Medicine

Wnt Signaling Regulation in Bone of Postmenopausal Women With Type 2 Diabetes

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

About eLife's process

Reviewed preprint posted
October 19, 2023 (this version)

Posted to bioRxiv
October 7, 2023

Sent for peer review
July 3, 2023

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Abstract

Type 2 diabetes (T2D) is associated with higher fracture risk, despite normal or high bone mineral density. We reported that bone formation genes (SOST and RUNX2) and advanced glycation end-products (AGEs) were impaired in T2D. Thus, we investigated Wnt signaling regulation and its association with AGEs accumulation in T2D. We obtained bone tissue from 15 T2D and 21 non-diabetic postmenopausal women undergoing hip arthroplasty. Bone histomorphometry revealed a trend of low mineralized volume in T2D. We showed that gene expression of Wnt agonists LEF-1 and WNT10B were lower in T2D. Accordingly, WNT5A and SOST gene expression were higher, while collagen (COL1A1) was lower in T2D. Importantly, AGEs content was associated with SOST and WNT5A, but inversely correlated with LEF-1 and COL1A1. Finally, SOST was also associated with glycemic control and disease duration. These findings suggest that Wnt signaling and AGEs could be the main determinants of bone fragility in T2D.

eLife assessment

The reviewers believe that this study provides **valuable** insights into understanding bone fragility in T2D patients through the use of human samples, laying the groundwork for future research. The employed methods were **solid**, demonstrating the feasibility of studying human samples. However, reviewers identified some minor weaknesses in the form of a limited number of subjects and a relatively small set of genes included in the analysis.

Introduction

Type 2 diabetes (T2D) is a metabolic disease, with an increasing worldwide prevalence, characterized by chronic hyperglycemia and adverse effects on multiple organ systems, including bones [1]. Patients with T2D have an increased fracture risk, particularly at the hip, compared to individuals without diabetes. A recent meta-analysis reported that individuals with T2D have 1.27 relative risk (RR) of hip fracture compared to non-diabetic controls [2]. Fragility fractures in patients with T2D occur at normal or even higher bone mineral density compared to healthy subjects, implying compromised bone quality in diabetes. T2D is associated with a reduced bone turnover[3], as shown by lower serum levels of biochemical markers of bone formation, such as procollagen type1 amino-terminal propeptide (P1NP) and osteocalcin, and bone resorption, C-terminal cross-linked telopeptide (CTX) in diabetic patients compared to nondiabetic individuals [4–7]. Accordingly, dynamic bone histomorphometry of T2D postmenopausal women showed a lower bone formation rate, mineralizing surface, osteoid surface, and osteoblast surface[8]. Our group recently demonstrated that T2D is also associated with increased *SOST* and decreased *RUNX2* genes expression, compared to non-diabetic subjects[9]. Moreover, we have proved in a diabetic model that a sclerostin-resistant *Lrp5* mutation, associated with high bone mass, fully protected bone mass and strength even after prolonged hyperglycemia [10]. Sclerostin is a potent inhibitor of the canonical Wnt signaling pathway, a key pathway that regulates bone homeostasis [11]. Therefore, we hypothesize that impairment of Wnt pathway through increased sclerostin activity is responsible for low bone turnover and contributes to bone fragility in diabetes.

Diabetes and chronic hyperglycemia are also characterized by increased advanced glycation end-products (AGEs) production and deposition [12]. AGEs may interfere with osteoblast differentiation, attachment to the bone matrix, function, and survival [13,14]. AGEs also alter bone collagen structure and reduce the intrinsic toughness of bone, thereby affecting bone material properties [9,15,16]. In this work, we propose to dissect Wnt signaling in bone of individuals with T2D and to investigate its relationship with AGEs accumulation. Results confirmed that T2D downregulates Wnt beta/catenin signaling and reduces collagen mRNA levels, in association with AGEs accumulation.

