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Diabetes and bone - from cell to human

Edited by
Peter Vestergaard, Antonino Catalano and Jakob Starup-Linde

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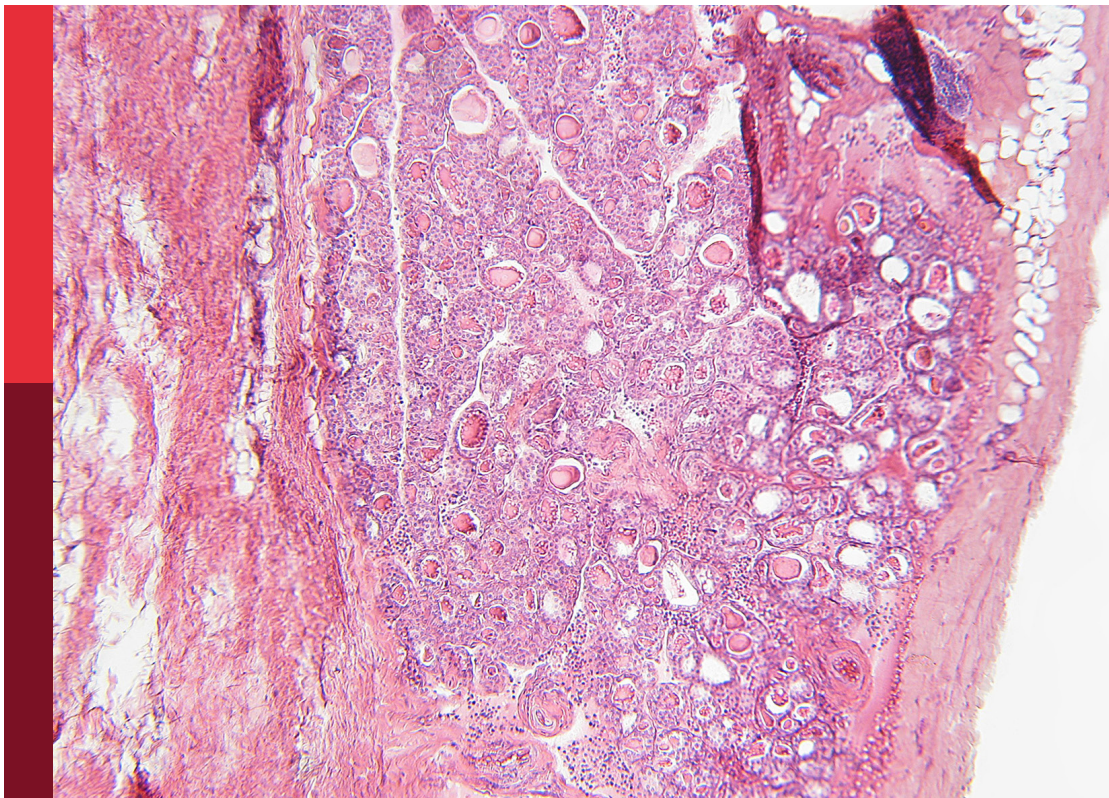

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Diabetes and bone - from cell to human

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Editorial: Diabetes and bone - from cell to human

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bone, diabetes, fractures, alendronate, antidiabetics medication

Editorial on the Research Topic
Diabetes and bone - from cell to human

The interaction between increased glucose levels and fluctuations in these as seen in diabetes is complex. Changes at the cellular level – even within minutes – may have profound effects on the clinical level leading to an increased risk of fractures. The treatments used to counteract hyperglycemia can modify the effects at the cellular, individual and population levels, but they cannot completely undo the negative effects of the hyperglycemic state. Differences in insulin levels and insulin resistance may influence the effects of hyperglycemia between type 1 and type 2 diabetes but may also play a role within type 2 diabetes, where different phenotypes may exist.

This topic spans the levels from observational studies on the cut-off levels for an effect of HbA1c on bone turnover (Joad et al.) and prevalence of morphometric vertebral fractures in diabetes and pre-diabetes (Hulten et al.) over interventional studies on the effect of diet on bone turnover (Fuglsang-Nielsen et al.) to pharmacoepidemiological studies on fracture risk related to various drugs used in diabetes (Al-Mashhadi et al., Al-Mashhadi et al., Zhang et al., Viggers et al.), even antiosteoporotic therapy may modify the risk of developing diabetes, showing the potential bone pancreas interplay (Viggers et al.), thus demonstrating the necessity to analyse the problem of the bone fragility in diabetes using many different techniques from different fields of science. This also shows that although epidemiological techniques can answer research questions that cannot be answered by preclinical and *in vitro* studies, such as the interaction between diabetes, its treatment, and fracture risk, preclinical studies can elucidate the mechanisms underlying the clinical problem of fractures, which cannot be understood in detail using epidemiology.

On the other hand, interventional studies point out that although understanding and describing a problem is essential, it is also necessary to know whether it is possible to modulate pathophysiological processes and thus potentially prevent fractures in diabetes, modulate possible hypoglycemia, which can lead to falls and fractures, and potentially reverse or prevent complications such as impaired eyesight, which can lead to an increased fracture risk.

We hope that you, too, will feel as inspired and enlightened as we did in editing this Research Topic.

Author contributions

All authors have drafted and revised the editorial. All authors have approved the final version of the submitted editorial.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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Effects of Anti-Diabetic Drugs on Fracture Risk: A Systematic Review and Network Meta-Analysis

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Purpose: Available data on the effects of anti-diabetic drugs on fracture risk are contradictory. Therefore, our study aimed to analyze all available data on the effects of anti-diabetic drugs on fracture risk in type 2 diabetes mellitus (T2DM) patients.

Methods: Embase, Medline, ClinicalTrials.gov, and Cochrane CENTRAL were searched for relevant trials. All data analyses were performed with STATA (12.0) and R language (3.6.0). Risk ratio (RR) with its 95% confidence interval (CI) was calculated by combining data for the fracture effects of anti-diabetic drugs, including sodium–glucose co-transporter 2 (SGLT2) inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) receptor agonists, meglitinides, α-glucosidase inhibitors, thiazolidinediones, biguanides, insulin, and sulfonylureas.

Results: One hundred seventeen eligible randomized controlled trials (RCTs) with 221,364 participants were included in this study. Compared with placebo, trelagliptin (RR 3.51; 1.58–13.70) increased the risk of fracture, whereas albiglutide (RR 0.29; 0.04–0.93) and voglibose (RR 0.03; 0–0.11) decreased the risk of fracture. Other medications were comparable in terms of their effects on fracture risk, and no statistical significance was observed. In terms of fractures, voglibose (0.01%) may be the safest option, and trelagliptin (13.64%) may be the worst. Sensitivity analysis results were consistent with those of the main analysis. No statistically significant differences were observed in the regression coefficients of age (1.03; 0.32–2.1), follow-up duration (0.79; 0.27–1.64), and sex distribution (0.63; 0.15–1.56).

Conclusions: We found varied results on the association between the use of anti-diabetic drugs and fracture risk. Specifically, trelagliptin raised the risk of fracture, whereas voglibose and albiglutide showed benefit with statistical difference. Other drugs were comparable in terms of their effects on fracture risk. Some drugs (omarigliptin, sitagliptin, vildagliptin, saxagliptin, empagliflozin, ertugliflozin, rosiglitazone, pioglitazone, and nateglinide) may increase the risk of fracture, while others (such as dulaglutide, exenatide, liraglutide, semaglutide, lixisenatide, linagliptin, alogliptin, canagliflozin, dapagliflozin, glipizide, gliclazide, glibenclamide, glimepiride, metformin, and insulin) may show benefits. The risk of fracture was independent of age, sex distribution, and the duration of exposure to anti-diabetic drugs. When developing individualized treatment

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strategies, the clinical efficacy of anti-diabetic drugs must be weighed against their benefits and risks brought about by individual differences of patients.

Systematic Review Registration: This Systematic Review was prospectively registered on the PROSPERO (https://www.crd.york.ac.uk/PROSPERO/, registration number CRD42020189464).

Keywords: anti-diabetic drug, fracture, type 2 diabetes mellitus, systematic review, meta-analysis

INTRODUCTION

Diabetes is a major global health problem. It affects nearly half a billion patients worldwide. Among diabetic patients, 90% suffer from type 2 diabetes mellitus (T2DM) (1, 2). Mounting evidence indicates that T2DM patients are at a higher risk of developing fragility fractures because their bone microenvironment is deteriorated by the disease (3, 4). T2DM patients with an increased bone mineral density (BMD) may suffer more from bone fractures. Many studies have suggested that a deteriorated bone quality, rather than a decreased BMD, may be the key factor influencing bone fragility in T2DM patients. From the perspective of clinical diagnosis, T2DM-related complications (for instance, neuropathy, macroangiopathy, and retinopathy) can be regarded as predictors of bone fractures, and drug therapies may have negative effects on bone quality (5). However, it is still not entirely clear why diabetes complications can lead to fragility fractures (6). Some studies have indicated that several mechanisms may be used to explain why patients with T2DM are more susceptible to fragility fractures, including oxidative stress, hyperglycemia, levels of insulin, risk of falls, functions of osteocalcin and adiponectin, variations in BMD, and treatment-induced hypoglycemia, all of which increase fracture risk in patients with T2DM (3, 6, 7). The fragility fractures caused by diabetes are fatally serious. They may require surgeries and may further develop into disabilities, paralysis, or deaths (8, 9). Therefore, the developed anti-diabetic treatment strategies should at least not increase the risk of bone fractures in the vulnerable population (10, 11).

Currently, multiple anti-diabetic drugs are available, but previous research did not integrate all related data into one analysis and compare the available anti-diabetic drugs head-to-head. Therefore, associations between fracture events and anti-diabetic drug effects have not been clearly elucidated (12–14). To address this problem, we herein utilized Bayesian meta-analysis, a validated and mature statistical method, to compare the effects of all available anti-diabetic drugs on fracture risk (15). This comprehensive review and meta-analysis aimed to evaluate the safety of anti-diabetic drugs in fracture events based on the data available from clinical trials. Our study may help clinical researchers investigate the risk of fracture related to the use of anti-diabetic drugs in future research.

METHODS

Search Strategy

This study was prospectively registered on the PROSPERO (https://www.crd.york.ac.uk/PROSPERO/, registration number CRD42020189464). A search for "Anti-diabetic drug", "Type 2 diabetes mellitus", "thiazolidinediones", "α-glucosidase", "bromocriptine-QR", "meglitinides", "GLP-1 receptor agonists", "biguanides", "sulfonylureas", "SGLT2 inhibitors", "insulin", and "DPP-4 inhibitors" was performed in Embase, Medline, ClinicalTrials.gov, and Cochrane CENTRAL to identify randomized controlled trials (RCTs) up to May 1, 2021, with English-language restriction.

Selection Criteria

Clinical trials were eligible if they met the following criteria: 1) RCTs; 2) duration ≥12 months; 3) the intervention or comparators were with anti-diabetic drugs, including sulfonylureas, dipeptidyl peptidase-4 (DPP-4) inhibitors, bromocriptine-QR, meglitinides, sodium–glucose co-transporter 2 (SGLT2) inhibitors, thiazolidinediones, biguanides, glucagon-like peptide-1 (GLP-1) receptor agonists, insulin, α-glucosidase, and placebo; 4) data on fracture were available.

Data Extraction and Quality Assessment

For the eligible studies, data were extracted by two reviewers (Y-SZ and YY) independently; the disagreements were resolved by two reviewers and, if necessary, consulted by a senior reviewer (B-CX). Cochrane risk-of-bias tool was used to estimate the risk of bias for eligible studies (16). The data on trials available, consisting of the first author, sample size, mean age, follow-up, intervention and comparators, HbA1c, and outcomes of interest, were extracted. Grading of Recommendations, Assessment, Development and Evaluation (GRADE) was performed to assess the quality of evidence for fracture outcomes included. The GRADE approach categorizes evidence into high, moderate, low, or very low quality.

Data Analysis

The Bayesian meta-analysis model was established by performing the Markov chain Monte Carlo methods (17). Random-effects model was used to account for heterogeneity between clinical trials for Bayesian analysis model, risk ratios (RRs) with its 95% confidence interval (CI) of anti-diabetic drugs on bone fracture were evaluated, RR value <1 favors "lower risk", RR value >1 favors "higher risk," and it permits all comparisons (direct/indirect comparisons) to be taken into calculating synchronously (18, 19). The posterior distributions of the parameters model were generated by four chains (100,000 per chain, 400,000 iterations) in the random-effects model (20). We checked heterogeneity by performing the I^2 statistic and verified the model fit by calculating residual deviance. In addition, we

calculated inconsistency of the direct and indirect comparisons by operating node-splitting method, and p-value <0.05 was defined as inconsistency. We calculated rank treatment of each anti-diabetic drug to estimate the safest probability. In addition, we calculated meta-regression analysis to discover the association with the fracture risk and age, fracture risk and sex distribution, and fracture risk and length of duration (21); and we performed sensitivity analysis to detect the influence of data (22). A comparison-adjusted funnel plot was drawn by using STATA software to analyze publication bias (23). All data analyses were performed with R language (3.6.0) (24).

RESULT

Study Characteristics and Quality

A total of 47,869 records were retrieved; after review of 812 records for eligibility, 117 RCTs were included. The interventions evaluated in the meta-analyses included nine types of anti-diabetic drugs: SGLT2 inhibitors, DPP-4 inhibitors, α-glucosidase inhibitors, thiazolidinediones, insulin, GLP-1 receptor agonists, meglitinides, biguanides, and sulfonylureas. The flowchart for selection of clinical trials is shown in **Figure 1**. All anti-diabetic drugs were connected to draw a network plot (**Figure 2**). Characteristics of the clinical trials with their quality analyses are shown in **Supplementary Tables 1**, **2**.

Statistical Analysis

The model fit calculated by residual deviance was agreeable (ratio 1.148, I^2 = 15%). The results of RRs are summarized in **Table 1**. The GRADE of quality evidence for anti-diabetic drugs on fracture outcomes is summarized in **Table 2**; all anti-diabetic drugs were graded as high/moderate quality in the present study.

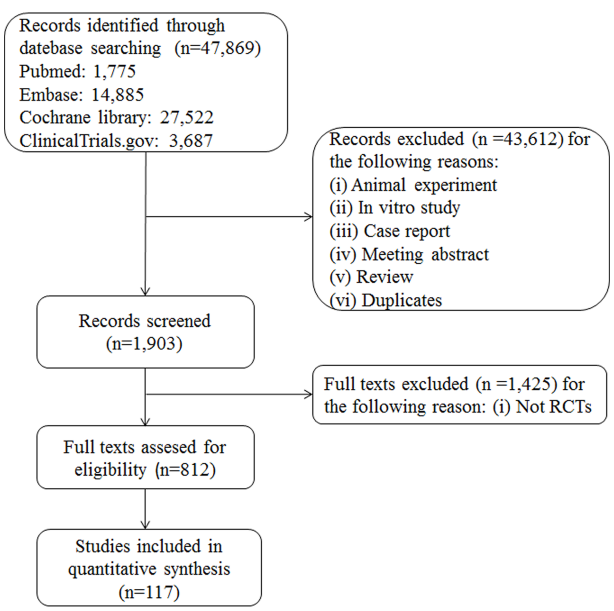

FIGURE 1 | Trial flow diagram.

Dipeptidyl Peptidase-4 Inhibitors

In the overall analysis, compared with placebo, we found varied results on the association between the use of DPP-4 inhibitors and fracture risk. Specifically, omarigliptin (RR 1.33; 0.21–8.24), sitagliptin (RR 1.29; 0.27–6.47), vildagliptin (RR 1.17; 0.23–6.16), and saxagliptin (RR 2.04; 0.38–12.09) raised the risk of fracture; whereas linagliptin (RR 0.9; 0.18–4.66) and alogliptin (RR 0.76; 0.12–4.87) reduced the risk. Additionally, trelagliptin (RR 3.51; 1.58–13.70) raised the risk of fracture with a statistical significance.

Glucagon-Like Peptide-1 Receptor Agonists

We found that GLP-1 receptor agonists showed benefits as compared with placebo. The effects of dulaglutide (RR 0.91; 0.17–4.88), exenatide (RR 0.95; 0.15–5.96), liraglutide (RR 0.73; 0.14–3.92), semaglutide (RR 0.66; 95% 0.13–3.41), and lixisenatide (RR 0.92; 0.2–6.3) were comparable and showed no statistically significant differences. Additionally, albiglutide (RR 0.29; 0.04–0.93) showed benefits with a statistical significance.

Sodium–Glucose Co-Transporter 2 Inhibitors

In the overall analysis, compared with placebo, canagliflozin (RR 0.62; 0.13–3.08) and dapagliflozin (RR 0.9; 0.16–5.14) decreased the risk of fracture; whereas empagliflozin (RR 1.19; 0.24–5.89) and ertugliflozin (RR 2.47; 95% 0.16–9.95) increased the risk of fracture, although the difference was not significant.

Sulfonylureas

In the overall analysis, the results showed that glipizide (RR 0.67; 0.12–3.74), gliclazide (RR 0.75; 0.05–9.46), glibenclamide (RR 0.98; 0.22–4.25), and glimepiride (RR 0.45; 0.09–2.17) showed benefits as compared with placebo. Unfortunately, the differences were not statistically significant.

Thiazolidinediones

In the overall analysis, the results suggest that rosiglitazone (RR 1.2; 0.21–6.83) and pioglitazone (RR 1.14; 0.31–4.25) increased the risk of facture as compared with placebo.

Others

In the overall analysis, compared with placebo, the results suggested that metformin (RR 0.81; 0.14–4.56), voglibose (RR 0.03; 0–0.11), and insulin (RR 0.68; 0.12–3.86) showed benefit, whereas nateglinide (RR 1.35; 0.24–7.55) raised the risk of fracture.

Ranking Probability

Based on surfaces under the cumulative probability cumulative ranking curves (SUCRAs), the probability ranking of anti-diabetic drugs is shown in **Supplementary Table 3**. In terms of the risk of inducing fracture, the safest treatment was voglibose (0.01%), and the worst treatment was trelagliptin (13.64%). According to GRADE, the quality of evidence for fracture outcomes was rated as high for most comparisons (**Table 2**). Quality of evidence was high for the overall ranking of anti-diabetic drug treatments.

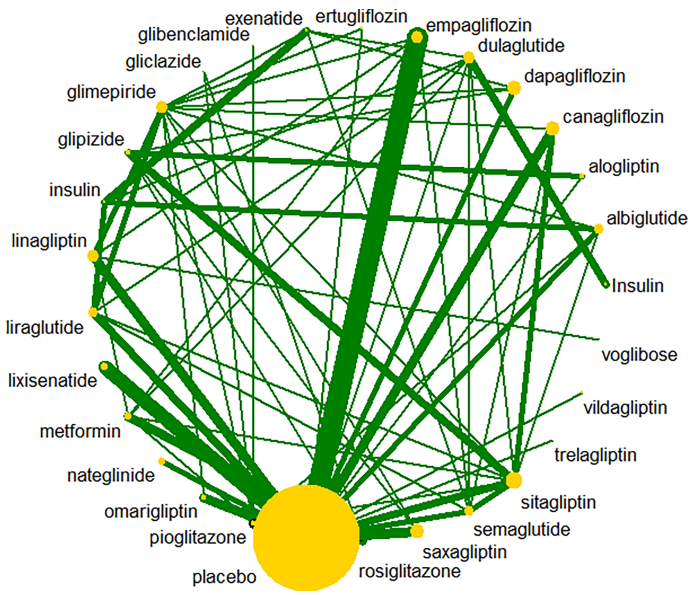

FIGURE 2 | Network plot for the Bayesian network meta-analyses.

Heterogeneity and Inconsistence Check

Inconsistency was detected in some direct/indirect comparisons (**Supplementary Table 4**), in sitagliptin versus liraglutide, sitagliptin versus glimepiride, empagliflozin versus linagliptin, omarigliptin versus glimepiride, ertugliflozin versus glimepiride, dulaglutide versus metformin, omarigliptin versus glibenclamide, and ertugliflozin versus glibenclamide. The global heterogeneity was 44% calculated by R software (**Supplementary Table 5**); no statistically significant heterogeneity was detected in the direct/indirect comparisons.

Funnel Plot and Publication Bias

As it is shown in **Figure 3**, it did not suggest any publication bias in the comparison-adjusted funnel plots.

Sensitivity Analysis and Meta-Regression

Almost all the results of sensitivity analysis were consistent with those of the main analysis (**Supplementary Table 6**). No significant differences were observed in the regression coefficients (RCs). The risk of fracture was independent of age (RC 1.03; 0.32–2.1), duration of treatment (RC 0.79; 0.27–1.64), and sex distribution (RC 0.63; 0.15–1.56), but fracture risk had no clear associations with plasma glucose, level, and drug doses in patients treated with anti-diabetic drugs.

DISCUSSION

Principal Findings

Previous studies have not fully demonstrated the effects of all anti-diabetic drugs on the risk of fracture in T2DM patients due to many limitations. One of the limitations is that data from these studies could not be integrated into a single analysis. As a result, the power of evidence of these studies seems weak due to the limited data, and no convincing results can be obtained. We found varied results on the association between fracture risk and the use of anti-diabetic drugs by assessing direct comparisons, indirect comparisons, inconsistency, and heterogeneity. Unfortunately, the results of trelagliptin and voglibose were obtained based on one RCT with one fracture event. However, in this study, a comprehensive analysis was performed to detect the association between fracture risk and the use of anti-diabetic drugs by integrating data from 221,364 participants treated with nine types of anti-diabetic drugs. Compared with previous meta-analyses, the Bayesian model adopted in this study could obtain more accurate statistical results because it describes indirect comparisons between trials (25). Therefore, trelagliptin and voglibose should not be excluded. Trelagliptin raised the risk of fracture, whereas voglibose and albiglutide showed benefits with statistically significant differences. In terms of the risk of inducing fracture, voglibose (0.01%) may be the safest option, and trelagliptin (13.64%) may be the worst. RCTs with varied durations, age distributions, and sex distributions were included, but fracture risk was independent of age and sex distributions and the duration of exposure to anti-diabetic drugs.

Glucagon-Like Peptide-1 Receptor Agonists and Fracture

In terms of fracture risk, GLP-1 receptor agonists showed benefits. A few researchers raised the doubts that GLP-1 receptor agonists may have effects on fracture risk. GLP-1 increased bone density by inhibiting bone resorption and promoting bone formation (26). But the research (included trials >12 weeks) did not support an association between the

TABLE 1 | Estimated relative treatment effects as risk ratios (RRs) and its corresponding 95% confidence intervals (CIs).

Treatment	albiglutide	dulaglutide	exenatide	lixisenatide	liraglutide	semaglutide	vildagliptin	omarigliptin	trelagliptin	saxagliptin	alogliptin	sitagliptin	linagliptin	dapagliflozin	ertugliflozin	canagliflozin	empagliflozin	glipizide	glimepiride	glibenclamide	gliclazide	pioglitazone	rosiglitazone	nateglinide	metformin	insulin	voglibose	placebo
albiglutide	NA	0.43 (0.07, 2.49)	0.41 (0.06, 2.83)	0.36 (0.05, 2.11)	0.53 (0.08, 3.14)	0.59 (0.1, 3.41)	0.34 (0.05, 2.04)	0.29 (0.04, 2.05)	0.01 (0, 0.04)	0.19 (0.03, 1.15)	0.52 (0.07, 3.52)	0.3 (0.05, 1.65)	0.43 (0.07, 2.42)	0.43 (0.07, 2.67)	0.16 (0, 2.6)	0.64 (0.11, 3.45)	0.33 (0.06, 1.79)	0.58 (0.09, 3.73)	0.86 (0.15, 4.75)	0.4 (0.08, 1.92)	0.52 (0.03, 9.09)	0.34 (0.06, 1.92)	0.32 (0.05, 2.15)	0.29 (0.04, 1.84)	0.48 (0.07, 3.21)	0.58 (0.09, 3.76)	1.12 (1.01, 2.56)	0.29 (0.04, 0.93)
dulaglutide	2.33 (0.4, 14.65)	NA	0.96 (0.29, 3.19)	0.83 (0.25, 2.62)	1.24 (0.46, 3.3)	1.38 (0.51, 3.85)	0.78 (0.26, 2.29)	0.69 (0.18, 2.6)	0.01 (0, 0.05)	0.45 (0.13, 1.39)	1.2 (0.32, 4.52)	0.7 (0.28, 1.78)	1.01 (0.36, 2.79)	1.01 (0.32, 3.19)	0.38 (0.01, 1.05)	1.48 (0.55, 3.94)	0.77 (0.28, 2.06)	1.35 (0.42, 4.48)	2 (0.77, 5.36)	0.93 (0.42, 2.06)	1.22 (0.12, 15.06)	0.79 (0.29, 2.22)	0.76 (0.22, 2.64)	0.68 (0.2, 2.25)	1.12 (0.34, 3.86)	1.34 (0.55, 3.39)	1.91 (1.09, 7.52)	0.91 (0.17, 4.88)
exenatide	2.44 (0.35, 17.87)	1.04 (0.31, 3.46)	NA	0.86 (0.21, 3.41)	1.29 (0.36, 4.69)	1.43 (0.41, 5.11)	0.81 (0.21, 3.06)	0.72 (0.15, 3.34)	0.01 (0, 0.05)	0.46 (0.11, 1.86)	1.25 (0.27, 5.82)	0.73 (0.21, 2.56)	1.05 (0.3, 3.64)	1.05 (0.28, 3.94)	0.39 (0.01, 1.78)	1.54 (0.43, 5.34)	0.8 (0.23, 2.79)	1.41 (0.35, 5.86)	2.09 (0.68, 6.55)	0.97 (0.32, 2.97)	1.26 (0.11, 18.2)	0.82 (0.23, 3.02)	0.79 (0.18, 3.52)	0.7 (0.16, 2.99)	1.16 (0.27, 5.18)	1.39 (0.43, 4.67)	2.05 (1.16, 7.44)	0.95 (0.15, 5.96)
lixisenatide	2.81 (0.47, 18.77)	1.21 (0.38, 4)	1.16 (0.29, 4.84)	NA	1.5 (0.48, 4.93)	1.67 (0.55, 5.31)	0.95 (0.29, 3.25)	0.83 (0.21, 3.39)	0.02 (0, 0.06)	0.54 (0.15, 1.83)	1.45 (0.37, 6.02)	0.85 (0.29, 2.53)	1.22 (0.41, 3.71)	1.22 (0.36, 4.39)	0.45 (0.01, 1.24)	1.79 (0.63, 5.22)	0.92 (0.33, 2.72)	1.63 (0.48, 6.04)	2.41 (0.86, 7.45)	1.12 (0.49, 2.74)	1.48 (0.14, 19.54)	0.96 (0.33, 2.97)	0.92 (0.25, 3.58)	0.81 (0.25, 2.92)	1.35 (0.37, 5.33)	1.63 (0.46, 6.21)	2.33 (1.99, 6.09)	0.9 (0.2, 6.3)
liraglutide	1.87 (0.32, 11.82)	0.8 (0.3, 2.17)	0.78 (0.21, 2.8)	0.67 (0.2, 2.1)	NA	1.11 (0.41, 3.06)	0.63 (0.2, 1.93)	0.55 (0.14, 2.13)	0.01 (0, 0.04)	0.36 (0.11, 1.14)	0.97 (0.26, 3.72)	0.57 (0.22, 1.48)	0.81 (0.29, 2.25)	0.81 (0.25, 2.66)	0.3 (0.01, 1.03)	1.19 (0.45, 3.18)	0.61 (0.23, 1.66)	1.09 (0.33, 3.69)	1.6 (0.64, 4.22)	0.74 (0.34, 1.67)	0.97 (0.1, 12.74)	0.64 (0.23, 1.83)	0.61 (0.17, 2.19)	0.54 (0.17, 1.83)	0.9 (0.28, 3.22)	1.08 (0.36, 3.33)	1.43 (1.07, 3.24)	0.73 (0.14, 3.92)
semaglutide	1.89 (0.29, 10.48)	0.73 (0.26, 1.98)	0.7 (0.2, 2.45)	0.6 (0.19, 1.81)	0.9 (0.33, 2.44)	NA	0.57 (0.18, 1.72)	0.5 (0.13, 1.84)	0.01 (0, 0.03)	0.32 (0.1, 0.99)	0.88 (0.23, 3.13)	0.51 (0.2, 1.24)	0.73 (0.26, 1.94)	0.73 (0.23, 2.32)	0.27 (0.01, 0.96)	1.07 (0.42, 2.66)	0.56 (0.22, 1.41)	0.99 (0.31, 3.17)	1.45 (0.57, 3.78)	0.67 (0.32, 1.42)	0.88 (0.09, 11.16)	0.58 (0.21, 1.56)	0.55 (0.16, 1.93)	0.49 (0.15, 1.59)	0.81 (0.24, 2.81)	0.98 (0.31, 3.17)	1.35 (1.06, 3.85)	0.66 (0.13, 3.41)
vildagliptin	2.97 (0.49, 19.28)	1.28 (0.44, 3.8)	1.23 (0.33, 4.67)	1.05 (0.31, 3.51)	1.58 (0.52, 4.91)	1.76 (0.58, 5.47)	NA	0.88 (0.22, 3.52)	0.02 (0, 0.06)	0.57 (0.16, 1.92)	1.53 (0.38, 6.17)	0.9 (0.33, 2.5)	1.29 (0.44, 3.77)	1.3 (0.38, 4.37)	0.48 (0.01, 1.35)	1.88 (0.67, 5.41)	0.98 (0.35, 2.81)	1.74 (0.5, 6.15)	2.56 (0.96, 7.23)	1.18 (0.51, 2.87)	1.55 (0.15, 20.08)	1.01 (0.38, 2.85)	0.97 (0.27, 3.67)	0.86 (0.25, 3.06)	1.43 (0.4, 5.39)	1.72 (0.57, 5.43)	2.12 (1.13, 6.07)	1.17 (0.23, 6.16)
omarigliptin	3.4 (0.49, 24.58)	1.45 (0.38, 5.48)	1.39 (0.3, 6.51)	1.2 (0.29, 4.7)	1.81 (0.47, 6.92)	2 (0.54, 7.43)	1.14 (0.28, 4.46)	NA	0.02 (0, 0.07)	0.65 (0.15, 2.57)	1.74 (0.38, 8.18)	1.03 (0.29, 3.64)	1.47 (0.41, 5.28)	1.47 (0.36, 5.92)	0.54 (0.02, 1.77)	2.15 (0.62, 7.53)	1.11 (0.32, 3.89)	1.97 (0.48, 8.4)	2.9 (0.86, 10.13)	1.34 (0.46, 4.07)	1.76 (0.15, 25.3)	1.16 (0.32, 4.13)	1.11 (0.24, 4.85)	0.98 (0.24, 4.1)	1.63 (0.37, 7.2)	1.95 (0.46, 8.4)	2.80 (1.28, 8.02)	1.33 (0.21, 8.24)
trelagliptin	4.9 (2.32, 19.08)	5.60 (2.24, 14.25)	5.28 (1.41, 13.61)	4.62 (1.43, 13.19)	3.71 (1.66, 14.84)	5.76 (2.08, 19.37)	4.39 (1.09, 13.25)	3.76 (1.28, 11.43)	NA	2.588 (1.89, 6.84)	4.60 (2.41, 16.63)	3.95 (1.8, 12.84)	5.72 (2.61, 22.46)	5.82 (2.81, 14.09)	3.71 (1.82, 10.4)	4.83 (2.8, 18.06)	4.46 (1.57, 12.97)	5.77 (2.93, 25.60)	8.22 (4.85, 29.1)	3.62 (1.63, 14.85)	6.67 (3.43, 29.04)	4.44 (1.74, 14.92)	4.34 (1.48, 13.05)	3.88 (1.48, 12.97)	4.63 (2.53, 14.47)	6.72 (2.5, 25.69)	7.43 (2.53, 22.23)	3.51 (1.58, 13.70)
saxagliptin	5.28 (0.87, 35.44)	2.25 (0.72, 7.64)	2.18 (0.54, 9.21)	1.87 (0.55, 6.55)	2.78 (0.87, 9.49)	3.09 (1.01, 10.46)	1.75 (0.52, 6.38)	1.54 (0.39, 6.58)	0.03 (0, 0.11)	NA	2.71 (0.72, 10.84)	1.58 (0.56, 4.8)	2.26 (0.78, 7.15)	2.27 (0.69, 8.19)	0.85 (0.03, 2.82)	3.32 (1.17, 10.23)	1.72 (0.6, 5.3)	3.05 (0.98, 10.31)	4.5 (1.65, 13.89)	2.08 (0.88, 5.48)	2.75 (0.27, 37.1)	1.78 (0.61, 5.76)	1.7 (0.47, 6.86)	1.52 (0.45, 5.75)	2.5 (0.7, 10.16)	3.03 (0.85, 12.4)	2.21 (1.29, 6.86)	2.04 (0.38, 12.09)
alogliptin	1.94 (0.28, 14.28)	0.84 (0.22, 3.13)	0.8 (0.17, 3.75)	0.69 (0.17, 2.72)	1.03 (0.27, 3.91)	1.14 (0.32, 4.34)	0.65 (0.16, 2.6)	0.57 (0.12, 2.66)	0.01 (0, 0.04)	0.37 (0.09, 1.4)	NA	0.58 (0.18, 1.94)	0.84 (0.23, 3)	0.84 (0.22, 3.31)	0.31 (0.01, 1.83)	1.23 (0.35, 4.23)	0.64 (0.18, 2.23)	1.13 (0.38, 3.46)	1.67 (0.49, 5.93)	0.77 (0.26, 2.32)	1.01 (0.09, 14.19)	0.66 (0.19, 2.4)	0.64 (0.15, 2.71)	0.56 (0.14, 2.36)	0.93 (0.22, 3.99)	1.13 (0.26, 4.91)	1.39 (1.02, 6.18)	0.76 (0.12, 4.87)
sitagliptin	3.29 (0.6, 19.83)	1.42 (0.56, 3.61)	1.37 (0.39, 4.73)	1.17 (0.4, 3.4)	1.77 (0.68, 4.65)	1.96 (0.8, 4.9)	1.11 (0.4, 3.06)	0.97 (0.27, 3.5)	0.02 (0, 0.07)	0.63 (0.21, 1.79)	1.71 (0.51, 5.68)	NA	1.43 (0.57, 3.62)	1.44 (0.5, 4.22)	0.54 (0.02, 1.58)	2.1 (0.91, 4.85)	1.09 (0.45, 2.63)	1.92 (0.75, 5.12)	2.84 (1.22, 6.98)	1.32 (0.69, 2.58)	1.73 (0.18, 21.35)	1.12 (0.46, 2.82)	1.08 (0.35, 3.37)	0.96 (0.32, 3.01)	1.59 (0.53, 4.98)	1.91 (0.63, 5.91)	2.69 (1.28, 9.04)	1.29 (0.27, 6.47)
linagliptin	2.33 (0.41, 14.11)	0.99 (0.36, 2.79)	0.95 (0.27, 3.29)	0.82 (0.27, 2.45)	1.24 (0.45, 3.4)	1.37 (0.52, 3.78)	0.78 (0.27, 2.27)	0.68 (0.19, 2.45)	0.01 (0, 0.05)	0.44 (0.14, 1.29)	1.19 (0.33, 4.35)	0.7 (0.28, 1.76)	NA	1 (0.34, 3.06)	0.38 (0.01, 1.38)	1.47 (0.59, 3.63)	0.76 (0.32, 1.85)	1.34 (0.44, 4.3)	1.99 (0.97, 4.25)	0.92 (0.46, 1.88)	1.21 (0.12, 14.75)	0.79 (0.31, 2.06)	0.75 (0.23, 2.53)	0.67 (0.22, 2.11)	1.11 (0.36, 3.66)	1.34 (0.43, 4.4)	2.04 (1.09, 3.254)	0.9 (0.18, 4.66)
dapagliflozin	2.31 (0.38, 15.1)	0.99 (0.31, 3.16)	0.95 (0.25, 3.53)	0.82 (0.23, 2.74)	1.23 (0.38, 3.97)	1.36 (0.43, 4.31)	0.77 (0.23, 2.65)	0.68 (0.17, 2.74)	0.01 (0, 0.05)	0.44 (0.12, 1.46)	1.19 (0.3, 4.59)	0.69 (0.24, 2.02)	1 (0.33, 2.98)	NA	0.37 (0.01, 1.32)	1.46 (0.5, 4.28)	0.76 (0.26, 2.24)	1.35 (0.42, 4.31)	1.98 (0.73, 5.63)	0.91 (0.38, 2.28)	1.2 (0.11, 15.72)	0.78 (0.26, 2.4)	0.75 (0.2, 2.89)	0.67 (0.19, 2.42)	1.11 (0.3, 4.31)	1.32 (0.38, 4.89)	1.96 (1.37, 6.13)	0.9 (0.16, 5.14)
ertugliflozin	6.42 (0.38, 16.51)	2.66 (0.25, 8.78)	2.58 (0.21, 9.27)	2.21 (0.19, 7.26)	3.33 (0.3, 7.35)	3.68 (0.34, 6.2)	2.07 (0.19, 6.61)	1.85 (0.15, 4.93)	0.04 (0, 0.17)	1.18 (0.1, 8.95)	3.25 (0.26, 7.85)	1.87 (0.18, 5.42)	2.66 (0.26, 8.2)	2.72 (0.24, 8.63)	NA	3.92 (0.38, 12.78)	2.04 (0.2, 6.22)	3.65 (0.32, 12.41)	5.29 (0.6, 16.59)	2.45 (0.26, 7.53)	3.44 (0.14, 9.5)	2.13 (0.2, 6.24)	2.06 (0.17, 7.17)	1.81 (0.16, 6.16)	3.02 (0.25, 6.06)	3.61 (0.3, 11.97)	6.13 (2.37, 19.91)	2.47 (0.16, 9.95)
canagliflozin	1.57 (0.29, 9.22)	0.68 (0.25, 1.82)	0.65 (0.19, 2.3)	0.56 (0.19, 1.58)	0.84 (0.31, 2.24)	0.93 (0.38, 2.36)	0.53 (0.18, 1.49)	0.47 (0.13, 1.62)	0.01 (0, 0.03)	0.3 (0.1, 0.86)	0.81 (0.24, 2.82)	0.48 (0.21, 1.1)	0.68 (0.28, 1.69)	0.69 (0.23, 2.02)	0.26 (0.01, 0.65)	NA	0.52 (0.22, 1.23)	0.92 (0.32, 2.8)	1.35 (0.6, 3.2)	0.63 (0.34, 1.19)	0.82 (0.09, 10.05)	0.54 (0.22, 1.37)	0.51 (0.16, 1.71)	0.46 (0.16, 1.38)	0.76 (0.24, 2.5)	0.91 (0.3, 2.9)	1.32 (1.07, 3.64)	0.62 (0.13, 3.08)
empagliflozin	3.03 (0.56, 18)	1.31 (0.49, 3.51)	1.26 (0.36, 4.35)	1.08 (0.37, 3.05)	1.63 (0.6, 4.34)	1.79 (0.71, 4.63)	1.02 (0.36, 2.89)	0.9 (0.26, 3.13)	0.12 (0, 0.36)	0.58 (0.19, 1.67)	1.56 (0.45, 5.47)	0.92 (0.38, 2.21)	1.31 (0.54, 3.15)	1.32 (0.45, 3.83)	0.49 (0.02, 1.02)	1.93 (0.82, 4.55)	NA	1.77 (0.59, 5.4)	2.61 (1.17, 6.02)	1.21 (0.66, 2.29)	1.58 (0.16, 19.15)	1.04 (0.42, 2.61)	0.99 (0.31, 3.2)	0.88 (0.3, 2.67)	1.46 (0.47, 4.68)	1.76 (0.57, 5.56)	2.64 (1.83, 8.36)	1.19 (0.24, 5.89)
glipizide	1.72 (0.27, 11.65)	0.74 (0.22, 2.38)	0.71 (0.17, 2.89)	0.61 (0.17, 2.1)	0.92 (0.27, 3)	1.01 (0.32, 3.24)	0.57 (0.16, 2.01)	0.51 (0.12, 2.09)	0.01 (0, 0.04)	0.33 (0.1, 1.02)	0.89 (0.29, 2.64)	0.52 (0.2, 1.33)	0.74 (0.23, 2.26)	0.74 (0.23, 2.38)	0.27 (0.01, 0.82)	1.09 (0.36, 3.17)	0.57 (0.19, 1.69)	NA	1.48 (0.5, 4.43)	0.68 (0.27, 1.72)	0.89 (0.08, 11.76)	0.59 (0.18, 1.82)	0.56 (0.16, 1.98)	0.5 (0.14, 1.83)	0.82 (0.23, 2.92)	0.99 (0.26, 3.73)	1.37 (1.10, 3.87)	0.67 (0.12, 3.74)
glimepiride	1.16 (0.21, 6.71)	0.5 (0.19, 1.29)	0.48 (0.15, 1.47)	0.41 (0.13, 1.17)	0.62 (0.24, 1.55)	0.69 (0.26, 1.76)	0.39 (0.14, 1.05)	0.34 (0.1, 1.16)	0.01 (0, 0.02)	0.22 (0.07, 0.61)	0.6 (0.17, 2.06)	0.35 (0.14, 0.82)	0.5 (0.24, 1.03)	0.51 (0.18, 1.38)	0.19 (0.01, 0.71)	0.74 (0.31, 1.66)	0.38 (0.17, 0.85)	0.68 (0.23, 2.02)	NA	0.46 (0.24, 0.87)	0.61 (0.06, 7.19)	0.4 (0.16, 0.94)	0.38 (0.12, 1.23)	0.34 (0.11, 1.01)	0.56 (0.18, 1.81)	0.67 (0.22, 1.99)	1.12 (1.04, 2.96)	0.45 (0.09, 2.17)
glibenclamide	2.51 (0.52, 13.15)	1.08 (0.48, 2.37)	1.03 (0.34, 3.17)	0.89 (0.37, 2.05)	1.34 (0.6, 2.92)	1.49 (0.71, 3.12)	0.85 (0.35, 1.97)	0.74 (0.25, 2.18)	0.01 (0, 0.03)	0.48 (0.18, 1.13)	1.29 (0.43, 3.82)	0.76 (0.39, 1.46)	1.09 (0.53, 2.16)	1.09 (0.44, 2.65)	0.41 (0.01, 1.32)	1.59 (0.84, 2.91)	0.83 (0.44, 1.52)	1.46 (0.58, 3.77)	2.16 (1.15, 4.19)	NA	1.31 (0.15, 14.9)	0.86 (0.43, 1.7)	0.82 (0.29, 2.3)	0.73 (0.3, 1.78)	1.2 (0.44, 3.39)	1.45 (0.55, 3.91)	7.51 (2.10, 25.51)	0.98 (0.22, 4.32)
gliclazide	1.91 (0.11, 30.33)	0.82 (0.07, 8.37)	0.79 (0.05, 9.35)	0.68 (0.05, 7.16)	1.03 (0.08, 10.26)	1.14 (0.09, 11.34)	0.64 (0.05, 6.52)	0.57 (0.04, 6.47)	0.01 (0, 0.04)	0.36 (0.03, 3.75)	0.99 (0.07, 11.22)	0.58 (0.05, 5.53)	0.82 (0.07, 8.15)	0.83 (0.06, 8.84)	0.29 (0.01, 0.73)	1.21 (0.1, 11.71)	0.63 (0.05, 6.16)	1.12 (0.09, 11.91)	1.65 (0.14, 15.9)	0.76 (0.07, 6.89)	NA	0.66 (0.06, 5.7)	0.63 (0.05, 5.83)	0.56 (0.04, 5.99)	0.93 (0.08, 8.88)	1.1 (0.08, 11.96)	1.49 (1.22, 4.07)	0.75 (0.05, 9.46)
pioglitazone	2.93 (0.52, 17.49)	1.26 (0.45, 3.46)	1.21 (0.33, 4.35)	1.04 (0.34, 3.07)	1.57 (0.55, 4.32)	1.73 (0.64, 4.72)	0.99 (0.35, 2.64)	0.86 (0.24, 3.08)	0.02 (0, 0.06)	0.56 (0.17, 1.65)	1.51 (0.42, 5.36)	0.89 (0.35, 2.15)	1.27 (0.49, 3.23)	1.28 (0.42, 3.84)	0.47 (0.01, 1.14)	1.86 (0.73, 4.58)	0.96 (0.38, 2.39)	1.71 (0.55, 5.42)	2.52 (1.06, 6.11)	1.17 (0.59, 2.32)	1.52 (0.18, 16.92)	NA	0.96 (0.3, 3.05)	0.85 (0.27, 2.64)	1.41 (0.45, 4.46)	1.69 (0.54, 5.41)	2.15 (1.17, 6.33)	1.14 (0.31, 4.25)
rosiglitazone	3.08 (0.47, 21.18)	1.32 (0.38, 4.46)	1.27 (0.28, 5.55)	1.09 (0.28, 4.06)	1.64 (0.46, 5.87)	1.83 (0.52, 6.27)	1.03 (0.27, 3.75)	0.9 (0.21, 4.09)	0.02 (0, 0.08)	0.59 (0.15, 2.15)	1.57 (0.37, 6.77)	0.93 (0.3, 2.85)	1.33 (0.39, 4.37)	1.34 (0.35, 5.06)	0.49 (0.01, 1.79)	1.94 (0.59, 6.26)	1.01 (0.31, 3.21)	1.79 (0.5, 6.41)	2.64 (0.81, 8.65)	1.22 (0.43, 3.42)	1.59 (0.17, 18.42)	1.04 (0.33, 3.35)	NA	0.89 (0.23, 3.48)	1.48 (0.75, 2.89)	1.77 (0.46, 7.06)	2.47 (1.91, 8.73)	1.2 (0.21, 6.83)
nateglinide	3.46 (0.54, 22.29)	1.48 (0.44, 4.89)	1.42 (0.33, 6.07)	1.23 (0.34, 4.08)	1.85 (0.55, 5.93)	2.04 (0.63, 6.53)	1.16 (0.33, 3.97)	1.02 (0.24, 4.21)	0.02 (0, 0.07)	0.66 (0.17, 2.23)	1.78 (0.42, 7.23)	1.04 (0.33, 3.13)	1.49 (0.47, 4.63)	1.5 (0.41, 5.25)	0.55 (0.02, 1.24)	2.19 (0.73, 6.38)	1.13 (0.37, 3.34)	2.01 (0.55, 7.32)	2.95 (0.99, 9.15)	1.37 (0.56, 3.34)	1.79 (0.17, 23.68)	1.18 (0.38, 3.64)	1.12 (0.29, 4.36)	NA	1.66 (0.43, 6.51)	2 (0.53, 7.56)	2.71 (1.31, 7.34)	1.35 (0.24, 7.55)
metformin	2.08 (0.31, 14.28)	0.89 (0.26, 2.94)	0.86 (0.19, 3.67)	0.74 (0.19, 2.69)	1.11 (0.31, 3.91)	1.23 (0.36, 4.18)	0.7 (0.19, 2.49)	0.61 (0.14, 2.7)	0.01 (0, 0.04)	0.4 (0.1, 1.43)	1.07 (0.25, 4.51)	0.63 (0.2, 1.88)	0.9 (0.27, 2.8)	0.9 (0.23, 3.34)	0.33 (0.01, 0.94)	1.32 (0.4, 4.15)	0.69 (0.21, 2.13)	1.22 (0.34, 4.3)	1.8 (0.55, 5.65)	0.83 (0.29, 2.27)	1.08 (0.11, 12.96)	0.71 (0.22, 2.22)	0.68 (0.35, 1.33)	0.6 (0.15, 2.32)	NA	1.2 (0.31, 4.73)	1.40 (7.89, 4.51)	0.81 (0.14, 4.56)
insulin	1.74 (0.27, 11.65)	0.75 (0.29, 1.8)	0.72 (0.21, 2.31)	0.61 (0.16, 2.19)	0.93 (0.3, 2.77)	1.02 (0.32, 3.27)	0.58 (0.18, 1.76)	0.51 (0.12, 2.16)	0.01 (0, 0.04)	0.33 (0.08, 1.18)	0.89 (0.2, 3.78)	0.52 (0.17, 1.58)	0.75 (0.23, 2.35)	0.76 (0.2, 2.66)	0.28 (0.01, 0.83)	1.1 (0.34, 3.38)	0.57 (0.18, 1.76)	1.01 (0.27, 3.78)	1.49 (0.5, 4.46)	0.69 (0.26, 1.82)	0.91 (0.08, 11.8)	0.59 (0.18, 1.87)	0.57 (0.14, 2.2)	0.5 (0.13, 1.89)	0.83 (0.21, 3.24)	NA	1.37 (1.16, 3.96)	0.68 (0.12, 3.86)
voglibose	0.08 (0, 0.28)	0.02 (0, 0.11)	0.02 (0, 0.11)	0.03 (0, 0.09)	0.03 (0, 0.14)	0.04 (0, 0.15)	0.03 (0, 0.09)	0.02 (0, 0.08)	0.01 (0, 0.03)	0.01 (0, 0.05)	0.03 (0, 0.14)	0.02 (0, 0.08)	0.03 (0, 0.11)	0.02 (0, 0.11)	0.01 (0, 0.05)	0.04 (0, 0.16)	0.02 (0, 0.08)	0.04 (0, 0.15)	0.07 (0, 0.23)	0.03 (0, 0.1)	0.04 (0, 0.15)	0.02 (0, 0.08)	0.02 (0, 0.08)	0.03 (0, 0.08)	0.04 (0, 0.13)	0.04 (0, 0.15)	NA	0.03 (0, 0.11)
placebo	2.57 (1.3, 9.22)	1.1 (0.2, 5.79)	1.06 (0.17, 6.6)	1.31 (0.16, 4.89)	1.37 (0.26, 7.1)	1.52 (0.29, 7.76)	0.86 (0.16, 4.44)	0.75 (0.12, 4.71)	0.01 (0, 0.05)	0.49 (0.08, 2.64)	1.32 (0.21, 8.12)	0.77 (0.15, 3.73)	1.11 (0.21, 5.47)	1.11 (0.19, 6.2)	0.4 (0.01, 1.67)	1.62 (0.32, 7.92)	0.84 (0.17, 4.13)	1.49 (0.27, 8.53)	2.2 (0.46, 10.81)	1.02 (0.23, 4.46)	1.33 (0.11, 20.63)	0.87 (0.24, 3.24)	0.83 (0.15, 4.81)	0.74 (0.13, 4.2)	1.23 (0.22, 7.06)	1.48 (0.26, 8.53)	7.38 (2.68, 24.04)	NA

