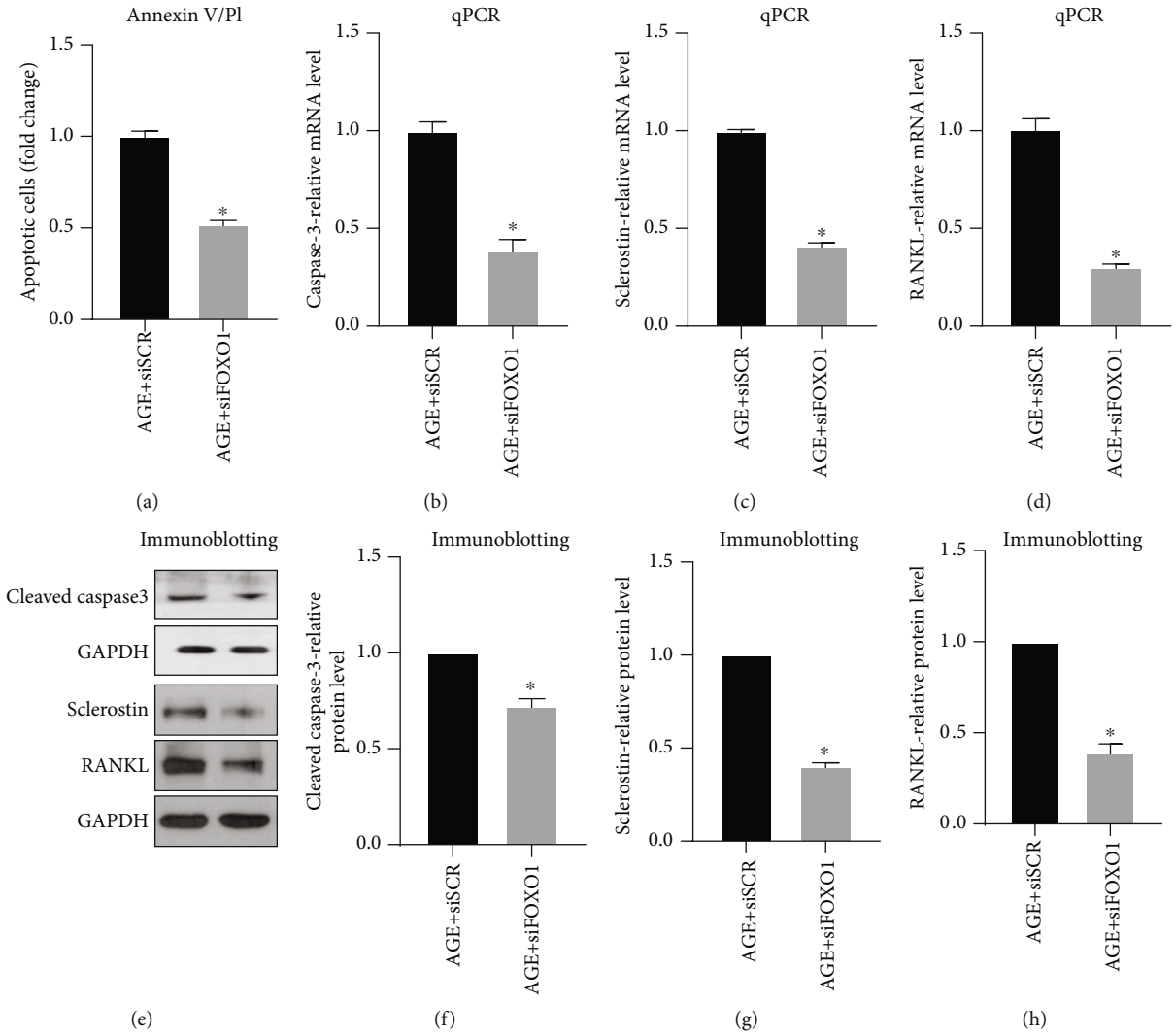

Figure 4: FOXO1 plays as a key regulator of AGE-induced MLO-Y4 cell apoptosis and dysfunction. MLO-Y4 cells were transfected with scramble siRNA or siFOXO1 to detect FOXO1 silencing's effect on AGE-induced MLO-Y4 cell apoptosis and dysfunction. (a) Annexin V/PI assays were performed to detect apoptosis. qPCR was performed to examine the effect of FOXO1 silencing on expression of caspase-3, sclerostin, and RANKL. (b) Quantifications of caspase-3-relative mRNA expression. (c) Quantifications of sclerostin-relative mRNA expression. (d) Quantifications of RANKL-relative mRNA expression. Western blot was performed to detect cleaved caspase-3, sclerostin, and RANKL protein expressions. (e) Western blot representative images (upper GAPDH is for cleaved caspase-3, bottom GAPDH is for sclerostin and RANKL). (f) Quantifications of cleaved caspase-3-relative expression. (g) Quantifications of sclerostin-relative expression. (h) Quantifications of RANKL-relative expression. All error bars are standard deviation of the mean. $^{*}p < 0.05$ compared to relative scramble siRNA control.

AGE-induced endogenous FOXO1 nuclei localization. However, this could also be related to FOXO1's posttranscriptional modifications. Phosphorylation has been well known for its regulation of FOXO1's downstream targets [40]. Besides phosphorylation, the previous study has shown that FOXO1's binding activities are heavily linked with its acetylated status [25]. It could be possible that AGE regulates the downstream genes of FOXO1 by altering its posttranscriptional modification. This will become a future interest in our study.

In summary, our experiments show that AGE significantly induced osteocyte apoptosis and caused its dysfunction. AGE upregulates p53 and its downstream target Bax then leads to a p53-mediated mitochondria intrinsic apoptosis through the activation of caspase-3. AGE upregulates sclerostin and RANKL expressions indicating that AGE impairs osteocytes' biological function. Decreased apoptosis and expression of cleaved caspase-3, sclerostin, and RANKL in FOXO1-silenced MLO-Y4 cells provide critical insight as to FOXO1 being an important regulator during this progress. The direct binding of FOXO1 to the caspase-3 promoter provides mechanistic insight into how osteocyte apoptosis is induced, and the binding of FOXO1 to the sclerostin and RANKL provides the mechanism of