Materials and Methods

Study subjects

We enrolled a total of 36 postmenopausal women (15 with T2D and 21 non-diabetic controls)undergoing hip arthroplasty for osteoarthritis, consecutively screened to participate in this study between 2020 and 2022. Diabetes status was confirmed by the treating diabetes physician. Participants were diagnosed with diabetes when they had fasting plasma glucose (FPG)

≥126 mg/dl or 2-h plasma glucose (2-h PG) ≥200 mg/dl during a 75-g oral glucose tolerance test (OGTT); or hemoglobin A1c (HbA1c) ≥6.5% in accordance with the American Diabetes Association diagnostic criteria. Eligible participants were <65 years of age. Exclusion criteria were any diseases affecting bone or malignancy. Additionally, individuals treated with medications affecting bone metabolism such as estrogen, raloxifene, tamoxifen, bisphosphonates, teriparatide, denosumab, thiazolidinediones, glucocorticoids, anabolic steroids, and phenytoin, and those with hypercalcemia or hypocalcemia, hepatic or renal disorder, hypercortisolism, current alcohol or tobacco use were excluded. The study was approved by the Ethics Committee of the Campus Bio-Medico University of Rome and all participants provided written informed consent.

Specimen preparation

Femoral head specimens were obtained during hip arthroplasty. As described previously [9], trabecular bone specimens were collected fresh and washed multiple times in sterile PBS until the supernatant was clear of blood. Bone samples were stored at – 80 °C until analysis.

Bone histomorphometry

Trabecular bone from femur heads were fixed in 10% neutral buffered formalin for 24 h and stored in 70% ethanol. Tissues were embedded in methylmethacrylate and sectioned by the Washington University Musculoskeletal Histology and Morphometry Core. Unstained and TRAP-stained (Sigma) slides were imaged at ×20 high resolution using a NanoZoomer 2.0 with bright field and FITC/TRITC (Hamamatsu Photonics). Images were then analyzed via Bioquant Osteo software according to the manufacturer's instructions and published standards (v18.2.6, Bioquant Image Analysis Corp., Nashville, TN).

RNA extraction and gene expression by RT-PCR

Total RNA from trabecular bone samples was extracted using TRIzol (Invitrogen) following the manufacturer's instructions. The concentration and purity of the extracted RNA were assessed spectrophotometrically (TECAN, InfiniteM200PRO), and only samples with 260/280 absorbance ratio between 1.8 and 2 were used for reverse transcription using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's recommendations. Transcription products were amplified using TaqMan real-time PCR (Applied Biosystems, Carlsbad, CA) and a standard protocol (95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute; followed by 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds). β-Actin expression was used as an internal control (housekeeping gene).

Relative expression levels of Sclerostin (SOST), Dickkopf-1 (DKK-1), Wnt ligands (WNT5a and WNT10b), T-cell factor/ lymphoid enhancer factor 1 (LEF-1), and collagen type I alpha 1 chain (COL1A1) were calculated using the $2^{-\Delta Ct}$ method.

Statistical analysis

Data were analyzed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA). Patients' characteristics were described using means and standard deviations or medians and interquartile ranges, as appropriate, and percentages. Group data are presented in boxplots with median and interquartile range; whiskers represent maximum and minimum values. Mann-Whitney test was used to compare variables between groups. Data were analyzed using nonparametric Spearman correlation analysis and the correlation coefficients (r) were used to assess the relationship between variables.

Results

Subject characteristics

Clinical characteristics of study subjects are presented in **Table 1**. T2D and non-diabetic subjects did not differ in age, BMI and menopausal age (**table 1**). As expected, fasting glucose was significantly higher in T2D compared to non-diabetic subjects [112.00 (Interquartile ranges-IQR 26.00) mg/dl, *vs*. 94.00 (19.5) mg/dl, respectively; p=0.009]. Median HbA1c was determined in all T2D subjects within three months before surgery [(6.95(0.90%)] (**Table 1**). Median disease duration in T2D subjects was [14.50(11.00)] years. Diabetes medications included monotherapy with metformin (n=12) and combination therapy with metformin plus insulin and glinide (n=3). There were no differences in serum calcium, creatinine and serum blood urea nitrogen (**Table 1**).