Comparisons should be read from left to right. The estimate is located at the intersection of the treatments in the column heads and the treatments in the row heads. An RR value >1 favors the column-defining treatment. An RR value <1 favors the row-defining treatment; NA, not applicable.

TABLE 2 | GRADE of quality evidence for glucose-lowering medications on fracture outcomes.

Medications	Risk of Bias	Inconsistency	Indirectness	Imprecision	Publication bias	Quality	Risk of fracture
Alogliptin	Not serious	Not serious	Undetected	Not serious	Not serious	High	Low
Linagliptin	Not serious	Serious	Undetected	Not serious	Not serious	Moderate	Low
Saxagliptin	Not serious	Not serious	Not serious	Not serious	Not serious	High	High
Sitagliptin	Not serious	Serious	Not serious	Not serious	Not serious	Moderate	High
Vildagliptin	Not serious	Not serious	Not serious	Not serious	Not serious	High	High
Omarigliptin	Not serious	Serious	Not serious	Not serious	Not serious	Moderate	High
Trelagliptin	Not serious	Not serious	Undetected	Not serious	Not serious	High	Very high
Albiglutide	Not serious	Not serious	Not serious	Not serious	Not serious	High	Very low
Dulaglutide	Not serious	Serious	Not serious	Not serious	Not serious	Moderate	Low
Exenatide	Not serious	Not serious	Not serious	Not serious	Not serious	High	Low
Liraglutide	Not serious	Serious	Not serious	Not serious	Not serious	Moderate	Low
Lixisenatide	Not serious	Not serious	Undetected	Not serious	Not serious	High	Low
Semaglutide	Not serious	Not serious	Not serious	Not serious	Not serious	High	Low
Canagliflozin	Not serious	Not serious	Not serious	Not serious	Not serious	High	Low
Dapagliflozin	Not serious	Not serious	Not serious	Not serious	Not serious	High	Low
Empagliflozin	Not serious	Not serious	Not serious	Not serious	Not serious	High	High
Ertugliflozin	Not serious	Serious	Not serious	Not serious	Not serious	Moderate	High
Glimepiride	Not serious	Not serious	Not serious	Not serious	Not serious	High	Low
Gliclazide	Not serious	Not serious	Undetected	Not serious	Not serious	High	Low
Glipizide	Not serious	Not serious	Not serious	Not serious	Not serious	High	Low
Rosiglitazone	Not serious	Not serious	Undetected	Not serious	Not serious	High	High
Pioglitazone	Not serious	Not serious	Not serious	Not serious	Not serious	High	High
Metformin	Not serious	Serious	Not serious	Not serious	Not serious	Moderate	Low
Voglibose	Not serious	Not serious	Undetected	Not serious	Not serious	High	Very low
Nateglinide	Not serious	Not serious	Undetected	Not serious	Not serious	High	High
Glibenclamide	Not serious	Not serious	Not serious	Not serious	Not serious	High	Low
Insulin	Not serious	Not serious	Not serious	Not serious	Not serious	High	Low

GRADE, Grading of Recommendations, Assessment, Development and Evaluation.

use of GLP-1 receptor agonists and the risk of fracture (27). The latest Bayesian network meta-analysis (included trials >26 weeks) suggested that exenatide showed benefits (28). Notably, according to bone biology, it is not likely that any intervention of less than 52 weeks will affect fracture risk, and therefore only RCTs with a duration of at least 52 weeks were considered in our analyses. Most of these results could not provide powerful evidence. Although GLP-1 receptor agonists did show benefits in animal models, researchers could not draw any conclusion confidently due to the limited clinical data (29, 30).

Dipeptidyl Peptidase-4 Inhibitors and Fracture Risk

In terms of fracture risk, DPP-4 inhibitors showed varied results, and trelagliptin raised the risk of fracture with a statistical significance. A previous meta-analysis supported

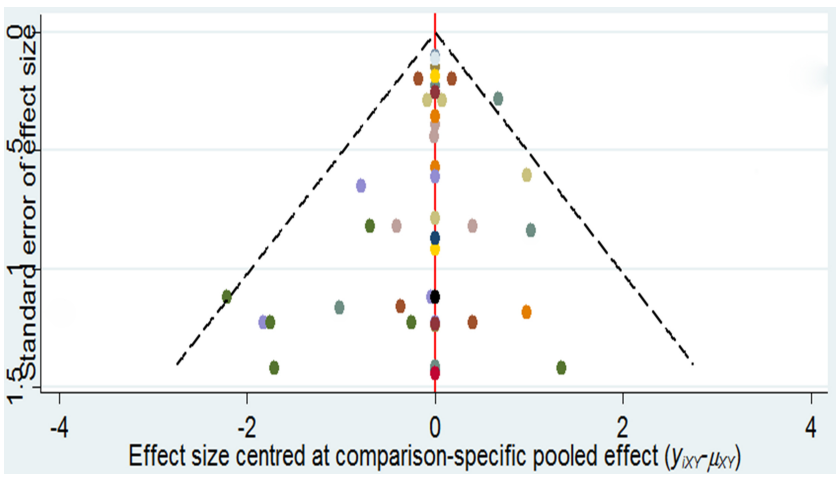

FIGURE 3 | Comparison-adjusted funnel plots of the network.

that DPP-4 inhibitors have neutral effects on fracture risk (27). An update meta-analysis suggested that DPP-4 inhibitors do modify the risk of fracture (31). Long-term treatment with DPP-4 inhibitors does not increase or decrease the risk of fracture (32). These findings agree with those of our Bayesian meta-analysis. Unfortunately, although our results showed that trelagliptin increased the risk of fracture, this could not be supported by available evidence. More clinical trials are needed to clarify the effect of trelagliptin on fracture events.

Sodium–Glucose Co-Transporter 2 Inhibitors and Fracture Risk

SGLT2 inhibitors did not modify the risk of fracture with statistically significant differences. A systematic review suggested that canagliflozin is linked to an increased fracture rate (33), a conclusion that is similar with our results. One study suggested that SGLT2 inhibitors have neutral effects on fracture risk (34). In a clinical trial, SGLT2 inhibitors exhibited better benefits than other anti-diabetic drugs in T2DM patients suffering from chronic kidney disease (10). Therefore, SGLT2 inhibitors could be considered in anti-diabetic strategies for patients susceptible to fracture.

Thiazolidinediones and Fracture Risk

Our results suggested that pioglitazone and rosiglitazone raised the risk of fracture, but no statistically significant difference was observed. Many studies showed that rosiglitazone and pioglitazone increased the risk of bone fractures (35–37). One study suggested that pioglitazone treatment does not increase the risk of fractures (38). But these studies could not provide powerful evidences due to the limited data. Therefore, thiazolidinediones should be considered carefully in patients susceptible to fracture.

Sulfonylureas and Fracture Risk

For sulfonylureas, our results showed that that glipizide, gliclazide, glibenclamide, and glimepiride decreased the risk of fracture. One study suggested that sulfonylureas could increase the risk of fractures in the old patients with T2DM (39). Many studies have indicated that sulfonylureas have neutral effects on bone metabolism and BMD, and that they increase the amount of falling events due to the high risk of hypoglycemic episodes (40). The few available preclinical and clinical data indicate that sulfonylureas do not have detrimental effects on the bone (41). Therefore, sulfonylureas could be considered in the development of anti-diabetic strategies.

Other Anti-Diabetic Drug and Fracture Risk

Among other anti-diabetic drugs evaluated, metformin, voglibose, and insulin showed benefits, whereas nateglinide raised the risk of fracture. Several recent studies have indicated that metformin is associated with a reduced risk of fracture (36, 42, 43), while previous studies have reported an increased risk of falling among patients using insulin (12). Therefore, metformin could be considered in patients susceptible to fracture. Nevertheless, more clinical trials are needed to clarify the effects of voglibose, insulin, and nateglinide on fracture events.

Limitations

The following limitations of this Bayesian model should be considered. Firstly, voglibose might not be suitable for all T2DM patients due to individual differences; the probability ranking of treatments should be taken into account in selecting suitable medications. Secondly, a random-effects model was used to reduce the influence of the constraint on common variances, but this method increases the possibility of introducing biases due to heterogeneity in the included RCTs (such as doses and plasma glucose). Thirdly, a significant difference in inconsistency was noted in some direct or indirect comparisons. Inconsistency could be generated by the data available from the existing clinical trials that suffer from methodological limitations including insufficient primary endpoints and fracture events (44). Fourthly, the effects of some anti-diabetic drugs, such as licogliflozin, chlorpropamide, bromocriptine-QR, tolbutamide, and acarbose on the fracture risks, could not be evaluated due to the limited data from clinical trials. Finally, some RCTs could not be retrieved due to database or language restrictions.

CONCLUSIONS

This comprehensive review and analysis might be helpful for researchers in investigating the relative risk of fracture related to the use of anti-diabetic drugs in future research. Further clinical trials on the association between bone fracture events and the use of anti-diabetic drugs are important since fragility fracture can seriously affect patients with diabetes. Unfortunately, the possible mechanisms of trelagliptin, voglibose, and albiglutide in promoting bone formation or inhibiting bone absorption in T2DM patients are still unclear, and there is still a lack of clinical studies to demonstrate the efficacy of trelagliptin, voglibose, and albiglutide in patients with T2DM-related fractures. Overall, we observed varied results on the association between the use of anti-diabetic drugs and fracture risk. Trelagliptin raised the risk of fracture, whereas voglibose and albiglutide showed benefits with statistically significant differences. Some anti-diabetic drugs (omarigliptin, sitagliptin, vildagliptin, saxagliptin, empagliflozin, ertugliflozin, rosiglitazone, pioglitazone, and nateglinide) may increase the risk of fracture; while others (dulaglutide, exenatide, liraglutide, semaglutide, lixisenatide, linagliptin, alogliptin, canagliflozin, dapagliflozin, glipizide, gliclazide, glibenclamide, glimepiride, metformin, and insulin) may show benefits. Many preclinical studies considered that various anti-diabetic drugs may have either aggravating or repairing effects on bone quality. Therefore, when developing T2DM treatment strategies, the clinical efficacy of various anti-diabetic drugs must also be weighed against their benefits and risks brought about by the individual differences of patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

Y-SZ, Y-DZ, and YY contributed equally. Conceptualization: Y-SZ, B-CX, and Y-DZ. Data extraction: YY, Y-DZ, and B-CX. Formal analysis: Y-SZ and S-CC. Funding acquisition: B-CX. Investigation: Y-SZ, B-CX, and YY. Methodology: Y-SZ and YY. Project administration: Y-SZ. Resources: Y-SZ, Y-DZ, and YY. Software: B-CX and Y-DZ. Supervision: B-CX, S-CC, and Y-DZ. Validation: Y-DZ and S-CC. Writing—original draft: Y-SZ, Y-DZ, and YY. Writing—review and editing: Y-SZ, YY, and B-CX. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.735824/full#supplementary-material

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Chondroitin Sulfate Alleviates Diabetic Osteoporosis and Repairs Bone Microstructure *via* Anti-Oxidation, Anti-Inflammation, and Regulating Bone Metabolism

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Diabetic osteoporosis (DOP) belongs to secondary osteoporosis caused by diabetes; it has the characteristics of high morbidity and high disability. In the present study, we constructed a type 1 diabetic rat model and administered chondroitin sulfate (200 mg/kg) for 10 weeks to observe the preventive effect of chondroitin sulfate on the bone loss of diabetic rats. The results showed that chondroitin sulfate can reduce blood glucose and relieve symptoms of diabetic rats; in addition, it can significantly increase the bone mineral density, improve bone microstructure, and reduce bone marrow adipocyte number in diabetic rats; after 10 weeks of chondroitin sulfate administration, the SOD activity level was upregulated, as well as CAT levels, indicating that chondroitin sulfate can alleviate oxidative stress in diabetic rats. Chondroitin sulfate was also found to reduce the level of serum inflammatory cytokines (TNF-α, IL-1, IL-6, and MCP-1) and alleviate the inflammation in diabetic rats; bone metabolism marker detection results showed that chondroitin sulfate can reduce bone turnover in diabetic rats (decreased RANKL, CTX-1, ALP, and TRACP 5b levels were observed after 10 weeks of chondroitin sulfate administration). At the same time, the bone OPG and RUNX 2 expression levels were higher after chondroitin sulfate treatment, the bone RANKL expression was lowered, and the OPG/RANKL ratio was upregulated. All of the above indicated that chondroitin sulfate could prevent STZ-induced DOP and repair bone microstructure; the main mechanism was through anti-oxidation, anti-inflammatory, and regulating bone metabolism. Chondroitin sulfate could be used to develop anti-DOP functional foods and diet interventions for diabetes.

Keywords: chondroitin sulfate, diabetes, osteoporosis, bone microstructure, bone metabolism

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INTRODUCTION

There are 463 million people with diabetes worldwide currently, an average of 1 in 11 adults (20–79 years old), and by 2045, the diabetes population will jump to 700 million (1, 2). Long-term high blood glucose can cause microvascular injury, endangering the kidneys, heart, eyes, peripheral nerves, brain, feet, as well as bone (3).

Diabetic osteoporosis (DOP) is a bone complication caused by diabetes, characterized by lowered bone mineral density (BMD), changes of bone microstructure, and raised bone fragility. DOP greatly reduces the quality of living of patients, subjecting them to heavy economic burden (4). As the number of people with diabetes increases globally, the number of DOP is also increasing annually. Clinical data showed that about 50% to 65% of people with diabetes have decreased BMD and increased incidence of fractures, and nearly 35% of them have been diagnosed as osteoporosis (5).

The present approaches for DOP are oral hypoglycemic drugs or insulin injections, supplemented by calcium preparations, bisphosphonates, etc. (6). However, adverse drug reactions have always been a major challenge related to drug treatment goals. Therefore, exploring more safer and effective strategies are highly crucial.

Chondroitin sulfate (CS) is a natural sticky polysaccharide made from the cartilage of animals. The content of CS is different in the cartilage of different species and ages. As a drug for the treatment of joint diseases, it was used in conjunction with glucosamine to relieve pain and promote cartilage regeneration, which can fundamentally improve joint problems (7). It was reported that CS has anti-inflammation effects (8), has anti-psoriasis effects (9), enhances immunity (10), lowers blood lipid (11), and has anti-tumor effects (12). It also has a preventive effect on diabetic nephropathy.in streptozotocin (STZ)-induced diabetic mice (13), and CS was also reported to increase bone formation in ovariectomized rats (14). Whether it has a protective effect on DOP has not been studied. Our previous study found that the CS could increase the BMD of diabetic rats, but the mechanisms were not clear. So, in present study, we researched the protective effect and mechanisms of CS on DOP, which will provide a new approach for DOP treatment.

MATERIALS AND METHODS

Chemicals

CS power was isolated and purified from the cartilage of giant salamander according to the method of Zhu (15); the content of CS in the experimental materials was 95%. STZ was obtained from Acmec Biochemical Company (Shanghai, China).

Animals

Fifty-six-day old male rats were obtained from Cheng Du Dashuo Experimental Animal Company (Cheng Du, China, license no. SCXK 2020-030). Animals were kept in independent ventilated cages in a standardized animal room with constant temperature and humidity. Water and food intake of animals was not restricted. Animals were feed with diets prepared by the American Society of Nutrition (AIN93) standard. Animal experiment operations were approved by Shaanxi University of Technology Animal Ethics Committee (approval No. 2020-74).

Diabetes Induction in SD Rats, Animal Grouping, and Treatment

After 7 days of acclimatization, except for 10 animals in the control group, the remaining animals were intraperitoneally injected with 45 mg/kg of STZ after fasting overnight, and the dosage of STZ was based on our previous study (16, 17); STZ was dissolved in citric acid buffer solution at pH 4.3, and 10 animals in the control group were injected with citric acid buffer solution. After 72 h, animal's blood glucose was detected, and animals with blood glucose higher than 11.1 mmol/L were selected for subsequent experiments.

Then, animals were regrouped into four groups: Group 1, control group (n = 10), rats were given deionized water by gavage every day; Group 2, type 1 diabetic group (n = 10), named T1DM group, type 1 diabetic rats were given deionized water by gavage every day; Group 3, CS-treated group (n = 10), named CS group, type 1 diabetic rats were given CS (200 mg/kg/day) by gavage every day; Group 4, metformin group (n = 10), named Met group, type 1 diabetic rats were given metformin (200 mg/kg/day) by gavage every day. The dose of CS was selected by pre-experiment.

During the experiment, animals were weighed every week, water and diet of animals were recorded, and the blood glucose of animals was measured every week. After 10 weeks of administration, animals were anesthetized with isoflurane and were sacrificed by cervical dislocation; blood was collected, and serum was separated and stored at −80°C. At the same time, femurs, vertebrae, and tibias of animals were collected.

BMD Measurement

The BMD of femur and vertebrae of each rat were obtained by using small-animal dual-energy X-ray absorptiometry (InAlzyer, Korea).

Bone Micro-CT Measurement

Femur tissues of rats were collected and scanned by a Locus SP micro-CT (GE Healthcare, Danderyd, Sweden) with a resolution of 6.5μm. The processing and analysis software were MICVIEW 3D reconstruction processing software and ABA-specific bone analysis software. The data of cortex volume, bone surface area, trabecular number, and bone volume were obtained by using ABA-specific bone analysis software.

Bone Turnover Marker Detection

According to instructions listed in ELISA test kits (Elabscience, Wuhan, China), a microplate reader (Elx808, Winooski, USA) was used to detect the content of bone turnover markers (CTX-1, OPG, ALP, TRACP 5b, RANKL, osteocalcin, and RUNX 2) in serum of each group of rats.

Oxidative Stress Index Detection

According to instructions listed in kits (Beyotime, Shanghai, China), SOD activity, MDA content, CAT activity, and GSH content in serum were detected by an ultraviolet and visible spectrophotometer (Alpha1860S, Shanghai, China).

Inflammatory Cytokine Detection

Based on instructions listed in ELISA test kits (Elabscience, Wuhan, China), the microplate reader (Elx808, Winooski, USA) was used to detect the content of serum inflammatory cytokines (IL-6, TNF-α, MCP-1, and IL-1).

Pathological Analysis of Femur Bone Tissue

Femur of each rat was fixed in 3.8% paraformaldehyde solution for 48 h, then rinsed with PBS, placed in 10% EDTA solution for 5 weeks, and then made into 4-micron-thick paraffin sections by a tissue slicer (Leica, Wetzlar, Germany), and the sections were stained with hematoxylin and eosin, sealed with neutral gum. After that, a slide was placed under a microscope to observe femur pathological changes. Bone morphometric parameters including trabecular separation (Tb·Sp), bone volume per tissue volume (BV/TV), and trabecular thickness (Tb·Th) were analyzed by image pro plus (IPP) 6.0 software.

Pathological Observation of Tibia Bone Marrow Adipocytes

Tibia of each rat was fixed in 3.8% paraformaldehyde solution for 48 h, then rinsed with PBS; femur was placed in 10% EDTA solution for 5 weeks and then made into 4-micron-thick paraffin sections by a tissue slicer (Leica, Wetzlar, Germany), and sections were stained with hematoxylin and eosin, sealed with neutral gum. After that, the slide was placed under a microscope to observe bone marrow adipocytes in tibia. Adipocytes were counted in each field, and the diameter of adipocytes was measured with IPP 6.0 software.

Femur Osteoclast Observation-TRAP Staining

Four-micron-thick femur paraffin sections were stained with TRAP staining kit, based on the instructions listed in the kit. The stained slides were sealed with neutral gum. After that, a slide was placed under a microscope (200× magnification) to observe osteoclasts in the femur. Osteoclasts were purple-red after staining, five fields of view were selected for each slice, and the number of osteoclasts in each field was counted.

Immunohistochemical

Femur paraffin slides (4 μm thick) were soaked in xylene for 20 min, and then slides were dehydrated with gradient ethanol and placed in a water bath at 95°C in citrate antigen retrieval solution (pH 6) for 1 h. After cooling to room temperature, slides were washed with PBS; 1% BSA solution was added to the slides to block endogenous peroxidase, and then the slides were washed with PBS, incubated with primary antibodies (OPG, RANKL, and RUNX2, respectively) (Santa Cruz Biotech, USA) for 1.5 h at 37°C in a constant temperature incubator, double washed with PBS, incubated with secondary antibody (Santa Cruz Biotech, USA) for 2 h, washed with PBS three times, and then incubated with DAB. After that, slides were counterstained with hematoxylin, and sealed with neutral gum. IHC-stained slides were observed under a microscope (Olympus, Germany) with 200× magnification. Five fields of view were chosen for each slice, and IPP software was used to count the area of positive staining, and percentage of positive area was calculated.

Statistical Analysis

Statistical analyses were performed with ANOVA by SPASS 19.0; all of the data were shown as mean ± SD, and statistical significance was compared between groups using the LSD method. $p < 0.05$ was considered significant, and $p < 0.01$ was considered extremely significant.

RESULTS

Chondroitin Sulfate Relieved the Symptoms of Hyperglycemia, Polydipsia, and Polyphagia Caused by Diabetes in SD Rats

The blood glucose, water intake, and food intake in type 1 diabetic rats were significantly increased compared with control ($p < 0.01$) (**Figures 1A, C, D**), while the body weight was decreased (**Figure 1B**); this result was consistent with basic pathological changes of diabetes, indicating that the type 1 diabetes animal model was successfully established. After 10 weeks of CS or metformin administration, the symptoms of diabetes in rats were effectively alleviated, reflected in lowered blood glucose, increased body weight, and reduced water and food intake. This indicates that CS can reduce blood glucose of diabetic rats and relieve the symptoms of diabetes.

Chondroitin Sulfate Increased BMD of Type 1 Diabetic Rats

Compared with control group, the BMD (lumbar vertebrae and femur) in diabetic rats was lowered significantly ($p < 0.01$) (**Figure 2**). After 10 weeks of CS or metformin administration, the BMD in lumbar vertebrae and femur was increased (CS *vs*. T1DM group, $p < 0.01$; Metformin *vs*. T1DM groups, $p < 0.01$) (**Figure 2**), indicating that CS could increase the BMD of type 1 diabetic rats.