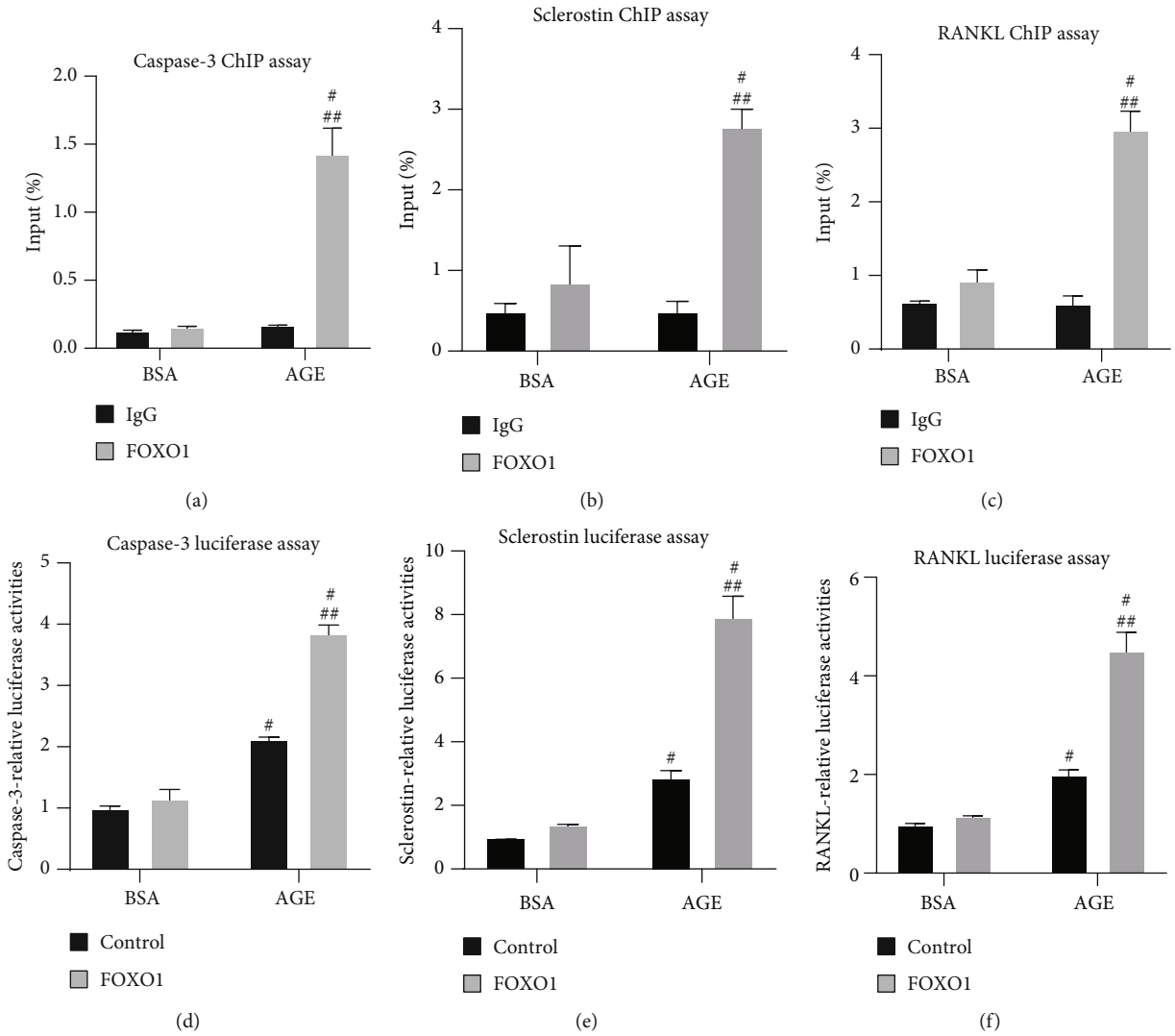

Figure 5: FOXO1 directly binds to the caspase-3, sclerostin, and RANKL promoter regions. ChIP assays were performed in MLO-Y4 cells with pulldown by FOXO1 antibody and PCR amplification of a region of the caspase-3, sclerostin, and RANKL promoter flanking the FOXO1 consensus response element. Results were compared to both its relative control IgG and the FOXO1 binding of the BSA control group. (a) Quantifications of caspase-3 ChIP assay. (b) Quantifications of sclerostin ChIP assay. (c) Quantifications of RANKL ChIP assay. MLO-Y4 cells were cotransfected with empty vector or FOXO1 vector and caspase-3, sclerostin, and RANKL luciferase reporter and Renilla control construct. Results were compared to control (empty vector) and FOXO1 expression plasmid in the BSA control group. (d) Quantifications of caspase-3 luciferase assay. (e) Quantifications of sclerostin luciferase assay. (f) Quantifications of RANKL luciferase assay. All error bars are standard deviation of the mean. $^{\#}p < 0.05$ compared to BSA control group. $^{\#\#}p < 0.05$ compared to the BSA FOXO1 group.

osteocytes dysfunction. Our results show for the first time that FOXO1 plays as a critical regulator in the AGE-induced osteocyte apoptosis and dysfunction. These results lead to a better understanding of FOXO1's role in diabetes-induced metabolism disorder in the bone tissue and provide an insight mechanism of how diabetes results in an enhanced risk of osteoporosis.

Data Availability

No data were used to support this study.

Conflicts of Interest

Citong Zhang, Wei Wei, Minghan Chi, Yao Wang, Xue Li, Manlin Qi, and Yanmin Zhou declare that they have no conflict of interest.

Authors' Contributions

CZ and YZ are involved with the study design. CZ, WW, MC, YW, XL, and MQ are involved in the study conduct. Data interpretation is performed by CZ, WW, and YZ. CZ and YZ drafted and revised the manuscript.

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Supplementary Materials

The siRNA transfection efficiency by means of knockdown efficiency of FOXO1. *(Supplementary Materials)*

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Research Article

Type 2 Diabetes Mellitus Increases the Risk to Hip Fracture in Postmenopausal Osteoporosis by Deteriorating the Trabecular Bone Microarchitecture and Bone Mass

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T2DM is linked to an increase in the fracture rate as compared to the nondiabetic population even with normal or raised bone mineral density (BMD). Hence, bone quality plays an important role in the pathogenesis of skeletal fragility due to T2DM. This study analyzed the changes in the trabecular bone microstructure due to T2DM at various time points in ovariectomized and nonovariectomized rats. Animals were divided into four groups: (I) control (sham), (II) diabetic (sham), (III) ovariectomized, and (IV) ovariectomized with diabetes. The trabecular microarchitecture of the femoral head was characterized using a micro-CT. The differences between the groups were analyzed at 8, 10, and 14 weeks of the onset of T2DM using a two-way analysis of variance and by post hoc multiple comparisons. The diabetic group with and without ovariectomies demonstrated a significant increase in trabecular separation and a decrease in bone volume fraction, trabecular number, and thickness. BMD decreased in ovariectomized diabetic animals at 14 weeks of the onset of T2DM. No significant change was found in connectivity density and degree of anisotropy among groups. The structural model index suggested a change towards a weaker rod-like microstructure in diabetic animals. The data obtained suggested that T2DM affects the trabecular structure within a rat's femoral heads negatively and changes are most significant at a longer duration of T2DM, increasing the risk to hip fractures.