Bone Histomorphometry

Surgical samples from 8 T2D and 9 non-diabetic subjects were used for histomorphometry analysis. We found no significant differences in BV/TV and osteoid volume, while mineralized volume/total volume (Md.V/TV) trended lower in T2D subjects relative to controls [(0.249% (0.121) vs 0.352% (0.184); p=0.053] (**Table 2**).

Gene Expression

SOST mRNA was significantly higher in T2D than in non-diabetic subjects (**Fig. 1A**, p<0.0001), whereas there was no difference in *DKK1* gene expression between the two groups (**Fig. 1B**). Of note, SOST mRNA transcript was very low in the majority of non-diabetic subjects (**Fig. 1A**). *LEF-1*(**Fig. 1C**, p=0.0136), *WNT10B* (***Fig. 1D***, p=0.0302) and *COL1A1* (**Fig.1F**, p= 0.0482) mRNA transcripts were significantly lower in T2D compared to non-diabetic subjects; Conversely, *WNT5A* was higher in T2D relative to non-diabetics (**Fig. 1E**, p=0.0232).

Correlation analysis

As shown in **figure 2**, AGEs were inversely correlated with *LEF-1* (**Fig. 2A**, p=0.0255) and *COL1A1* mRNA abundance (**Fig. 2B**, p=0.0004), whereas they were positively correlated with *SOST* (**Fig. 2C**, p<0.0001) and *WNT5A* mRNA (**Fig. 2D**, p=0.0322). There was no correlation between AGEs content and *WNT10B* (**Fig. 2E**; p=0.1938) or *DKK1* gene expression (**Fig. 2F**; p=0.9349). Likewise, we did not find any significant correlation between *LEF-1*, *WNT5A*, *WNT10B*, *DKK-1*, *COL1A1* expression in bone and glycemic control in T2D individuals (**Supplemental Figure 1A-D**). However, there were positive correlations between *SOST* and glycemic control (**Figure 3A**, p=0.0043), *SOST* and disease duration (**Figure 3B**, p=0.00174), and *WNT5A* and fasting glucose levels (**Figure 3C**, p=0.0037)

Discussion

We show that key components of the Wnt/beta-catenin signaling are abnormally expressed in the bone of postmenopausal women with T2D. *LEF-1*, a transcription factor that mediates responses to Wnt signal and Wnt target gene itself, and *WNT10B*, an endogenous regulator of Wnt/β-catenin signaling and skeletal progenitor cell fate, are both downregulated in bone of postmenopausal women with T2D. Consistently, expression of the Wnt inhibitor, *SOST* is increased, suggesting suppression of Wnt/β-catenin signaling in the bone of T2D individuals. Interestingly, our data suggest that sclerostin expression is very low in healthy postmenopausal women not affected by osteoporosis. Our data also show that the expression of *WNT5A*, a non-canonical ligand linked to

	T2D subjects (n=15)	Non-diabetic subjects (n=21)	P value
Age (years)	73.00 (13.00)	73.00 (10.50)	0.644
BMI (kg/m^2)	30.81 (9.56)	25.00 (7.50)	0.117
Menopausal age (years)	50.00 (10.25)	52.00 (5.00)	0.344
Fasting glucose levels (mg/dl)	112.00 (26.00)	94.00 (19.05)	**0.009
Disease duration (years)	14.50 (11.00)	-	-
HbA1c (%)	6.95 (0.90)	-	-
Serum calcium (mg/dl)	9.05 (0.75)	9.15 (0.55)	0.535
Creatinine (mg/dl)	0.730 (0.39)	0.780 (0.23)	0.919
Serum blood urea nitrogen (mg/dl)	42.0017.00)	37.00 (14.75)	0.235

Table 1.

Clinical features of the study subjects

Results were analyzed using unpaired T-test with Welch's correction. Results are presented as median and interquartile ranges (IQR).