Chondroitin Sulfate Repaired Bone Micro-CT Structure of Type 1 Diabetic Rats

Femur micro-CT scanning results showed that trabecular structure in the model group was sparse, and some trabecular areas disappeared (**Figure 3A**). After 10 weeks of administration of CS or metformin, trabecular structure was repaired. Micro-CT metrological data (**Figures 3A–E**) showed that cortical bone volume, bone surface area, number of bone trabecular, and bone volume in type 1 diabetic group were significantly lower than those

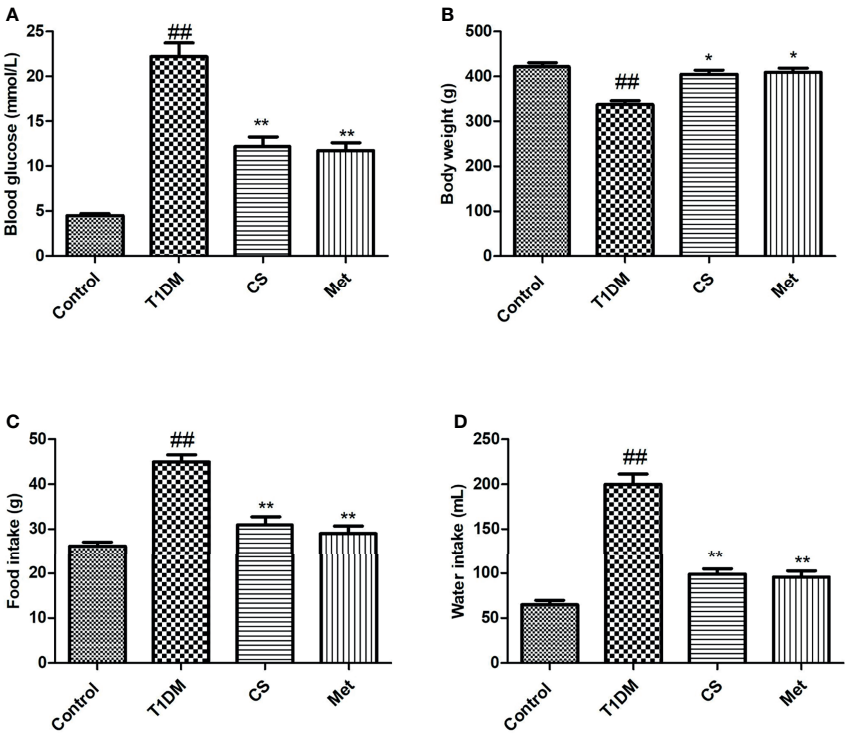

FIGURE 1 | Blood glucose, body weight, food intake, and water intake at the end of the 10th week in different groups. **(A)** Blood glucose in different groups. **(B)** Body weight in different groups. **(C)** Food intake in different groups. **(D)** Water intake in different groups. ##indicates $p < 0.01$ compared with control; **indicates $p < 0.01$ compared with the diabetic group; *indicates $p < 0.05$ compared with the diabetic group.

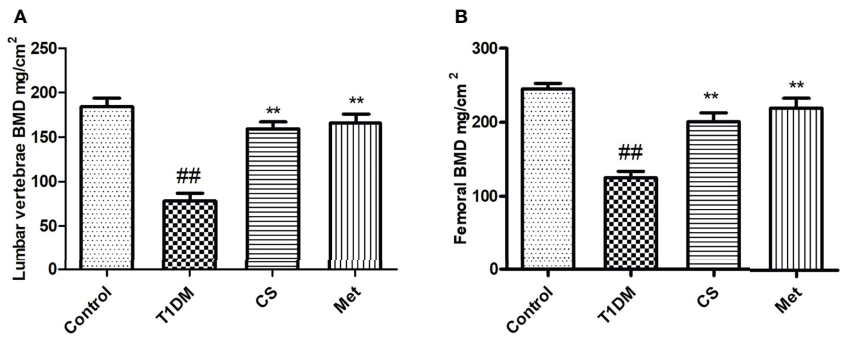

FIGURE 2 | The bone mineral density of different groups at the end of the 10th week. **(A)** The BMD of lumbar vertebrae in different groups. **(B)** The femoral BMD in different groups. ##indicates $p < 0.01$ compared with control. **indicates $p < 0.01$ compared with the diabetic group.

in the control group ($p < 0.01$). After 10 weeks of CS or metformin administration, the above indexes were upregulated, and the differences were significant compared with the diabetic group.

Chondroitin Sulfate Regulated Bone Turnover of Type 1 Diabetic Rats

As **Table 1** shows, some of the serum bone turnover markers (OPG, RUNX 2, osteocalcin, and TRACP 5b) and OPG/RANKL ratio levels were lowered in type 1 diabetic rats compared with control ($p < 0.01$), and those were significantly increased in the CS and Met group compared with the type 1 diabetic group. Other serum bone turnover markers such as RANKL, CTX 1, and ALP levels were higher in the type 1 diabetic group compared with the control ($p < 0.01$), which were significantly decreased in CS and Met groups, indicating that CS could regulate bone turnover of type 1 diabetic rats.

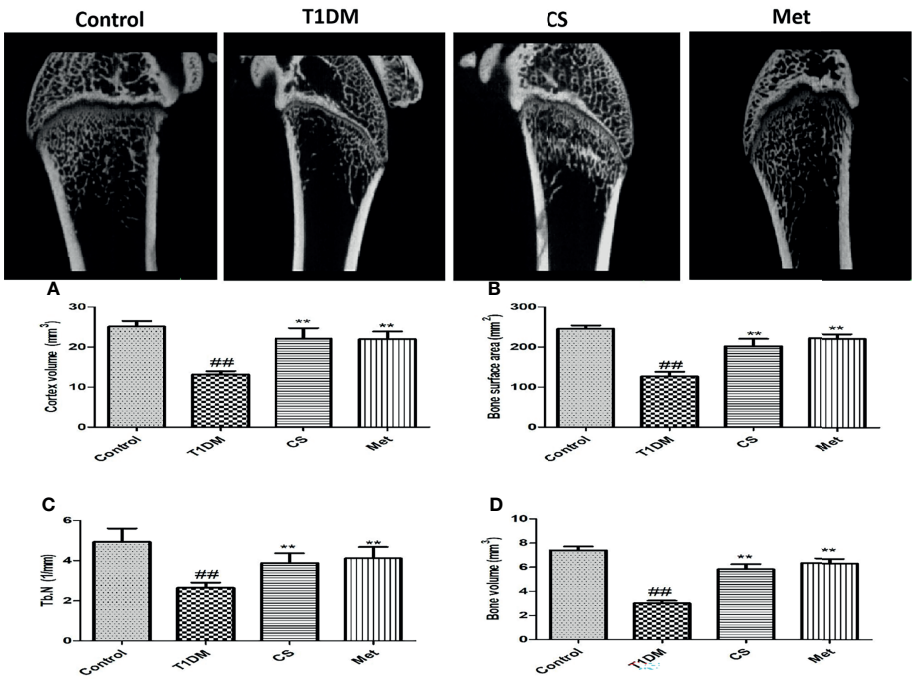

FIGURE 3 | **(A)** The femur micro-CT structure of different groups at the end of the 10th week. **(B)** Cortex volume in different groups. **(C)** Bone surface area in different groups. **(D)** Trabecular number in different groups. **(E)** Bone volume in different groups. ##indicates $p < 0.01$ compared with control. **indicates $p < 0.01$ compared with the diabetic group.

TABLE 1 | Bone turnover markers in different groups at the end of the 10th week.

	Control	T1DM	CS	Met
OPG (ng ml^{-1})	9.31 ± 2.07	2.88 ± 1.05##	7.16 ± 2.25**	7.56 ± 2.72**
RANKL (ng ml^{-1})	3.08 ± 1.12	10.20 ± 2.50##	4.36 ± 1.31**	5.80 ± 2.07**
OPG/RANKL ratio	3.02 ± 1.23	0.29 ± 0.11##	2.14 ± 0.96**	3.02 ± 1.23**
RUNX 2 (ng ml^{-1})	10.69 ± 2.96	3.64 ± 1.36##	8.49 ± 2.71**	8.42 ± 2.73**
Osteocalcin (ng ml^{-1})	30.11 ± 6.67	9.60 ± 2.95##	23.31 ± 5.73**	24.24 ± 4.75**
TRACP 5b (U dl^{-1})	3.25 ± 1.31	6.47 ± 1.50##	4.92 ± 1.11*	4.59 ± 1.24*
ALP (U dl^{-1})	75.93 ± 13.15	167.31 ± 18.55##	96.99 ± 19.86**	92.90 ± 14.31**
CTX 1 (ng ml^{-1})	41.73 ± 9.68	107.14 ± 23.21##	60.18 ± 16.38**	54.39 ± 17.35**

*##indicates p < 0.01 compared with control, **indicates p < 0.01 compared with the diabetic group, *indicates p < 0.05 compared with the diabetic group.*

Chondroitin Sulfate Downregulated Inflammatory Cytokines of Type 1 Diabetic Rats

Serum inflammatory cytokine levels in type 1 diabetic rats increased dramatically compared with the control group ($p < 0.01$). After 10 weeks of CS or metformin treatment, the serum inflammatory cytokine levels were lowered in CS or Met groups (CS *vs.* T1DM group, $p < 0.01$; Metformin *vs.* T1DM groups, $p < 0.01$) (**Figure 4**), indicating that CS has an inhibitory effect on inflammation induced by type 1 diabetes in SD rats.

Chondroitin Sulfate Alleviated Oxidative Stress in Type 1 Diabetic Rats

Oxidative stress existed in diabetic rats; serum SOD, GPX, and CAT activity levels were lowered, and MDA level was raised in type 1 diabetic rats. Ten weeks after CS or metformin treatment, SOD, GPX, and CAT activity levels were raised, and MDA level was lowered (CS *vs.* T1DM, $p < 0.01$; Met *vs.* T1DM, $p < 0.01$) (**Figure 5**). The results indicated that CS could alleviate oxidative stress in T1DM rats.

Chondroitin Sulfate Repaired Bone Microstructure of Type 1 Diabetic Rats

Compared with the control group, the femoral bone of diabetic rats was sparse and fractured, the spacing of trabecular bone became wider, and the trabecular bone became thinner (**Figures 6A–D**). The femoral structure was repaired after 10 weeks of CS or metformin administration. The results of bone morphometric data (**Figures 6E–G**) showed that the femoral thickness (Tb·Th) and the percentage of bone trabecular area

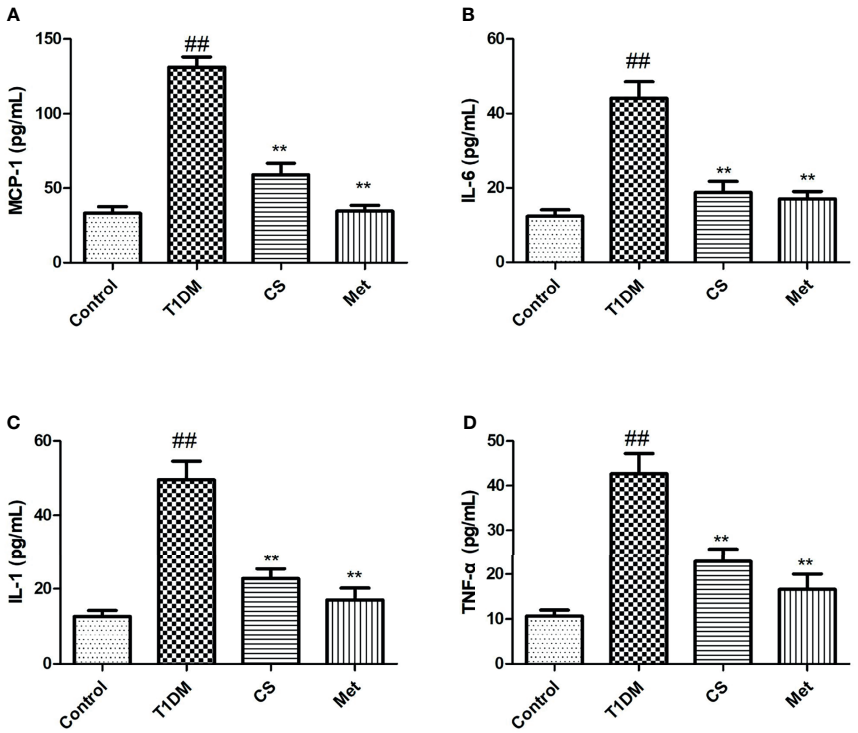

FIGURE 4 | The serum inflammatory cytokine levels in different groups at the end of the 10th week. **(A)** MCP-1 levels in serum; **(B)** IL-6 levels in serum; **(C)** IL-1 levels in serum; **(D)** TNF-a levels in serum. ##indicates $p < 0.01$ compared with control. **indicates $p < 0.01$ compared with the diabetic group.

(BV/TV) in CS or metformin treatment groups were significantly increased compared with the type 1 diabetic group ($p < 0.01$), and the trabecular separation (Tb·Sp) was decreased in CS or metformin treatment groups compared with the type 1 diabetic group ($p < 0.01$).

Chondroitin Sulfate Inhibited Bone Marrow Lipogenesis of Type 1 Diabetic Rats

Compared with the control group, the number of bone marrow adipocytes of diabetic rats increased significantly, and the bone marrow adipocyte density and adipocyte diameter were larger than the control ($p < 0.01$) (**Figure 7**). Ten weeks after CS or metformin treatment, the bone marrow adipocyte density and adipocyte diameter decreased (CS *vs.* T1DM, $p < 0.01$; Met *vs.* T1DM, $p < 0.01$) (**Figure 7**). The results indicated that CS could inhibit bone marrow lipogenesis of type 1 diabetic rats.

Chondroitin Sulfate Inhibited Osteoclastogenesis of Type 1 Diabetic Rats

After TRAP staining, the osteoclasts were stained purplish red. There was an increased number of femoral osteoclasts in type 1 diabetic group rats compared with control ($p < 0.01$) (**Figure 8**). Ten weeks after CS or metformin treatment, osteoclast number was decreased in the CS or Met group (CS group *vs.* T1DM group, $p < 0.01$; Met group *vs.* T1DM group, $p < 0.01$) (**Figure 8E**), indicating that CS could inhibit osteoclasts proliferation in type 1 diabetic rats.

Chondroitin Sulfate Upregulated OPG and RUNX 2 and Downregulated RANKL Expression in Bone Tissues

Pathological analysis showed that bone OPG and RUNX 2 expression levels were lowered in type 1 diabetic rats (T1DM *vs.* Control, $p < 0.01$); RANKL level in bone tissue was higher in type 1 diabetic rats (T1DM *vs.* Control, $p < 0.01$) (**Figure 9**). After 10 weeks of CS or metformin administration, the bone OPG and RUNX 2 levels increased (CS or Met group *vs.* T1DM group, $p < 0.01$) and the RANKL level decreased (CS or Met *vs.* T1DM group, $p < 0.01$) (**Figures 9A–C**).

DISCUSSION

DOP is a serious metabolic bone disease associated with diabetes. It has the characteristics of reduced BMD, destruction of bone microstructure, increased brittleness, reduced strength, and easy to fracture, which seriously affects the quality of life of people with diabetes (18, 19). Clinical treatment of DOP is to use chemical drugs such as vitamin D3, diphosphonate, calcitonin, and calcium to inhibit bone absorption, promote bone formation, and improve bone mineralization on the premise of

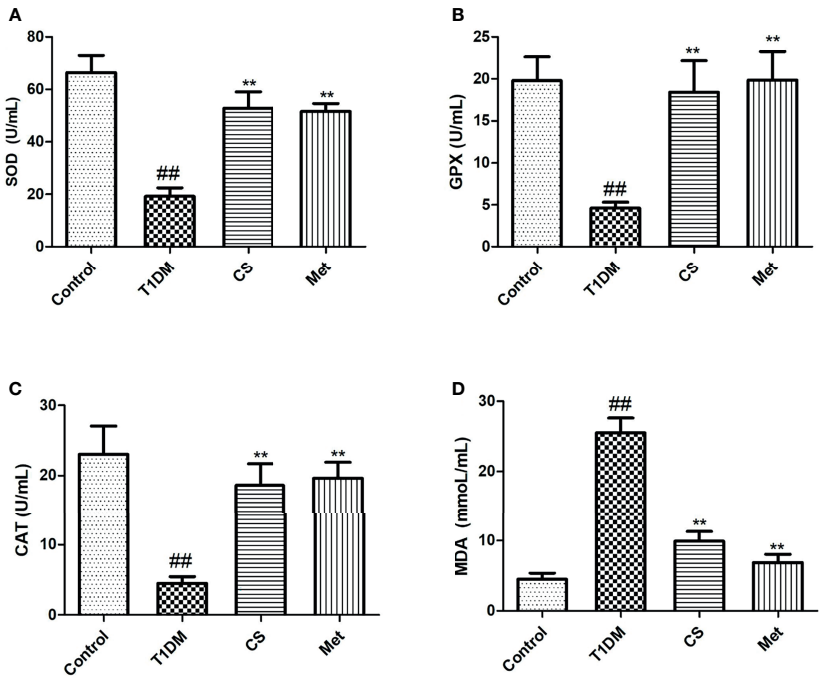

FIGURE 5 | The level of serum oxidative stress parameters in different groups at the end of the 10th week. **(A)** The level of SOD activities in different groups. **(B)** The level of glutathione peroxidase (GPX) activities in different groups. **(C)** The level of catalase (CAT) activities in different groups. **(D)** The level of malondialdehyde (MDA) content in different groups; ##indicates $p < 0.01$ compared with control, **indicates $p < 0.01$ compared with the diabetic group.

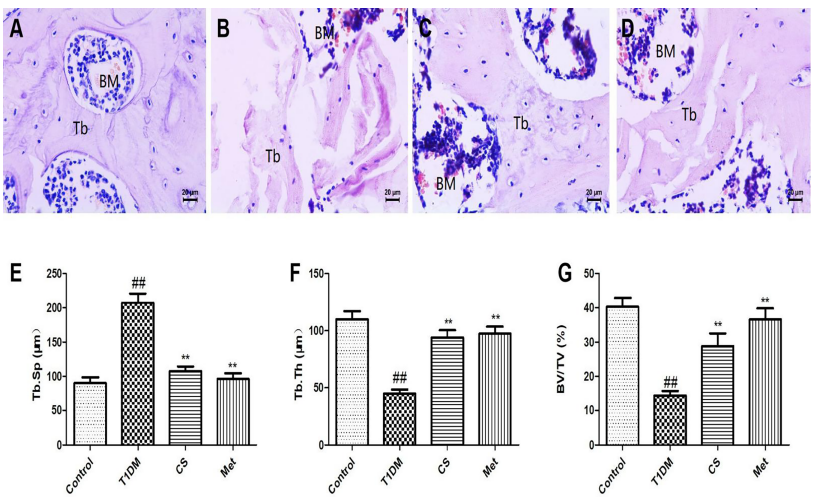

FIGURE 6 | The pathological sections of femur of rats in each group at the end of the 10th week; paraffin section of femur was stained with hematoxylin and eosin, 400×. **(A)** The control group femur. **(B)** The diabetic group femur. **(C)** The CS group femur. **(D)** The Met group femur. **(E)** The trabecular separation (Tb·Sp) in different groups. **(F)** The femoral thickness (Tb·Th) in different groups. **(G)** The percentage of bone trabecular area (BV/TV) in different groups; ##indicates $p < 0.01$ compared with control. **indicates $p < 0.01$ compared with the diabetic group. BM. Bone marrow; Tb. Trabecular bone.

controlling blood glucose. However, using chemical drugs long term can also lead to side effects, such as gastrointestinal diseases (20). Therefore, natural anti-osteoporosis drugs are much popular.

In the present study, we used the method of STZ injection to construct a rat model of type 1 diabetes, and intragastric administration of CS for 10 weeks. We detected the BMD, bone micro-CT, and bone pathology, and the results indicated

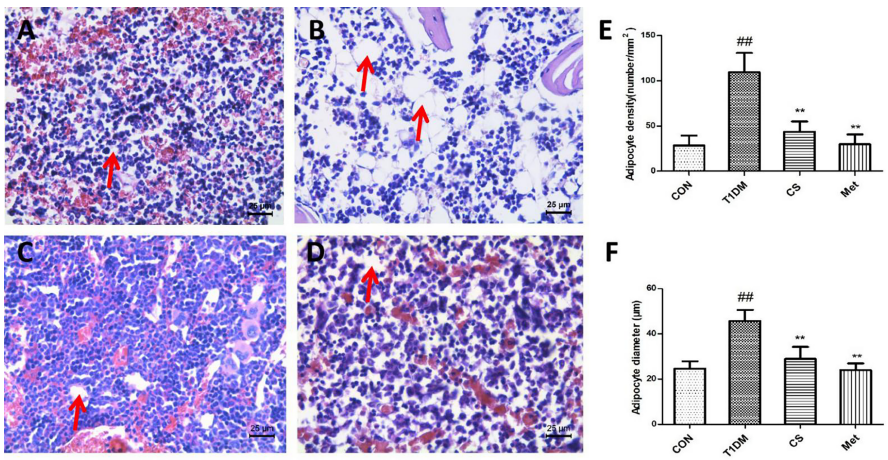

FIGURE 7 | Adipocytes in the bone marrow cavity of the tibia in different groups at the end of the 10th week, H&E staining. **(A)** The control group tibia bone marrow. **(B)** The diabetic group tibia bone marrow. **(C)** The CS group tibia bone marrow. **(D)** The Met group tibia bone marrow. **(E)** Adipocyte density in different groups. **(F)** Adipocyte diameter in different groups. ##indicates $p < 0.01$ compared with control. **indicates $p < 0.01$ compared with the diabetic group. Red arrows indicate adipocytes.

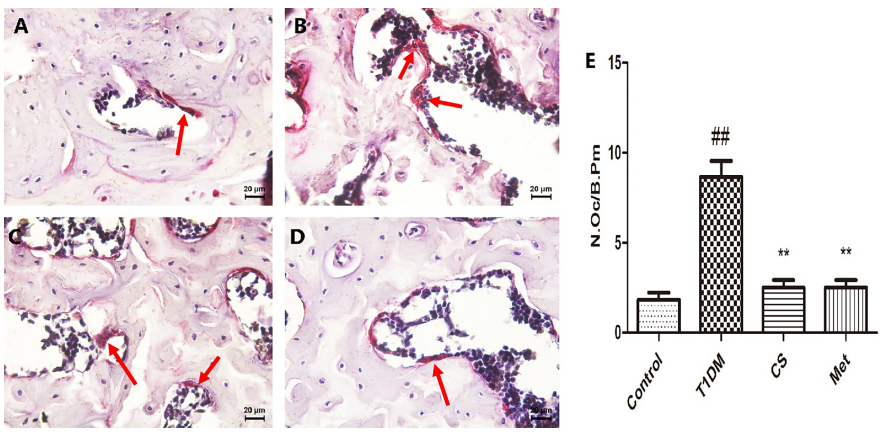

FIGURE 8 | The osteoclasts in femoral bone tissues of different groups at the end day of the 10th week. **(A)** The femoral bone tissues of control group rat. **(B)** The femoral bone tissues of diabetic group rat. **(C)** The femoral bone tissues of CS group rat. **(D)** The femoral bone tissues of Met group rat. **(E)** Number of osteoclasts per unit field in different groups. Bone tissues were stained by TRACP method, osteoclasts are multinucleate cells, and the red arrows point to osteoclasts. ##indicates $p < 0.01$ compared with control. **indicates $p < 0.01$ compared with the diabetic group.

that CS could inhibit bone loss, increase BMD, repair bone microstructure of type 1 diabetic rats, and thus prevent DOP.

DOP is closely related to oxidative stress. Hyperglycemia induced reactive oxygen species (ROS) production *in vivo* and hindered the proliferation and differentiation of osteoblasts (21–23). It has been confirmed that a sharp increase in the level of ROS induces death of osteoblasts, resulting in bone structure damage and BMD reduction (24). In the present study, we found that the SOD, GPX, and CAT activity levels were upregulated after 10 weeks of CS administration, indicating that CS could inhibit oxidative stress in type 1 diabetic rats; this may be one of important reasons why CS could prevent bone loss in type 1 diabetic rats.

Inflammation is closely related to osteoporosis, which is another main cause of DOP (25). The level of inflammatory cytokines in diabetes was significantly higher than that in healthy people, and accumulation of inflammatory cytokines could mediate oxidative stress damage, prompting osteoclast proliferation, increasing bone absorption, and thus causing osteoporosis (26). In this study, we found that CS could inhibit the inflammation in type 1 diabetic rats, and inflammatory cytokines were downregulated by CS. Many other natural products have also been reported to relieve DOP through an anti-inflammatory manner (23, 27–29).

Both osteoblasts and bone marrow adipocytes were derived from bone marrow mesenchymal stem cells (30). As the number of

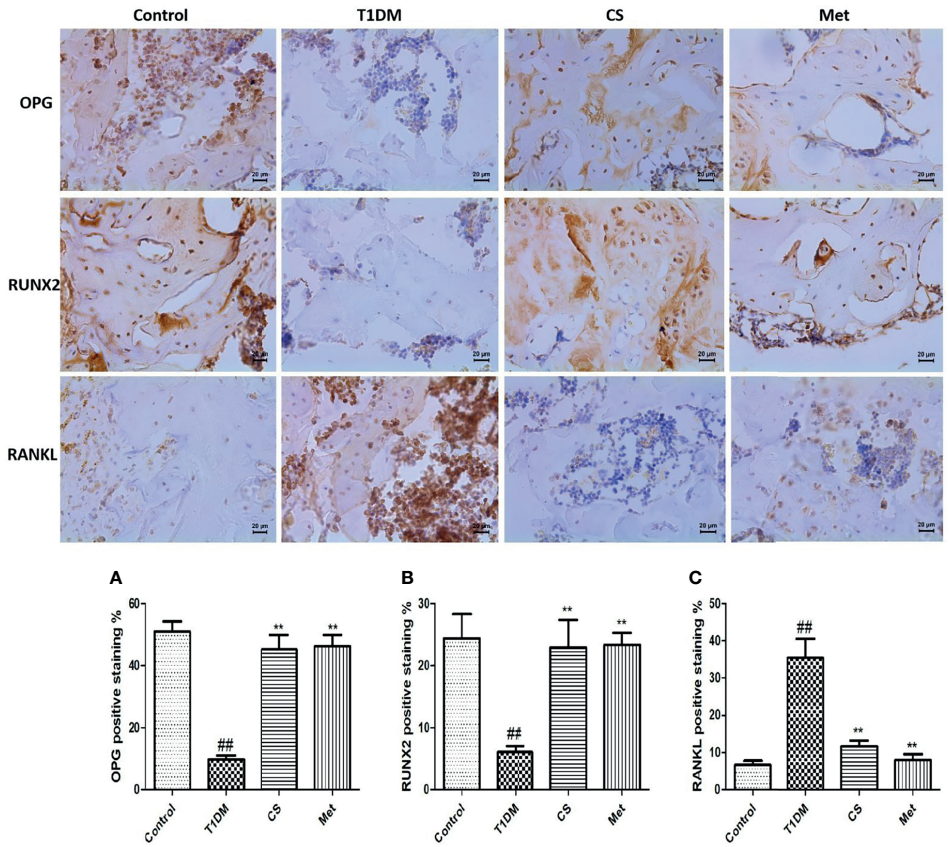

FIGURE 9 | Immunohistochemical staining of femur and positive staining percentage of OPG, RUNX2, and RANKL in each group. **(A)** The positive staining percentage of OPG in the femur of each group. **(B)** The positive staining percentage of RUNX 2 in the femur of each group. **(C)** The positive staining percentage of RANKL in the femur of each group. ##indicates $p < 0.01$ compared with control. **indicates $p < 0.01$ compared with the diabetic group.

bone marrow adipocytes increased, that of osteoblasts will decrease. Changes in the number and size of bone marrow adipocytes were positively correlated with osteoporosis, so they were important criteria for evaluating the efficacy of osteoporosis drugs (31–33). In diabetes, hyperglycemia promotes the differentiation of bone marrow adipocytes and inhibits osteoblast differentiation (34). As indicated in the present study, the bone marrow adipocyte number and size in type 1 diabetic rats were higher than those in control group rats, which were downregulated by 10 weeks of CS administration. Our previous study also found that trace elements of zinc, black rice anthocyanin, and lycopene were all having a preventive effect on DOP, and all of them can inhibit bone marrow adipocyte generation (4, 23, 35), which is consistent with the results of this study.

Bone tissue structure is an important basis for evaluating bone health (36). In this study, we applied bone micro-CT and bone tissue pathology techniques to evaluate bone structure; the results indicated that CS can improve bone structure lesions caused by diabetes, and it can effectively restore bone morphological parameters and upregulate BMD.

The biochemical markers of bone turnover (BMBT) were metabolite of bone tissue. BMBT includes bone formation and bone resorption markers, which were important indicators for laboratory diagnosis of osteoporosis (37, 38). Not only can it quickly reflect the process of bone formation and bone resorption, but it also can be used to reveal pathogenesis of metabolic bone disease, predict the rate of bone loss and fracture risk, and diagnose osteoporosis; at the same time, it can be used to quickly reflect the therapeutic effect of anti-osteoporosis drugs (39). In our present study, the bone formation markers (osteocalcin and ALP) as well as bone resorption markers (TRACP 5b and CTX 1) all increased in type 1 diabetic rats, which indicated that diabetic rats had a higher bone turnover rate. This study showed that CS could downregulate the BMBT in diabetic rats and inhibit the higher bone turnover rate.

RANKL was a member of the TNF superfamily involved in immune regulation and bone metabolism, and it was an important activator of osteoclast differentiation and maturation (40). RANKL activates osteoclast differentiation, thus promoting bone resorption by competing with OPG to bind to receptor RANK. Overexpression of RANKL can lead to excessive activation of osteoclasts, which can lead to osteoporosis (41). OPG was also a member of the TNF superfamily. It was highly expressed in testis and bone marrow (42). OPG binds to RANKL and blocks binding of RANKL to RANK. At the same time, OPG can inhibit osteoclast

differentiation and maturation, and promote osteoclasts apoptosis (43). When the ratio of OPG to RANKL increases, bone formation activity of osteoblasts increases, and bone metabolism tends to be in a positive balance. When the ratio of OPG to RANKL decreases, the bone resorption activity of osteoclasts increases, and bone metabolism tends to be in a negative balance (44). In the present study, the level of OPG/RANKL ratio in diabetic rats was greatly decreased compared with control, indicating increased bone resorption activity; after 10 weeks of CS treatment, the OPG/RANKL ratio was upregulated, indicating increased bone formation activity. One of the mechanisms of CS against DOP is by upregulating the OPG/RANKL ratio. In this study, we detected the bone OPG, RANKL, and RUNX 2 proteins using an immunohistochemical method, which has the advantage of localizing the protein, but in a quantitative aspect, it is inferior to Western blotting; in subsequent experiments, we will supplement the Western blotting experiment to confirm the above results.

As a first-line drug for diabetes, metformin is a chemically synthesized drug. The main side effects are gastrointestinal reactions, such as nausea and vomiting (45), as well as gut microbiota dysbiosis (46). Secondly, metformin will interfere with the absorption of vitamin B12 and folic acid. Studies have shown that up to 30% of patients taking metformin have B12 deficiency (47, 48). In this study, CS showed a good anti-DOP effect, similar to metformin. However, the CS used in this study was naturally extracted and has the advantages of high safety and less side effects compared with metformin. This study revealed the role of CS in DOP treatment through animal experiments. Later, we will further reveal its molecular mechanism through *in vitro* cell experiments and further confirm its effect through human experiments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Shaanxi University of Technology Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

Study design: SQ, HZ, DC, TY, and XL. Study conduct: SQ, HZ, MS, ZS, YH, and SC. Data collection and analysis: SQ, HZ, MS, ZS, YH, and SC. Animal model construction: SQ and HZ. Histopathological experiment: SQ, MS, ZS, and SC. Manuscript draft: SQ. Manuscript revision: HZ. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Alendronate Use and Risk of Type 2 Diabetes: A Nationwide Danish Nested Case-Control Study

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Objective: A link has been proposed between glucose homeostasis and bone metabolism. Bisphosphonates are first-line treatment of osteoporosis, and we aimed to investigate whether the risk of developing type 2 diabetes was associated with prior use of alendronate.

Research Design and Methods: We conducted a population-based nested case-control study through access to all discharge diagnoses (ICD-10 system) from the National Danish Patient Registry along with all redeemed drug prescriptions (ATC classification system) from the Health Service Prescription Registry. All cases with a diagnosis of type 2 diabetes between 2008 and 2018 were matched on sex and age with 3 randomly selected controls by incidence-density sampling. Exposure was defined as ever use of alendronate and further grouped as effective and compliant use. ORs were calculated by conditional logistic regression analysis with adjustment for several confounders and test for trend for dose-response relationship.

Results: We included 163,588 patients with type 2 diabetes and 490,764 matched control subjects with a mean age of 67 years and 55% male subjects. The odds of developing type 2 diabetes were lower among ever users of alendronate (multiple adjusted OR: 0.64 [95% CI 0.62-0.66]). A test for trend suggested a dose-response relationship between longer effective use of alendronate and lower risk of type 2 diabetes.

Conclusion: These results suggest a possible protective effect of alendronate in a dose-dependent manner against development of type 2 diabetes.

Keywords: diabetes, type 2 diabetes, bone, alendronate, bisphosphonate

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INTRODUCTION

Type 2 diabetes and osteoporosis are emerging global health problems with increased morbidity and mortality and continuous demand for disease prevention and management (1, 2). Bisphosphonates, e.g. alendronate, are first-line treatment of osteoporosis; the treatment sufficiently suppresses bone resorption with few adverse effects (3).

Antiresorptive therapies, e.g. alendronate, were initially hypothesized to decrease insulin sensitivity by decreasing osteocalcin levels (4). Contrarily, current research is pointing towards a possible protective effect of alendronate on the risk of developing type 2 diabetes as well as reducing insulin consumption (5–9). In this population-based study we hypothesized that alendronate use was not associated with the development of incident type 2 diabetes and examined a potential dose-dependent relationship.

RESEARCH DESIGN AND METHODS

The STROBE statement guideline for reports of case-control studies was followed (a STROBE checklist can be found in **Supplemental Table S1**) (10).

Study Design and Setting

We conducted a population-based nested case-control study using information from Danish nationwide registries. Case subjects were people with type 2 diabetes, and controls were subjects without diabetes mellitus. For each case subject, 3 age- and gender-matched control subjects were randomly selected from the general population by incidence-density sampling (11). Exposure was use of alendronate prior to index date. As the time of data collection was set between January 1st 1998 and December 31st 2018. We estimated outcome (type 2 diabetes diagnosis) from January 1st 2008 until December 31st 2018 and exposure (alendronate use) from January 1st 1998 until end of exposure or index date.

Data Sources

Data were available and anonymized by Statistics Denmark (*Danmarks Statistik*, project identifier no. 703382) and were obtained through National Danish registers. All Danish citizens are assigned a 10-digit personal identification number (PIN) which ensures a complete medical history of all contacts to the Danish health care system and drug prescriptions for each individual (12–14). The unique PIN was anonymized and linked to all registries used in this study. All Danish citizens have equal access to full health care provided by the Danish National Health Service, which includes free access to hospitals and partial compensation of drug expenses. All authorized Danish research organizations can apply for access to the registries. An ethics committee approval is not required for epidemiological studies in Denmark, as we had no access to personally identifiable information. However, the registries are subject to control by the Danish Data Protection Agency.

Data on diagnoses were obtained from the Danish National Patient Registry (14). The registry covers all contacts to the hospitals on both in- and outpatient basis. The data include all relevant physician-assigned discharge diagnoses on individual level, coded according to the International Classification of Diseases, Tenth Revision (ICD-10).

Information on drug prescriptions were coded according to the Anatomical Therapeutical Chemical (ATC) classification and recorded from 1996 by the Danish National Health Service Prescription Registry (13, 15). To ensure adequate registration, we collected data from 1998.

Data on sex and date of birth as well as emigration and death (if applicable) were retrieved from the Danish Civil Registration system, which ensures high-fidelity subject identification and matching with respect to emigration and death (12, 16).

Participants

The study population included subjects alive and residing in Denmark with no emigration history on January 1st 2008. Index date was set as date of diabetes classification for case subjects and a "dummy" date was set for each control subject with respect to emigration and death, i.e. control subjects had to be at risk (alive and Danish resident) at the time of index date to be included. We excluded subjects with classified diabetes before January 1st 2008, those with type 1 diabetes and individuals of age below 50 years at index date (**Supplemental Figure S1**). A 50 years age cut off was chosen as the average age for menopause in Denmark is 51.7 years and the risk of osteoporosis increases afterwards (17). In addition, 12 subjects had misinformed death date and were excluded. Thus, the cohort included adult individuals with age ≥ 50 years without diabetes and new-onset type 2 diabetes between January 1st 2008 and December 31st 2018.