1. Introduction

Diabetes mellitus (DM) although identified more than half a century ago as being associated with bone frailty has come to the forefront only within the last decade as an important osteoporosis risk factor. Diabetics suffering from both types are at an increased risk of osteoporosis and fractures [1, 2]. Despite this increased fracture risk, bone fragility remains an underappreciated complication of DM.

Postmenopausal osteoporosis, due to estrogen deficiency, is the most common type of primary osteoporosis in women. Estrogen deficiency imbalances the bone remodeling cycle resulting in increased bone resorption and 25-30% destruction in bone mass during a 5 to 10-year period [3].

In the United States, 10 million individuals > 50 years of age are estimated to have osteoporosis of the hip (based on a T-score of ≤−2.5), with about 1.5 million osteoporotic fractures each year [3]. The socioeconomic costs of osteoporosis and associated fractures are high and are expected to increase remarkably over the next decades due to increasing life expectancy. Osteoporotic hip fractures are associated with increased mortality and reduced quality of life and more problematic in patients with diabetes due to compromised bone fracture healing [4]. A recent report has suggested that anxiety negatively affects the HRQoL in patients with diabetes of long duration [5].

The standard diagnostic technique for assessing osteoporosis and monitoring therapy is dual X-ray absorptiometry (DXA) measuring bone mineral density (BMD). BMD can predict femoral bone strength and fracture risk to some extent, but BMD values of patients with and without femur fractures overlap [4, 6]. Osteoporotic changes and increased

fractures reported in type 1 DM (T1DM) are linked to a decrease in bone mineral density [2]. However, data on skeletal abnormalities in type 2 DM (T2DM) appear contradictory and the exact explanation to this is still unknown. Individuals with T2DM have low, normal, or increased bone mineral density and yet associated with increased fractures at various skeletal sites [7–9]. Hence, the bone quantity measured as bone mineral density is not the only factor that contributes to osteoporosis, but changes in bone quality should also be considered while predicting fracture risk.

Bone quality involves the analysis of the bone microstructure. In a high-quality bone, the trabeculae are well-connected, greater in number, thicker, and more plate-like [10]. There are less fatigue damage, a higher level of mineralization, and balanced porosity in cortical bones, and the health of osteocytes plays a great role [11]. Osteoporosis deteriorates the microstructure of the bone particularly at trabecular sites, especially in the vertebrae, ribs, and hips.

We hypothesize here that diabetes mellitus is a chronic disease which may also reduce bone quality over a period of time. The aim of the current study is to investigate the effect of T2DM on postmenopausal osteoporosis. Data available for the effect of T2DM on bones is variable, and there is not much data available for combined effects of estrogen deficiency and diabetes.

As the increased risk of fractures in diabetes has a distinct propensity for the proximal end of the femur, the study has evaluated microstructural changes at the femoral head that occurred due to T2DM and lack of estrogen (postmenopausal) and due to the combined effect of T2DM and postmenopausal osteoporosis at various time points. Ovariectomized (OVX) rats were used as a model for postmenopausal osteoporosis.

Microcomputed tomography (micro-CT) is used to investigate changes of the trabecular bone at the femoral head. Micro-CT is a noninvasive technique and regarded as a valuable technique for investigating the microarchitecture of the bone.

2. Materials and Methods

2.1. Animal Model. Three-month-old female Wistar rats ($n = 96$) were obtained from the animal house facility at United Arab Emirates University for this study. The animals were singly housed in cages under the standard conditions with a 12 h alternating light and dark cycle (22–24°C), in 50–60% humidity and provided with free access to standard rat chow and water ad libitum during the two weeks of acclimatization. All efforts were made to minimize animal suffering and to reduce the number of animals used. All animal procedures were approved by the animal ethical committee at Unite Arab Emirates University (ERA_2017_5597).

2.2. Experimental Design. The experimental animals were randomly divided into four groups ($n = 8$ for each group): Gp. I—control/sham-operated, Gp. II—sham-T2DM, Gp. III—OVX, and Gp. IV—T2DM+OVX.

2.3. Establishment of Type 2 Diabetic Rat Model. Animals ($n = 48$) were fed a high-calorie diet (D12492 diet; Research Diets, Inc., USA) for 3 weeks followed by the injection of two lower doses of streptozotocin (STZ) (30 mg/kg intraperitoneally) which was administered at weekly intervals. [12]. Three days after the last injection, tail vein blood glucose was measured after fasting for 5 h using a blood glucose meter (Accu-Chek Performa; Roche Diagnostics, USA). Rats having blood glucose > 15 mmol/liter were considered diabetic and were used for our study [12]. Insulin resistance in diabetic animals was further confirmed through an insulin tolerance test [12]. The high-fat diet was continued throughout the experimental period in the diabetic group of animals. The blood glucose concentrations and body weight were monitored fortnightly.

2.4. Surgery. Rats underwent bilateral ovariectomies two weeks after the onset of diabetes to make them sex hormone deficient and to stimulate the accelerated loss of bone. The procedure was carried out as per standard protocol [13]. Briefly, the operation was made after placing an anesthetized animal on its dorsal surface. The area of surgery was cleaned with ethanol (Merck, India). A small transverse peritoneal incision was made with a surgical scalpel blade on the middle part of the abdomen slightly towards the right, just near to the second right nipple of the rat to open the peritoneal cavity of the rat. The adipose tissue was pulled away until the right uterine tube and the ovary surrounded by a variable amount of fat were identified. The procedure was repeated for the left ovary through the same incision. After identifying the ovaries and uterine horns, the distal uterine horns were ligated and the ovaries were removed. The distal uterine horns were returned to the peritoneal cavity after removal of ovaries, and the skin was stitched back. The control rats underwent sham procedures only. A postoperative follow-up was carried out on all animals.

All animals ($n = 96$) were sacrificed at 6, 8, and 12 weeks ($n = 32$ for each time interval) after surgery to see the effect of 8, 10, and 14 weeks of the onset of diabetes on bone samples. All bones were dissected out, cleaned, fixed, and kept in the storage for further analysis. Right femurs were dissected out for this study.