	T2D subjects (n=8)	Non-diabetic subjects (n=9)	P value
BV/TV (%)	0.248 (0.249)	0.358 (0.184)	0.120
Md.V/BV (%)	0.994(0.014)	0.995 (0.013)	0.998
Md.V/TV (%)	0.249 (0.210)	0.352 (0.184)	0.053
OV/BV (%)	0.009 (0.013)	0.004 (0.012)	0.704
OV/TV (%)	0.001 (0.005)	0.001 (0.005)	0.896
OS/BS (%)	0.026 (0.018)	0.035 (0.139)	0.525

Table 2.

Histomorphometric parameters of trabecular bone of the study subjects

Results were analyzed using unpaired T-test with Welch's correction and are presented as median and interquartile ranges (IQR).

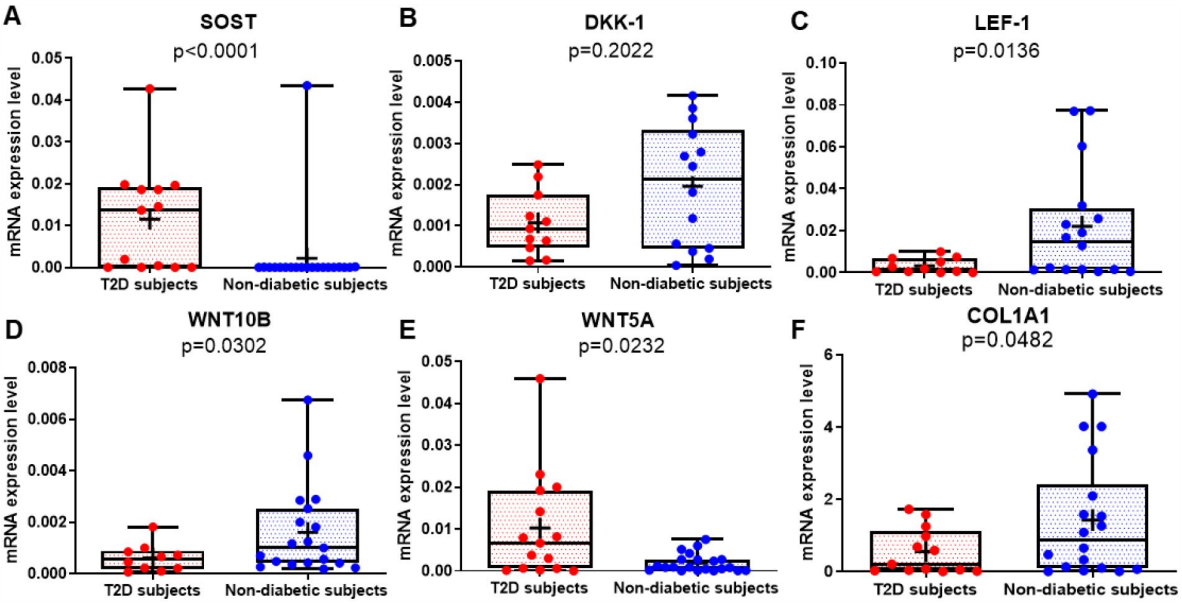


Figure 1.

Gene expression analysis in trabecular bone samples. (A) SOST mRNA levels resulted higher in T2D subjects versus Nondiabetic subjects (p<0.0001). (B) DKK-1 mRNA expression level was not different between groups (p=0.2022). (C) LEF-1 mRNA levels resulted lower in T2D subjects versus nondiabetics subjects (p=0.0136). (D) WNT10B mRNA expression level was lower in T2D subjects versus nondiabetic subjects (p=0.0302). (E) WNT5A mRNA resulted higher in T2D subjects versus nondiabetics subjects (p=0.0232). (F) COL1A1 mRNA levels resulted lower in T2D subjects versus Nondiabetic subjects (p=0.0482). Data are expressed as logarithmic scale. Medians and interquartile ranges, differences between non-diabetics and T2D subjects were analyzed using Mann-Whitney test.