Identification of Type 2 Diabetes Case Subjects

In order to classify subjects with type 2 diabetes, we identified all subjects with diabetes mellitus between 2008-2018 either by any ICD-10 code (main or secondary) related to diabetes (E10, E11, E12, E13, E14, G63.2, H28.0, H36.0, M14.2, O24, R73) or by an ATC code of glucose-lowering drugs used in diabetes (A10A or A10B) based on a previously published algorithm (18–20). Thereby, all people with diabetes were defined either from a hospital visit or by prescription of glucose-lowering drugs. The diabetes diagnosis and concordance between actual use and prescription of diabetes related medications are in general high (21–26). The diabetes cohort was further classified in type 1 and type 2 diabetes. In Denmark, all patients with type 1 diabetes will eventually be in contact with the hospital and no other glucose-lowering drugs than insulin are recommended. Consequently, type 1 diabetes was defined by at least one E10 ICD-10 code (type 1 diabetes) and at least one A10A ATC code (insulins and analogues) and no A10B ATC code (blood glucose-lowering drugs exclusive insulins); all other individuals with diabetes were classified as type 2 diabetes.

Selection of Population-Based Control Subjects

Three control subjects without diabetes mellitus were randomly selected for each case subject and matched by sex and year of birth in order to ensure age and gender adjustment. The control subjects were selected using the incidence-density sampling technique, i.e. control subjects had to be alive and at risk of diabetes at the time the corresponding case was diagnosed with diabetes (time of case occurrence).

Exposure; Alendronate Use

All prescriptions in Denmark are logged, stored and linked to the unique civil registry number. The prescription database includes data on redeemed drugs and corresponding dates, doses and pack sizes according to the ATC classification system (27). Within the database, we identified all prescriptions of alendronate with the ATC code "M05BA04". For alendronate exposure, *ever* use (yes/no), *effective* use (cumulative drug dose), and *compliance* were recorded. *Ever* use was defined as any prescription of alendronate before the index date. Effective use was calculated using a Defined Daily Dose (DDD) of 10 mg, based on the World Health Organization Collaborating Centre for Drug Statistics Methodology. To calculate treatment duration, the number of daily doses at the last dispensation date was added to this date, and the date of first drug dispensation was subtracted. Compliance was then assessed using the medication possession ratio (MPR); by dividing the cumulative dose (DDDs) by the treatment duration. MPR was grouped in <0.5, 0.5-0.8 and ≥0.8, the latter being defined as compliant use.

Identification of Potential Confounding Factors

Potential and measurable risk factors related to type 2 diabetes and alendronate use were selected based on available literature. We identified potential confounders by means of ICD-10 and ATC codes in the period before index date starting from the 1st of January 1998 to index date (**Supplemental Table S2** for specifications).

As a proxy of smoking status, we used ICD-10 codes related to lung diseases, of which some were directly and others indirectly associated with tobacco exposure, as well as nicotine poisoning and psychiatric tobacco-related diagnoses (20). In addition, we identified ATC codes corresponding to treatments for tobacco dependence (ever), e.g. nicotine replacement therapy, or drugs for obstructive airway diseases (after the age of 40). Due to potential underestimation, we classified this factor as *heavy smoking*. We evaluated alcohol consumption by either one relevant ICD-10 or ATC code covering diseases and drugs with direct affiliation to alcohol, e.g. intoxication, alcohol abuse, alcoholic liver disease, alcoholic cardiomyopathy, alcoholic polyneuropathy, alcoholic gastritis, alcohol-induced pancreatitis or alcohol related psychiatric disorders etc. (28). We classified this factor as *alcohol abuse.* Obesity was evaluated by ICD-10 codes of obesity or use of anti-obesity pharmaceuticals by ATC codes. Information on chronic and acute pancreatitis were obtained from ICD-10 codes. Hyper- and hypothyroidism were assessed by either ICD-10 or ATC codes. Comorbidity was assessed by use of Charlson Comorbidity Index (CCI) (29) based on discharge diagnoses registered by ICD-10 codes (**Supplemental Table S3**).

Data on socioeconomic status was obtained from Statistics Denmark. We assessed income as the amount of DKK (Danish kroner) from the year preceding the year of index and adjusted for inflation to a 2018 level using the consumer price index from Statistics Denmark. Lastly, we converted the income to euro € at a rate of 1 € = 7.467 DKK (exchange rate December 2018) and grouped into quintiles for analysis. Marital status was available through the Danish Civil Registration System and assed from the year prior the year of index. It was defined and grouped according to the classification from Statistics Denmark: married, divorced, widowed or unmarried.

Statistical Analysis

Outcome and exposure were binary variables of type 2 diabetes (case vs. control) and alendronate use, respectively. Exposure was further grouped in categorical variables of duration intervals and compliance. Subject characteristics in tables are presented as numbers and percentages (%), means and standard deviations (SD), % and SD or medians and interquartile range (IQR). In addition, 95% confidence intervals (CI) were calculated, either from means of continues outcomes or proportions of binary outcomes and presented in the text. Unpaired t-test, Chi-square test and Wilcoxon Mann-Whitney median test were performed to compare continuous and dichotomous characteristics between cases and controls. A conditional logistic regression model was used to estimate the effect of alendronate exposure–*ever* use, *effective* use and *compliant* use, respectively–on type 2 diabetes as odds ratios (OR) with 95% CI. A trend test (conditional logic regression model) was performed on effective use, excluding non-users from the analysis to evaluate a possible dose-response relationship between longer duration of alendronate use and risk of type 2 diabetes. We conducted sensitivity analyses excluding heavy smokers, alcohol abusers, prior pancreatitis, glucocorticoid users, obese individuals and those with age above 65. All analyses were conducted in STATA 16.1 (StataCorp, College Station, Texas, US).

RESULTS

Study Population Characteristics

Supplemental Figure S1 presents a flow diagram of the study population selection process. A total of 654,352 individuals were included in the study (163,588 case subjects and 490,764 control subjects). The distribution of sex (55% male subjects) and mean age (66.7 years) were equal among cases and controls confirming a balanced matching. Descriptive subject characteristics can be found in **Table 1**. Subjects with type 2 diabetes were more likely to be heavy smokers, alcohol abusers and obese compared to controls. In addition, pancreatitis, hyperthyroidism, hypothyroidism and previous use of glucocorticoids were more prevalent among subjects with type 2 diabetes compared to control subjects. Lastly, people with type 2 diabetes had a higher degree of comorbidity compared to controls.

Regarding socioeconomic status, 2,935 subjects had unknown information on social status; of these, 59.11% were subjects with type 2 diabetes, and 40,89% were control subjects. Control subjects were more likely to be married than subjects with type 2 diabetes (62.62% [95% CI 62.48-62.75] vs 56.96% [95% CI 56.72-57.20]. In addition, control subjects had a higher income before index date compared to people with type 2 diabetes.

TABLE 1 | Characteristics of case subjects (type 2 diabetes) and control subjects.

	All subjects n = 654,352	Type 2 diabetes n = 163,588	Control subjects n = 490,764	*P*-value*
Age (years), mean ± SD	66.67 ± 10.00	66.67 ± 10.00	66.67 ± 10.00	–
Age category (years), n (%)				–
50-59	198,452 (30.33)	49,613 (30.33)	148,839 (30.38)	–
60-69	231,028 (35.31)	57,757 (35.31)	173,271 (35.31)	–
70-79	161,268 (24.65)	40,317 (24.65)	120,951 (24.65)	–
≥ 80	63,604 (9.72)	15.901 (9.72)	47,703 (9.72)	–
Sex, % ± SD				–
Female	44.89 ± 0.50	44.89 ± 0.50	44.89 ± 0.50	
Male	55.11 ± 0.50	55.11 ± 0.50	55.11 ± 0.50	
Heavy Smoking, % ± SD	25.84 ± 0.44	32.69 ± 0.47	23.56 ± 0.42	< 0.01
Alcohol abuse, % ± SD	4.50 ± 0.21	6.40 ± 0.24	3.87 ± 0.19	< 0.01
Obesity, % ± SD	8.80 ± 0.28	17.14 ± 0.38	6.03 ± 0.24	< 0.01
Pancreatitis, % ± SD	0.67 ± 0.08	1.61 ± 0.13	0.36 ± 0.06	< 0.01
Hyperthyroidism, % ± SD	2.35 ± 0.15	2.96 ± 0.17	2.15 ± 0.14	< 0.01
Hypothyroidism, % ± SD	4.85 ± 0.21	6.0 ± 0.24	4.45 ± 0.21	< 0.01
Glucocorticoid use, % ± SD	26.88 ± 0.44	31.99 ± 0.47	25.17 ± 0.43	< 0.01
Hypertension	57.68 ± 0.49	76.43 ± 0.42	51.42 ± 0.50	< 0.01
CCI, mean ± SD	0.51 ± 1.18	0.88 ± 1.53	0.38 ± 1.00	< 0.01
CCI categories, n (%)				
0-0.99	490,586 (74.97)	96,372 (58.91)	395,214 (80.33)	< 0.01
1-1.99	75,546 (11.55)	30,758 (18.80)	44,788 (9.13)	< 0.01
≥ 2	88,220 (13.48)	36,458 (22.29)	51,762 (10.55)	< 0.01
Income, € in thousands, median (IQR)	30,9 (22,1-47,9)	28,6 (21,4-42,8)	32,1 (22,4-49,5)	<0.01
Income, € in thousands				
1st Quintile, median (IQR)	16,4 (14,1-18,3)	16,3 (14,0-18,2)	16,4 (14,2-18,3)	<0.01
2nd Quintile, median (IQR)	23,9 (22,2-25,4)	24,0 (22,2-25,5)	23,9 (22,1-25,4)	<0.01
3rd Quintile, median (IQR)	30,9 (28,7-33,9)	31,0 (28,6-33,6)	31,0 (28,8-34,0)	<0.01
4th Quintile, median (IQR)	43,9 (40,4-47,9)	44,0 (40,2-48,0)	44,0 (40,4-47,9)	<0.01
5th Qiuntile, median (IQR)	66,2 (58,2-83,1)	64,8 (57,6-80,3)	66,4 (58,3-83,8)	<0.01
Marital status, n (%)				
Married	400,477 (61.20)	93,176 (57.96)	307,301 (62.62)	<0.01
Divorced	65,984 (10.08)	17,781 (10.87)	48,203 (9.82)	<0.01
Unmarried	91,784 (14.03)	25,687 (15.70)	66,097 (13.47)	<0.01
Widowed	93,172 (14.24)	25,209 (15.41)	67,963 (13.85)	<0.01
Unknown	2,935 (0.45)	1,735 (1.06)	1,200 (0.24)	<0.01

*All characteristics were evaluated in the time from 1998 until index date. Data are presented as numbers (n, %), mean with SD or median with IQR. *P-values represent analyses by Chi2-test or Wilcoxon Mann-Whitney median test, significance level was set at 5%.*

Lastly, a higher proportion of control subjects were in the 5th income quintile compared to type 2 diabetes [21.72% (95% CI 21.60-21.83) vs 14.85% (96% CI 14.68-15.03)].

Characteristics of Alendronate Users

Descriptive characteristics of people exposed vs unexposed to alendronate can be found in **Supplemental Table S4**. In total, we identified 31,976 users of alendronate prior to or at index date with a median exposure time of 2.55 years (IQR 0.75-5.26). Of these, 25,169 were control subjects and 6,807 were type 2 diabetes patients corresponding to 5.13% (95% CI 5.07-5.19) and 4.16% (95% CI 4.06-4.26), respectively. Median exposure time was 2.31 years (IQR 0.68-4.98) for type 2 diabetes patients and 2.61 years (IQR 0.78-5.32) for control subjects. In total, 20,786 subjects (65.01%) were still users of alendronate at index date with a higher proportion among control subjects; corresponding to 66.32% (95% CI 65.73-66.90) of control subjects and 60.14% (95% CI 58.97-61.31) of type 2 diabetes subjects.

The proportion of females with alendronate use was in general higher than males with alendronate use [8.95% (95% CI 8.84-9.05) vs 1.58% (95% CI 1.54-1.62)]. However, the proportion of alendronate users was lower among female type 2 diabetes subjects than among female control subjects [78.60% (95% CI 77.60-79.56) vs 83.15% (95% CI 82.68-83.61)]. The highest percentage of alendronate users was found in the age group 70-79 years (39.25%) in both patients with type 2 diabetes (38.64%) and control subjects (39.42%). However, male users of alendronate were younger than female users [mean age in years: 67.11 (95% CI 66.86-67.35) vs 70.21 (95% CI 70.09-70.32)].

Ever Use of Alendronate

The ORs for developing incident type 2 diabetes after alendronate use are presented in **Table 2**. Patients with type 2 diabetes were less likely than matched control subjects to have ever used alendronate and the association became more pronounced after multiple adjustment. The crude OR was significantly lower among those who used alendronate at index date compared to those who had stopped prior to index date [OR: 0.82 (95% CI 0.73-0.93)] but became insignificant after adjustment (OR: 0.94 [95% CI 0.81-1.09]).

Stratification by sex and the ORs of incident type 2 diabetes are presented in **Table 3**. Female subjects were more likely to

TABLE 2 | Risk of type 2 diabetes presented as crude and adjusted ORs grouped in ever, effective and compliant users.

	Cases, n (%) n = 163,588 (100)	Controls, n (%) n = 490,764 (100)	Crude OR (95% CI) (age/sex-match)	Adjusted OR (95% CI) All confounders¥
Never users of alendronate	156,781 (95.84)	465,595 (94.87)	1.00 (ref.)	1.00 (ref.)
Ever users of alendronate	6,807 (4.16)	25,169 (5.13)	**0.79** (0.77-0.81)	**0.64** (0.62-0.66)
Effective use				
< 6 months	1,657 (1.01)	5,563 (1.09)	1.00 (ref.)	1.00 (ref.)
0.5-1.9 years	1,945 (1.19)	6,751 (1.38)	0.93 (0.87-1.01)	0.94 (0.87-1.02)
2-3.9 years	1,422 (0.87)	5,605 (1.14)	**0.82** (0.76-0.89)	**0.87** (0.80-0.95)
4-5.9 years	827 (0.51)	3,656 (0.74)	**0.73** (0.66-0.80)	**0.79** (0.72-0.88)
6-7.9 years	516 (0.32)	2,003 (0.41)	**0.83** (0.74-0.93)	0.89 (0.79-1.00)
>8 years	440 (0.27)	1,791 (0.36)	**0.78** (0.70-0.88)	**0.84** (0.74-0.95)
Compliant use				
MPR < 0.5	564 (8.29)	1,750 (6.95)	1.00 (ref.)	1.00 (ref.)
MPR 0.5-0.8	1,090 (16.01)	3,835 (15.24)	**0.88** (0.79-0.99)	0.93 (0.82-1.05)
MPR > 0.8	5,153 (75.70)	19,584 (77.81)	**0.82** (0.74-0.90)	0.90 (0.80-1.00)

Conditional logistic regression analysis of ORs (95% CI) for development of type 2 diabetes when exposed to alendronate.
¥Adjusted for smoking, alcohol, obesity, pancreatitis, hypothyroidism, hyperthyroidism, use of glucocorticoids, CCI, income and marital status. Estimates in bold represent p < 0.05.

TABLE 3 | Risk of type 2 diabetes stratified by sex.

	Females		Males	
	Crude OR	Adjusted OR	Crude OR	Adjusted OR
Never users	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref)
Ever users	**0.74** (0.72-0.76)	**0.60** (0.58-0.62)	1.03 (0.97-1.10)	**0.71** (0.67-0.77)
Effective use				
< 6 months	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
0.5-1.9 years	0.95 (0.87-1.03)	0.96 (0.87-1.05)	0.90 (0.77-1.05)	0.90 (0.77-1.06)
2-3.9 years	**0.82** (0.75-0.90)	**0.88** (0.87-0.97)	0.87 (0.73-1.03)	0.87 (0.73-1.05)
4-5.9 years	**0.75** (0.68-0.84)	**0.82** (0.74-0.92)	**0.70** (0.56-0.87)	**0.72** (0.57-0.90)
6-7.9 years	**0.84** (0.74-0.95)	0.89 (0.78-1.01)	0.91 (0.69-1.19)	0.96 (0.73-1.28)
>8 years	**0.83** (0.73-0.94)	0.88 (0.77-1.01)	**0.70** (0.51-0.98)	0.73 (0.51-1.03)
Compliant use				
MPR < 0.5	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
MPR 0.5-0.8	**0.85** (0.74-0.96)	0.91 (0.79-1.04)	**0.88** (0.79-0.99)	0.93 (0.82-1.05)
MPR > 0.8	**0.80** (0.71-0.89)	0.90 (0.79-1.01)	**0.82** (0.74-0.90)	0.90 (0.80-1.00)

Conditional logistic regression analysis of OR (95% CI) for development of type 2 diabetes when exposed to alendronate.
¥Adjusted for smoking, alcohol, obesity, pancreatitis, hypo- and hyperthyroidism, use of glucocorticoids, CCI, income and marital status. Estimates in bold represent p < 0.05.

ever use alendronate compared to male subjects, and the odds for type 2 diabetes were correspondingly lower among female ever users (**Table 3**).

In an analysis including all used types of oral administrated bisphosphonates, i.e. etidronate (ATC-code M05BA01), risedronate (ATC-code M05BA07), ibandronate (ATC-code M05BA06) and pamidronate (ATC-code M05BA03), the results did not change [crude OR: 0.79 (95% CI 0.77-0.81), adjusted OR: 0.63 (95% CI 0.61-0.65)].

Alendronate Duration and Compliance

Table 2 presents crude and adjusted ORs for *effective* use (<6 months as reference) and *compliant* use (MPR<0.5 as reference), which are also illustrated in **Figure 1**. The ORs for incident type 2 diabetes decreased with longer effective use and the lowest OR was found among effective alendronate use of 4-6 years compared to those with less than 6 months of use. The trend test revealed a dose-response relationship between longer effective use in years and lower risk of type 2 diabetes (p<0.007). Additionally, a trend towards a more pronounced association with compliant users were observed (MPR>0.8, p=0.052). The risk of type 2 diabetes was lower among those who used alendronate <6 months compared to never users of alendronate [adjusted OR: 0.70 [95% CI 0.66-0.74)].

In female subjects, ever use of alendronate was significantly associated with a decreased OR after 2-6 years of use (**Table 3**). In male subjects, use of alendronate was associated with a decreased OR after 4-6 years. In addition, the crude ORs were significantly lower in compliant female alendronate users compared to non-compliant users, however, the ORs became insignificant after multiple adjustment.

Sensitivity Analyses

In sensitivity analyses with the exclusion of obese subjects, alcohol users, pancreatitis, steroid users or heavy smokers ever use of alendronate still revealed a lower OR of incident type 2 diabetes. In particular, the OR decreased further when excluding heavy smokers (adjusted OR: 0.59; 95% CI 0.57-0.62). Similarly, when excluding all individuals aged above 65 years (n=339,604), the OR remained significantly low [adjusted OR: 0.56 (95% CI

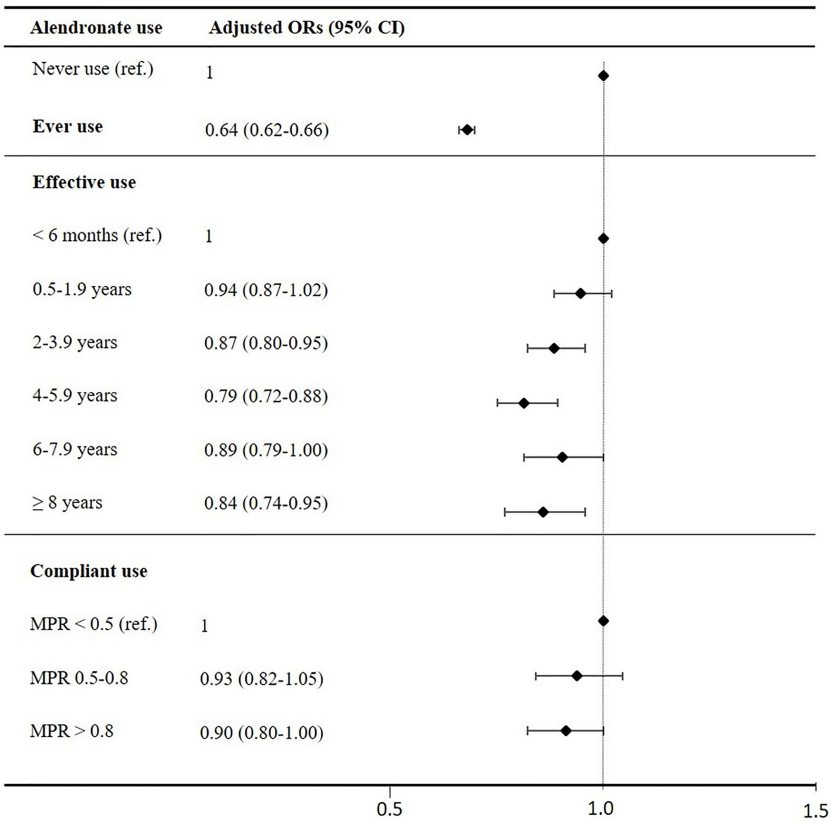

FIGURE 1 | Adjusted ORs for development of type 2 diabetes. ORs are adjusted for heavy smoking, alcohol abuse, obesity, pancreatitis, hyperthyroidism, hypothyroidism, steroid use, CCI, income, social status and presented with lower and upper CI 95% as error bars. The lowest categories (never users, effective users with use below 6months and compliant users with MPR below 0.5) as reference line (OR=1).

0.52-0.61)]. Stratification by hypertension status revealed a lower OR among people without hypertension [adjusted OR: 0.53 (95% CI 0.47-0.59)]. No change in OR was found between people with and without dyslipidemia.

DISCUSSION

In this large nationwide nested case-control study, we found that patients with type 2 diabetes were less likely than matched control subjects to have ever used alendronate. The largest risk reduction observed was almost 40% among ever users of alendronate compared to non-users. In addition, we present a significant and prominent dose-dependent association between longer effective use and decreased risk of type 2 diabetes. To our knowledge, this is the first case-control study investigating the association between alendronate use and type 2 diabetes.

The risk of incident type 2 diabetes was significantly lower among those with less than 6 months of alendronate use compared to never users. This may suggest that alendronate acts somewhat promptly on glucose metabolism, with its effects becoming more prominent after several years. We chose to only include alendronate as exposure, as this is first line recommendation and the most frequent used bisphosphonate in both research and clinics settings. Few clinical trials have investigated the association between alendronate use and glucose metabolism. Fard et al. conducted a randomized controlled trial with 60 postmenopausal women aged 45-60 years enrolled to receive either 70 mg alendronate per week or placebo for 12 weeks (5). They found reduced fasting plasma glucose, insulin concentration and increase in insulin sensitivity measured by the Matsuda Index in the alendronate group (both compared to baseline and to the control group). This short 12-week intervention period confirms our finding of a possible protective effect already after 6 months. How alendronate influences on glucose metabolism is not clarified. *In vitro* studies suggest that alendronate decreases adipogenesis and activates lipolysis (30, 31), conditions that may be altered in subjects with decreased insulin sensitivity. Furthermore, a bone-resorption-specific impact on insulin signaling by Osteocalcin has been suggested, though the evidence for an effect in humans is very limited and the hypothesis is based on an animal model (32). Schwartz et al. performed *post hoc* analyses of three randomized controlled trials with 3-4 years of follow-up but did not find any changes in diabetes incidence after treatment with alendronate, zoledronic acid or denosumab (33). However,

the daily administration of alendronate was only 5 mg during the first 2 years and increased to 10 mg for the last 2 years. According to our dose-dependent findings 5 mg daily may be an inadequate dose to reveal a significant effect of alendronate on fasting blood glucose and risk of type 2 diabetes.

In Denmark, it is recommended to re-evaluate treatment after 5 years to consider discontinuing based on BMD evaluation. Individuals using alendronate for a longer time were in general more comorbid estimated by a CCI. It is possible that individuals with osteoporosis but without pre-diabetes have a higher possibility of discontinuing treatment after 5 years compared to those with greater risk of type 2 diabetes as a result of potentially healthier bones. This may explain why the OR increased in the 6-8 years exposure group. Unfortunately, we did not have access to neither blood samples nor bone scans and consequently no measures of hemoglobin A1c or BMD were available. However, we found that the risk of developing type 2 diabetes were lower among those who continued alendronate treatment, suggesting a possible sustained protection together with long term effects, corresponding to a known long half-life of alendronate (34).

Although research concerning alendronate and type 2 diabetes are conflicting, our results are consistent with several previous studies. Vestergaard et al. conducted a cohort study on 103,562 individuals exposed to alendronate and found an OR of 0.69 (95% CI 0.57-0.83) for type 2 diabetes after adjustment for corticosteroid use (6). In addition, the study reported a decreasing risk of developing type 2 diabetes with increasing doses of alendronate. Another cohort study by Toulis et al. suggested a 50% risk reduction of type 2 diabetes among users of alendronate for more than 1 year compared to those who did not use alendronate (9). We chose to include all with a prescription of alendronate as exposed individuals and found significantly lower adjusted odds for diabetes after 2 years (compared to those with less than 6 months of use). In addition, we were able to adjust for several relevant confounders, including heavy smoking, alcohol abuse, socioeconomic status and several other comorbidities and medication uses.

One notable strength in the present study is the high quality and validity of the Danish National Registers based on the unique identification number assigned to all Danish citizens (12, 14, 35, 36). Furthermore, the identification of people diagnosed with diabetes in Denmark was nationwide without any selection bias. We present data from a large cohort that enabled us to match exactly 3 controls to every case randomly by incidence-density sampling on age and gender to eliminate bias and ensure uniform risk and exposure time. We expected subjects exposed to alendronate to be relatively unhealthy compared to those who did not receive alendronate due, for instance, to risk factors for osteoporosis (37), e.g. smoking as presented in **Supplemental Table S4**. Contrarily, it is possible that people with osteoporosis and relatively longer exposure duration are healthier, have higher tolerance for alendronate, and may have lower BMI (38) and, consequently, lower risk of type 2 diabetes. This may give rise to healthy survivor bias as seen in many previous cohort studies, and so we chose a case-control setup to minimize that bias. Our sensitivity analyses suggest that the risk of type 2 diabetes decrease further when heavy smokers and people with hypertension are excluded from the analysis. Smoking is a risk factor for type 2 diabetes but as are obesity, alcohol and pancreatitis of which the ORs did not change after sensitivity analyses. It may be that smoking impacts on alendronates mechanism of action, as has been addressed recently (39), although this warrants further research.

One important limitation of this case-control study is the retrospective design which hinders the inference of causation between alendronate exposure and type 2 diabetes. Another limitation is the data collection and diabetes classification. We excluded people from the type 2 diabetes group if they had ever received an E10 (Type 1 diabetes mellitus) diagnosis and no glucose-lowering drugs other than insulins (ATC A10B). Thus, people initially misdiagnosed as having type 1 diabetes or possibly severe cases of type 2 diabetes were lost in this investigation. All Danish citizens with type 1 diabetes will eventually be in contact with the hospital and will thereby be given an ICD-10 E10 code. In contrast, general practitioners outside the hospital will most often be responsible for treatment of people with type 2 diabetes. Thus, only complicated cases of type 2 diabetes will be treated in the hospital and receive an ICD-10 E11 (type 2 diabetes mellitus) code. However, individuals who have never been in contact with the hospital but have ever received A10B medications were classified as type 2 diabetes and included in the cohort. By this classification, we were unable to identify naïve or mild cases of type 2 diabetes, e.g. people who have never received an ICD code or glucose-lowering drugs and have thus been treated with life-style interventions only. Although the Danish registries contain a wide range of information, we did not have access to over-the-counter-medicine, e.g. vitamin D supplementation, and we were unable to correct for lifestyle factors such as diet and exercise, which might have been associated with our identified outcome and exposure. In addition, the registries did not include data on smoking habits and alcohol consumptions; however, we estimated some of these baseline characteristics by ICD-10 and ATC codes. Consequently, we only obtained these characteristics from those with already developed concomitant disease or with prescribed medical therapy. We have information on diagnoses of adiposity, but not information on BMI in the included people. The cohort consists of divergent patient groups and people with osteoporosis may have a lower BMI with possible protection against development of type 2 diabetes (40), a bias that may lead to confounding by indication.

This study supports the hypothesis of an interaction between glucose homeostasis and bone metabolism. It is possible that people with osteoporosis and increased risk of type 2 diabetes could benefit from alendronate concerning the risk of developing type 2 diabetes after osteoporosis diagnosis. In addition, it is still unclear whether other anti-resorptive therapies, e.g., denosumab, show similar tendencies.

In conclusion, our data support previous studies suggesting a possible protective effect of alendronate on development of type 2 diabetes in a dose-dependent manner. In addition, it seems that smoking may suppress this protective effect. However, the underlying mechanism needs further exploration, and so we propose future research to prospectively evaluate glucose

metabolism in people with and without type 2 diabetes exposed to alendronate.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: All authorized Danish research organizations can apply for access to the registries. An ethics committee approval is not required for epidemiological studies in Denmark, as we had no access to personally identifiable information. However, the registries are subject to control by the Danish Data Protection Agency. Requests to access these datasets should be directed to https://www.dst.dk/en.

AUTHOR CONTRIBUTIONS

All authors contributed to the article according to the ICJME requirements for co-authorship. All authors had full access to all data used in the study, critically revised the paper for intellectual content and approved submitted versions and the final version of the paper. RV and PV designed the study. RV performed data management and statistical analyses with assistance from PV. RV and PV interpreted the data. RV wrote the paper. ZA-M and JS-L made critical revisions of data management, design, data interpretation and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.771426/full#supplementary-material

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Hemoglobin A1c Threshold for Reduction in Bone Turnover in Men With Type 2 Diabetes Mellitus

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Background: Emerging data suggest that type 2 diabetes mellitus (T2D) is associated with an increased risk for fractures despite relatively normal or increased bone mineral density (BMD). Although the mechanism for bone fragility in T2D patients is multifactorial, whether glycemic control is important in generating this impairment in bone metabolism remains unclear. The purpose of our study is to identify a hemoglobin A1c (A1c) threshold level by which reduction in bone turnover begins in men with T2D.

Method: A cross-sectional analysis of baseline data was obtained from 217 men, ages 35–65, regardless of the presence or absence of hypogonadism or T2D, who participated in 2 clinical trials. The following data were obtained: A1c by HPLC, testosterone and estradiol by LC/MS, bone turnover markers Osteocalcin [OC], C-terminal telopeptide [CTx], and sclerostin by ELISA, and BMD by DXA. Patients were grouped into 4 categories based of A1c (group I: <6%, group II: 6.0–6.4%, group III: 6.5–6.9%, and group IV: ≥7%). Threshold models were fit to the data using nonlinear regression and group comparisons among the different A1c categories performed by ANOVA.

Results: Threshold model and nonlinear regression showed an A1c cut-off of 7.0, among all choices of A1cs, yields the least sum of squared errors. A comparison of bone turnover markers revealed relatively lower OC ($p = 0.002$) and CTx ($p = 0.0002$) in group IV (A1c ≥7%), compared to the other groups. An analysis of men with T2D ($n = 94$) showed relatively lower OC ($p=0.001$) and CTx ($p=0.002$) in those with A1c ≥7% compared to those with <7%, respectively. The significance between groups persisted even after adjusting for medications and duration of diabetes.

Conclusion: An analysis across our entire study population showed a breakpoint A1c level of 7% or greater is associated with lower bone turnover. Also in men with T2D, an A1c ≥7% is associated with low bone turnover.

Keywords: Hemoglobin A1c, type 2 diabetes mellitus, bone turnover markers, CTX, osteocalcin

INTRODUCTION

It is well-recognized that patients with type 2 diabetes mellitus (T2D) are at an increased risk for fractures despite normal or relatively higher BMD (1–3). Although there are multiple mechanisms hypothesized as contributors to bone fragility in patients with T2D such as poor glucose control, accumulation of advanced glycation end products (AGEs), use of antidiabetic medications, and the presence of microvascular complications (2, 4), it is well-established that these individuals have suppressed bone turnover, primarily reduced bone formation (5–7). At this juncture, it remains unclear how glycemic control by itself influences bone turnover in the presence or absence of antidiabetic medications (6, 8). *In vitro* studies have shown that hyperglycemia is toxic to the osteoblast leading to reduced bone formation (8), which ultimately also results in reduced bone resorption. It is recommended by the ADA to maintain an A1c of less than 7% to prevent microvascular and other complications from diabetes; however, there is no data that identifies an A1c threshold level for impairment in bone metabolism in patients with T2D. The bone is a dynamic organ and changes in bone turnover precede alterations in bone structural and biomechanical properties. The objective of this study is to identify the hemoglobin A1c (A1c) threshold by which reduction in bone turnover begins in the entire population of male volunteers and subsequently analyze the effect of this threshold on bone turnover in men with T2D. We hypothesize that the reduction in bone turnover is influenced by glycemic control; those with worse control will have greater suppression in bone turnover, while well-controlled diabetics will have bone turnover markers similar to non-diabetics. Furthermore, we hypothesize that there is a cut-off of A1c level at which this abnormality in bone turnover commences and above which bone turnover markers will show significant reduction.

METHODS

Study Population

This is a secondary analysis using the baseline data from 2 clinical trials in veterans who volunteered to be screened for the study evaluating the effect polymorphisms in the CYP19A1 gene on the response to testosterone therapy in men with hypogonadism (NCT: 01378299) between October 2011 to November 6, 2016 (9, 10) and part of the population of men who screened between May 2018 to October 2019 for the study on the effect of aromatase inhibitors and weight loss in obese men with hypogonadism (NCT: 03490513) (11, 12). There were 105 men from the former and 112 from the latter study were included in this analysis. Inclusion criteria in both studies have been published elsewhere, but briefly, the first one included men between 40 and 75 y who have an average fasting total testosterone (T) level from 2 measurements taken between 8 and 11 AM on 2 occasions 30 min apart of less than 300 ng/dl and with no medical problems that may prevent them from finishing the study. Exclusion criteria included: treatment with bone-acting drugs (e.g., bisphosphonates, denosumab, teriparatide, glucocorticoids, sex-steroid compounds, selective estrogen receptor modulators, androgen deprivation therapy, and anticonvulsants) and finasteride. Additional exclusion criteria included: osteoporosis and history of fragility fractures or diseases known to affect bone metabolism such as: hyperparathyroidism, chronic liver disease, uncontrolled or untreated hyperthyroidism, and significant renal impairment (creatinine of >1.5 mg/dl). Those with a history of prostate cancer, breast cancer, and untreated sleep apnea also met the criteria for exclusion. The inclusion criteria for the second study include men between 35 and 65 y with BMI of 35 kg/m^2 or more who have an average fasting total T level from 2 measurements of less than 300 ng/dl, taken between 8 and 10 AM on 2 separate days within 1 month and with symptoms consistent with androgen deficiency as assessed by the quantitative Androgen Deficiency in Aging Male (qADAM) questionnaire. LH should be less than 9.0 mIU/L and estradiol (E2) should be 14 pg/ml or more. Since the primary study includes lifestyle intervention to promote weight loss by dietary behavioral modification and supervised exercise program in addition to either aromatase inhibitors or placebo, those with: 1) cardiopulmonary disease (e.g., recent myocardial infarction or MI defined as MI within 6 months at the time of study entry, unstable angina, and stroke) or unstable disease (e.g., NYHA Class III or IV congestive heart failure), severe pulmonary disease requiring steroid pills or the use of supplemental oxygen (that would contraindicate exercise or dietary restriction), unstable weight (i.e., ± 2 kg) in the last 3 months, and diabetes mellitus with a fasting blood glucose of more than 160 mg/dl, and/or Hemoglobin A1c (A1c) more than 9.5% were excluded from participation and clinical/biochemical evidence of pituitary or hypothalamic disease. The rest of exclusion criteria were as the first study as detailed above. Based on the review of baseline data of participants from the above two studies, our study included a mix of hypogonadal (n = 134) and non-hypogonadal men (n = 83).

All participants provided written informed consent in accordance with the guidelines in the Declaration of Helsinki for the ethical treatment of human subjects. The study was conducted at the New Mexico VA Health Care System and at the Michael E. DeBakey VA Medical Center. The protocol was approved by the Institutional Review Boards of the University of New Mexico and of the Baylor College of Medicine. Participants were recruited from patients attending the Endocrine, Urology and Primary Care Clinics of the New Mexico VA Health Care System and the Michael E. DeBakey VA Medical Center. Recruitment was accomplished either through flyers or letters to physicians about patients who may qualify for the study.

Bone Mineral Density (aBMD) by Dual Energy X-Ray Absorptiometry (DXA)

BMD was measured by DXA of lumbar spine and proximal femur using Hologic Discovery (Hologic Inc, Bedford, MA, USA). Regions of interest in the lumbar spine include L1–L4 vertebrae while that of the femur include the total hip and femoral neck. The coefficients of variation (CV) at our center are ~1.1% for the lumbar spine and 1.2% for the proximal femur (13).