2.5. Micro-CT Measurements. Femurs were first fixed in buffered formalin, then stored in phosphate-buffered saline (PBS) to be examined by cabinet cone-beam micro-CT (μCT 50, SCANCO Medical AG, Brüttisellen, Switzerland). The test was carried out with a cone beam originating from a 5 μm focal-spot X-ray tube set at a voltage of 70 kVp with a current intensity of 200 μA. The standard 0.5 mm thick aluminum filter was used. The scanned region was approx. 10 mm, and the field of view was 10.24 mm. The integration time was set to 800 ms. The photons were detected by a CCD-based area detector, and the projection data was computer-reconstructed into an image matrix using Scanco 3D-viewer V4.2. For each scan, a stack of 2000 cross-sections was reconstructed. The reconstructed images were 2048×2048 pixels each.

2.6. Evaluation of Trabecular Structure within the Femoral Head. The samples were segmented based on their grey-scale values in the CT slices. An algorithm was developed to generate a cubical mask in the center of the femoral heads. A volume of interest containing only trabecular bone was extracted for morphometric analysis. The trabecular thickness was computed from the segmented sample using the maximum fitted sphere methods [14, 15]. The pore diameters were then computed applying the same method on the inversed segmented image. Please note that this is not the true pore size but the local thickness of the pores. The script for performing the analysis was started with a one-click operation in the SCANCO evaluation program.

The following structural parameters were measured for the morphometric analysis of trabecular bone: TV—total volume (mm^3); BV—bone volume (mm^3); BV/TV—relative bone volume (%); bone surface density which is measured as the ratio of the segmented bone surface to the total volume of the region of interest BS/TV; Conn-Dens—connectivity density, normed by TV ($1/mm^3$); SMI—structure model index (0 for parallel plates, 3 for cylindrical rods); Tb.N—-trabecular number (1/mm); Tb.Th—trabecular thickness (mm); Tb.Sp: trabecular separation = marrow thickness (mm). These indices are calculated without assuming anything about the shape of the bone (i.e., without plate model assumption). All parameters were calculated three dimensionally (3D) based on counting voxels. Bone mineral density was calculated as mean 1 includes voxel values of everything within the volume of interest (mixture of bone and background) scan which were calibrated for bone in units of mg HA/ccm.

2.7. Statistical Analysis. Data were analyzed by two-way analysis of variance and unpaired t-tests using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Bonferroni's multiple comparison test was used to assess the difference between the groups and also if there is any change in defined trabecular structural parameters over different time periods. Data are expressed as the means ± SD. Statistical significance was established at $p < 0.05$. Adjusted p value ($^{*}p < 0.05$, $^{**}p < 0.01$).

3. Results

The blood glucose levels (mean ± SD) for diabetic rats range between 21.30 ± 8.16 to 26.50 ± 1.02 (mmol/liter) and 19.98 ± 8.05 to 24.63 ± 6.58 (mmol/liter) for Gp. II and Gp. IV, respectively. The blood glucose level for nondiabetic rats in Gp. I was 6.3 ± 0.26 to 6.62 ± 0.53 (mmol/liter) and 6.15 ± 2.03 to 6.49 ± 0.88 (mmol/liter) in Gp. III (see complete data in the supplementary file (available here)).

Graphs for all the measured structural parameters and 3D images of the micro-CT scans of the trabecular bone from each of the four groups at three different time points are shown in Figures 1 and 2, respectively. Table 1 shows the mean ± SD values and percentage differences for the significant data for all the measured structural parameters. 3D evaluation of trabecular structure within a rat's femur head was compared over the period of 8, 10, and 14 weeks of the onset of T2DM in Gp. I—control/sham-operated, Gp. II—sham-T2DM, Gp. III—OVX, and Gp. IV—T2DM+OVX. The total number of animals in each group was 8 for any one-time period.

The BV/TV decreased $p < 0.05$ in Gp. II and Gp. IV bone samples when compared with controls in Gp. I after 10 and 14 weeks of the onset of diabetes, respectively. The BV/TV in Gp. II (14 wks) sham-diabetic animals was significantly less ($p < 0.05$) than nondiabetic ovariectomized animals from Gp. III (14 wks). A statistically significant decrease in BV/TV was also observed in Gp. IV when compared with Gp. III ($^{**}p < 0.01$) at 14 weeks of the onset of DM (Figure 1(a)).

The BS/TV increased in Gp. III at 14 weeks of the onset of diabetes which was found when compared with respective control in GP. I ($p < 0.05$) (Figure 1(b)).

The Tb.N decreased ($p < 0.05$) in Gp. II at 10 and 14 weeks of the onset of DM when compared with the dataset obtained from 8-month diabetic animals from the same group. The Tb.N was found to decrease ($p < 0.01$) in Gp. IV after 14 weeks of exposure to diabetes as compared to its respective control in Gp. III. However, the Tb. N in the 14-week bone samples obtained from Gp. III was significantly higher ($p < 0.05$) when compared with those from Gp. I and Gp. II and with those obtained from Gp. IV ($p < 0.01$) (Figure 1(c)). The Tb.Th (Figure 1(d)) in Gp. II and Gp. IV at 14 weeks was significantly lower ($p < 0.05$) than control in Gp. I.

The porosity within the trabecular bone was measured as Tb.Sp. It increased ($p < 0.05$) in Gp. II at 10 weeks when compared with its respective control in Gp. I and also when compared with that in Gp. III at 14 weeks ($p < 0.01$). Additionally, increased trabecular separation was found in the diabetic group (Gp. II) with an increase in the duration of diabetes and the change was significant ($p < 0.05$) between 8 and 10 weeks of the onset of diabetes in Gp. II. Trabecular separation in Gp. IV was significantly higher ($p < 0.001$) than that in Gp. III at 14 weeks of the onset of diabetes (Figure 1(e)). The data for Gp. III was heterogeneous, and though the trabecular separation increased with time (8-10 wks), the change was not significant, and at 14 weeks, the bone samples showed a decrease in the trabecular separation when compared with controls (Gp. I) ($p < 0.05$ at the same time point).

Mean 1 which represents vBMD decreased significantly in 14-week bone samples from Gp. IV when compared with those from Gp. I and Gp. III with $p < 0.05$ and $p < 0.01$, respectively (Figure 1(f)).

Higher negative values for SMI were found in the nondiabetic control groups—Gp. I and Gp. III. The SMI was found to increase in Gp. IV after 8 and 14 weeks of the onset of diabetes when compared with those in Gp. II and Gp. III at the same time points (Figure 1(g)).

No significant difference has been found in any comparison for connectivity density (Figure 1(h)) and the degree of anisotropy (DA) (Figure 1(i)). The interaction between different groups and the effect of the duration of time on different groups were also considered to be nonsignificant ($p > 0.05$). No statistically significant change in any of the structural parameters was found with time alone in control (Gp. I).