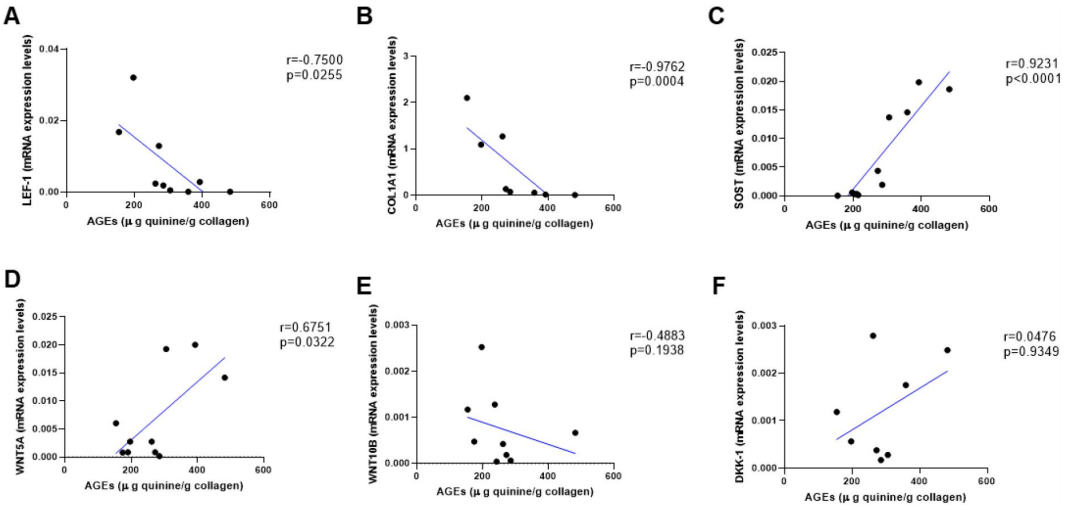


Figure 2.

Relationship between AGEs (äg quinine/g collagen) bone content and mRNA level of the Wnt signaling key genes in T2D and non-diabetic subjects. (A) LEF-1 negatively correlated with AGEs (r=-0.7500; p=0.0255). (B) COL1A1 negatively correlated with AGEs (r=-0.9762; p=0.0004). (C) SOST mRNA level expression positively correlated with AGEs (r=0.9231; p<0.0001). (D) WNT5A mRNA expression level positively correlated with AGEs (r=0.6751; p=0.0322). (E) WNT10B mRNA expression level was not correlated with AGEs (r=-0.4883; p=0.1938). (F) DKK1 mRNA expression level was not correlated with AGEs (r=0.0476; p=0.9349). Data were analyzed using nonparametric Spearman correlation analysis and r represents the correlation coefficient.

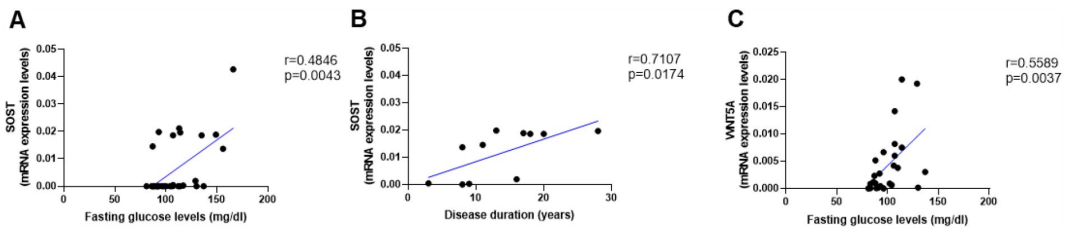


Figure 3.

Relationship between fasting glucose levels (mg/dl) and disease duration with SOST and WNT5A mRNA levels. (A) SOST positively correlated with fasting glucose levels (r=0.4846; p=0.0043). (B) SOST positively correlated with disease duration (r=0.7107; p=0.0174). (C) WNT5A positively correlated with fasting glucose levels (r=0.5589; p=0.0037). Data were analyzed using nonparametric Spearman correlation analysis and r represents the correlation coefficient.

inhibition of Wnt/beta-catenin signaling was increased, whereas *COL1A1* was decreased. The latter finding is consistent with reduced bone formation and suppression of Wnt signaling in T2D. We have previously reported upregulation of *SOST* and downregulation of *RUNX2* mRNA in another cohort of postmenopausal women with T2D [9]. Of note, the cohort of T2D subjects studied here had glycated hemoglobin within therapeutic targets, implying that the changes in gene transcription we identified persist in T2D bone despite good glycemic control.