Type 2 Diabetes Mellitus

The presence of T2D was ascertained from diagnosis in the chart, the intake of medication for T2D, Hemoglobin A1c values ≥6.5% and fasting blood glucose of ≥126 mg/dl. Definition of T2D was made if at least of one these rules is present (6, 10).

Biochemical Measurements

Fasting blood samples were collected at baseline; serum samples were extracted and stored at −80°C until analysis. Baseline serum T levels represent an average of 2 determinations measured at the end of the study, by liquid chromatography/mass spectrometry (LC/MS) (Mayo Clinic Laboratories, Mayo Clinic, Rochester, MN). Testosterone intra-assay CVs are 7.4, 6.1, 9.0, 2.3 and 0.9% at 0.65, 4.3, 48, 118 and 832 ng/dl, respectively. Inter-assay CVs are 8.9, 6.9, 4.0, 3.6 and 3.5% at 0.69, 4.3, 45, 117 and 841 ng/dl, respectively. The detection range is 0.5–2,000 ng/dl. E2 was measured by LC/MS (Mayo Clinic Laboratories, Mayo Clinic, Rochester, MN) with assay sensitivity of 0.23 to 405 pg/ml, intra-assay CV of 1.4 to 11.8%, and inter-assay CV of 4.8 to 10.8% (6). A1c was assessed by high performance liquid chromatography (Tosoh G8, South San Francisco, CA, USA). Fasting glucose was measured using a Unicel *DxC 800* Auto-analyzer (Beckman Coulter, Fullerton, CA, USA). The following were measured using enzyme-linked immunosorbent assay kits: C-terminal telopeptide of type I collagen (CTx), marker of bone resorption (Crosslaps; Immunodiagnostic System Inc., Gaithersburg, MD), osteocalcin, marker of bone formation, (Metra OC; Quidel Corporation, San Diego, CA), and sclerostin (TECO medical Sclerostin HS Enzyme Immunoassay Kit, Quidel Corp, San Diego, CA). The coefficients of variation (CVs) for the above assays in our laboratory are <10% and <3.5% for A1c (6).

Statistical Analysis

Results are expressed as means ± SD. A p-value of 0.05 was considered statistically significant. A threshold model was fit to data using non-linear regression with a threshold as an adjustable parameter, optimal threshold A1c = 7%. Patients were grouped into 4 categories based of A1c levels (group I: <6%, group II: 6.0–6.4%, group III: 6.5–6.9%, and group IV: ≥7%). Group comparisons between those with T2D and those without T2D were analyzed using analysis of variance (ANOVA) without and with adjustments for covariates such as age, testosterone levels, estradiol levels, duration of T2D, and medications. Correlations among the different variables were analyzed by simple correlation analysis. The data were managed using Excel 2010 (Microsoft, Redmond, WA) and were analyzed using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Table 1 shows the clinical characteristics of the 217 male participants. The mean age of the participants was 55 ± 9 years old with mean BMI of 36.15 ± 6.44 kg/m^2, and mean A1c was 6.1 ± 1.5%. Among the 94 subjects with T2D, 15 were not on any medication, 23 were on metformin only, 10 were on insulin only, and 2 were on sulfonylurea only. A total of 29 subjects were on insulin in combination with other agents, the most common combination agent being Metformin (n = 30), followed by sulfonylurea (n = 9). Our study included 2 patients on sodium-glucose co-transporter 2 (SGLT2) inhibitors (Empagliflozin), 7 on Glucagon-like peptide-1 receptor agonsits (GLP1 agonists), 4 on Dipeptidyl peptidase IV (DDP4) inhibitors, 0 on Thiazolidinediones (TZDs), and 15 were on combination with different agents except for insulin.

Bone Turnover Markers

Entire Study Population

We hypothesized that there is an A1c cut-off above which bone turnover markers will show significant reduction. To determine A1c cut-offs for lower osteocalcin and CTx levels, we used a threshold model and nonlinear regression. This shows that an A1c cut-off of 7.0, among all choices of A1c cut-offs, yields the least sum of squared errors in each case (See **Figures 1A, B**).

TABLE 1 | Clinical characteristics of the study population according to hemoglobin A1c levels.

Hemoglobin A1c (%)	Group I <6.0 (n = 90)	Group II 6.0–6.4 (n = 48)	Group III 6.5–6.9 (n = 24)	Group IV ≥7.0 (n = 55)	P-value
Age (years)	*52.9 ± 9.5	54.6 ± 8.3	58.5 ± 8.7	58.0 ± 8.7	**0.003**
BMI (kg/m^2)	35.3 ± 6.4	37.6 ± 7.2	36.4 ± 4.7	36.2 ± 6.0	0.27
Whole Body BMD (g/cm^2)	1.152 ± 0.110	1.150 ± 0.132	1.182 ± 0.085	1.145 ± 0.110	0.41
Lumbar spine BMD (g/cm^2)	1.103 ± 0.148	1.132 ± 0.149	1.146 ± 0.127	1.139 ± 0.172	0.85
Total Hip BMD (g/cm^2)	1.088 ± 0.143	1.111 ± 0.127	1.108 ± 0.093	1.117 ± 0.135	0.95
Femoral Neck BMD (g/cm^2)	0.888 ± 0.157	0.932 ± 0.153	0.864 ± 0.106	0.882 ± 0.150	0.19
Osteocalcin (ng/ml)	6.92 ± 4.30	6.10 ± 3.18	6.26 ± 2.89	**3.90 ± 2.64	**0.002**
CTx (ng/ml)	0.33 ± 0.17	0.34 ± 0.19	0.30 ± 0.14	**0.18 ± 0.12	**0.0002**
Sclerostin (ng/ml)	0.73 ± 0.27	0.73 ± 0.21	0.77 ± 0.24	0.75 ± 0.21	0.87
Testosterone (ng/dl)	301.74 ± 119.18	295.50 ± 113.74	273.14 ± 92.75	275.73 ± 81.64	0.44
Estradiol (pg/ml)	23.79 ± 24.70	25.58 ± 15.20	20.38 ± 18.32	22.20 ± 14.60	0.79
25-hydroxyvitamin D (ng/ml	26.07 ± 9.31	24.76 ± 10.41	24.33 ± 7.84	25.85 ± 11.59	0.82
PTH (pg/ml)	56.84 ± 28.76	54.01 ± 24.07	55.66 ± 22.47	47.41 ± 29.74	0.30

*BMI, body mass index; CTx, C-telopeptide; BMD, bone mineral density; PTH, parathyroid hormone; values for BMD adjusted to age, BMI and testosterone levels; post-hoc analysis for significant comparisons: *p group I vs. III and IV, **group IV vs I, II and III. Bolded p-values are significant.*

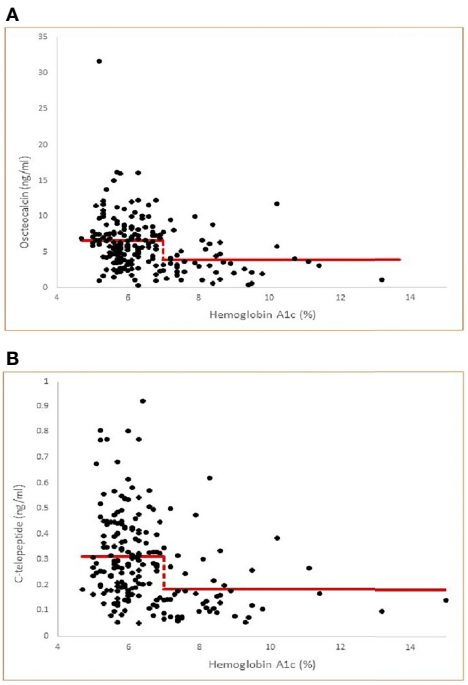

FIGURE 1 | **(A)** Threshold model of Osteocalcin versus Hemoglobin A1c by nonlinear regression (multiple correlation r=0.31, p<0.001) with least squares fitted (optimal) threshold A1c=7.0. Model Osteocalcin values= 6.57 for A1c< 7.0 and 3.86 for A1c ≥ 7.0. The threshold model is statistically significant compared to a null hypothesis of no threshold (P<0.001). **(B)** Threshold model of C-telopeptide versus Hemoglobin A1c by nonlinear regression (multiple correlation r=0.31, p<0.001 with least squares fitted (optimal) threshold A1c=7.0. Model C-telopeptide values= 0.311 for A1c < 7.0 and 0.182 for A1c ≥ 7.0. The threshold model is statistically significant compared to a null hypothesis of no threshold (p<0.001).

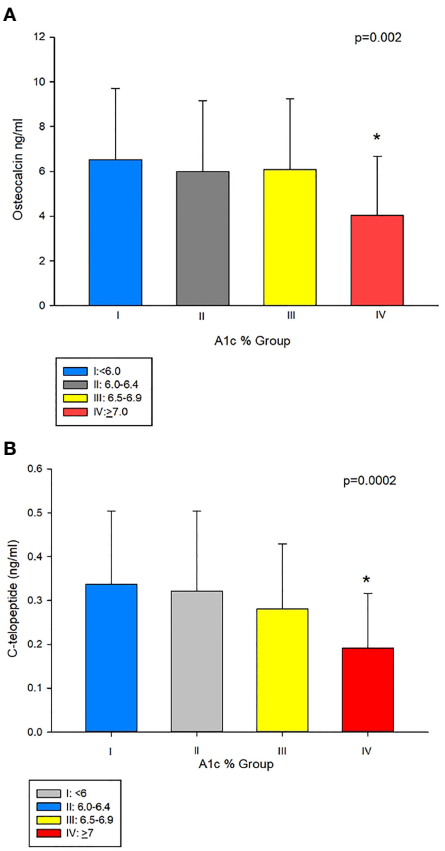

FIGURE 2 | **(A)** Osteocalcin (OC) in group IV (A1c<7%), compared to groups with A1cs ≥7%, i.e. I II, and III (4.04 ± 2.64 vs 6.53 ± 3.18, 5.99 ± 3.16 and 6.09 ± 3.16 ng/mL, respectively, p = 0.002). **(B)** C-telopeptide (CTx) was lower in group IV compared to groups I, II, and III (0.1 9 ± 0.12 ng/mL vs 0.34 ± 0.17, 0.32 ± 0.18 and 0.28 ± 0.14 ng/mL, respectively, p=0.0002). **Post-hoc* analysis: p<0.05 showed significant difference in OC and CTx in group IV compared group I, II and III.

To further examine this hypothesis, we divided our subjects into 4 groups based of A1c values (%) (group I: <6, group II: 6.0–6.4, group III: 6.5–6.9, and group IV: ≥7) modified according to clinical guidelines for good control which is <7 as per American Diabetes Association (14) and <6.5 as per American Association of Clinical Endocrinology (15). **Table 1** shows the clinical characteristics of the study population according to A1c grouping. Those in group I were younger than in groups II, III, and IV, the difference of which was significant compared to groups III and IV. As shown in **Table 1** and **Figure 2A**, patients in group IV (with A1c of ≥7%) had significantly lower osteocalcin levels compared to groups I, II, and III (with A1c of <7%) (p = 0.002). CTx was also significantly lower in group IV compared to groups I, II, and III (p = 0.0002) (**Table 1** and **Figure 2B**). Posthoc analysis showed no significant difference in osteocalcin and CTx levels between groups I, II, and III. There were no significant differences in sclerostin levels across the different A1c groups.

Subjects With T2D

A separate analysis of a subset of patients with T2D (n = 94) showed that those with A1c of ≥7% had significantly longer duration of T2D compared to those with A1c of <7% (**Table 2**). Significantly lower

TABLE 2 | Clinical characteristics of the participants with T2D according to hemoglobin A1c levels.

Hemoglobin A1c (%)	<7%(n = 39)	≥7%(n = 55)	P
Age	59.1 ± 7.1	58.0 ± 8.7	0.53
BMI	36.2 ± 4.9	36.2 ± 6.0	0.94
Duration of T2D (years)	4.1 ± 3.8	8.7 ± 5.7	**0.0002**
Use of medication for T2D	25/38	53/55	**0.0001**
Whole Body BMD (g/cm^2)	1.171 ± 0.104	1.145 ± 0.110	0.11
Lumbar spine BMD (g/cm^2)	1.155 ± 0.149	1.125 ± 0.167	0.50
Total hip BMD (g/cm^2)	1.108 ± 0.106	1.117 ± 0.135	0.80
Femoral neck BMD (g/cm^2)	0.871 ± 0.127	0.882 ± 0.150	0.58
osteocalcin (ng/ml)	5.90 ± 2.66	3.90 ± 2.64	**0.001**
CTx (ng/ml)	0.28 ± 0.16	0.18 ± 0.12	**0.002**
Sclerostin (ng/ml)	0.80 ± 0.24	0.75 ± 0.21	0.34
Testosterone (ng/dl)	276.7 ± 117.7	275.7 ± 81.6	0.96
Estradiol (pg/ml)	22.0 ± 18.2	22.2 ± 14.6	0.97
25-hydroxyvitamin D (ng/ml)	25.0 ± 8.9	25.9 ± 11.6	0.72
PTH (pg/ml)	53.8 ± 20.0	47.4 ± 29.7	0.30

BMI, body mass index; T2D, type 2 diabetes mellitus, CTx, C-telopeptide; SCL, Sclerostin; BMD, bone mineral density; PTH, parathyroid hormone; p-values for BMD are adjusted for age, BMI and testosterone levels. Bolded p-values are significant.

osteocalcin (p=0.001), (**Table 2**, **Figure 3A**) and CTx (p <0.002), (**Table 2** and **Figure 3B**) were also observed in those with A1c ≥7% compared to those <7%. The significance between the groups persisted even after adjusting for duration of T2D (p = 0.02 for osteocalcin and p = 0.01 for CTx) and for medication use, (p = 0.02 for osteocalcin and p = 0.05 for CTx), see **Supplemental Table 1**. Analyses adjusted for total testosterone and estradiol showed significantly lower osteocalcin and CTx in subjects with A1c ≥7% than those with A1c of <7% (p <0.001 for both osteocalcin and CTx), see **Supplemental Table 1**. Mean PTH level did not differ between the patients with T2D who have A1c of <7% compared to those with A1c of ≥7% with or without adjustment for 25-hydroxyvitamin D level. There was no significant difference in sclerostin levels between those with A1c of <7% and those with A1c of ≥7% (**Table 2**).

Simple correlation analysis between A1c and the different clinical and laboratory parameters in patients with T2D showed negative correlations for both osteocalcin (r = −0.29, p = 0.008) and CTx (r = −0.22, p = 0.048), (**Table 3**). There were also significant negative correlations between osteocalcin and duration of T2D (r = −0.25, p = 0.04) and the use of medications for T2D (r = −0.32, p = 0.003). Similarly, negative correlations were observed between CTx with the duration of T2D (r = −0.35, p = 0.003) and the use of medications (r = −0.41, p = 0.0001). There was no correlation between sclerostin and A1c; but it is significantly positively correlated with age (r = 0.42, p = 0.002) and 25-hydroxyvitamin D (r = 0.26, p = 0.03), see **Table 3**.

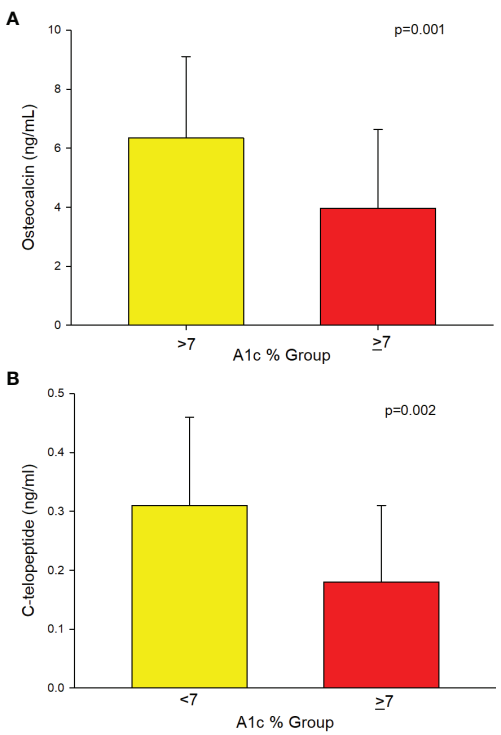

FIGURE 3 | **(A)** Analysis of the subgroup of men with T2D (n=94) showing lower Osteocalcin (3.95 ± 2.68 vs. 6.34 ± 2.77, p=0.001) and **(B)** showing lower C-telopeptide (0.18 ± 0.13 vs. 0.31 ± 0.15, p=0.002) in those with A1c ≥7% compared to those ≤7%, respectively.

TABLE 3 | Simple correlation analysis between osteocalcin and CTX with the different Clinical and laboratory parameters in patients with T2D.

	Osteocalcin		CTx		Sclerostin	
	r	p	r	p	r	p
Age (years)	0.14	0.21	0.16	0.14	0.42	**0.002**
BMI (kg/m^2)	−0.02	0.85	−0.02	0.90	0.14	0.22
Duration of T2D (years)	−0.25	**0.04**	−0.35	**0.003**	0.17	0.16
Hemoglobin A1c (%)	−0.29	**0.008**	−0.22	**0.048**	−0.07	0.57
Testosterone (ng/dl)	0.09	0.41	−0.02	0.87	−0.11	0.37
Estradiol (pg/ml)	−0.07	0.59	−0.06	0.66	−0.21	0.12
25-hydroxyvitamin D (ng/ml)	0.06	0.59	0.06	0.69	0.26	**0.03**
PTH (pg/ml)	0.21	0.09	0.21	0.09	0.03	0.80
Use of medications for T2D	−0.32	**0.003**	−0.41	**0.0001**	0.21	0.07

BMI, body mass index; T2D, type 2 diabetes mellitus, CTx, C-telopeptide; BMD, bone mineral density; PTH, parathyroid hormone. Bolded p-values are significant.

To evaluate the effect of the use of medications for T2D on the different bone biomarkers, we divided our patients with T2D into those who are on medications and those who are not (**Table 4**). As expected, those on medications had T2D for significantly longer period of time and had significantly higher A1c than those not on medications (**Table 4**). More importantly, those on medications had significantly lower osteocalcin and CTx levels compared to those not on medications (**Table 4**). To further evaluate the effect of medications on bone turnover of subjects that are at goal with their T2D, we analyze the subgroup of subjects who have A1c of less than 7% (n = 38) of which 15 were not on medications and 23 were on different antidiabetic medications. There was no significant difference in osteocalcin levels between those who were vs. those who were not on medications (5.55 ± 3.0 vs 6.53 ± 1.91 ng/ml, p = 0.31); and no significant difference in CTx levels between those who were vs. those who were not on medications (0.26 ± 0.16 vs 0.32 ± 0.16 ng/ml, p = 0.29).

Bone Mineral Density (BMD)

BMD analysis on the spine, total hip and femoral neck adjusted for age, BMI, and T levels showed no significant difference in areal BMD at all sites in patients based on A1c categories (**Table 1**) and in the subset of men with T2D (**Table 2**).

TABLE 4 | Clinical and laboratory parameters according to the use of medications for T2DM.

	Without medications N = 15	With medications N = 79	P
Age (years)	56.0 ± 9.9	58.9 ± 7.7	0.21
Duration of T2D (years)	2.02 ± 3.2	7.7 ± 5.4	**0.003**
Hemoglobin A1c (%)	6.60 ± 0.68	7.95 ± 1.74	**0.004**
Osteocalcin (ng/ml)	6.64 ± 2.0	4.40 ± 2.90	**0.01**
CTx (ng/ml)	0.39 ± 0.15	0.21 ± 0.13	**0.0001**
Sclerostin (ng/ml)	0.66 ± 0.18	0.79 ± 0.23	0.07
PTH (pg/ml)	61.77 ± 27.72	47.77 ± 26.02	0.12

T2D, type 2 diabetes mellitus; CTx, C-terminal telopeptide of collagen I; PTH, parathyroid hormone. Bolded p-values are significant.

DISCUSSION

Our results show that in men with T2D, an A1c ≥7% is associated with low bone turnover suggesting that impairment in bone metabolism in these patients starts around the cut-off established by the American Diabetes Association as goal for good control (14). The cut-off of <7% for A1c is established as a goal to minimize or reduce the risk for microvascular (retinopathy, nephropathy, and neuropathy) and possibly also macrovascular complications (MI or coronary artery disease and stroke) common among patients with poorly-controlled T2D. Similar to the other organs in the body, our results suggest that the potential harmful effects of poor glycemic control on bone among patients with T2D likely also starts around this A1c level.

Bone disease associated with T2D is characterized by skeletal fragility despite a relatively normal or higher than normal BMD (2, 3, 16). Although a few studies reported increased cortical porosity by high-resolution peripheral quantitative computer tomography, some reported preserved trabecular bone microarchitecture (17). Thus, the increased fracture risk in T2D is likely from a defect in bone quality rather than bone quantity. There are several mechanisms proposed as contributors to skeletal fragility in patients with T2D and include but not limited to: accumulation of advanced glycation end products (AGEs) which can adversely affect biomechanical properties (18), reactive oxygen species(ROS), use of antidiabetic medications, and the presence of microvascular complications (2, 4); there could be more of these factors present in those with poorly-controlled T2D. However, low bone turnover has become the hallmark of bone derangement associated with T2D with reduced bone formation as the primary defect (5). This abnormality is confirmed from studies in both human and animal models (5, 7, 19, 20). Two large meta-analysis demonstrated significantly lower CTx and osteocalcin in people with T2D compared to those without T2D (7, 21). Transiliac crest bone biopsies in 5 patients with T2D and 4 subjects without T2D revealed reduction in osteoid surface, bone formation rate, osteoblast surface, mineralizing surface, and cortical width T2D compared to those withoutT2D (5). Moreover, circulating osteoblast precursors were also lower in patients with T2D compared to those without T2D. There were also significant reduction in serum markers of bone formation such as osteocalcin and procollagen I intact N-terminal (P1NP) coupled with decreased serum marker of bone resorption, i.e., CTx, in patients with T2D compared to those without T2D (5). These findings suggest inactive bone remodeling which results in failure to repair microcracks and replace old with new bone leading to increase buildup of bone with poor quality.

Our study shows negative correlation between blood glucose control (as assessed by A1c) and bone turnover markers, i.e., higher A1c is associated with reduction in bone turnover markers. Furthermore, our study also identifies an A1c of 7% as the threshold by which reduction in bone turnover occurs among those with T2D. A prior report suggested that T2D patients with low bone formation marker (N-amino terminal propeptide of type 1 procollagen or P1NP) have higher risk for osteoporotic fractures (20). Given the importance of bone remodeling in bone health maintenance, it is critical to identify and cut-off for blood sugar control at which reduction in markers of bone turnover occurs. Although the test accuracy for A1c could be affected by conditions that affect red blood cell turnover, non-enzymatic glycation of hemoglobin, assay variablity and ethinicity, for the most part, it is still considered as a reliable index of the average blood glucose over a period of 12 weeks and has been used in most studies to investigate the effect of glycemic control on target organs. Sclerostin positively correlated with both age and 25-hydroxyvitamin D; associations which have been previously reported (22, 23). On the other hand, there was no significant difference in sclerostin levels across the A1c groups in the entire study population and in those with T2D between A1c of <7% and those with A1c of ≥7%.

Several studies have examined the relationship between blood glucose control using A1c and fracture risk (24–31). While some reported linear relationship between fractures and A1c, others did not (27, 31, 32). Others also showed that there was a significant interaction between the use of insulin and A1c such that, those who are on insulin with tight glycemic control had a higher risk for any clinical fracture (29, 30). These findings were hypothesized due to higher incidence of hypoglycemic events resulting in falls.

A secondary analysis of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial failed to find a difference in the incidence of nonvertebral fractures over a period of 3.8 years of follow-up between those in the intensive glycemia and standard glycemia groups (32). While most of the above studies showing an increased risk for fracture with poor glycemic control are done in the elderly population (mostly in their 70s), the ACCORD trial had relatively younger participants, i.e., in their 60s which may partially contribute to the variable findings. In addition, the median A1c, was 6.4% among those in the intensive treatment group, while it was 7.5% in the standard group which suggests that the standard treatment group is not really in poor glycemic control. Although one can say that these inconsistent findings can also be due to a host of factors such differences in assessing glycemic control (single vs. multiple A1c measurements), and the cut-offs used, duration of T2D, diabetes complications, BMI, and fall risks, there seems to be more evidence suggesting that glucose control could play a role in preventing adverse bone complications.

Our results also demonstrated that in the entire population, those T2D patients with A1c of 7% or better (group III and some in group II) have bone turnover (osteocalcin and CTx) that is not different from those without T2D, i.e., group I. While this finding suggests that not all patients with T2D will have low bone turnover, this also implies that it is not the diagnosis of T2D itself but the glycemic control that determines bone complications from T2D. This is supported by the findings that those with T2D with A1c of <7% have significantly higher bone markers than those with ≥7%. Although it appears that medications may have an effect on bone turnover markers, an analysis in those with A1c of <7% showed that there was no significant difference in bone markers between those who were, and were not on medications, suggesting that subjects who were on anti-diabetic drugs have low bone turnover because of poor glucose control rather than from the treatment. In addition, whereas some of our subjects are hypogonadal, and low testosterone (with consequent low estradiol level) may alter bone turnover (33), our analyses are adjusted for testosterone and estradiol levels. Of note, there is no

significant difference in testosterone and estradiol levels between those with A1c <7% compared to those with ≥7%.

It is likely that the suppression in bone turnover, the hallmark of diabetic bone disease, occurs much earlier than the structural and biomechanical changes that predispose them to fractures. A1c is a more stable marker of glycemic control compared to fasting and postprandial blood glucose levels, hence, finding an A1c threshold by which bone turnover become suppressed will be useful in identifying who could be potentially predisposed to future skeletal complications from T2D. To our knowledge, there are no other studies to date in men with T2D looking at threshold A1c at which bone health gets affected. Some studies have demonstrated differences in fracture risk between men and women with T2D (34–36). It is possible that there are gender differences in what constitutes as the threshold A1c for bone complications. However, in these studies no particular A1c threshold was reported.

Our study has limitations. We used a single timepoint A1c, i.e., only at the time of baseline visit, thus, we have no data on the long-term blood sugar control of our subjects. Also, our population is composed only of men. Nevertheless, our study is the first to recognize that the A1c recommended by the American Diabetes Association as target to prevent a host of non-skeletal complications from diabetes (14) is also the same A1c level where bone turnover appears to be reduced in men with T2D compared with lower A1c levels in a cross-sectional setting where A1c is measured in a single time point.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Boards of the University of New Mexico and of the Baylor College of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SJ and RA-V: conceptualization. SJ, CQ, and RA-V: formal analysis. SJ, DV, and RA-V: investigation. SJ, EB, FD, GG, AF-G, GC, LA, RC, VR, DV, and RA-V: writing, reviewing and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.788107/full#supplementary-material

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The Efficacy of Alendronate Versus Denosumab on Major Osteoporotic Fracture Risk in Elderly Patients With Diabetes Mellitus: A Danish Retrospective Cohort Study

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Objective: Patients with diabetes mellitus have an increased risk of fractures; however, the underlying mechanism is largely unknown. We aimed to investigate whether the risk of major osteoporotic fractures in diabetes patients differs between subjects initiated with alendronate and denosumab, respectively.

Methods and Research Design: We conducted a retrospective nationwide cohort study through access to all discharge diagnoses (ICD-10 system) from the National Danish Patient Registry along with all redeemed drug prescriptions (ATC classification system) from the Health Service Prescription Registry. We identified all subjects with a diabetes diagnosis between 2000 and 2018 and collected data on the first new prescription of anti-osteoporotic treatment between 2011 and 2018. Exposure was defined as either alendronate or denosumab treatment initiated after diabetes diagnosis. Outcome information was collected by identification of all major osteoporotic fracture (MOF) diagnoses, i.e., hip, spine, forearm, and humerus, from exposure until 2018 or censoring by emigration or death. The risk of fracture was calculated as hazard ratios (HR) using multiply adjusted Cox proportional models with death as a competing risk.

Results: We included 8,745 subjects initiated with either alendronate (n = 8,255) or denosumab (n = 490). The cohort consisted of subjects with a mean age of 73.62 (SD ± 9.27) years, primarily females (69%) and suffering mainly from type 2 diabetes (98.22%) with a median diabetes duration at baseline of 5.45 years (IQR 2.41–9.19). Those in the denosumab group were older (mean 75.60 [SD ± 9.72] versus 73.51 [SD ± 9.23] years), had a higher proportion of women (81% versus 68%, RR 1.18 [95% CI 1.13–1.24], and were more comorbid (mean CCI 2.68 [95% CI 2.47–2.88] versus 1.98 [95% CI 1.93–2.02]) compared to alendronate initiators. In addition, denosumab users had a higher prevalence of previous fractures (64% versus 46%, RR 1.38 [95% CI 1.28–1.48]).

The adjusted HR for any MOF after treatment initiation with denosumab was 0.89 (95% CI 0.78–1.02) compared to initiation with alendronate.

Conclusion: The risk of incident MOF among subjects with diabetes was similar between those initially treated with alendronate and denosumab. These findings indicate that the two treatment strategies are equally effective in preventing osteoporotic fractures in subjects with diabetes.

Keywords: diabetes, fracture, alendronate, denosumab, osteoporosis, bone

INTRODUCTION

Osteoporosis is an emerging global health problem characterized by microarchitectural deterioration of bone tissue with increased bone fragility and higher fracture risk leading to increased morbidity and mortality (1–3). Diabetes mellitus is a chronic metabolic imbalance associated with increased risk of fractures that cannot be sufficiently predicted by reduced bone mineral density (BMD) (4, 5). In patients with type 1 and type 2 diabetes, the fracture risk may be increased by 7- and 1.3-fold, respectively (4). A current meta-analysis found a relative risk of hip fracture of 4.93 in type 1 diabetes and 1.33 in type 2 diabetes (6). In addition, the relative risk of non-vertebral fractures was found increased by 1.92 and 1.19 in type 1 and type 2 diabetes, respectively (6). Vertebral fractures are often asymptomatic and complex to assess, and thus, data on vertebral fracture risk are sparse (7). Compromised insulin pathways are assumed to cause a deficit in bone structure, reduced osteoblast activity, and a lower number of osteoclasts (8).

Bisphosphonates sufficiently suppress bone resorption by direct inhibition of osteoclast activity, and alendronate, an oral bisphosphonate, is currently the most commonly used treatment of osteoporosis (9). Denosumab is a relatively new treatment of osteoporosis approved as treatment in Denmark in 2010 (10). It is a monoclonal antibody against the receptor activator of nuclear factor-κB ligand (RANKL) which prevents the interaction of RANKL with its receptor, resulting in inhibition of the osteoclast-mediated bone resorption (11, 12).

A more pronounced effect on BMD by denosumab compared to bisphosphonates has been suggested in clinical trials examining postmenopausal women (13–16). In postmenopausal women, it is estimated that alendronate and denosumab increase BMD by 4.7% and 6.0% at the total hip and 6.2% and 9.2% in lumbar spine, respectively (9, 17). In addition, the risk of fractures is reduced by approximately 20%–50% by alendronate and 20%–70% by denosumab; alendronate with the highest protective effect on hip fractures and denosumab on vertebral fractures (17, 18). However, the association between BMD and fracture prediction is not well established (19, 20). Changes in BMD and reduction in fracture risk among users of either alendronate or denosumab are overall similar between subjects with and without diabetes (13, 21, 22). Both alendronate and denosumab treatments are associated with a decreased bone turnover with a more pronounced decrease during denosumab treatment (15). Meta-analyses have shown decreased bone turnover markers in people with diabetes (3, 23, 24). However, bone-specific alkaline phosphatase is reported as normal or increased, suggesting that the bone matrix may become hypermineralized (25). Yet, it is unknown whether a lowering of bone turnover is beneficial and thus alendronate may be superior to denosumab. However, to our knowledge, no studies have investigated potential discrepancies in fracture risk between alendronate and denosumab use in subjects with diabetes.

We aimed to compare the efficacy of alendronate and denosumab treatment on the risk of any new major osteoporotic fracture (MOF), i.e., hip, spine, forearm, and humerus, in subjects with diabetes. We hypothesized that the risk of any MOF was similar after initiation of denosumab compared to alendronate in subjects with diabetes.

RESEARCH DESIGN AND METHODS

The STROBE statement guideline for reports of observational studies was followed (a STROBE checklist is found in **Supplemental Table S1**) (26).

Study Design and Setting

We conducted a retrospective nationwide cohort study using information from the Danish national registries. We identified all patients with diabetes between 2000 and 2018 to ensure identification of all individuals with preexisting diabetes and enable an estimation of diabetes duration before exposure. We chose to collect data on exposure of alendronate and denosumab between 2011 and 2018 as denosumab became available as treatment in Denmark in 2010. Outcome information was collected by identifying all fracture-related diagnoses from exposure date until 2018 or censoring date.

Data Sources

All data were provided and anonymized by Statistics Denmark (*Danmarks Statistik*, project identifier no. 703382) and were obtained through National Danish registries. All Danish citizens are assigned a 10-digit personal identification number which ensures a complete medical history of all contacts to the Danish healthcare system and drug prescriptions for each individual (27–29). The unique person identification number (PIN) has been anonymized and linked to all registries used in this study. All Danish citizens have equal access to full healthcare provided by the Danish National Health Service, which includes free access to hospitals and partial compensation of drug

expenses. All authorized Danish research organizations can apply for access to the registries.

Data on diagnoses were obtained from the Danish National Patient Registry (29). The registry covers all contacts to the hospitals on both in- and outpatient bases. The data include all relevant physician-assigned discharge diagnoses on the individual level, coded according to the International Classification of Diseases, Tenth Revision (ICD-10).

Information on drug prescriptions was coded according to the Anatomical Therapeutical Chemical (ATC) classification and recorded from 1996 by the Danish National Health Service Prescription Registry (28, 30). To ensure adequate registration, we collected data from January 1, 2000.

Data on sex and date of birth as well as emigration and death (if applicable) were retrieved from the Danish Civil Registration system, which ensures high-fidelity subject identification with respect to emigration and death (27, 31).

Study Population

The study population included subjects alive and residing in Denmark with no emigration history on January 1, 2011. We excluded subjects with classified diabetes before January 1, 2000, and individuals of age below 50 years at the index date (initiation of exposure as defined below) (**Supplemental Figure S1**). We chose age 50, as the average age for menopause in Denmark is 51.7 years with a corresponding increase of osteoporosis afterward (32). We excluded subjects treated with other anti-osteoporotic drugs (including alendronate and denosumab) before exposure. Thus, the final cohort consisted of adult individuals with new-onset diabetes between January 1, 2000, and December 31, 2018, who were initiated with either alendronate or denosumab at age ≥ 50 years and after diabetes diagnosis.

Identification of Diabetes Subjects

Subjects with diabetes mellitus were identified between years 2000 and 2018 either by any ICD-10 code (main or secondary) related to diabetes (E10, E11, E12, E13, E14, G63.2, H28.0, H36.0, M14.2, O24, R73) or by an ATC code of glucose-lowering drugs used in diabetes (A10A or A10B) based on a previously published algorithm (**Supplemental Table S2**) (33–37). The diabetes diagnosis and concordance between actual use and prescription of diabetes-related medications are in general high (38–43). Consequently, all people with diabetes were defined either from a hospital visit or by prescription of glucose-lowering drugs.

In Denmark, all patients with type 1 diabetes will eventually be in contact with the hospital and no other glucose-lowering drugs than insulin were recommended in the study period. Consequently, type 1 diabetes was defined by at least one E10 ICD-10 code (type 1 diabetes) and at least one A10A ATC code (insulins and analogues) and no A10B ATC code (blood glucose-lowering drugs exclusive of insulins); all other individuals with diabetes were classified as type 2 diabetes subjects.

Exposure: Treatment With Alendronate or Denosumab

All drug prescriptions in Denmark are logged, stored, and linked to the unique PIN. The prescription database includes data on redeemed drugs and corresponding dates, doses, and pack sizes according to the ATC classification system (44).

We defined exposure as a first-ever dispense of either alendronate or denosumab after age 50, after diabetes diagnosis, and after January 1, 2011, using the ATC codes "M05BA04" and "M05BX04", respectively. The date of the first dispensing of alendronate or denosumab during the study period was set as the index date. We excluded all subjects with any recorded dispensing of other anti-osteoporotic medication (i.e., raloxifene, ipriflavone, strontium ranelate, teriparatide, calcitonin, and other bisphosphonates) before the index date (**Supplemental Table S2**).