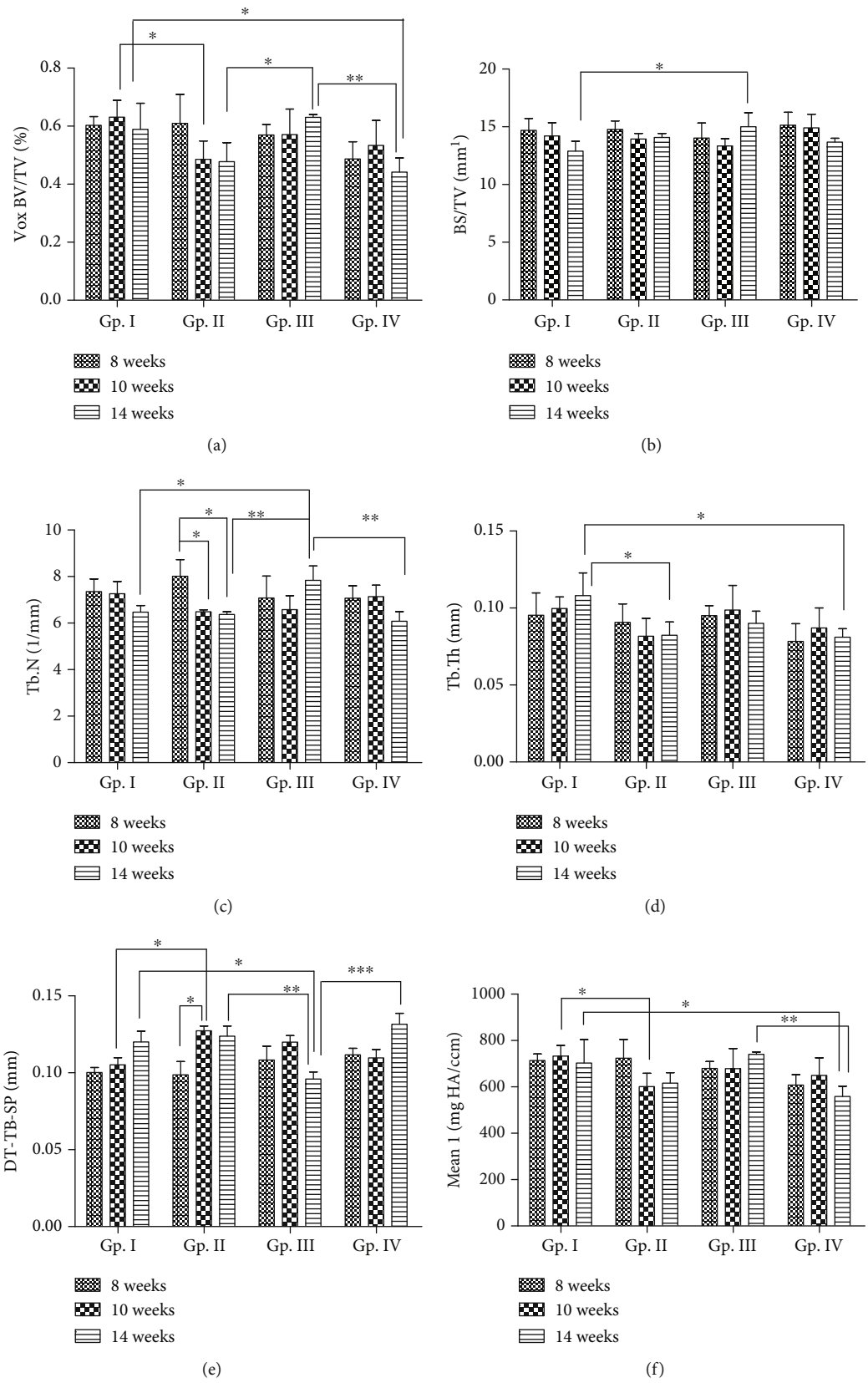

Figure 1: Continued.