High circulating sclerostin has been reported in diabetes [17,18], and increased sclerostin is associated with fragility fractures [19]. Aside from confirming higher SOST expression, we also show that other Wnt/β-catenin osteogenic ligands are abnormally regulated in the bone of T2D postmenopausal women. WNT10B is a positive regulator of bone mass; transgenic overexpression in mice results in increased bone mass and strength [20], whereas genetic ablation of *Wnt10b* is characterized by reduced bone mass [21,22], and decreased number and function of osteoblasts [21]. More to the point, *Wnt10b* expression is reduced in the bone of diabetic mice [23]. Therefore, the reduced *WNT10B* in human bone we found in the present study further supports the hypothesis of reduced bone formation in T2D. Accordingly, LEF-1 gene expression was also downregulated confirming that Wnt/beta-catenin pathway is decreased in T2D. Importantly, the overexpression of LEF-1 induces the expression of osteoblast differentiation genes (osteocalcin and COL1A1) in differentiating osteoblasts [24]. In fact, in this study we also demonstrated that a downregulation of LEF-1 in T2D bone goes along with a downregulation of COL1A1, strengthen data of a reduced production of bone matrix most likely as the result of reduced osteoblasts synthetic activity in diabetes [8,25]. Reduced *RUNX2* in T2D postmenopausal women also confirms previous findings (PMID: 32777114)[9] and further supports the notion of reduced osteoblast differentiation or function in diabetes. On the other hand, the contribution of upregulated WNT5A in diabetic bone is more complex. WNT5A regulates Wnt/beta-catenin signaling depending on the receptor availability [26]. Non-canonical WNT5A activates β-catenin-independent signaling, including the Wnt/Ca^{++} [27] and planar cell polarity pathways [28]. Heterozygous Wnt5a null mice have low bone mass with impaired osteoblast and osteoclast differentiation [29]. Wnt5a inhibits Wnt3a protein by downregulating beta/catenin-induced reporter gene expression [26]. In line with these findings, we showed that there was an increased gene expression of WNT5A in bone of T2D postmenopausal women confirming a downregulated Wnt/beta-catenin signaling and impaired osteoblasts function. We have previously shown that AGEs content is higher in T2D bone compared to non-diabetic bone, even in patients with well-controlled T2D [9]. Here we show that AGEs accumulation is positively correlated with SOST and WNT5A gene expression, and negatively correlated with LEF-1, WNT10B, and COL1A1 mRNA. These findings are consistent with the hypothesis that AGEs accumulation is associated with impaired Wnt signaling and low bone turnover in T2D. We did not find any abnormalities in histomorphometric parameters in our subjects with T2D, consistent with our previous report [9]. Reduced osteoid thickness and osteoblast number were reported in premenopausal T2D women with poor glycemic control compared to non-diabetic subjects but not in the group with good glycemic control [30]. Therefore, good glycemic control appears to prevent or rescue any changes in static histologic parameters of bone turnover that might be caused by uncontrolled diabetes.

Our study has some limitations. One is the cross-sectional design; another one is the relatively small number of T2D subjects enrolled. Moreover, we measured the mRNA abundance of the genes of interest, and we cannot assume that the differences we found reflect differences in protein abundance. Although osteoarthritis may affect some of the genes we studied [31], all study subjects were affected by variable degree of osteoarthritis, and the effect of such potential confounder is not likely to be different between T2D and control subjects. Finally, we did not use the tetracycline double-labeled technique to investigate dynamic bone parameters.

The main strength of our study is that this study is the first to explore the association of AGEs on Wnt pathway in postmenopausal T2D women. Moreover, we measured the expression of several Wnt genes directly on bone samples of postmenopausal T2D women.

In conclusion, our data show that, despite good glycemic control, T2D decreases expression of COL1A1 and Wnt genes that regulate bone turnover, in association with increased AGEs content. These results may help understand the mechanisms underlying bone fragility in T2D.