We considered subjects as exposed to the initiated drug on the index date, equivalent to the intention-to-treat approach used in randomized controlled trials. To calculate the crude treatment duration, the number of daily doses at the last dispensation date was added to this date, and the date of first drug dispensation was subtracted. The cumulative treatment dose was calculated using a defined daily dose (DDD) of 10 and 0.33 mg for alendronate and denosumab, respectively, based on the World Health Organization Collaborating Centre for Drug Statistics Methodology. Compliance was then assessed using the medication possession ratio (MPR), by dividing the cumulative dose (DDDs) by the treatment duration. MPR was grouped in intervals of a) <0.5, b) 0.5–0.8, and c) ≥0.8, the latter being defined as compliant use. Effective use was defined as the cumulative dose in days if MPR <0.8 and by the crude treatment duration if MPR ≥0.8.

Outcome: Major Osteoporotic Fractures (MOFs)

Any fracture of the spine, hip, humerus, or forearm was defined as a MOF (45). The primary outcome was any MOF identified by primary or secondary diagnoses during hospitalization by ICD-10 codes (**Supplemental Table S3**) during the follow-up period (between 2011 and 2018). MOF was further categorized into the specific type, i.e., fracture of the spine, hip, humerus, and forearm.

Identification of Covariates

Covariates at baseline were identified by means of ICD-10 and ATC codes in the period from start date of data collection (January 1, 2000) until the index date (**Supplemental Table S2**). Age at baseline was calculated based on date of birth and date of initiation of treatment.

A history of fracture was identified as any fracture by ICD-10 codes before treatment exposure.

As a proxy for smoking status, we used ICD-10 codes related to lung diseases, of which some were directly and others indirectly associated with tobacco exposure, as well as nicotine poisoning and psychiatric tobacco-related diagnoses (37). In addition, we identified ATC codes corresponding to treatments for tobacco dependence (ever), e.g., nicotine replacement therapy, or initiation of drugs for obstructive airway diseases after the age of 40. Due to potential underestimation, we classified this factor as *heavy smoking*.

We evaluated alcohol consumption by either one relevant ICD-10 or ATC code covering diseases and drugs with direct affiliation to alcohol, e.g., intoxication, alcohol abuse, alcoholic

liver disease, alcoholic cardiomyopathy, alcoholic polyneuropathy, alcoholic gastritis, alcohol-induced pancreatitis, or alcohol related psychiatric disorders (37, 46). We classified this factor as *alcohol abuse*.

Obesity was evaluated by ICD-10 codes of obesity or use of anti-obesity pharmaceuticals by ATC codes. Information on chronic and acute pancreatitis was obtained from ICD-10 codes.

Hyper- and hypothyroidism were assessed by either ICD-10 or ATC codes.

Hypertension was defined by any ICD-10 code related to hypertension and/or prescription of any antihypertensive drug. Hypoglycemia was assessed by a related ICD-10 code.

Comorbidity was assessed by use of the Charlson Comorbidity Index (CCI) (47) based on discharge diagnoses registered by ICD-10 codes with a general high accuracy (**Supplemental Table S3**) (48). As alendronate is more or less contraindicated when peptic ulcers or renal impairment is present, we chose to exclude peptic ulcers and nephrological diseases (including those in late diabetes complications) from the index and estimated these as separate variables (**Supplemental Table S2**).

In addition, we identified any prescription of insulins, statins, opioids, glucocorticoids, and anxiolytics by ATC codes up till/at baseline.

Data on socioeconomic status was obtained from Statistics Denmark. We assessed income as the amount of DKK (Danish kroner) from the year preceding the year of index and adjusted for inflation to a 2018 level using the consumer price index from Statistics Denmark. Lastly, we converted the income to euro € at a rate of 1 € = 7.467 DKK (exchange rate December 2018) and grouped into quintiles for analysis.

Marital status was available through the Danish Civil Registration System and assessed from the year prior to the year of index. It was defined and grouped according to the classification from Statistics Denmark: married, divorced, widowed, or unmarried.

Statistical Analysis

The study period was defined as time from exposure initiation, i.e., initiation of treatment with either alendronate or denosumab (index date), until the date of a MOF outcome, death, emigration, or December 31, 2018, whichever occurred first.

Descriptive statistics are presented as numbers (n) and percentages (%), means and standard deviations (SD), or medians and interquartile ranges (IQR). Unpaired t-tests and chi-square tests were used to compare continuous and dichotomous variables across exposure groups. Differences in exposure groups are presented as mean differences or risk ratios (RR) with 95% confidence intervals (CI).

We plotted exposure-specific cumulative incidence curves for any first MOF, considering death as a competing risk by performing a competing risk regression analysis fitted by Fine and Gray's proportional sub-distribution hazard models (49) with death as a competitive event and alendronate exposure as comparator. Crude and adjusted hazard rate ratios (HR) with 95% CI were estimated for each outcome. We examined the assumption of proportionality by graphical log-log plots, and no violation was identified. With respect to multicollinearity, we performed a multiple adjustment. Interactions were evaluated and found significant between age and a history of fracture with no difference in results after incorporating the main effects and interaction effect in our primary analysis. Thus, we made a subgroup analysis stratified by fracture history, age (< and ≥75 years), and sex.

We performed several sensitivity analyses. Firstly, we excluded all subjects with type 1 diabetes from the cohort. In addition, we included censoring at any discontinuation of treatment due to a switch from the initial treatment to another anti-osteoporotic drug, i.e., per-protocol approach. As denosumab has a faster clearance than alendronate (50), we further stratified this sensitivity analysis (censoring at switch in treatment) on effective use. Moreover, we performed a sensitivity analysis including censoring at switch in treatment and at discontinuation (last date of drug prescription with addition of amount of DDD in the dispense) and made a modified analysis by censoring 1 year after discontinuation of treatment. Furthermore, we performed a sensitivity analysis only on subjects with high adherence, i.e., MPR ≥0.8. As glucocorticoids are known to impact on bone quality, we performed a sensitivity analysis only including those who used glucocorticoids up till/at baseline.

Lastly, we identified and displaced subjects with a switch in treatment from alendronate to denosumab within 6 months to the denosumab group.

All analyses were conducted in STATA 16.1 (StataCorp, College Station, Texas, US).

Resource Availability

Data were available and anonymized by Statistics Denmark. All authorized Danish research organizations can apply for access.

Approval by the ethics committee is not required for epidemiological studies in Denmark. We had no access to personally identifiable information and the registries are subject to control by the Danish Data Protection Agency.

RESULTS

Baseline Characteristics

We identified 8,745 elderly subjects with new onset diabetes mellitus with initiated anti-osteoporotic treatment of either alendronate (n = 8,255) or denosumab (n = 490) after diabetes diagnosis and without any history of anti-osteoporotic treatment.

Table 1 shows baseline characteristics of subjects initiated with alendronate and denosumab. In general, the cohort consisted of elderly subjects with mean (± SD) age 73.62 (± 9.27) years suffering mainly from type 2 diabetes (98.22% [95% CI 97.92–98.48]) with a median (IQR) diabetes duration at baseline of 5.45 years (2.41–9.19).

Subjects initiated with denosumab were older, mean (± SD) 75.60 (± 9.72) versus 73.51 (± 9.23) years ($p < 0.001$), had a higher proportion of women in the cohort (81% versus 68%, RR 1.18 [95% CI 1.13–1.24), and were more comorbid (mean CCI 2.26 [96% CI 2.07–2.44] versus 1.78 [95% CI 1.74–1.82]) compared to alendronate initiators. In addition, denosumab

exposed individuals had a higher prevalence of previous fractures (64% versus 46%, RR 1.38 [95% CI 1.28–1.48]), a higher proportion of renal impairment (11% versus 6%, RR 1.93 [95% CI 1.47–2.53]), and a higher prevalence of peptic ulcers (16% versus 7%, RR 2.14 [95% CI 1.72–2.66]). There was no difference in marital status or income, either on total income or within each quintile, between subjects initiated with alendronate and denosumab. A higher proportion of those treated with denosumab had hyperthyroidism (5.10% versus 3.08%, RR 1.66 [95% CI 1.11–2.48]), and they were more frequently users of insulin (22.65% versus 18.69%, RR 1.21 [95% CI 1.02–1.44]), opioids (84.29% versus 76.83%, RR 1.10 [95% CI 1.05–1.14]), and anxiolytics (92.86% versus 88.26%, RR 1.05 [95% CI 1.03–1.08]) compared to the alendronate group. There was no

TABLE 1 | Baseline characteristics of subjects initiated with alendronate and denosumab after diabetes diagnosis in Denmark from 2011 to 2018.

	All subjects n = 8,745	Alendronate n = 8,255	Denosumab n = 490
Age (years), mean ± SD	73.62 (9.27)	73.51 (9.23)	75.60 (9.72)
Age category (years), n (%)			
50–59	755 (9)	720 (9)	35 (7)
60–69	2,196 (25)	2,100 (25)	96 (20)
70–79	3,481 (40)	3,293 (40)	188 (38)
≥80	2,313 (26)	2,142 (26)	171 (35)
Sex, n (%)			
Female	6,043 (69)	5,647 (68)	396 (81)
Male	2,702 (31)	2,608 (32)	94 (19)
Type 2 diabetes, n (%)	8,589 (98)	8,114 (98)	475 (97)
Diabetes duration in years, median (IQR)	5.45 (2.41-9.19)	5.43 (2.41-9.18)	5.57 (2.34-9.52)
History of any fracture, n (%)	4,141 (47)	3,828 (46)	313 (64)
CCI, mean ± SD	1.81 (1.89)	1.78 (1.88)	2.26 (2.07)
CCI categories, n (%)			
0	2,609 (30)	2,491 (30)	118 (24)
1	1,963 (22)	1,879 (23)	84 (17)
≥2	4,173 (48)	3,885 (47)	288 (59)
Peptic ulcer, n (%)	507 (6)	455 (6)	52 (11)
Renal impairment, n (%)	693 (8)	615 (7)	78 (16)
Income, € in thousands, median (IQR)	26.13 (19.85-32.54)	26.11 (19.85-32.58)	26.44 (19.92-31.53)
1st quintile, n (%)	1,749 (20)	1,645 (20)	104 (21)
2nd quintile, n (%)	1,749 (20)	1,677 (20)	72 (15)
3rd quintile, n (%)	1,749 (20)	1,637 (20)	112 (23)
4th quintile, n (%)	1,749 (20)	1,637 (20)	112 (23)
5th quintile, n (%)	1,749 (20)	1,659 (20)	90 (18)
Marital status, n (%)			
Married	4,241 (49)	4,015 (49)	226 (56)
Divorced	1,358 (16)	1,280 (16)	78 (16)
Unmarried	606 (6.93)	574 (7)	32 (7)
Widowed	2,534 (29)	2,380 (29)	154 (31)
Unknown	6 (0)	6 (0)	0 (0)
Heavy smoking, n (%)	3,116 (36)	2,927 (35)	189 (39)
Alcohol abuse, n (%)	747 (9)	708 (9)	39 (8)
Obesity, n (%)	1,543 (18)	1,457 (18)	86 (18)
Pancreatitis, n (%)	298 (3)	283 (3)	15 (3)
Hyperthyroidism, n (%)	279 (3)	254 (3)	25 (5)
Hypothyroidism, n (%)	629 (7)	589 (7)	40 (8)
Glucocorticoid use, n (%)	5,027 (57)	4,757 (58)	270 (55)
Statin use, n (%)	6,791 (78)	6,424 (78)	367 (75)
Insulin use, n (%)	1,654 (19)	1,543 (19)	111 (23)
Hypoglycemia, % ± SD	193 (2)	177 (2)	16 (3)
Hypertension, n (%)	7,996 (91)	7,543 (91)	453 (92)
Opioid use, n (%)	6,755 (77)	6,342 (77)	413 (84)
Anxiolytics, n (%)	7,741 (89)	7,286 (88)	455 (93)
Initiation year, n (%)			
2011	1,098 (13)	1,018 (12)	80 (16)
2012	1,052 (12)	989 (12)	63 (13)
2013	1,076 (12)	1,018 (12)	58 (12)
2014	1,100 (13)	1,044 (13)	56 (11)
2015	1,072 (13)	1,021 (12)	51 (10)
2016	1,113 (13)	1,050 (13)	63 (13)
2017	1,155 (13)	1,099 (13)	56 (11)
2018	1,079 (12)	1,016 (12)	63 (13)

All characteristics were evaluated in the time from 2000 until the index date (exposure start). Data are presented as numbers (n, %), mean with SD or median with IQR.

significant difference in smoking, alcohol, obesity, pancreatitis, hypothyroidism, glucocorticoid use, statin use, hypoglycemia, or hypertension between exposure groups.

Risk of Major Osteoporotic Fractures

Median (IQR) follow-up time was 2.67 (1.17–4.62) years among alendronate initiators and 2.36 (0.95–4.53) years among denosumab initiators. Deaths during follow-up were more frequent in the denosumab group (27% versus 34%, RR 1.29 [95% CI 1.14–1.47]).

Median treatment duration in days (defined by cumulative DDD) of alendronate and denosumab was 560 days (IQR 182–1,218) and 727 days (IQR 363–1,455), respectively.

Table 2 and **Figure 1** present risk of MOFs during the study period. A new MOF occurred in 49% (n = 238) and 39% (n = 3,256) of denosumab and alendronate initiators, respectively. Crude HR for any MOF during the study period among initiators of denosumab was 1.26 (95% CI 1.10–1.44) with initiators of alendronate as reference. The risk was entirely attenuated in the fully adjusted model (HR 0.89 [95% CI 0.89–1.02]). Stratification by age (75-year cutoff), sex, and a history of any fracture did not change the risk of any MOF significantly (**Table 2**).

Hip fractures were the most prevalent type of MOF in both exposure groups followed by fractures of the forearm, spine, and humerus (**Table 2**). The risk of hip fracture as first MOF was similar between groups (adjusted HR 0.93 [95% CI 0.75–1.16]).

Sensitivity Analysis

Table 3 presents data from 6 sensitivity analyses.

The risk of any MOF did not change after excluding subjects with type 1 diabetes from the cohort (adjusted HR 0.89 [95% CI 0.78–1.02)]) or those with low adherence (MPR <0.8 (adjusted HR 0.89 [0.77–1.02)]). Neither were there any difference in the risk of MOF between alendronate and denosumab initiators when only including subjects with use of glucocorticoids up till/at baseline.

In total, 4,078 (47%) subjects discontinued the original treatment before end of follow-up (**Supplemental Table S5**). Of these, 3,484 subjects discontinued without any prescription of other anti-osteoporotic treatment; 149 from the denosumab group and 3,284 from the alendronate group, corresponding to 30% and 42%, respectively. Of those who discontinued, 445 replaced the original treatment with another anti-osteoporotic treatment before end of follow-up; 274 subjects switched from alendronate to denosumab, 7 subjects switched from denosumab to alendronate and 165 subjects switched to a third anti-osteoporotic drug of which all were alendronate initiators (1 subject switched from alendronate to denosumab and lastly to a third drug). Baseline characteristics of subjects discontinuing the original treatment did

TABLE 2 | Risk of MOF and stratification by age, sex, history of any fracture, and MOF type.

	Exposure	MOF, n (%)	Hazard ratios (HR) and 95% CI			
			Crude	Adjusted 1[a]	Adjusted 2[b]	Adjusted 3[c]
Overall	Denosumab	238 (49)	1.26 (1.10–1.44)	1.17 (1.03–1.34)	0.92 (0.80–1.05)	0.89 (0.78–1.02)
	Alendronate	3,256 (39)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Age category						
<75 years	Denosumab	88 (37)	1.18 (0.95–1.47)	1.14 (0.92–1.42)	0.93 (0.81–1.06)	0.80 (0.64–1.00)
	Alendronate	1,504 (46)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
≥75 years	Denosumab	150 (63)	1.23 (1.04–1.46)	1.20 (1.02–1.42)	0.98 (0.83–1.16)	1.97 (0.82–1.16)
	Alendronate	1,752 (54)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Sex						
Female	Denosumab	203 (85)	1.19 (1.03–1.38)	1.17 (1.01–1.35)	0.93 (0.80–1.08)	0.90 (0.77–1.04)
	Alendronate	2,416 (74)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Male	Denosumab	35 (15)	1.29 (0.91–1.82)	1.20 (0.85–1.26)	0.85 (0.61–1.19)	0.86 (0.63–1.26)
	Alendronate	840 (26)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
History of any fracture						
Yes	Denosumab	218 (92)	0.90 (0.78–1.05)	0.89 (0.77–1.03)	–	0.87 (0.75–1.01)
	Alendronate	2,863 (88)	1 (reference)	1 (reference)	–	1 (reference)
No	Denosumab	20 (8)	1.23 (0.78–1.94)	1.12 (0.72–1.75)	–	1.13 (0.72–1.77)
	Alendronate	393 (12)	1 (reference)	1 (reference)	–	1 (reference)
Type of first MOF						
Spine	Denosumab	45 (19)	1.13 (0.84–1.53)	1.14 (0.84–1.53)	0.90 (0.67–1.21)	0.82 (0.59–1.15)
	Alendronate	684 (21)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Hip	Denosumab	98 (41)	1.31 (1.06–1.62)	1.20 (0.97–1.48)	1.07 (0.87–1.32)	0.93 (0.75–1.16)
	Alendronate	1,289 (40)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Humerus	Denosumab	33 (14)	1.31 (0.92–1.87)	1.20 (0.97–1.48)	0.95 (0.77–1.17)	0.91 (0.63–1.29)
	Alendronate	434 (13)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
Forearm	Denosumab	62 (26)	1.25 (0.97–1.62)	1.13 (0.87–1.46)	0.87 (0.67–1.13)	0.87 (0.66–1.14)
	Alendronate	849 (26)	1 (reference)	1 (reference)	1 (reference)	1 (reference)

MOF, n (%) represents number and % of MOFs in each category by exposure. Adjusted HRs (95% CIs) with alendronate exposure as reference with exclusion of stratified category in adjusted analyses.

[a]*Adjusted for sex and age.*

[b]*Adjusted for sex, age, history of fracture.*

[c]*Multiple adjustment for sex, age, history of fractures, diabetes duration, insulin, hypoglycemia, anxiolytics, statin, opioid, smoking, alcohol, glucocorticoid, pancreatitis, hypo- and hyperthyroidism, peptic ulcer, renal impairment, CCI, income, and marital status.*

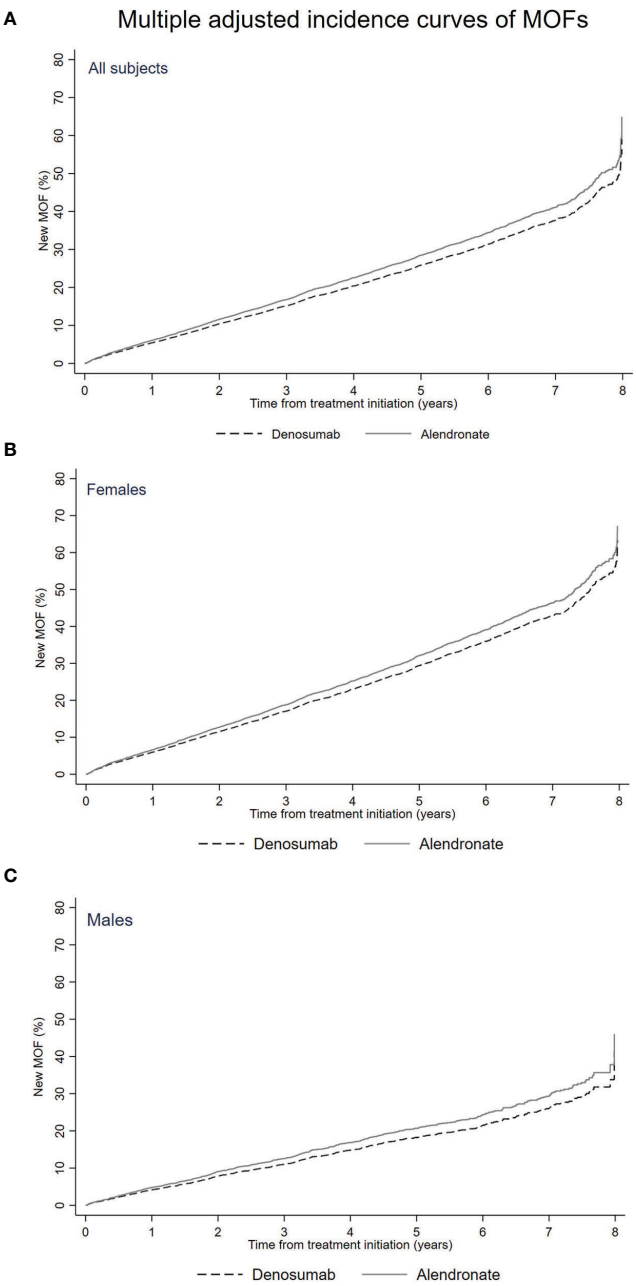

FIGURE 1 | Multiple adjusted cumulative incidence curve of any first MOF following initiation of alendronate or denosumab (primary analysis). **(A)** All subjects. **(B)** Females. **(C)** Males.

not differ from the original cohort (**Supplemental Table S5**). The numbers and risks of any MOF did not change after censoring at switch in treatment, at discontinuation, or 1 year after discontinuation. After displacing subjects with a switch in treatment from alendronate to denosumab within 6 months to the denosumab exposure group (n = 81), the risk of MOF did not change.

Stratification by type of MOF and by effective use did not reveal any differences in the risk of MOF between exposure groups.

DISCUSSION

This cohort study examined the risk of any first MOF among subjects with diabetes after initiation with anti-osteoporotic treatments of denosumab or alendronate. The risk of any MOF after treatment initiation between users of alendronate and denosumab was similar during the follow-up period, although the estimates moved toward a protective effect of denosumab after multiple adjustments. Hip fractures were the most frequent

TABLE 3 | Risk of MOF in sensitivity analyses.

	Exposure	MOF, n (%)	Hazard ratios (HR) and 95% CI			
			Crude	Adjusted 1[a]	Adjusted 2[b]	Adjusted 3[c]
1, Type 2 diabetes	Denosumab	232 (49)	1.25 (1.10–1.44)	1.17 (1.02–1.34)	0.92 (0.80–1.05)	0.89 (0.78–1.02)
	Alendronate	3,204 (39)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
2, MPR ≥ 0.8	Denosumab	222 (49)	1.23 (1.07–1.41)	1.17 (1.02–1.34)	0.90 (0.79–1.04)	0.89 (0.77–1.02)
	Alendronate	2,777 (40)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
3, Censor at switch	Denosumab	209 (43)	1.33 (1.15–1.53)	1.24 (1.08–1.43)	0.92 (0.80–1.07)	0.89 (0.76–1.03)
	Alendronate	2,683 (33)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
4, Censor switch and discontinuation	Denosumab	209 (43)	1.42 (1.20–1.68)	1.34 (1.13–1.59)	1.02 (0.86–1.21)	0.97 (0.82–1.16)
	Alendronate	2,683 (33)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
5, Censor 1 year discontinuation	Denosumab	209 (43)	1.34 (1.15–1.56)	1.26 (1.08–1.46)	0.92 (0.81–1.10)	0.91 (0.78–1.06)
	Alendronate	2,683 (33)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
6, Switch < 6 months	Denosumab	271 (47)	1.22 (1.08–1.39)	1.15 (1.02–1.30)	0.92 (0.81–1.04)	0.89 (0.79–1.01)
	Alendronate	3,223 (39)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
7, Glucocorticoid users	Denosumab	123 (46)	1.44	1.32 (1.09–1.59)	1.01 (0.84–1.22)	0.99 (0.83–1.20)
	Alendronate	1,602 (34)	1 (reference)	1 (reference)	1 (reference)	1 (reference)

Risk of MOF in 5 sensitivity analyses. 1, Only including subjects with type 2 diabetes. 2, Only including subjects with high compliance/drug adherence (MPR ≥ 0.8). 3, Censoring at switch in anti-osteoporotic treatment. 4, Censoring at switch in or discontinuation of anti-osteoporotic treatment. 5, Censoring at switch in anti-osteoporotic treatment and 1 year after discontinuation. 6, Subjects displaced to denosumab users if a switch from alendronate to denosumab was set within 6 months of treatment. 7, Only including users of glucocorticoid up till/at baseline. MOF, n (%) represents numbers and % of MOFs in each category by exposure. Adjusted HRs (95% CIs) with alendronate exposure as reference.

[a]*Adjusted for sex and age.*

[b]*Adjusted for sex, age, history of fracture.*

[c]*Multiple adjustment for sex, age, history of fractures, diabetes duration, insulin, hypoglycemia, anxiolytics, statin, opioid, smoking, alcohol, glucocorticoid, pancreatitis, hypo- and hyperthyroidism, peptic ulcer, renal impairment, CCI, income, and marital status.*

type of MOF in both alendronate and denosumab initiators without difference in risk.

In clinical practice, increased BMD is expected as an adequate response to therapy and results in a significant reduction in fracture risk (51). Long-term studies suggest larger BMD gain after denosumab use compared to alendronate with no difference in safety and adverse events (15, 16). In addition, a large cohort study found similar fracture risks between users of denosumab and alendronate (52). However, these studies did not include analyses on subjects with diabetes. As BMD is often inappropriately high in patients with type 2 diabetes (4), a proper response to anti-osteoporotic therapy in patients with diabetes is restricted to assessment of fracture risk, an endpoint hard to evaluate in clinical trials as it requires long-term follow-up. Our study supports the overall hypothesis of no difference between alendronate and denosumab on fracture risk in diabetes. However, to our knowledge, no other studies have examined the risk of fractures between denosumab and alendronate users in this setting.

Several studies on diabetic animals have provided solid evidence of a reduction in fracture risk with anti-osteoporotic treatments (53, 54), based on their ability to increase BMD and bone strength. Although diabetes is characterized by low bone turnover, further reduction of bone turnover with antiresorptive therapies does not seem to negatively affect the potential to prevent fractures (55). A systematic review from 2018 identified 9 studies and found no differences in the efficacy of anti-osteoporotic medications on fracture risk and BMD changes in patients with diabetes; however, no eligible studies were identified to evaluate denosumab (56). In humans, available data are scarce and mainly obtained from *post hoc* analyses of large osteoporosis RCTs. In a *post hoc* analysis, 3 years of alendronate treatment increased BMD at all sites compared to a placebo group, and the increase was similar in subjects without incident diabetes (57). In addition, treatment with denosumab increased BMD at all sites irrespective of the presence of diabetes and reduced the risk for new vertebral fractures; however, the study revealed a higher incidence of non-vertebral fractures (mostly forearm and ribs) in subjects with diabetes (22). We did not find a higher risk of forearm fractures among initiators of denosumab compared to alendronate.

The effect of transition from alendronate to denosumab or risedronate has been investigated previously. A recent observational study investigated switching from bisphosphonates to denosumab but did not find any BMD improvement after 6 months of denosumab treatment in patients with type 2 diabetes with prior bisphosphonate use (58). An RCT comparing women with suboptimal adherence to alendronate therapy found a higher BMD increase and reduced bone turnover 12 months after a switch to denosumab compared to risedronate (59). Another RCT found that discontinuation of alendronate did not affect fracture risk after 5 years without treatment (60). As alendronate has a long half-life, a potential benefit from a switch may, in part, be the long-lasting or an additive effect of alendronate. In our sensitivity analysis, no change in risk of any MOF was found after censoring those with a switch in treatment or discontinuation. In addition, we did not observe any differences after stratification by effective use or after exclusion of those with low adherence to the initiated treatment. Lastly, we did not observe a difference in the risk of any MOF after displacing subjects with a switch in treatment from alendronate to denosumab within 6 months of treatment.

Falls is another possible cause of fractures in patients with diabetes (61). It has been suggested that denosumab improves

muscle mass and strength, and thus, may have the potential to reduce fall risk, which may in turn lower the risk of fractures (62). However, the aforementioned higher incidence of forearm and rib fractures in denosumab uses may also indicate a higher fall rate (22). On the other hand, current research suggests a possible protective effect of alendronate on the risk of developing type 2 diabetes as well as reducing insulin consumption and improving insulin sensitivity in prediabetes (37, 63–67); factors which could decrease the risk of late diabetes complications and, thereby, fracture risk (68).

One notable strength of the current study is the utility of the Danish National Registers based on the unique personal identification number assigned to all Danish citizens with high quality and validity (27, 29, 69, 70). Furthermore, the identification of people with diabetes in Denmark was nationwide without any selection bias. Another strength was the ability to include a high number of potential confounders. It is highly possible that denosumab initiation is preferred in older patients and in those with peptic ulcers and renal impairment. However, we were able to adjust for these covariates by means of ICD-10 codes, and the risk of MOF did not change when only including subjects with high adherence to the drug or after displacement of switchers from alendronate to denosumab within 6 months of treatment.

Though few adverse events have been reported after initiation of alendronate (71), these events are rarely reported after initiation of denosumab (17). This may lead to differences in treatment indication, or consequently, a switch in treatment from alendronate to denosumab as seen in our cohort. However, we would expect most changes in treatment due to adverse events to occur within 6 months after treatment initiation. In addition, we compare a newer agent with an established treatment and cannot dismiss the possibility of residual confounding. For example, we did not have access to laboratory results, e.g., glycemic control, BMI, or BMD measurements, all of which may influence on bone microarchitecture and fracture risk (72). Furthermore, some fractures, especially spine fractures, may go undetected, and this may have led to an underreporting of MOFs in our analysis. However, underreporting of vertebral fractures is expected to be similar between the two groups; therefore, we do not expect this to affect the results in either direction. In addition, the median follow-up time was just above 2 years and may as well underestimate the evaluation of fracture risk. A higher proportion of denosumab users had renal impairment and peptic ulcers compared to alendronate initiators and could potentially have a lower BMD at treatment initiation. Although we were able to adjust for two of these factors, it is possible that these are incompletely measured by ICD-10 codes, allowing confounding by indication. Those initiated with alendronate were in general less comorbid than those initiated with denosumab, while a higher proportion of deaths occurred in the denosumab group, which may lead to a healthy survivor bias. We chose to perform a competitive regression analysis as well as adjusting for a highly validated comorbidity index to minimize this bias (48). Lastly, as we excluded subjects with diabetes before January 1, 2000, and individuals of age below 50 years at index date (year 2011 as the earliest), naïve type 1 diabetes patients included in this cohort were older than a typical type 1 diabetes patient underestimating the proportion of subjects with type 1 diabetes compared to type 2 diabetes. As patients with type 1 diabetes have a higher fracture risk compared to type 2 diabetes, this might underestimate the fracture rate (4). However, excluding individuals with type 1 diabetes from the cohort did not affect our results.

Although the Danish registries contain a wide range of validated information, we did not have access to over-the-counter-medicine, e.g., vitamin D supplementation, or information of lifestyle factors such as diet and exercise. In addition, the registries did not include data on smoking habits and alcohol consumptions; however, we estimated some of these baseline characteristics using ICD-10 and ATC codes as proxies. Consequently, we only obtained these covariates from subjects with already developed concomitant disease or with prescribed medical therapy.

In conclusion, subjects with diabetes initiated with denosumab have a similar risk of a new major osteoporotic fracture as subjects initiated with alendronate. The risk was not associated with sex, age, or a history of fractures. Alendronate appears to be the first choice in treatment of osteoporosis irrespectively of the presence of diabetes. To our knowledge, there are no specific treatment recommendations available for osteoporosis in the presence of diabetes, and it is our hope that the current findings may encourage attention to the cross-link between bone health and diabetes. We propose future research to prospectively evaluate anti-osteoporotic treatments in patients with diabetes, e.g., by basic metabolic research, acute intervention trials and randomized controlled trials including head-to-head comparison of the effects of denosumab and alendronate on bone indices in subjects with diabetes. As BMD is an insufficient measure of fracture risk, more data are needed to clarify whether there are any differences in the efficacy of anti-osteoporotic drugs on other bone indices and fracture risk in subjects with diabetes.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because only authorized Danish research organizations can apply for access. Requests to access the datasets should be directed to p.vestergaard@rn.dk.

AUTHOR CONTRIBUTIONS

All authors contributed to the article according to the ICJME requirements for coauthorship. All authors had full access to all data used in the study, critically revised the paper for intellectual content, and approved submitted versions and the final version of the paper. RV and PV designed the study. RV performed the data management and statistical analyses with assistance from all coauthors. RV and PV interpreted the data. RV wrote the paper. ZA-M and JS-L made ongoing critical revisions of data management, design, and data interpretation and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.826997/full#supplementary-material

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Prevalent Morphometrically Assessed Vertebral Fractures in Individuals With Type 2 Diabetes, Prediabetes and Normal Glucose Metabolism: The Maastricht Study

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Background: Type 2 diabetes (T2D) is frequently reported to be associated with an increased fracture risk. Epidemiological data on prevalent morphometric vertebral fractures (VFs) in T2D are sparse and even less is known in the prediabetic state.

Purpose: To determine the association between prevalence and severity of morphometric VFs and glucose metabolism state: normal glucose metabolism (NGM), impaired glucose metabolism (prediabetes) or T2D.

Methods: This study included cross-sectional data from 3625 participants of the Maastricht Study who had a vertebral fracture assessment on lateral Dual Energy X-Ray Absorptiometry images. VFs were classified based on morphometric assessment into mild, moderate and severe VFs (respectively 20–24%, 25–39% or ≥40% reduction in expected vertebral body height). Logistic regression models were used to investigate the association between glucose metabolism status and the prevalence and severity of VFs. Analyses were adjusted for subject characteristics and life-style factors.

Results: T2D individuals were older (62.8 ± 7.5 years old) and less often female (30.5%) compared to the NGM group (57.7 ± 8.5 years old, and 58.8% female, respectively).

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At least one mild, moderate or severe prevalent VF was found in 8.6% of the men and 2.2% of the women in the T2D group, in 9.4% and 8.4% in the prediabetes group and in 9.1% and 4.8% in the NGM group, respectively. After adjustment T2D in women was associated with a lower probability of having a prevalent VF compared to NGM [adjusted OR 0.25 (95% CI 0.09-0.65)], while this was not the case for prediabetes. Furthermore, women with T2D had a significantly lower probability of a prevalent moderate or severe VF [adjusted OR 0.32 (95% CI 0.11-0.96)]. In men there was no significant association between T2D or prediabetes and prevalent VFs.

Conclusion: Women with T2D had a lower probability of prevalent VFs compared to women with a normal glucose metabolism, while this was not the case for men with T2D and participants with prediabetes.

Keywords: bone, type 2 diabetes, vertebral fracture (VF), vertebral fracture assessment (VFA), dual energy X-ray absorptiometry (DEXA)

INTRODUCTION

Type 2 diabetes mellitus (T2D) is a chronic disease characterized by macro- and microvascular complications. The impact of diabetes on bone metabolism may lead to a deterioration of bone microarchitecture and lower bone strength. These alterations could be regarded as a skeletal complication of T2D which, in combination with increased risk of falling, may lead to an increased fracture risk (1). Interestingly, a higher risk of fractures has been reported in T2D despite a normal or even higher areal bone mineral density (aBMD) compared to non-diabetic individuals (2–4).

This increased fracture risk has largely been shown for hip and non-vertebral fractures (5–7), while literature on prevalent vertebral fractures (VFs) in T2D is sparse and inconclusive. In a recent combined meta-analysis of individual data obtained from cohort studies and previously published studies, a decreased risk of prevalent VFs in T2D compared to no diabetes was reported (8). However, it was reported that information of the type of treatment individuals with T2D were receiving and comorbidities was lacking and that there could have been bias due to loss to follow-up of participants in the individual data analysis. Furthermore, the ascertainment of prevalent VFs differed among included studies. Lastly, the possible difference in prevalent VF risk between men and women was not fully elucidated, since some studies included only men or only women.

It is, however, of great importance to identify prevalent VFs in individuals with clinical risk factors for fractures, since the presence of a prevalent VF is strongly associated with the risk of subsequent vertebral and non-vertebral fractures (9–11) and mortality risk (12, 13).

Abbreviations: aBMD, Areal bone mineral density; BMI, Body mass index; CI, Confidence intervals; DXA, Dual energy X-ray absorptiometry; eGFR, Estimated glomerular filtration rate; FPG, Fasting plasma glucose; HbA1c, Glycated hemoglobin A1c; MVC, Microvascular complication; NGM, Normal glucose metabolism; NGT, Normal glucose tolerance; OR, Odds ratio; OGTT, Oral glucose tolerance test; T2D, Type 2 diabetes mellitus; VF, Vertebral fracture.

Because individuals with T2D tend to fracture at a higher BMD T-score compared to healthy individuals without diabetes, it has been suggested that individuals with T2D should be systematically assessed for the presence of a VF, preferably by the assessment of lateral spine images of modern dual energy X-ray absorptiometry (DXA) devices (14, 15) if there is an indication for BMD testing based on the clinical fracture risk profile.

In this study, we aimed to assess prevalent morphometric VFs using lateral DXA images in participants of the Maastricht Study, an extensive phenotyping study on determinants of type 2 diabetes, its complications, and its comorbidities (16). In addition, we aimed to compare the presence and severity of prevalent VFs between participants with normal glucose metabolism (NGM), prediabetes and T2D.