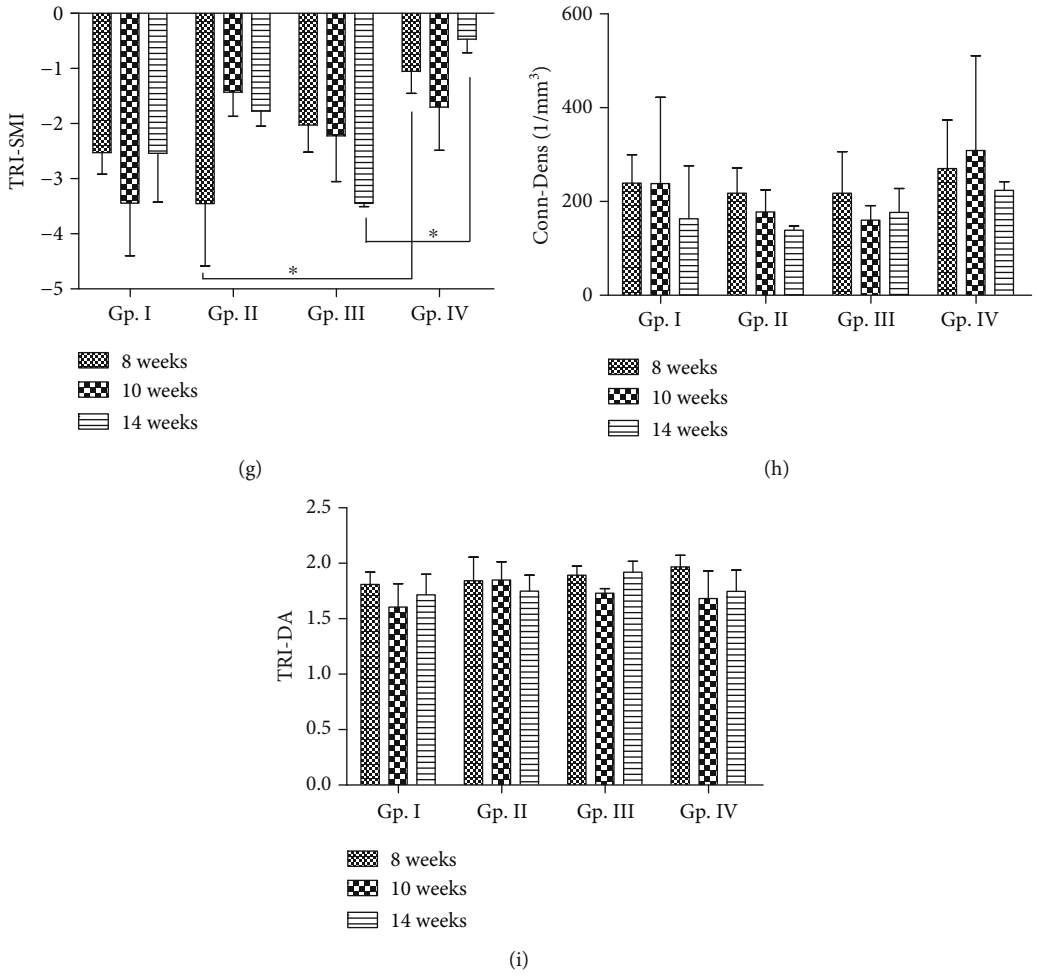

Figure 1: Plots of changes in various structural parameters of trabecular bone: (a) bone volume/total volume BV/TV, (b) bone surface density (BS/TV), (c) trabecular number (Tb.N), (d) trabecular thickness (Tb.Th), (e) trabecular separation (Tb.Sp), (f) mean 1 (vBMD), (g) structural model index (SMI), (h) connectivity density (Conn-Dens), and (i) degree of anisotropy (DA) of a rat's femur head from Gp. I—control/sham-operated, Gp. II—sham-T2DM, Gp. III—OVX, and Gp. IV—T2DM+OVX which were compared over the period of 8, 10, and 14 weeks of onset of diabetes. Two-way ANOVA was performed with Bonferroni's posttest multiple comparison test using GraphPad Prism 5. Adjusted p value ($^{*}p < 0.05$, $^{**}p < 0.01$). Error bars = mean ± SD.

4. Discussion

Fragility fractures are a common complication of osteoporosis affecting the elderly population predominantly women after estrogen loss in postmenopausal age. The bone loss associated with estrogen deficiency is generally attributed to an imbalance between bone resorption and formation results in the loss of bone mass and deterioration of trabecular bone microarchitecture [16–18].

The importance of PMO is very clear as with the increase in the aging population, the complications such as the hip fractures will treble to over six million a year by 2050 [19]. T2DM is more common with advancing age and, therefore, frequently coexists with age-related bone loss [20, 21]. Diabetes has an increased prevalence of risk factors for falls and subsequent injuries, including poor vision, peripheral neuropathy, and stroke. Data has shown that an increased tendency of falls and a higher risk of injury following a fall do not fully account for the greater risk of fracture in diabetes and people suffering from T2DM are at an increased risk of fragility fractures despite normal or increased bone mineral density. Hence, recent research is focused on analyzing changes in the bone microarchitecture that deteriorates bone quality and could be an important factor contributing to diabetic osteopathy.

Patients suffering from diabetes have an increased incidence of fragility fractures at an early age as compared to the nondiabetic population [21–23]. The estimated risk ratio for diabetes and hip fracture is 1.38 (95% CI, 1.25–1.53) for T2DM [22]. Trabecular bone loss is more prominent in PMO due to its large surface to volume ratio, and it shows a higher turnover rate than the cortical bone [18, 24, 25]. This study characterized using micro-CT the changes in the trabecular architecture within the head of a rat's femur, due to different duration of T2DM, and investigated the effect of T2DM on postmenopausal osteoporosis. The data in this study were collected by using an animal model; however, further human studies will be necessary to confirm these results.

The ovariectomized rat model used in this study is a well-established animal model of postmenopausal osteoporosis

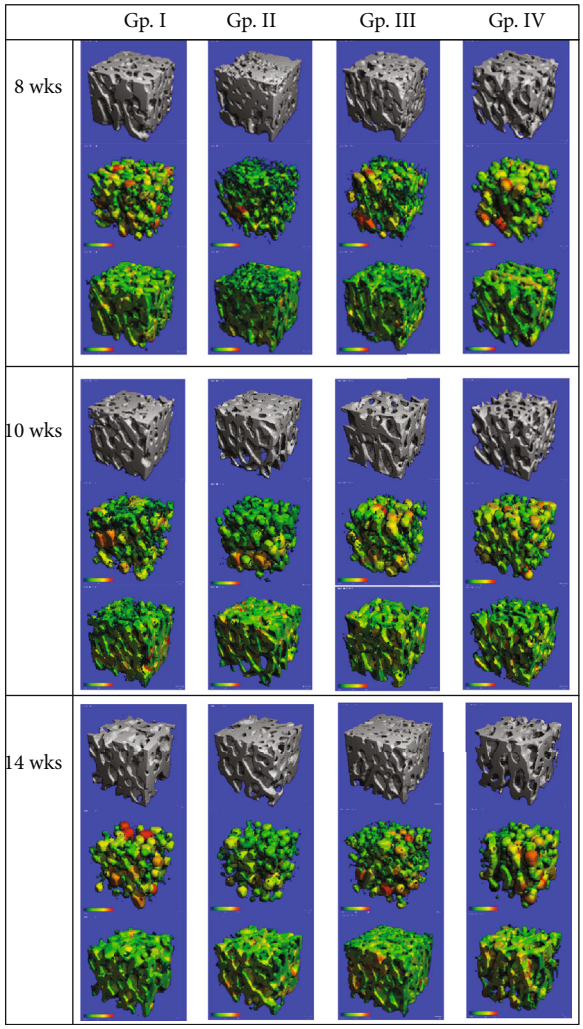

Figure 2: Representation of 3D microarchitecture of the trabecular bone at the head of the femur from 12 rats from four groups: Gp. I—control/sham-operated, Gp. II—sham-T2DM, Gp. III—OVX, and Gp. IV—T2DM+OVX obtained by micro-CT examination at 6, 8, and 10 weeks after surgery.

because the bone loss in these animals is considered to mimic that of postmenopausal women [24]. The study examined the changes in the trabecular bone microarchitecture within the head of the femur from control/sham-operated, sham-T2DM, OVX, and T2DM+OVX female mature rats using micro-CT. Micro-CT examination is a noninvasive, nondestructive way of examining the microarchitecture of the bone at high resolution [26, 27].

Bone structural parameters measured were similar to histomorphometry analysis, such as the bone volume fraction (BV/TV), bone surface density (BS/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). Nonmetric parameters such as the structural model index (SMI), connectivity density (Conn-Dens), degree of anisotropy (DA), mean 1, or vBMD were also measured. [27].