Data Availability

All data are available upon request to the corresponding authors

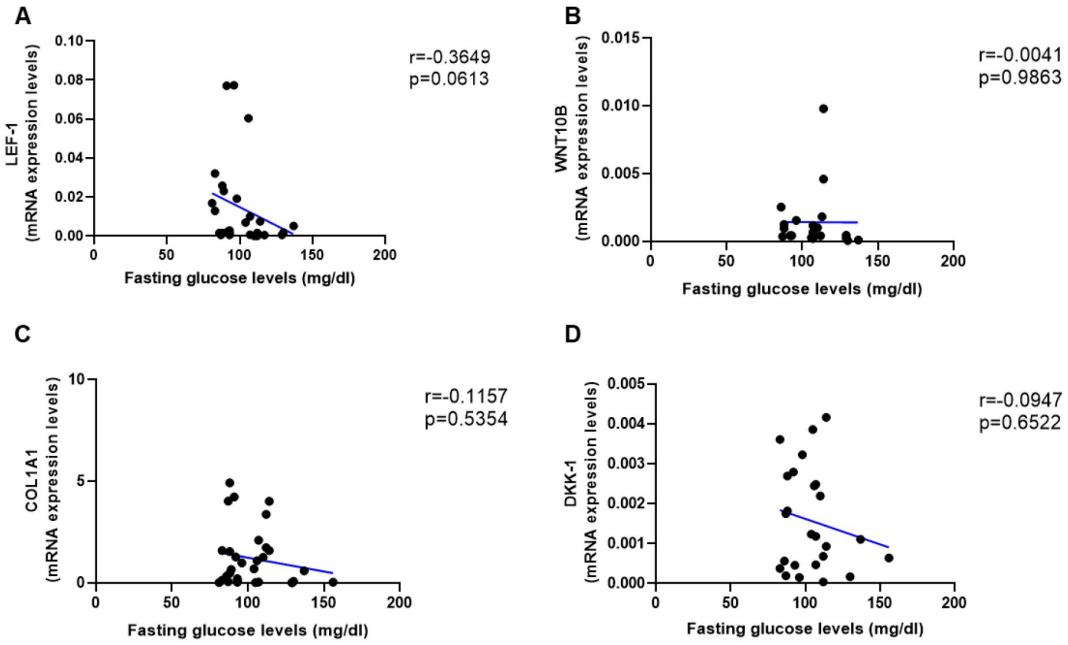
Acknowledgements

This work was supported by an internal grant of Campus Bio-Medico University of Rome

Author Contributions

Conceptualization, N.N, R.P., M.M and R.S. ; methodology, G.L., F.C., M.F., V.V., F.T., A.B., S.T..; software, G.L., C.P., A.B., and E.S.; validation, N.N. and G.L..; formal analysis, N.N. and G.L.; investigation, N.N., R.S., M.M. and R.P.; resources, N.N, G.V., R.P. M.M., and R.S.; data curation, G.L. and N.P..; writing—original draft preparation, G.L., M.F., N.P., R.C. and N.N.; writing—review and editing, N.N., C.P., E.S., R.S. and R.C.; visualization, N.N. and R.S..; supervision, N.N., R.P., and M.M.; project administration, N.N. and G.L.; funding acquisition, N.N. and R.S. All authors have read and agreed to the published version of the manuscript.

Legend of supplementary figures



Supplementary figure 1.

Relationship between fasting glucose levels (mg/dl) and LEF-1, WNT5A, WNT10B, DKK-1, COL1A1 mRNA levels. (A-E) Data showed no correlation between fasting glucose levels (mg/dl) and (A) LEF-1 (r=-0.3649; p=0.0613), (B) WNT10B (r=-0.0041; p=0.9863), (C) COL1A1 (r=-0.1157; p=0.5354), (D) DKK-1 (r=-0.0947; p=0.6522) mRNA levels. Data were analyzed using nonparametric Spearman correlation analysis and r represents the correlation coefficient.