MATERIALS AND METHODS

The Maastricht Study: Population and Design

We used data from The Maastricht Study, an observational prospective population-based cohort study. The rationale and methodology have been described previously (16). In brief, the study focuses on the etiology, pathophysiology, complications and comorbidities of T2D and is characterized by an extensive phenotyping approach. Eligible for participation were all individuals aged between 40 and 75 years and living in the southern part of the Netherlands. Participants were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry *via* mailings. Recruitment was stratified according to known T2D status, with an oversampling of individuals with T2D, for reasons of efficiency. The present report includes cross-sectional data from the first 7689 participants, who completed the baseline survey between November 2010 and December 2017. The examinations of each participant were performed within a time window of three months. The study has been approved by the institutional medical ethical committee (NL31329.068.10) and

the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

To determine glucose metabolism status, all participants, except those who used insulin, underwent a standardized 2-h 75g oral glucose tolerance test (OGTT) after an overnight fast. For safety reasons, participants with a fasting glucose level above 11.0 mmol/L, as determined by a finger prick, did not undergo the OGTT. For these individuals (n=13), fasting glucose level and information about diabetes medication were used to determine glucose metabolism status. Glucose metabolism status was defined according to the WHO 2006 criteria into normal glucose tolerance (NGT), impaired fasting glucose (fasting plasma glucose (FPG) 6.1-6.9 mmol/l and 2h plasma glucose <7.8 mmol/l) or impaired glucose tolerance (FPG <7.0 mmol/l and 2h plasma glucose 7.8 - 11.1 mmol/l), which were both considered as prediabetes, and T2D (FPG ≥7.0 mmol/l or 2h plasma glucose ≥11.1mmol/l) (17). Individuals without type 1 diabetes on diabetes medication were classified as having T2D (16).

Methods

The participants were invited for a DXA in the period of October 2016 until July 2019. In this cross-sectional study we used data from all participants of The Maastricht Study who had a BMD measurement at the lumbar spine and hip and lateral spine imaging by DXA (Hologic QDR 4500, Hologic, Bedford, MA, USA). BMD measurements were classified according to the lowest value of T-score in the total hip/femoral neck or lumbar spine: osteoporosis as T-score ≤−2.5, osteopenia as T-score between −2.5 and −1.0, and normal BMD as T-score ≥−1.0.

The densitometric lateral spine images were used for vertebral fracture assessment (VFA). The BMD measurements and lateral spine images were performed in a clinical facility and evaluated by experienced radiologists, who were blinded with reference to the glucose metabolism status of the participants, using a standardized land marking protocol. First, the image quality was evaluated, and the vertebrae were labeled, starting with the identification of the fourth lumbar vertebra. Subsequently, the evaluable vertebrae were determined. A vertebra was considered evaluable if the posterior and anterior cortices and both endplates were fully and clearly visible. If this was not the case, the vertebra was not evaluated. The standardized land marking protocol uses 95 points to represent the circumferential vertebral borders, including right/left/central endplate margins and anterior/posterior margins.

For the purpose of this study, the vertebral shape and the appearance of the end plate were evaluated to differentiate between VFs and vertebrae with other deformities, e.g. degenerative changes or Scheuermann's disease, by two trained clinicians (CS, SB) who were blinded with reference to the glucose metabolism status of the participants. Subsequently, VFs were graded by morphometric assessment according to Genant et al. (18) as grade 0, <20% reduction in expected vertebral body height at the anterior, mid, or posterior location; grade 1, 20–24% (mild VF); grade 2, 25–39% (moderate VF); or grade 3, ≥40% (severe VF) reduction, respectively. Patients were classified according to the most severe VF as those without VFs, those with at least one mild VF, or those with at least one moderate or severe VF.

Covariates

For the current study age, sex, smoking status, BMD, body mass index (BMI), educational level, and time gap (time in months between baseline visit and DXA scan) were all considered as potential confounders. BMI was calculated by dividing weight in kilogram by height in meters squared, which were measured while wearing light clothing without shoes, using a scale and stadiometer to the nearest 0.1 cm and 0.5 kg, respectively. Smoking status, fracture history, and educational level were assessed using a questionnaire. Smoking status was categorized into never, former and current, and educational level was categorized into low, medium and high.

Statistical Analyses

General characteristics and the presence, severity and number of prevalent VFs were calculated for all three groups, being NGM, prediabetes and T2D, separately. This data was additionally stratified for men and women, due to the skewed distribution of men and women per group. Categorical variables are presented as number of participants (%) and continuous variables are presented as mean values [standard deviation (SD)].

Logistic regression was used to investigate the association between glucose metabolism status and the presence of prevalent VFs, using both crude and adjusted models. To test for interaction between glucose metabolism status and sex, glucose metabolism status and BMI, and glucose metabolism status and BMD, interaction terms (e.g. dummy-coded glucose metabolism status variables * sex, etc.) were incorporated into the logistic regression models. A $P_{interaction}$<0.10 was considered to be statistically significant, as is common in statistical interaction testing (19). The main analysis investigated the association between glucose metabolism status and the presence of at least one prevalent VF. Furthermore, the relationship between glucose metabolism status and moderate and severe VFs versus no VFs was studied. Both analyses were stratified by sex. The results from these analyses are presented as odds ratios (OR), with 95% confidence intervals (CI). For glucose metabolism status, NGM was set as the reference group.

Additionally, a logistic regression analysis was performed within the T2D cohort to study the association between diabetes related characteristics [glycated hemoglobin A1c (HbA1c), diabetes duration and the presence of microvascular complications (MVCs)] and the presence of prevalent VFs.

Multiple models were created for the logistic regression analyses, providing ORs adjusted for the potential confounders depending on the number of events per analysis. For every ten events, meaning cases of a VF, one potential confounder was added to the model.

Lastly, a sensitivity analysis was performed including only participants with screen-detected T2D (T2D detected in the Maastricht Study by OGTT) from the study population.

All analyses were performed with IBM Statistical Package for Social Sciences for Macintosh, version 25.0 (IBM SPSS, IBM

Corp, Armonk, NY, USA). *P*-values<0.05 were considered statistically significant.

RESULTS

The general characteristics of the study population are summarized in **Table 1**. A total of 3,626 participants were included in this study (2346 NGM (64.7%), 546 prediabetes (15.1%) and 734 T2D (20.2%)) (**Supplemental Figure 1**). The mean age was 59 ± 8.5 years and 1,853 (51.1%) were female. Since a significant interaction effect (*P*-value = 0.012) between sex and glucose metabolism status was found, all results are presented separately for men and women. In men (n=1,773), 966 (54.4%) had NGM, 297 (16.8%) prediabetes and 510 (28.8%) T2D. In women (n=1,853), 1380 (74.5%) had NGM, 249 (13.4%) prediabetes and 224 (12.1%) T2D.

Table 2 shows the number, severity and location of prevalent VFs per glucose metabolism status group. At least one mild, moderate or severe prevalent VF was found in 8.6% of the men and 2.2% of the women in the T2D group, in 9.4% and 8.4% in the prediabetes group and in 9.1% and 4.8% in the NGM group, respectively.

In women, T2D was associated with a lower probability of having at least one prevalent VF compared to NGM [adjusted OR 0.25 (95% CI 0.09-0.65)], while this was not the case for prediabetes (**Table 3**).

Furthermore, T2D was associated with a significantly lower probability of a moderate or severe VF [adjusted OR 0.32 (95%

TABLE 1 | General characteristics of the study population.

	Men (N = 1773)			Women (N = 1853)		
	NGM (n = 966)	**Prediabetes (n = 297)**	**T2D (n = 510)**	**NGM (n = 1380)**	**Prediabetes (n = 249)**	**T2D (n = 224)**
Age (years)	58.7 (8.4)	61.8 (7.6)	63.3 (7.2)	57.1 (8.5)	60.6 (8.7)	61.5 (7.9)
BMI (kg/m^2)	26.1 (3.2)	27.8 (3.4)	29.3 (4.2)	25.0 (3.8)	27.7 (4.7)	29.7 (5.0)
Educational level						
Low	226 (23.4%)	92 (31.0%)	194 (38.0%)	434 (31.4%)	111 (44.6%)	133 (59.4%)
Medium	263 (27.2%)	73 (24.6%)	138 (27.1%)	402 (29.1%)	67 (26.9%)	57 (25.4%)
High	477 (49.4%)	132 (44.4%)	178 (34.9%)	544 (39.4%)	71 (28.5%)	34 (15.2%)
Smoking status						
Never	413 (42.8%)	90 (30.3%)	144 (28.2%)	592 (42.9%)	91 (36.5%)	90 (40.2%)
Former	431 (44.6%)	179 (60.3%)	301 (59.0%)	628 (45.5%)	129 (51.8%)	105 (46.9%)
Current	122 (12.6%)	28 (9.4%)	65 (12.7%)	160 (11.6%)	29 (11.6%)	29 (12.9%)
Alcohol use						
None	77 (8.0%)	22 (7.4%)	92 (18.0%)	254 (18.4%)	64 (25.7%)	104 (46.4%)
Low	670 (69.4%)	197 (66.3%)	317 (62.2%)	760 (55.1%)	120 (48.2%)	93 (41.5%)
High	219 (22.7%)	78 (26.3%)	101 (19.8%)	366 (26.5%)	65 (26.1%)	27 (12.1%)
History of CVD	110 (11.4%)	50 (16.8%)	153 (30.0%)	160 (11.6%)	35 (14.4%)	39 (17.4%)
History of fractures	414 (42.9%)	121 (40.7%)	178 (34.9%)	455 (33.0%)	82 (32.9%)	64 (28.6%)
Family history of fractures	397 (41.1%)	106 (35.7%)	183 (35.9%)	768 (55.7%)	125 (50.2%)	102 (45.5%)
Family history of osteoporosis	57 (5.9%)	17 (5.7%)	33 (6.5%)	246 (17.8%)	42 (16.9%)	36 (16.1%)
Medication use						
Antihyperglycemic drugs	0 (0%)	0 (0%)	377 (73.9%)	0 (0%)	0 (0%)	153 (68.3%)
Insulin	0 (0%)	0 (0%)	95 (18.6%)	0 (0%)	0 (0%)	38 (17.0%)
Oral antihyperglycemic drugs	0 (0%)	0 (0%)	354 (69.4%)	0 (0%)	0 (0%)	138 (61.6%)
Blood pressure lowering drugs	250 (25.9%)	129 (43.4%)	366 (71.8%)	240 (17.4%)	109 (44.0%)	153 (68.3%)
Psychoactive drugs	23 (2.4%)	6 (2.0%)	17 (3.3%)	77 (5.6%)	20 (8.1%)	24 (10.7%)
Anti-osteoporosis treatment	4 (0.4%)	2 (0.7%)	2 (0.4%)	29 (2.1%)	7 (2.8%)	3 (1.3%)
Glucocorticoids	3 (0.3%)	2 (0.7%)	6 (1.2%)	4 (0.3%)	1 (0.4%)	6 (2.7%)
Diabetes-related characteristics						
HbA1c (mmol/mol)	35.1 (3.9)	37.6 (4.3)	51.1 (11.4)	35.1 (3.8)	37.6 (4.4)	50.5 (11.8)
HbA1c (%)	5.4 (0.4)	5.6 (0.4)	6.8 (1.0)	5.4 (0.4)	5.6 (0.4)	6.8 (1.1)
Diabetes duration at inclusion (years)	0 (0)	0 (0)	7.0 (7.4)	0 (0)	0 (0)	5.5 (6.4)
Retinopathy	2 (0.2%)	6 (2.0%)	21 (4.1%)	7 (0.5%)	1 (0.4%)	5 (2.2%)
Impaired vibration sensation	57 (5.9%)	32 (10.8%)	83 (16.3%)	52 (3.8%)	17 (6.8%)	27 (12.1%)
Nephropathy[a]	107 (11.1%)	44 (14.8%)	139 (27.3%)	149 (10.8%)	38 (15.3%)	42 (18.8%)
eGFR <60ml/min	50 (5.2%)	29 (9.8%)	51 (10.0%)	124 (9.0%)	25 (10.1%)	32 (14.3%)
Albuminuria >30mg/24h	68 (7.0%)	17 (5.7%)	103 (20.2%)	30 (2.2%)	14 (5.6%)	17 (7.6%)
BMD LS (g/cm^2)	1.09 (0.18)	1.14 (0.19)	1.19 (0.20)	0.97 (0.16)	1.00 (0.17)	1.05 (0.19)
BMD TH (g/cm^2)	0.98 (0.13)	1.01 (0.13)	1.03 (0.16)	0.84 (0.12)	0.86 (0.13)	0.90 (0.14)
BMD FN (g/cm^2)	0.80 (0.12)	0.82 (0.12)	0.84 (0.17)	0.72 (0.11)	0.73 (0.11)	0.75 (0.13)
Normal BMD	430 (44.5%)	136 (45.8%)	261 (51.2%)	388 (28.1%)	78 (31.3%)	100 (44.6%)
Osteopenia	472 (48.9%)	146 (49.2%)	234 (45.9%)	703 (50.9%)	129 (51.8%)	98 (43.8%)
Osteoporosis	64 (6.6%)	15 (5.1%)	15 (2.9%)	289 (20.9%)	42 (16.9%)	26 (11.6%)

Values show mean (SD) or number (%).

VF, vertebral fracture; NGM, normal glucose metabolism; T2D, type 2 diabetes; eGFR, estimated glomerular filtration rate; BMD, bone mineral density; LS, lumbar spine; TH, total hip; FN, femoral neck; VFA, vertebral fracture assessment; CVD, cardiovascular disease; BMI, body mass index.

[a]*defined as an estimated glomerular filtration rate (eGFR) below 60 ml/min/1.73 m2, albuminuria, or both.*

TABLE 2 | Number, severity and location of prevalent vertebral fractures in men and women with normal glucose metabolism, prediabetes and type 2 diabetes.

	Men (N = 1773)			Women (N = 1853)		
	NGM (n = 966)	**Prediabetes (n = 297)**	**T2D (n = 510)**	**NGM (n = 1380)**	**Prediabetes (n = 249)**	**T2D (n = 224)**
Presence of VFs						
By number of VFs						
No VF	878 (90.9%)	269 (90.6%)	466 (91.4%)	1314 (95.2%)	228 (91.6%)	219 (97.8%)
≥ 1 VF	88 (9.1%)	28 (9.4%)	44 (8.6%)	66 (4.8%)	21 (8.4%)	5 (2.2%)
1 VF	61 (6.3%)	23 (7.7%)	36 (7.1%)	58 (4.2%)	15 (6.0%)	5 (2.2%)
2 VFs	20 (2.1%)	4 (1.3%)	8 (1.6%)	5 (0.4%)	4 (1.6%)	0 (0%)
3 VFs	7 (0.7%)	0 (0%)	0 (0%)	1 (0.1%)	2 (0.8%)	0 (0%)
> 3 VFs	0 (0%)	1 (0.3%)	0 (0%)	2 (0.1%)	0 (0%)	0 (0%)
By severity						
Mild VF	39 (4.0%)	8 (2.7%)	17 (3.3%)	24 (1.7%)	8 (3.2%)	1 (0.4%)
Moderate VF	43 (4.5%)	18 (6.1%)	22 (4.3%)	37 (2.7%)	10 (4.0%)	4 (1.8%)
Severe VF	6 (0.6%)	2 (0.7%)	5 (1.0%)	5 (0.4%)	3 (1.2%)	0 (0%)
By location						
Thoracic VF	66 (6.8%)	19 (6.4%)	36 (7.1%)	49 (3.6%)	15 (6.0%)	4 (1.8%)
Lumbar VF	33 (3.4%)	11 (3.7%)	11 (2.2%)	23 (1.7%)	8 (3.2%)	1 (0.4%)

Values show mean (SD) or number (%).
VF, vertebral fracture; NGM, normal glucose metabolism; T2D, type 2 diabetes.

CI 0.11-0.96)] (**Table 4**). In men there was no significant association between T2D or prediabetes and prevalent VFs (**Tables 3**, **4**).

In men with T2D, we did not find a significant association between HbA1c, diabetes duration or the presence of MVCs with the presence of a prevalent VF. In women, the number of participants with T2D and a VF (n=5 (2.2%)) was too low for further analysis.

The results from the sensitivity analysis, including only screen-detected T2D, did not show any significant results. Neither men nor women with screen-detected T2D had a significantly altered probability of prevalent VFs compared to participants in the NGM group (**Supplemental Table 1**). Likewise, the probability of prevalent moderate or severe VFs was not significantly associated with the presence of screen-detected T2D, in neither men nor women (**Supplemental Table 2**).

DISCUSSION

In this study, we aimed to investigate the association between glucose metabolism status and the presence and severity of prevalent morphometrically identified VFs on lateral DXA images. Women with T2D had a lower probability of prevalent VFs compared to women with a normal glucose metabolism, while this was not the case for men with T2D and participants with prediabetes.

Our results are in line with the sensitivity analysis of the meta-analysis by Koromani et al. (8), which reported a lower odds of prevalent VFs with T2D in men and women (OR: 0.84; 95% CI: 0.74 – 0.95) based on individual participant data (IPD) of five cohorts (20–24), and two published studies (25, 26). In this sensitivity analysis 2 studies causing heterogeneity were excluded from the meta-analysis. For one study, reason for exclusion was that participants were recruited from a tertiary center, and consequently participants had an abnormally low BMD (T-score of -2.0 for women, -2.4 for men). The other excluded study relied on a national database for the ascertainment of VFs, which possibly causes T2D to be diagnosed with VFs more often, since they are usually under stricter supervision. As in our study, the included studies reported that the OR for VFs was significantly lower among women but not among men.

Our study confirms the results of the meta-analysis by Koromani et al. (8), and provides additional certainty due to

TABLE 3 | Odds of prevalent vertebral fractures in prediabetes and type 2 diabetes, stratified by sex.

Men (N = 1773)	**All VFs (N = 160)**	**No VF (N = 1613)**	**Crude OR (95% CI)**	**Adjusted OR* (95% CI)**
NGM (n=966)	88 (9.1%)	878 (90.9%)	Reference	Reference
Prediabetes (n=297)	28 (9.4%)	269 (90.6%)	1.04 (0.66-1.62)	0.99 (0.63-1.58)
T2D (n=510)	44 (8.6%)	466 (91.4%)	0.94 (0.65-1.38)	0.87 (0.57-1.34)
Women (N = 1853)	**All VFs (N = 92)**	**No VF (N = 1761)**	**Crude OR (95% CI)**	**Adjusted OR* (95% CI)**
NGM (n=1380)	66 (4.8%)	1314 (95.2%)	Reference	Reference
Prediabetes (n=249)	21 (8.4%)	227 (91.6%)	1.83 (1.10-3.06)	1.22 (0.70-2.11)
T2D (n=224)	5 (2.2%)	219 (97.8%)	0.46 (0.18-1.14)	0.25 (0.09-0.65)

Values show numbers (%).
**Logistic regression analysis, adjusted for age, smoking status, bone mineral density, body mass index, educational level, and time gap (time in months between baseline visit and DXA scan).*
VF, vertebral fracture; NGM, normal glucose metabolism; T2D, type 2 diabetes; OR, odds ratio; CI, confidence interval.

TABLE 4 | Odds of prevalent moderate and severe vertebral fractures in prediabetes and type 2 diabetes, stratified by sex.

Men (N = 1709)	Moderate or severe VF (N = 94)	No VF (N = 1613)	Crude OR (95% CI)	Adjusted OR* (95% CI)
NGM (n=927)	49 (5.3%)	878 (94.7%)	Reference	Reference
Prediabetes (n=289)	20 (6.9%)	269 (93.1%)	1.33 (0.78-2.28)	1.26 (0.72-2.21)
T2D (n=493)	27 (5.5%)	466 (94.5%)	1.04 (0.64-1.67)	1.01 (0.59-1.72)
Women (N = 1820)	**Moderate or severe VF (N = 59)**	**No VF (N = 1760)**	**Crude OR (95% CI)**	**Adjusted OR* (95% CI)**
NGM (n=1356)	42 (3.1%)	1314 (96.9%)	Reference	Reference
Prediabetes (n=241)	13 (5.4%)	228 (94.6%)	1.78 (0.94-3.38)	1.15 (0.58-2.27)
T2D (n=223)	4 (1.8%)	219 (98.2%)	0.57 (0.20-1.61)	0.32 (0.11-0.96)

**Logistic regression analysis, adjusted for age, smoking status, bone mineral density, and body mass index.*
VF, vertebral fracture; NGM, normal glucose metabolism; T2D, type 2 diabetes; OR, odds ratio; CI, confidence interval.

the comprehensive design of The Maastricht Study. Firstly, in the cohorts and studies analyzed by Koromani et al. (8), VFs were assessed on lateral radiographs, except for one cohort using lateral DXA images (22), and only moderate and severe VFs were included. In our study mild, moderate and severe VFs were assessed on lateral DXA images. Furthermore, only in two of the five meta-analysis cohorts, women and men were studied (20, 23). However, in the other three cohorts and in one published study only women (21, 22, 24, 26) were included, and in one study only men were included (25). Additionally, some studies included in the meta-analysis by Koromani et al. (8) relied on GP records or self-reported T2D diagnosis (20, 21, 25, 26), which may lower the validity of diabetes classification. In our study, glucose metabolism status was independently investigated using the seven-point OGTT, providing accurate data on glucose metabolism status of all participants. Lastly, in two studies included in the meta-analysis, ORs were either not adjusted or only adjusted for a limited number of confounders (25, 26). In the Maastricht Study cohort, all participating men and women were extensively phenotyped, allowing us to accurately study men and women separately and to adjust our results adequately for potential confounders.

Based on the findings of our study in combination with individual participant data analyses reported by Koromani et al. (8) women with T2D have a lower probability of moderate and severe VFs and likely also of mild prevalent VFs while this is not the case for men. The underlying mechanism of this finding is not yet fully elucidated, given the limited number of studies, but several factors could play a role. Firstly, it could be speculated that the lower probability of prevalent VFs in women with T2D may be related to higher estrogen levels in women with T2D compared to women without T2D. Women with T2D generally have a higher BMI compared to women with NGM, as was the case in our cohort. In the postmenopausal state, there is a linear relationship between a higher BMI and higher free estrogen levels (27, 28). Furthermore, higher levels of estradiol were reported to be independently associated with the risk of T2D, after adjustment for BMI, glucose and insulin levels (29). Estrogen levels are known to be inversely proportional to fracture risk in postmenopausal women, since estrogen deficiency stimulates bone resorption and does not allow adequate bone formation (30). Thus, since most of the women with T2D included in our study were postmenopausal, they were likely to have higher estrogen levels compared to the women with NGM related to a higher body fat mass, which could contribute to the lower probability of prevalent VFs (31). This may especially be the case in the women with T2D in our cohort, who are characterized by a well-regulated T2D (mean HbA1C 50.5 mmol/mol) and by a relatively short time since the diagnosis of T2D (mean 5.5 years) so that possible detrimental effects of longer T2D duration on bone may not be present yet. This notion would be consistent with our sensitivity analysis which shows that the lower odds of prevalent VFs in T2D women was not found in screen-detected women with T2D, who had a lower BMI (28.7 ± 4.7 kg/m^2) compared to women with previously diagnosed T2D (30.1 ± 5.1 kg/m^2). The finding that the OR for VFs was lower in women with T2D even after adjustment for BMI could be explained by the fact that higher estrogen levels are correlated to fat mass distribution, which is not completely accounted for by adjustment for BMI.

Another potential explanation may be that postmenopausal women with T2D with a relatively short and well-regulated T2D were reported to have a better trabecular bone quality compared to women without T2D represented by a greater plate-like and less rod-like trabecular network (32). Since vertebrae primarily consist of trabecular bone a better trabecular bone quality could result in a lower probability of prevalent VFs.

In the meta-analysis of Koromani et al. (8) the lower risk of prevalent VFs was mostly present among the elderly and obese individuals. Unfortunately, due to the limited number of women with VFs, we were not able to stratify our analyses for BMI or BMD categories or to perform further in-depth analyses in the T2D cohort.

It is noteworthy that although the association between glucose metabolism status and the presence of prevalent morphometric VFs was only significant in women, the proportion of men with a prevalent VF was higher than in women (9.1% and 8.6% in men with NGM and T2D, respectively versus 4.8% and 2.2% in women). This was also shown in a study by Waterloo et al. (33), who hypothesized that this could be related to the lifestyle of men in their younger years since high-energy trauma was reported to be the cause of clinical VFs twice as often in men compared to women (34).

The present study has some limitations. Firstly, this study has a cross-sectional design. A causal relationship between glucose metabolism status and the risk of prevalent VFs could therefore not be studied. Additionally, for some participants the baseline visit took place several years before the DXA was performed,

meaning we might not have a clear picture of the level of diabetes management at the time of the DXA. Furthermore, prevalent VFs were assessed by VFA on lateral DXA images, while the golden standard is radiography. As previously reported, the sensitivity of diagnosing VFs on DXA images is lower compared to X-ray, which could have led to an underestimation of prevalent VFs in our study (35). It is however unlikely that this explains the lower probability of VFs in women with T2D compared to NGM. In addition, a benefit of a VFA on DXA images is that no additional imaging was required for VF diagnosis and that DXA exposes individuals to a lower dose of radiation compared to X-ray. Moreover, it has been suggested that studies investigating VF prevalence in T2D could be biased by survivorship selection bias, resulting in a lower VF prevalence due to higher mortality in individuals with T2D compared to individuals without T2D (8). However, we believe that this is not an explanation for the lower probability of VFs in women with T2D in our study since the participants included in The Maastricht Study are thought to be relatively healthy compared to the average T2D individuals due to a participation bias (36, 37). Next, our results suggest a drastically lower OR for VFs in women with T2D (adjusted OR 0.25 (95% CI 0.09-0.65) compared to the sensitivity analysis by Koromani et al. (8) (OR: 0.84; 95% CI: 0.74 – 0.95), perhaps hinting at the possibility of unknown confounders for which we were not able to adjust. Regarding the diagnosis of T2D, we applied the WHO criteria by using a two-hour OGTT and we did not use HbA1C. This may be of influence to the number of participants classified as having T2D. However, HbA1C was reported to be insensitive for screening, especially with regard to undiagnosed diabetes and pre-diabetes (38). Lastly, due to the low proportion of VFs in T2D women, we were unable to perform in-depth analyses to shed light on the mechanism underlying our findings.

To conclude, we have found that women with T2D had a lower probability of prevalent VFs compared to women with a normal glucose metabolism, while this was not the case for men with T2D or for men and women with prediabetes.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the dataset used in the present study was derived from the Maastricht Study. Upon reasonable request and with permission of the Maastricht Study management team, this dataset is available from the corresponding author. Requests to access the datasets should be directed to JB, jvdbergh@viecuri.nl.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: VH, CSa, JD, and JB. Methodology: VH, CSa, JD, and JB. Validation: VH, JD, and JB. Formal analysis: VH and JD. Investigation: staff of the Maastricht Study. Resources: Maastricht Study management team. Data curation: VH, JD, and Maastricht Study management team. Writing—original draft: VH. Writing—review and editing: VH, CS, JD, NS, PG, CW, G-JD, RO, NR, RV, CSt, CK, MS, SB, and JB. Visualization: VH and JD. Supervision: JB. Project administration: Maastricht Study management team. Funding acquisition: JD and JB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.832977/full#supplementary-material

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The Effects of 12-Weeks Whey Protein Supplements on Markers of Bone Turnover in Adults With Abdominal Obesity – A *Post Hoc* Analysis

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Background: While osteoporosis is characterized by skeletal fragility due to increased bone turnover and low bone mineral density (BMD), subjects with abdominal obesity and type-2 diabetes have increased risk of bone fractures despite low bone turnover and increased BMD. Diets with increased protein content are reported to increase bone turnover in healthy adults and may be a point of interest in preserving bone strength in subjects with abdominal obesity and/or type-2 diabetes.

Methods: We examined the effect of 12-weeks dietary intervention on bone turnover in 64 adults with abdominal obesity using data from the MERITS trial. The trial was a randomized, controlled, double blinded study in which participants were allocated to receive either 60 g/d of whey protein hydrolysate or maltodextrin in combination with either high (30 g/d) or low dietary fiber intake (10 g/d). Primarily, we assessed changes in plasma markers of bone turnover Procollagen type 1 N-terminal propeptide (p1NP), C-terminal telopeptide type-1 collagen (CTX), and parathyroid hormone (PTH) within the four intervention groups. In addition, we measured u-calcium and u-carbamide excretion, 25(OH)D, and BMD by whole body DXA scans. Finally, we compared changes in insulin resistance (Homeostasis-model assessment of insulin resistance, HOMA-IR) with changes in bone turnover markers.The trial was registered at www.clinicaltrials.gov as NCT02931630.

Results: Sixty-four subjects were included in the study. We did not find any effect of twelve weeks of high protein or high fiber intake on plasma levels of P1NP or CTX. There was a nonsignificant positive association between protein intake and PTH levels (p=0.06). U-calcium and u-carbamide increased in both protein groups. There was a positive association between change in HOMA-IR and PTH (p=0.042), while changes in P1NP and CTX did not associate to changes in HOMA-IR.

Conclusion: Twelve weeks of increased whey protein intake in subjects with abdominal obesity did not affect markers of bone turnover significantly, although tended to increase PTH levels. Dietary fiber intake did not affect bone turnover. We report a positive association between change in HOMA-IR and PTH supporting a hypothesis of insulin resistance as a potential key factor in the expanding field of bone fragility in T2D subjects.

Keywords: bone turnover, abdominal obesity, dietary protein, dietary fiber, insulin resistance

INTRODUCTION

A diet rich in protein may be beneficial to skeletal properties, since bone structure largely consists of proteins integrated within the organic collagen matrix during mineralization. Nevertheless, the effect of high protein diets remains poorly established when it comes to bone health (1).

Most studies on protein diets and bone health are performed on healthy or postmenopausal women, where an increased risk of fractures relates to increased bone turnover with resulting loss of bone mass. However, in dysmetabolic conditions dominated by insulin resistance such as abdominal obesity, metabolic syndrome or prediabetes and type 2 diabetes, the determination of bone properties seems to be quite different. Contrasting to postmenopausal osteoporosis, bone health is dominated by low bone turnover (2–7), which may lead to unrepaired micro-fractures and poor bone quality (8).

Formerly, obesity was considered a protective feature against fractures due to mechanical protective properties of subcutaneous fat in the hip region and an increased bone density from increased mechanical load. Within the last decades, this assumption has been challenged (9, 10). Although body mass index (BMI) is positively associated with BMD (10), the risk of fracture is not reduced with increasing BMI (11–13). More recent findings actually associate abdominal obesity (14–16) and T2D (4, 8, 17) with increased risk of fractures.

Some studies indicate that a diet rich in protein may increase bone turnover. In 2015, Kerstetter et al. found that 18 months of whey protein isolate supplements (45 g/d) versus isocaloric maltodextrin induced an increase in CTX and preserved fat-free mass in elderly men and women with BMI from 19 to 32 kg/m^2 (18). In 2017, Heer et al. found 60 days of increased protein intake (1.45 g/kg/d + 0.72 g/d branched chain amino acids versus 1 g protein/kg/d) to increase P1NP and CTX, N-terminal telopeptide (NTX) and Tartrate-resistant acid phosphatase (TRAP) in bedridden women (19).

Studies on long-term protein supplements and bone are primarily performed on subjects with osteoporosis - a disease in which the increased fracture risk is based on elevated bone turnover and thus very different from obesity and T2D (20). The effect of whey protein per se on bone turnover markers is only known in the acute setting, in which bone turnover is lowered which is the case for all macronutrients (21). Whey protein is known for its insulinotropic abilities compared to other protein sources in the acute settings (22). However, the long-term effect of protein intake on bone turnover in insulin resistant subjects is not elucidated. In the present study we investigated whether long-term whey protein intake stimulate bone turnover.

Using data from the MERITS trial (23), we examined if increased intake of whey protein for 12 weeks would increase bone turnover in prediabetic subjects with abdominal obesity.

Moreover, we aimed to examine the association between change in bone turnover and insulin resistance.

MATERIALS AND METHODS

Study Design and Participants

The present study was part of the MERITS trial, described in detail previously (23). The trial was a controlled intervention study in which 65 subjects with abdominal obesity completed 12 weeks of dietary supplement with either whey protein (WP) or maltodextrin (MD) in combination with either high (HiFi) or low (LoFi) dietary fiber diet. The primary aim of the MERITS study was to assess changes within lipid metabolism, whereas secondary aims included changes in bone turnover.

We recruited 73 subjects with age ≥40 years with abdominal circumference ≥94 cm (men) or ≥80 cm (women). Exclusion criteria included osteoporosis, diabetes, severe renal, cardiovascular, or psychiatric illness or medical treatment with hormonal replacement therapy or corticosteroids. We allowed for the continued use of regular medication, including vitamin D and calcium supplements, if no changes occurred during the trial or three months prior to inclusion.

By blocks of eight, we randomized the participants by age and sex into one of four groups: WP + high fiber (WP-HiFi), WP + low fiber (WP-LoFi), maltodextrin + high fiber (MD-HiFi), or maltodextrin + low fiber (MD-LoFi).

For a period of 12 weeks, the participants received powder supplements twice daily (2 x 30 g of WP or MD). Furthermore, participants were to substitute bread and cereal products of their normal diet with test products (bread and cereals) of either high or low fiber content aiming at a fiber intake of 30 g (HiFi) or 10 g (LoFi) fiber per day from test products.

Arla Foods Ingredients Group P/S (Viby, Denmark) provided the WP hydrolysate (Lacprodan® HYDRO.REBUILD) and MD (Glucidex® 19). Lantmännen (Vaasan bakery, Vilnius, Lithuania)/Lantmännen Cerealia AB (Järna, Sweden) provided bread and cereal products.

The test products were iso-energetic (WP and MD, HiFi and LoFi). Data on nutritional composition of the test products are given elsewhere (23). Calcium content within powders was

negligible. Participants were to refrain from any change in physical activity or any further change in dietary intake during the study, as no change in weight was intended.

The HiFi bread and cereals consisted of enzyme-treated wheat bran and refined wheat, while LoFi products were based only on refined wheat. Enzyme treatment was performed by DuPont Industrial Biosciences Aps (Brabrand, Denmark) to increase fiber solubility for improved baking properties.

The study was conducted in accordance with the Declaration of Helsinki of 1975 (as revised in 1983), approved by the Central Denmark Region Committees on Health Research Ethics (Journal no. 1-10-72-370-15) and registered at ClinicalTrials.gov (NCT02931630).

Study Visits

At trial initiation, participants attended the clinic after an overnight fast (minimum 10 h) by means of car or public transportation. Blood was drawn from an antecubital vein between 7.00 and 8.30 AM. A Whole-body Dual-Energy X-ray Absorptiometry (DXA) scan (Hologic Horizon A scanner, Hologic, Inc., Massachusetts, USA using Apex System Software version 5.6.0.5) was performed to assess body composition. Whole body BMD excluding the head region is reported in this manuscript. Participants collected 24-h urine samples using 3L containers in cooling bags, which were analyzed for carbamide and calcium. The procedures were repeated after 12 weeks of dietary intervention.

Participants picked up the intervention products at the test site with regular intervals.

Dietary adherence was assessed by self-reported 3-day weighed dietary records at the beginning, middle and end of the trial, and by measuring urinary carbamide and plasma alkylresorcinols (markers of dietary protein and fiber intake, respectively).

Blood Analysis

Blood samples were centrifuged at 2000 × *g* for 15 min at 4 °C, immediately frozen at −20 °C and moved to −80 °C within 8 h. All fasting values were calculated as the mean of three consecutive fasting blood samples.

Plasma glucose was measured on Cobas c111-system by standard enzymatic colorimetric assays using commercial kits (cat. 04657527, Roche Diagnostics GmbH). Intra-/inter-assay precision were between 0.8–1.1% and 0.5–0.6%. Plasma insulin was measured by ELISA technique using commercial kits (cat. K6219, Dako Denmark A/S) with intra-/inter-assay precision of 5.1–7.5% and 4.2–9.3%.

Serum CTX, P1NP, and PTH were measured on Cobas 6000 e 601 system using ELISA sandwich immunometric assays method (Roche Diagnostics GmbH). Intra-/inter-assay precision was 1.4–3.2%/1.9–3.4% for P1NP, 1.2–4.7%/1.5–5.7% for CTX, and 1.1–2.0%/1.7–3.4% for PTH.

Plasma ionized calcium, phosphate, magnesium, alkaline phosphatase, 25(OH)D, glomerular filtration rate (GFR) and urinary calcium and carbamide were analyzed at the Department of Clinical Biochemistry at Aarhus University Hospital, Denmark (DS/EN ISO 15189:2013 approved).