The BV/TV indicates the fraction of a given volume of interest (VOI, i.e., the total volume (TV)) that is occupied by the mineralized bone (bone volume). It was evaluated to detect relative changes if any in bone volume density that occurred following ovariectomy (OVX) and diabetes (T2DM). The results have shown that T2DM negatively affects bone volume density as it is significantly decreased in bones of diabetic animals with and without ovariectomy. The bone volume density significantly decreased with an increase in the duration of diabetes with $p < 0.01$ at 14 weeks of the onset of DM in ovariectomized animals with diabetes (Table 1).

The bone surface density (BS/TV) which is measured as a ratio of the segmented bone surface to the total volume of the region of interest [14, 15] was increased in ovariectomized rats without diabetes (Gp. III) after 14 weeks of exposure to diabetes compared to its respective control from Gp. I in this study. The bone surface is affected by the activity of osteoclasts and osteoblasts where resorption lacunae have more perimeter per unit length than osteoid covered or quiescent bone [28]. This represents increased bone turnover in ovariectomized animals as reported by earlier studies [29, 30].

The number of trabeculae (Tb.N) decreased after 14 weeks of the onset of diabetes in the postdiabetic ovariectomized group when compared with their respective ovariectomized nondiabetic controls. Additionally, we found a decrease in the number of trabeculae with an increase in the duration of diabetes in the nonovariectomized diabetic group. Trabecular thickness also decreased under the influence of DM in diabetic rats with or without ovariectomy as found in an earlier study [24].

However, unlike earlier studies [31–33], we found an increase in the trabecular number in samples obtained from an ovariectomized animal. This difference in results could be due to a transient stage of osteoporosis in our samples where trabeculae were not completely resorbed and were in the process of breakdown.

Trabecular separation means an increase in the distance between the adjacent trabeculae and requires perforation and removal of whole trabecular elements. This study is consistent with previous studies which showed that bones are becoming increasingly porous with an increase in trabecular separation in both diabetic groups with and without ovariectomies [34]. The trabecular separation increase was the most significant ($p < 0.001$) in ovariectomized animals at 14 weeks of the onset of diabetes. Trabecular separation increased with an increase in the duration of diabetes in the nonovariectomized group with significant change observed in bones at 10 weeks of the onset of DM as compared to bones at 8 weeks of the onset of DM. [34]. Pritchard et al. [34] also found an increase in the average whole size within the trabecular bone network at the distal radius and suggested it contributes to higher fracture risk in type 2 diabetic population. Kerckhofs et al., [35] on the contrary, found an increase in the thickness of trabeculae and no significant change in trabecular separation in the proximal tibia of diabetic mice when compared to controls.

Mean 1 or vBMD represents what is often called "volumetric bone mineral density," more precisely called apparent bone mineral density. Specifically, it is the total bone mineral content contained within the volume of interest divided by the total volume (TV) of the region of interest. It is the

Table 1: Structural parameters of trabecular bone within the femoral head for four groups at different time points. Data presented as the mean ± SD.

(a)

VOX BV/TV (%)	8 weeks	10 weeks	14 weeks
Gp. I	0.60 ± 0.02	0.63 ± 0.05	0.58 ± 0.09
Gp. II	0.61 ± 0.09	0.48 ± 0.06[a]	0.47 ± 0.06[b]
Gp. III	0.57 ± 0.03	0.57 ± 0.08	0.63 ± 0.00
Gp. IV	0.49 ± 0.05	0.53 ± 0.08	0.44 ± 0.04[c,d]

[a]% difference to Gp. I (-23.80) ($^{*}p < 0.05$). [b]% difference to Gp. I (-24.1) ($^{*}p < 0.05$). [c]% difference to Gp. III (-25.3) ($^{*}p < 0.05$). [d]% difference to Gp. III (-30.15) ($^{**}p < 0.01$).

(b)

TRI-BS/TV (mm^{-1})	8 weeks	10 weeks	14 weeks
Gp. I	14.69 ± 1.02	14.21 ± 1.14	12.88 ± 0.86
Gp. II	14.77 ± 0.74	13.93 ± 0.47	14.07 ± 0.35
Gp. III	14.02 ± 1.30	13.33 ± 0.62	15.00 ± 1.22[a]
Gp. IV	15.14 ± 1.11	14.90 ± 1.91	13.67 ± 0.32

[a]% difference to Gp. I (16.52) ($^{*}p < 0.05$).

(c)

DT-TB.N (1/mm)	8 weeks	10 weeks	14 weeks
Gp. I	7.35 ± 0.54	7.25 ± 0.52	6.47 ± 0.27
Gp. II	8.01 ± 0.72	6.48 ± 0.07[a]	6.37 ± 0.11[b]
Gp. III	7.07 ± 0.94	6.57 ± 0.60	7.83 ± 0.61[c,d,e]
Gp. IV	7.06 ± 0.53	7.13 ± 0.49	6.08 ± 0.41

[a]% difference to Gp. I (21.02) ($p < 0.05$). [b]% difference to 8 weeks in Gp. II (-19.10) ($p < 0.05$). [c]% difference to 8 weeks in Gp. II (-20.47) ($p < 0.05$). [d]% difference to Gp. II (22.91) ($p < 0.01$). [e]% difference to Gp. IV (28.78) ($p < 0.01$).

(d)

DT-Tb.Th (μm)	8 weeks	10 weeks	14 weeks
Gp. I	0.09 ± 0.01	0.09 ± 0.00	0.10 ± 0.01
Gp. II	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.00[a]
Gp. III	0.09 ± 0.00	0.09 ± 0.01	0.09 ± 0.00
Gp. IV	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.00[b]

[a]% difference to Gp. I (-20) ($p < 0.05$). [b]% difference to Gp. I (-20) ($p < 0.05$).

(e)

DT-Tb.Sp (μm)	8 weeks	10 weeks	14 weeks
Gp. I	0.10 ± 0.00	0.10 ± 0.00	0.12 ± 0.01
Gp. II	0.09 ± 0.01	0.12 ± 0.00[a,b]	0.12 ± 0.01
Gp. III	0.10 ± 0.01	0.11 ± 0.00	0.09 ± 0.00[c,d,e]
Gp. IV	0.11 ± 0.00	0.10 ± 0.00	0.13 ± 0.01

[a]% difference to Gp. I (20) ($p < 0.05$). [b]% difference to Gp. I (-25) ($p < 0.05$). [c]% difference to 8 weeks in Gp. II (20) ($p < 0.05$). [d]% difference to Gp. II (33) ($p < 0.01$). [e]% difference to Gp. IV (44) ($p < 0.001$).