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Reviewer #1 (Public Review):

Summary:
Leanza et al. investigated the regulation of Wnt signaling factors in the bone tissue obtained from individuals with or without type 2 diabetes. They showed that typical canonical Wnt ligands and downstream factors (Wnt10b, LEF1) are down-regulated, while Wnt5a and sclerostin mRNA are unregulated in diabetic bone tissue. Further, Wnt5a and sclerostin associated with the content of AGEs and SOST mRNA levels also correlated with glycemic control and disease duration.

Strengths:
- A strength of the study is the investigation of Wnt signaling in bone tissue from humans with type 2 diabetes. Most studies measure only serum levels of Wnt inhibitors, but this study takes it further and looks into bone specifically.
- The measurement of AGEs and its correlation to the Wnt signaling molecules is interesting and important. The correlation of sclerostin and Wnt5a with AGEs and disease duration suggests that inhibited Wnt signaling is paralleled by higher AGE levels and potentially weaker bone.
- The methodology in terms of obtaining the bone samples and the rigorous evaluation of RNA integrity is great and provides a solid basis for further analyses.

Weaknesses:
- A weakness may include the rather limited number of samples. Especially for some sub-analyses (e.g. RNA analyses), only a subset of samples was used.
- How was the sample size determined? It seems like more samples might have been necessary to obtain significant results for methods with a higher standard deviation (e.g.

histomorphometry).
- Why is the number of samples different for the mRNA measurements? In most cases, there were 9, but in some 8 and in some 10?

Overall, this study validates findings from the group that reported similar findings in 2020. This validates their methodology and shows that alterations in Wnt signaling are reproducible in human bone tissue.

Reviewer #2 (Public Review):

Summary:
This study reports the levels of expression of selected genes implicated in Wnt signaling in trabecular bone from femur heads obtained after surgery from post-menopausal women with (15 women) or without (21 women) type 2 diabetes. They found higher expression levels of SOST and WNT5A, and lower expression levels of LEF-1 and WNT10B in tissues from subjects with T2D, correlating with glycemia and advanced glycation products. No significant differences in bone density were observed. Overall, this is a cross-sectional, observational study measuring a limited set of genes found to vary with glycemia in postmenopausal women undergoing hip surgery.

Strengths:
The study demonstrates the feasibility of measuring gene expression in post-surgical trabecular bone samples, and finds differences associated with glycemia despite a relatively small number of subjects. It can form the basis for further research on the causes and consequences of changes in elements of the WNT signaling pathway in bone biology and disease.

Weaknesses:
The small number of targeted genes does not provide a comprehensive view of the transcriptional landscape within which the effects are observed. The gene expression changes are not associated with cellular or physiological properties of the tissue, raising questions about the biological significance of the observations.

Reviewer #3 (Public Review):

Summary:
The manuscript by Leanza and colleagues explores the regulation of Wnt signaling and its association with advanced glycation end products (AGEs) accumulation in postmenopausal women with type 2 diabetes (T2D). The paper provides valuable insights into the potential mechanisms underlying bone fragility in individuals with T2D. Overall, the manuscript is well-structured, and the methodology is sound. I would suggest some minor revisions to improve clarity.

Strengths:
The study addresses an important and clinically relevant question concerning the mechanisms underlying bone fragility in postmenopausal women with T2D.

The study's methodology appears sound, and the inclusion of postmenopausal women with and without T2D undergoing hip arthroplasty adds to the clinical relevance of the findings. Additionally, measuring gene expression and AGEs in bone samples provides direct insights into the study's objectives.

The manuscript presents data clearly, and the results are well-organized.

Weaknesses:
Title. The title could be more specific to better reflect the content of the study. Also, the abstract should concisely summarize the study's main findings, providing some figures.

Introduction: the introduction would benefit from the addition of a clearer, more focused statement of the research questions or hypotheses guiding this study.

Methods: more information is needed on the hystomorphometry analysis. Surgical samples from 8 T2D and 9 non-diabetic subjects were used for histomorphometry analysis. How did these subjects compare with the other subjects in the T2D and control groups? Were they representative? How were they selected?