(f)

Mean 1/v BMD	8 weeks	10 weeks	14 weeks
Gp. I	713.91 ± 28.29	733.09 ± 45.67	703.05 ± 100.41
Gp. II	723.56 ± 80.66	601.41 ± 57.03[a]	615.87 ± 45.78
Gp. III	679.41 ± 30.16	678.59 ± 87.01	740.24 ± 10.18
Gp. IV	607.56 ± 45.38	649.28 ± 76.17	558.88 ± 43.70[b,c]

[a]% difference to Gp. I (-17.97) ($p < 0.05$). [b]% difference to Gp. I (-20.5) ($p < 0.05$). [c]% difference to Gp. III (-24.5) ($p < 0.01$).

(g)

TRI-SMI	8 weeks	10 weeks	14 weeks
Gp. I	-2.52 ± 0.67	-3.44 ± 1.64	-2.53 ± 1.52
Gp. II	-3.45 ± 1.95$_a$	-1.42 ± 0.75	-1.77 ± 0.48
Gp. III	-2.02 ± 0.85	-2.22 ± 1.43	-3.44 ± 0.10[b]
Gp. IV	-1.05 ± 0.68	-1.70 ± 1.35	-0.46 ± 0.42

[a]% difference to Gp. IV (-69) ($p < 0.05$). [b]% difference to Gp. IV (-86) ($p < 0.05$).

(h)

Conn-Dens	8 weeks	10 weeks	14 weeks
Gp. I	239.30 ± 60.24	238.45 ± 183.92	163.451 ± 112.91
Gp. II	217.70 ± 54.22	177.56 ± 47.33	138.773 ± 8.98
Gp. III	217.53 ± 88.57	160.22 ± 31.02	176.888 ± 50.66
Gp. IV	270.09 ± 103.62	308.704 ± 201.93	223.662 ± 18.52

recommended method [27] for reporting BMD for a cancellous bone as it relates directly to bone strength. It shows trends similar to BV/TV.

Data available on skeletal abnormalities in T2DM and its direct relationship with BMD are contradictory, and the exact explanation of this is still unknown. In different studies, bone mineral density values have increased, decreased, or remained normal [36]. Petit and colleagues [8] reported a higher BMD in elderly patients with T2DM when compared to age-matched non-DM volunteers. In contrast, several other investigators reported a negative effect of T2DM on BMD [9, 37].

This study found that BMD tends to decrease with an increase in the duration of diabetes in nonovariectomized diabetic animals, and significant change ($p < 0.05$) was recorded at 8 weeks of the onset of DM when compared with its respective controls. Most significant ($p < 0.01$) negative changes in BMD were seen in the postovariectomized diabetic group after 14 weeks of the onset of DM when compared with its respective control in nondiabetic ovariectomized control. The results of this study show that T2DM affects the BMD negatively as reported in earlier studies [9, 37, 38].

However, we did not find any significant decrease in BMD in the head of the femur from the ovariectomized nondiabetic group as reported by others [30] for the proximal tibial metaphysis in the ovariectomized rats compared to the control group. Substantial heterogeneity in the data is most likely due to differences in the study design and use of the different animal models or skeletal sites.

The structure model index (SMI) is a parameter defined to describe a "plate-like" and "rod-like" architecture of trabeculae within the cancellous bone. It is calculated by means of three-dimensional image analysis based on a differential analysis of the triangulated bone surface. The relative proportion of rods to plates in the trabecular bone is thought to be important for the bone's mechanical competence, with plates considered to be mechanically superior to rods. The deterioration of the cancellous bone structure due to aging and disease is characterized by conversion from plate elements to rod elements. The most real trabecular structures will lie somewhere between the ideal plate and ideal rod structure, and the value lies between 0 and 3, depending on the volume ratio of rods and plates. It is possible that samples may have similar volume density but varying SMI number depending upon the number of the plate-like and rod-like architecture of trabaculae.

The results of our study show a significant increase in SMI values at the head of the femur obtained from postdiabetic ovariectomized animals when compared to nonovariectomized animals with diabetes and with ovariectomized animals without diabetes at eight and fourteen weeks, respectively. The increase in SMI value suggests that the trabecular architecture is changing from more plate-like to weaker rod-like as trabeculae undergo perforation and/or thinning mechanisms [39].

Higher negative values were found in the nondiabetic control groups Gp. I and Gp. III which correspond to high BV/TV values in these groups as found. Hildebrand et al. [40] also showed that the trabecular structure is predominantly plate-like in the head of the femur. Negative SMI values are also reported in an earlier study [40] in the distal femoral metaphysis with BV/TV greater than 35%. As bone volume fraction decreases, trabeculae tend to become more rod-like. However, at very high or low BV/TV values, the SMI may be outside of the defined range [27]. More negative values represent a more plate-like, stronger lattice and are being associated with the greatest strength and less fracture risk [41].

The connectivity density (Conn-Dens) represents one aspect of how trabeculae contribute to bone strength by estimating their interconnectivity. It represents how many branches between nodes can be cut before the structure is completely separated. The connectivity density tends to decrease with aging in each group in this study; however, no statistically significant difference was found within or in comparison to other groups.

The degree of anisotropy (TRI-DA) is described as 1 being isotropic and >1 being anisotropic. Bone tissue is described to be an anisotropic material which means that it can show different mechanical behaviors to applied load in different directions [42]. All the groups in this study showed an anisotropic bone structure with no significant change due to ovariectomies or due to diabetes in the degree of anisotropy within the head of the femur unlike other studies [2, 43] which showed an increase in the degree of anisotropy with an advanced stage of postmenopausal osteoporosis due to selective bone loss. The difference in the result could be due to the small sample size in each group in this study.

5. Conclusions

This study showed a significant effect of diabetes on trabecular bone architecture and on bone mineral density in the head of the femur obtained from mature female Wistar rats with and without ovariectomies. Hence, T2DM should be considered an important risk factor for hip fractures. From a clinical perspective, the elderly female population with T2DM is at higher risk of fracture; therefore, there is a need to correlate the measurement of bone mass with the measurements of structural parameters. Most changes in trabecular microstructure and in bone mineral density were found at the longer duration of diabetes. Better glycaemic control at an earlier stage of diabetes may prevent or delay the deterioration of bone microarchitecture and preserve both bone quality and bone quantity. A better understanding of the bone microstructure and metabolism will help us find various mechanisms of skeletal fragility involved in diabetes and would be helpful in improving its diagnosis, treatment, and assessment of the efficacy of the osteoporosis therapy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

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

Acknowledgments

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Supplementary Materials

Supplementary data shows the mean ± SD of body weight (gms) and blood glucose level (mmol/l) changes in GP. I, GP. II, GP. III, and GP. IV at 8, 10, and 14 weeks of the onset of diabetes before sacrifice. *(Supplementary Materials)*

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