All analyses were pre-specified at the project origin.

Statistical Analysis

The final analysis included only participants who completed the trial.

Baseline values are displayed as means with SD unless otherwise indicated.

Two-factor ANOVA was used to assess any effect or interaction of protein or fiber intake on markers of bone turnover. Any significant effect was followed by a pairwise comparison of groups corrected for multiple comparisons by Tukey-Kramer method. Estimates were adjusted for age and sex.

Linear regression analysis was used to determine associations between change in insulin resistance and bone turnover markers in all subjects. Assumptions of linear regression were checked using scatter plots, QQ plots and histograms of the residuals. The regression analyses were not corrected for multiple testing due to the exploratory nature of the study and the intent to generate new hypotheses.

Normality and variance across groups was checked by diagnostic plots of the residuals. If these were not met, the dependent variable was log-transformed.

Homeostasis model assessment of insulin resistance was calculated by the formula: Fasting plasma glucose (mmol/L) x fasting plasma insulin (mU/L)/22.5.

All statistics were performed using STATA/IC 15.1 (StataCorp LP College Station, TX, USA).

Power calculations for sample size were based on expected change in postprandial triglycerides, which was the primary aim of the MERITS trial (23).

RESULTS

Baseline characteristics by randomization group are listed in **Table 1**. Further baseline values of the various outcomes are displayed in **Table 2**.

Study flow chart and dietary intake reports have previously been reported (23). In brief, 64 participants were included in the present study (one participant excluded due to osteoporosis). Two subjects dropped out due to dislike of test products. Test products were otherwise well tolerated. By self-reported, weighed, 3-d dietary intake reports, compliance was deemed high. The mean protein intake in the WP groups was 141.6 (16.5) g/d versus 86.8 (18.1) g/d in MD groups. Mean fiber intake was 34.6 (4.9) g/d in HiFi groups versus 16.0 (5.2) g/d in LoFi groups. The reports were supported by levels of plasma alkylresorcinol and urinary carbamide as markers of fiber and protein intake (23).

Table 2 shows baseline values and changes following the 12-week intervention within the four groups. Twelve weeks of high protein and/or high fiber intake did not affect levels of P1NP or CTX. Likewise, we did not find any change in PTH. However, as 25(OH)D is known to strongly affect PTH levels, we adjusted for change in 25(OH)D, which modified the results, showing a negative trend between protein intake and PTH levels (p=0.06).

As expected, we found an increased u-calcium and u-carbamide in both protein groups.

TABLE 1 | Baseline characteristics by randomization group.

	WP-LoFi	WP-HiFi	MD-LoFi	MD-HiFi
Subjects (n)	15	16	16	17
Age (years)[1]	67 (60,69)	65 (59, 69)	62 (58, 68)	64 (56, 67)
Sex (male/female)	9/6	7/9	8/8	7/10
Smoking (n)	2	0	0	2
BMI, kg/m^2	28 (4)	29 (2)	30 (4)	29 (4)
Metabolic syndrome (n)	10	9	8	7
Obesity, BMI>30 (n)	6	5	9	5
Postmenopausal (n)	4	8	6	8
p-calcium, ionized (mmol/L)	1.27 (0.04)	1.28 (0.03)	1.26 (0.04)	1.27 (0.03)
p-magnesium (mmol/L)	0.86 (0.06)	0.86 (0.04)	0.85 (0.05)	0.88 (0.05)
p-phosphate (mmol/L)	0.94 (0.16)	1.00 (0.12)	0.91 (0.24)	0.96 (0.15)
p-alkaline phosphatase (U/L)	77.1 (20.0)	84.9 (26.7)	72.3 (18.5)	75.8 (20.9)
p-eGFR (ml/min)	84.5 (5.8)	77.9 (10.7)	82.8 (9.2)	82.1 (8.5)

Values are means (SD) unless otherwise specified. [1] Median (25th and 75th centile).
WP, whey protein; MD, Maltodextrin; LoFi, low fiber; HiFi, high fiber; eGR, estimated glomerular filtration rate.

There was a non-significant negative association (p=0.06) between change in dietary fiber intake and change in 25(OH)D.

The intervention did not affect insulin resistance within the four groups (additional data on insulin resistance reported elsewhere (24)). **Table 3** displays the association between change in insulin resistance and change in bone turnover markers by linear regression analysis in all subjects. There was a positive association between change in HOMA-IR and PTH, while changes in P1NP and CTX did not associate to changes in HOMA-IR. The change in PTH was not associated with changes in CTX or P1NP (p=0.24, p=0.26).

DISCUSSION

It is a common belief, that bone health is influenced by dietary habits. In the standard treatment of osteoporosis (25), a bone healthy diet aiming at avoiding calcium and vitamin D deficiency, is recommended. However, when minerals and vitamins are accounted for, the effect of everyday meals on bone health remains sparse. The introduction of circulating bone turnover markers has provided a simple way of assessing the acute bone response to various stimuli. Ingestion of food suppresses the immediate bone turnover (21, 26–28), however

TABLE 2 | Baseline values and changes following 12-weeks of dietary intervention.

	WP-LoFi	WP-HiFi	MD-LoFi	MD-HiFi	Two-factor ANOVA, p value		
					Protein group	Fiber group	Inter-action
P1NP, baseline[1](µg/L)	47.2 (32.9, 65.7)	44.8 (35.1, 57.6)	36.8 (30.3, 45.9)	56.5 (40.1, 61.3)			
P1NP, change(µg/L)	-0.43 (11.47)	0.51 (7.35)	1.43 (5.99)	-1.93 (10.47)	0.88	0.53	0.37
P1NP, change (adj.) (µg/L)[2]	-0.24 (2.38)	0.26 (2.29)	2.22 (2.36)	-2.26 (2.27)	0.93	0.37	0.26
CTX, baseline[1](µg/L)	0.38 (0.23, 0.53)	0.37 (0.30, 0.49)	0.34 (0.25, 0,38)	0.34 (0.29, 0.47)			
CTX, change(µg/L)	-0.02 (0.10)	-0.01 (0.07)	-0.01 (0.08)	0.02 (0.07)	0.23	0.30	0.69
CTX, change (adj.)(µg/L)[2]	-0.02 (0.02)	-0.01 (0.02)	-0.01 (0.02)	0.02 (0.02)	0.23	0.32	0.71
PTH, baseline[1] (pmol/L)	4.0 (3.5, 5.5)	4.2 (3.3, 4.7)	3.7 (3.4, 4.8)	3.9 (3.4, 4.9)			
PTH, change(pmol/L)	-0.1 (0.7)	-0.0 (0.8)	0.2 (0.8)	0.1 (0.6)	0.15	0.96	0.54
PTH, change (adj.)(pmol/L)[2]	-0.14 (0.17)	-0.07 (0.16)	0.43 (0.17)	-0.02 (0.17)	0.06	0.26	0.12
HOMA-IR, baseline	1.92 (0.89)	1.83 (0.83)	2.15 (0.81)	1.50 (0.82)			
HOMA-IR, change	0.05 (0.76)	-0.12 (0.36)	0.27 (0.53)	0.10 (0.37)	0.10	0.25	0.98
U-calcium, baseline (mmol/d)	4.12 (1.33)	3.88 (2.58)	3.63 (1.50)	4.36 (2.54)			
U-calcium, change (mmol/d)	1.12 (1.29)*	0.34 (1.09)	-0.03 (1.61)	-0.28 (1.83)	**0.03**	0.23	0.50
U-carbamide, baseline (mmol/d)	382 (103)	302 (117)	363 (150)	320 (132)			
U-carbamide, change (mmol/d)	208 (33)*[a]	212 (30)*[a]	-36 (31)[b]	60 (29)*[b]	**<0.01**	0.11	0.13
T-score, baseline	-0.9 (1.5)	-0.96 (1.0)	-0.6 (1.05)	-1.15 (1.17)			
T-score, change	0.06 (0.14)	-0.03 (0.12)	0.10 (0.28)*	0.05 (0.15)	0.25	0.20	0.74
BMD, baseline	1.09 (0.14)	1.07 (0.10)	1.10 (0.11)	1.05 (0.12)			
BMD, change	0.01 (0.01)	-0.003 (0.01)	0.005 (0.02)	0.005 (0.01)	0.31	0.33	0.23
25(OH)D, basline (nmol/L)	64.1 (26.4)	74.8 (26.7)	60.8 (25.3)	75.4 (22.5)			
25(OH)D, change (nmol/L)	2.3 (12.6)	1.1 (13.9)	11.8 (24.9)*	-3.8 (16.9)	0.71	0.06	0.12

All values are means (SD) unless otherwise stated. Values within a row with different superscript letters are statistically different (p<0.05). P-values <0.05 are displayed in bold.
[1]Median (25th and 75th centile).
[2]Adjusted for change in 25(OH)D. Values given as mean change (SE),
WP, whey protein; MD, Maltodextrin; LoFi, low fiber; HiFi, high fiber; P1NP, procollagen type 1 N-terminal propeptide; CTX, C-terminal cross-linked telopeptide type 1 collagen; PTH, parathyroid hormone; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; BMD, bone mineral density; 25(OH)D, 25-hydroxy vitamin D.

TABLE 3 | Multiple linear regression analysis of change in insulin resistance by HOMA-IR and change in bone markers.

	Coefficient	95% CI	p-value
ΔP1NP	-0.01	(-0.02, 0.01)	0.29
ΔP1NP adjusted (sex)	-0.01	(-0.02, 0.01)	0.33
ΔP1NP adjusted (sex, 25(OH)D)	-0.01	(-0.02, 0.01)	0.27
ΔCTX	0.42	(-1.27, 2.11)	0.62
ΔCTX adjusted (sex)	0.29	(-1.43, 2.01)	0.74
ΔCTX adjusted (sex, 25(OH)D)	0.25	(-1.48, 1.99)	0.77
ΔPTH	0.19	(0.01, 0.37)	**0.038**
ΔPTH adjusted (sex)	0.19	(0.02, 0.37)	**0.033**
ΔPTH adjusted (sex, 25(OH)D)	0.32	(0.01, 0.63)	**0.042**

P1NP, procollagen type 1 N-terminal propeptide; CTX, C-terminal cross-linked telopeptide type 1 collagen; PTH, parathyroid hormone; 25(OH)D, 25-hydroxy vitamin D. P-values <0.05 are displayed in bold.

the long-term effect of daily meals of varying composition on bone health is not well characterized.

Diets of increased protein content are generally advised to preserve bone mass (29–31), although this recommendation is primarily based on pooled evidence from studies showing no adverse effects and at best, a modest reduction in fracture risk (32–34). Most studies are performed in healthy subjects or subjects with postmenopausal osteoporosis.

In type 2 diabetes, abdominal obesity and metabolic syndrome, bone turnover is low - in contrast to postmenopausal osteoporosis, where bone turnover is increased. Fracture risk is however increased, and the low bone turnover is suggested to accumulate microfractures and make the bone more fragile. Thus, increasing bone turnover in type 2 diabetes, abdominal obesity and metabolic syndrome may be beneficial for bone health. Very little research, if any, explore the long-term effects of diet and especially enhanced protein intake on bone within weight stable subjects with type 2 diabetes, abdominal obesity or metabolic syndrome.

We hypothesized that the increase in protein intake would lead to increased bone turnover. This was however not the case. Both bone formation and degradation markers remained unchanged after 12 weeks of whey protein versus placebo. Although, we did observe a negative trend for an association between protein intake and PTH levels (p=0.06). In normal weight subjects, low protein diets are known to lead to secondary increase in PTH which is believed to relate to reduced intestinal calcium absorption and increased bone turnover (35). Likewise, in healthy women increased protein intake is reported to lead to decreased PTH (36), which is consistent with our findings.

We report an increase in urinary calcium excretion in the protein group. It is well known that increased protein intake induces increased urinary calcium excretion (37). Increased calcium absorption from the gut (seemingly independent of calcium intake) and bone degradation have been proposed as potential mechanisms, although this remains widely debated. An increased absorption of calcium from the gut may explain our borderline significant negative association between protein intake and PTH. In the current study, the calcium intake from test products was negligible, as WP hydrolysate only added 6 mg/d of extra calcium (data not displayed). The finding of an increased urinary calcium excretion in the protein group concurs with existing evidence.

Thus, a protein-rich diet in subjects with abdominal obesity appears to affect calcium homeostasis similarly to normal weight individuals.

The secondary hypothesis of the study was that insulin resistance and bone turnover are inversely associated. We did however not observe any difference in insulin sensitivity by HOMA-IR or bone markers between groups. In a similar dietary intervention study on type 2 diabetic subjects a high protein diet improved HbA1c but not HOMA-IR, indicating that primarily postprandial insulin sensitivity was affected (38), which is not reflected in HOMA-IR. In our study, postprandial insulin sensitivity assessed by Matsuda index was however unaffected (data shown elsewhere (24),). As the present study was primarily designed to identify changes in the lipid metabolism and not in bone markers or insulin resistance, we may not have had enough subjects to obtain significant changes, which naturally is a weakness of this *post hoc* study.

As formerly mentioned, insulin resistant subjects with type 2 diabetes are reported to have low bone turnover (3). We previously reported associations between decreased levels of bone turnover markers and insulin resistance in non-diabetic subjects with abdominal obesity (5). In the current study, we hoped to see bone turnover increase in subjects that became more insulin sensitive, and potentially *vice versa*, assuming that the regulatory mechanism is reversible. This was not the case, as we did not see any association between change in P1NP or CTX with change in HOMA-IR. We did find an association between increase in PTH and increase in insulin resistance in all subjects independent of the dietary intervention. This finding is interesting as it aligns with previous reports were hyperparathyroidism is associated with diabetes and insulin resistance (39). How the association between PTH and insulin resistance relates to a reduced bone turnover in patients with type 2 diabetes is not well understood, but a possible mechanism may be elicited by osteocytic dysfunction with excess production of sclerostin (8).

We report the findings of a *post hoc* analysis of a randomized controlled trial. Thus, no power calculation was performed on bone turnover markers which is a limitation of the current study. As we did not observe a trend on changes in markers of bone turnover, we do not expect that a larger sample would have revealed any differences in CTX or P1NP between the groups. It is possible that a population of increased insulin resistance and lower bone turnover at baseline, such as T2D subjects, would

have increased the chances of detecting changes in bone turnover. We assume our study duration is too short to induce changes in BMD. Furthermore, we have not measured BMD at regional sites (e.g. lumbar spine and hip). We found a nonsignificant increase in 25(OH)D within the LoFi groups (p=0.06), that was most likely due to lower baseline values in these groups. Any seasonable variation during the 13 months the trial ran (from May 2016 to June 2017), was expected similar within groups because of the continuous block randomization.

CONCLUSION

The current study did not find an effect of long-term supplementation of dietary protein or fiber on bone turnover in subjects with abdominal obesity. PTH tended to associate negatively with protein intake, although bone turnover markers remained unaffected. A high protein intake induced increased urinary calcium excretion, unrelated to increased calcium intake. We did find an association between measures of insulin resistance and levels of circulating PTH levels, which supports the hypothesis that insulin resistance may be key to understand the low bone turnover and increased bone fragility observed in subjects with T2D.

More long-term studies on diet and the bone turnover of subjects with type 2 diabetes, abdominal obesity and metabolic syndrome are needed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Central Denmark Region Committees on Health Research Ethics (Journal no. 1-10-72-370-15). The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RF-N, ER, SG, and KH conceived and designed the study. RF-N and ER conducted the study. RF-N, PV, and JS-L analyzed the data. RF-N wrote the initial manuscript. All authors critically reviewed and approved the final manuscript.

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The Positive Relationship Between the Low-Density Lipoprotein Cholesterol/Apoprotein B Ratio and Bone Turnover Markers in Patients With Type 2 Diabetes

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Background: Dyslipidemia may contribute to low bone turnover in patients with type 2 diabetes (T2D) through mediating oxidative stress and atherosclerosis. The low-density lipoprotein cholesterol/apoprotein B (LDL-C/Apo B) ratio is a surrogate marker of small and density low-density lipoprotein cholesterol (sd-LDL-C), a most harmful group of LDL-Cs. The present study aimed to investigate the association between the LDL-C/Apo B ratio and bone turnover in patients with T2D.

Methods: This study was a cross-sectional study enrolled patients with T2D from January 2021 to December 2021. Each participant was assessed for lipid profiles, bone turnover markers (BTMs), lumbar spine (L1-L4) and hip dual-energy X-ray absorptiometry (DXA) scans. Osteoporosis was diagnosed as a T-score lower than or equal to -2.5 at the spine or hip.

Results: A total of 335 patients with T2D were enrolled in the study, and the LDL-C/Apo B ratio ranged from 0.78 to 4.00. Along with the LDL-C/Apo B ratio tertile ascending, osteocalcin (OC), C-terminal telopeptide (CTx) and N-terminal propeptide of type-I procollagen (PINP) levels gradually increased (all $p < 0.05$). There were no differences in lumbar spine and hip T-score, proportion of osteoporosis (all $p > 0.05$) among the three subgroups. The LDL-C/Apo B ratio was positively correlated with lnOC ($r = 0.244$, $p < 0.001$), lnCTx ($r = 0.226$, $p < 0.01$) and lnPINP ($r = 0.211$, $p < 0.001$). These significant positive correlations persisted even when divided into male and female subgroups. Furthermore, three multiple linear regression analyses were constructed to investigate the independent association of the LDL-C/Apo B ratio with the BTMs levels. After adjusting for other clinical parameters, the LDL-C/Apo B ratio was still significantly associated with OC level ($\beta = 0.199$, $t = 3.348$, $p < 0.01$), CTx level ($\beta = 0.238$, $t = 4.084$, $p < 0.001$) and PINP level ($\beta = 0.162$, $t = 2.741$, $p < 0.01$).

Conclusion: The LDL-C/Apo B ratio was significantly and positively associated with BTMs in patients with T2D. In clinical practice, more attention should be paid to the patients with T2D whose LDL-C/Apo B ratio is relatively low for the purpose of maintaining bone health.

Keywords: type 2 diabetes, bone turnover, low-density lipoprotein cholesterol/apolipoprotein B ratio, small and density low-density lipoprotein cholesterol, osteocalcin, C-terminal telopeptide, N-terminal propeptide of type-I procollagen

INTRODUCTION

Type 2 diabetes (T2D) and its chronic complications have posed a great threat to global public health, among which bone fragility has attracted more and more attention due to its high incidence, high disability rate and serious impact on quality of life (1). Bone fragility may contribute to an up to three-fold increased risk of lip fractures and more common wrist and foot fractures in people with T2D than in healthy people (2). However, bone mineral density (BMD) is not sufficient to reflect alternations in bone fragility in patients with T2D, as studies have shown that these patients have a 5-10% increase in BMD compared to their peers without T2D (3). The maintenance of bone health requires the continuous replacement of worn bone tissue with newly synthesized calcified bone matrix throughout life, a process named as bone turnover (4). Bone turnover markers (BTMs) include osteocalcin (OC), C-terminal telopeptide (CTx) and N-terminal propeptide of type-I procollagen (PINP), and a series of studies have consistently concluded that BTMs were significantly reduced and were associated with increased risks of fragility fracture in patients with T2D compared to healthy controls (5). Therefore, it is of great significance to discover and timely intervene the risk factors of low bone turnover in patients with T2D to improve the prognosis of these patients.

T2D is usually accompanied by hyperlipidemia due to insulin resistance, and hyperlipidemia may be involved in multiple chronic complications of T2D (6). *In vitro* studies showed that oxidized lipids reduced bone turnover by inhibiting osteoblast differentiation and inducing osteoclast differentiation (7). Low-density lipoprotein cholesterol (LDL-C) is a heterogeneous group of lipoproteins, among which with smaller sizes and heavier densities were known as small and density LDL-C (sdLDL-C) (8). Compared with other LDL-Cs, sdLDL-C is characterized by low affinity with LDL receptor, long half-life, susceptible to oxidation, easy to penetrate into the artery wall and so on (9). The LDL-C/apolipoprotein B (LDL-C/Apo B) ratio is a surrogate marker of LDL particle size, and the smaller the LDL-C/Apo B ratio is, the more dominant SD-LDL particles are in LDL particles (10). Due to the methods of detecting sd-LDL-C are laborious, the LDL-C/Apo B ratio is commonly used in clinical work and scientific research (10). Clinical studies revealed that the LDL-C/Apo B ratio was significantly negatively associated with instability of atherosclerotic coronary atherosclerotic plaques (11) and restenosis of coronary stents (12). As bone is highly vascularized connective tissue, affecting the blood supply for bone tissue contributes to the decline of bone turnover (13). The LDL-C/Apo B ratio was closely related to atherosclerosis and oxidative stress, which both were major pathogenesis of low bone turnover in patients with T2D. Therefore, we speculated that the LDL-C/Apo B ratio might be closely associated with BTMs in patients with T2D; those with a lower LDL-C/Apo B ratio may have greater suppression in bone turnover than those with a higher LDL-C/Apo B ratio. However, few studies have explored the relationship between the two.

In the present study, we aimed to estimate the relationship of the LDL-C/Apo B ratio with BTMs in patients with T2D.

METHODS

Study Design and Participants

This was an observational cross-sectional study which was performed among patients diagnosed with T2D at the Second Affiliated Hospital of Nantong University between January 2021 to October 2021 (14). The main exclusion criteria were as follows: type 1 diabetes, previous use of steroids and anabolic steroids, previous use of antiosteoporosis drugs (e.g., vitamin D, calcium tablet, bisphosphonates, denosumab and selective estrogen receptor modulators), previous or current received antiandrogen therapy, previous use of proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, previous or current malignant tumors, chronic hepatitis and heart failure, acute diabetic complications, history of lumbar surgery, history of thyroid or parathyroid disease. Finally, total 335 patients with T2D were included in the present study. Written informed consent was given after each subject fully understood the present study protocol. The study followed the Declaration of Helsinki thoroughly and was approved by the medical research ethics committee of the Second Affiliated Hospital of Nantong University.

Basic Data Collection

Clinical data including the demographic data, lifestyle, medication history and diagnosis history of diseases were collected by interviewing and examining each participant upon enrollment. Body mass index (BMI) was calculated as the weight/height squared. Blood pressure was measured by a standard mercury sphygmomanometer, and the average of three recordings was recorded.

Laboratory Examination

Fasting blood samples and fresh first-void morning urine samples were collected for respective measurement of blood indexes, urinary albumin and urinary creatinine after enrollment. The urinary

albumin-to-creatinine ratio (UACR) was calculated as the ratio of urinary albumin to urinary creatinine. Lipid profiles such as triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL-C), LDL-C, apoprotein A (Apo A) and apoprotein B (Apo B) levels, glucose, uric acid (UA), creatinine (Cr) and cystatin C levels were measured using an automated biochemical analyzer (Model 7600, Hitachi) with the inter-assay and intra-assay coefficients of variation (CVs) < 2.8%, and the LDL-C/Apo B ratio was calculated as the ratio of LDL-C to Apo B ratio. According to the CKD-EPI creatinine-cystatin C equation, estimated glomerular filtration rate (eGFR) was calculated (2012) (15). Chronic kidney disease (CKD) was defined based on an eGFR < 60 ml/min/1.73 m^2 or a UACR ≥ 30 mg/g lasting more than 3 months (16). HbA1c levels were measured with an ion exchange-based high-performance liquid chromatography (HPLC) method in a hemoglobin analysis system (D-10, Bio-Rad) with the inter-assay and intra-assay CVs < 3.0%. Serum C-peptide (CP) levels were measured by the chemiluminescence method in an immunoassay system (DxI 800, Beckman Coulter) with the inter-assay and intra-assay CVs < 4.0%. In order to eliminate the influence of exogenous insulin, HOMA-IR_{CP} which was defined as (fasting glucose × fasting CP)/22.5 was adopted as an indicator of insulin resistance (17). Serum OC, CTx, PINP, parathyroid hormone (PTH) and vitamin D levels were analyzed on an automated immunoassay system (iSYS, Immunodiagnostic Systems Ltd., Boldon) using a chemiluminescence method.

The corresponding CVs from the manufacturers as follows: OC < 2.0%, CTx < 5.4%, PINP < 4.5%, PTH < 4.5%, and vitamin D < 4.0%.

Diagnosis of Peripheral Artery Disease

The ankle-brachial index (ABI) was detected in each participant using the color Doppler blood flow device (Chioy Medical, Beijing) under the operation of an experienced physician. Then peripheral artery disease (PAD) was diagnosed with reference to the Inter-Society Consensus for the Management of Peripheral Arterial Disease guideline and based the ABI values (18).

Measurement of Bone Mineral Density

All participants underwent spine (L1-L4) and hip dual-energy X-ray absorptiometry (DXA) scans on Prodigy Scanners (GE-Healthcare, Madison) by trained investigators, and the results were analyzed according to the manufacturer's recommendations. The calculation of T-score was based on the peak BMD in healthy young people of the same race and gender, and the calculation formula of T-score was (measured BMD - peak BMD in healthy young people of the same race and gender)/standard deviation of peak BMD in healthy young people of the same race and sex. The daily CV value of DXA was controlled below 0.24%. Average T-scores of L1-L4 and T-scores of hips were recorded for further analyses. Osteoporosis was diagnosed as a T-score lower than or equal to -2.5 at the spine or hip (19).

Statistical Analyses

The total participants were divided into three subgroups based on the LDL-C/Apo B ratio. Kolmogorov-Smirnov test was first conducted to test whether continuous variables conformed to normal distribution. In order to achieve a normal distribution for further analysis, a natural logarithm transformation (ln) was applied, such as lnOC, lnCTX and lnPNIP. The normally and skewed distributed continuous variables and the categorical variables were respectively described as mean ± SD, median (25 and 75% interquartile) and frequencies (percentages). We adopted the one-way analysis of variance, the Kruskal-Wallis test and the chi-square test to compare differences in normally distributed data, skewed distributed data and categorical data, respectively. Pearson's bivariate correlation analyses were applied to investigate the correlations of the LDL-C/Apo B ratio with BTMs in the total population and separately in men and women. Furthermore, we constructed three multiple linear regression analyses to investigate the independent association of the LDL-C/Apo B ratio with the BTMs levels. Before conducting the linear regression analyses, the case analyses screening outliers were carried out first. If the standardized residual values were not in the range of -3 to 3, the outliers should be considered. Later, the case records were retrieved to exclude the abnormal data as a result of typing errors, and if not, the corresponding data were removed. Data analyses were performed on SPSS statistical software 18.0 (IBM SPSS Inc., USA). A value of $p < 0.05$ was defined as statistical significance.

RESULTS

Clinical Characteristics of the Study Participants

Table 1 showed the clinical characteristics of the total population and the three subgroups based on the LDL-C/Apo B ratio tertiles. Along with the LDL-C/Apo B ratio ascending, lnOC, lnCTx and lnPINP levels gradually increased (all $p < 0.05$). BMI, proportion of peripheral artery disease, statins use, use of acarbose and TG level were the highest in T1, followed by T2 and T3, while TC and Apo B levels were the highest in T3, followed by T2 and T1 (all $p < 0.05$). Among the three subgroups, there were significant differences in percentage of hypertension, use of β-blocker and Apo A level (all $p < 0.05$). However, there were no differences in age, proportion of males, diabetic duration, systolic/diastolic blood pressure, use of other antidiabetic treatments and other antihypertensive treatments, HbA1c level, HOMA-IR_{CP} level, HDL-C level, LDL-C level, UACR level, eGFR level, proportion of diabetic kidney disease, PTH level, vitamin D level, lumbar spine and hip T-score, proportion of osteoporosis (all $p > 0.05$).

Relationships Between the LDL-C/Apo B Ratio and BTMs

As illustrated in **Table 2**, the LDL-C/Apo B ratio was positively associated with lnOC, lnCTx and lnPINP levels ($r = 0.244$, 0.226 and 0.211, respectively, all $p < 0.001$). These significant and positive correlations persisted even when divided into male and female subgroups.

Multiple Linear Regression Models Displayed Independent Associations of the LDL-C/Apo B Ratio With BTMs Levels

In **Table 3**, the LDL-C/Apo B ratio was significantly and positively associated with lnOC level (β = 0.244, t = 4.582, p < 0.001,

TABLE 1 | Clinical characteristics of the study participants.

Variables	Total	T1	T2	T3	*p* value
LDL-C/Apo B ratio	2.68 (2.43-2.89)	<2.54	2.54-2.80	>2.80	
LDL-C/Apo B ratio (range)	0.78-4.00	0.78-2.53	2.53-2.81	2.83-4.00	
n	335	112	115	108	
Age (years)	58.37 ± 12.70	58.80 ± 12.46	57.55 ± 13.28	58.79 ± 12.39	0.920
Male, *n* (%)	178 (53.1)	60 (53.6)	65 (56.5)	53 (49.1)	0.534
Diabetic duration (years)	6.0 (2.0-10.0)	6.5 (2.0-10.0)	6.0 (1.0-10.0)	7.0 (2.0-10.0)	0.843
BMI (kg/m^2)	25.54 ± 3.82	25.94 ± 4.46	25.77 ± 3.60	24.91 ± 3.30	0.027
Hypertension, *n* (%)	118 (35.2)	47 (42.0)	35 (30.4)	36 (33.3)	0.008
SBP (mmHg)	137.70 ± 19.69	136.56 ± 20.21	137.53 ± 18.45	139.06 ± 20.50	0.440
DBP (mmHg)	83.55 ± 11.33	83.08 ± 12.35	84.37 ± 11.02	83.18 ± 10.59	0.287
PAD, *n* (%)	164 (49.0)	65 (58.0)	58 (50.4)	41 (38.0)	0.011
Antidiabetic treatments					
Insulin treatment, *n* (%)	91 (27.1)	26 (23.2)	27 (23.5)	38 (35.2)	0.075
Metformin, *n* (%)	158 (47.2)	61 (54.5)	52 (45.2)	45 (41.7)	0.144
Acarbose, *n* (%)	23 (6.9)	15 (13.4)	5 (4.3)	3 (2.8)	0.003
Insulin-secretagogues, *n* (%)	106 (31.6)	36 (32.1)	42 (36.5)	28 (25.9)	0.233
Insulin-sensitizers, *n* (%)	34 (10.1)	11 (9.8)	12 (10.4)	11 (10.2)	0.988
DPP-4 inhibitors, *n* (%)	27 (8.1)	9 (8.0)	9 (7.8)	9 (8.3)	0.990
SGLT-2 inhibitors, *n* (%)	29 (8.7)	14 (12.5)	10 (8.7)	5 (4.6)	0.116
Antihypertensive treatments					
CCB, *n* (%)	84 (25.1)	36 (32.1)	26 (22.6)	22 (20.4)	0.099
ARB, *n* (%)	75 (22.4)	31 (27.7)	26 (22.6)	18 (16.7)	0.146
β-blockers, *n* (%)	23 (6.9)	14 (12.5)	4 (3.5)	5 (4.6)	0.014
Diuretics, *n* (%)	31 (9.3)	10 (8.9)	11 (9.6)	10 (9.3)	0.986
Statins medications, *n* (%)	22 (6.6)	18 (16.1)	3 (2.6)	1 (0.9)	<0.001
HbA1c (%)	9.27 ± 2.06	9.46 ± 2.15	9.19 ± 2.02	9.16 ± 2.06	0.701
HOMA-IR$_{CP}$	0.45 (0.24-0.72)	0.53 (0.29-0.74)	0.44 (0.28-0.79)	0.42 (0.21-0.68)	0.386
UACR (mg/g)	16.25 (8.00-44.20)	16.20 (8.85-42.05)	17.10 (7.98-53.85)	15.90 (7.40-37.10)	0.384
eGFR (ml/min/1.73m^2)	97.52 ± 28.02	96.74 ± 27.79	97.95 ± 28.70	97.83 ± 27.81	0.946
CKD, *n* (%)	45 (13.4)	21 (18.8)	13 (11.3)	11 (10.2)	0.126
TG (mmol/L)	1.66 (1.07-2.59)	2.09 (1.27-3.67)	1.64 (1.12-2.49)	1.28 (0.90-2.02)	<0.001
TC (mmol/L)	4.26 (3.72-5.02)	3.69 (2.98-4.38)	4.16 (3.80-4.78)	4.98 (4.37-5.46)	<0.001
HDL-C (mmol/L)	1.11 ± 0.26	1.01 ± 0.25	1.11 ± 0.24	1.23 ± 0.24	0.986
LDL-C (mmol/L)	2.76 ± 0.88	2.06 ± 0.71	2.85 ± 0.69	3.39 ± 0.67	0.914
Apo A (mmol/L)	1.10 (1.00-1.27)	1.04 (0.95-1.17)	1.09 (0.91-1.17)	1.08 (0.99-1.29)	<0.001
Apo B (mmol/L)	1.02 (0.87-1.18)	0.94 (0.74-1.07)	1.04 (0.91-1.17)	1.08 (0.99-1.29)	<0.001
lnOC	2.39 ± 0.47	2.27 ± 0.48	2.41 ± 0.42	2.48 ± 0.49	0.007
lnCTx	-0.88 ± 0.57	-0.97 ± 0.64	-0.85 ± 0.54	-0.81 ± 0.52	0.040
lnPINP	3.64 ± 0.47	3.54 ± 0.45	3.68 ± 0.42	3.70 ± 0.53	0.030
PTH (pg/mL)	34.95 (27.13-46.60)	33.55 (24.28-45.75)	37.80 (29.50-46.60)	34.10 (26.50-46.60)	0.105
Vitamin D (ng/mL)	16.42 ± 6.95	15.78 ± 6.90	16.22 ± 6.56	17.30 ± 7.39	0.364
Lumbar spine T-score	-1.35 (-2.28–0.225)	-1.85 (-2.63–0.75)	-1.60 (-2.00–0.80)	-1.10 (-2.40-0.00)	0.708
Hip T-score	-1.10 (-1.80–0.20)	-1.20 (-1.80–0.20)	-0.90 (-1.80-0.20)	-1.20 (-2.30–0.20)	0.439
OP, *n* (%)	111 (33.1)	38 (33.9)	32 (27.8)	41 (37.6)	0.458

Normally distributed values in the table are given as the mean ± SD, skewed distributed values are given as the median (25 and 75% interquartiles), and categorical variables are given as frequency (percentage).

LDL-C/Apo B ratio low-density lipoprotein cholesterol/apoprotein B ratio, BMI body mass index, SBP/DBP, systolic/diastolic blood pressure, PAD peripheral artery disease, Insulin-secretagogues insulin secretagogues, Insulin-sensitizers insulin sensitizing agents, DPP-4 inhibitors dipeptidyl peptidase-4 inhibitors, sodium-glucose co-transporter-2 inhibitors SGLT-2 inhibitors, CCB calcium channel blockers, ARB angiotensin receptor blockers, HbA1c glycosylated hemoglobin A1c, UACR urinary albumin-to-creatinine ratio, eGFR estimated glomerular filtration rate, CKD chronic kidney disease, TG triglyceride, TC total cholesterol, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, Apo A apolipoprotein A, Apo B apolipoprotein B, OC osteocalcin, CTx C-terminal telopeptide, PINP N-terminal propeptide of type-I procollagen, PTH parathyroid hormone, OP osteoporosis.

$R^2 = 0.059$), lnCTx level ($\beta = 0.226$, $t = 4.220$, $p < 0.001$, $R^2 = 0.051$) and lnPINP level ($\beta = 0.211$, $t = 3.933$, $p < 0.001$, $R^2 = 0.045$). After adding the other clinical covariates in each model step by step, the R^2 was gradually increased. In the model 1, after adjusting for age, sex, diabetic duration, BMI, systolic/diastolic blood pressure and smoking history, the LDL-C/Apo B ratio was significantly and positively associated with lnOC level ($\beta = 0.193$, $t = 3.426$, $p < 0.01$, $R^2 = 0.073$), lnCTx level ($\beta = 0.213$, $t = 3.885$, $p < 0.001$, $R^2 = 0.127$) and lnPINP level ($\beta = 0.158$, $t = 2.860$, $p < 0.01$, $R^2 = 0.109$). Antidiabetic treatments, antihypertensive treatments and statins medications were then added as clinical covariates in the model 2, and the LDL-C/Apo B ratio was significantly and positively associated with lnOC level ($\beta = 0.192$, $t = 3.238$, $p < 0.01$, $R^2 = 0.107$), lnCTx level ($\beta = 0.219$, $t = 3.833$, $p < 0.001$, $R^2 = 0.175$) and lnPINP level ($\beta = 0.155$, $t = 2.644$, $p < 0.01$, $R^2 = 0.131$). The fully adjusted model 3 further adjusted for HbA1c, HOMA-IR$_{CP}$ and eGFR, the LDL-C/Apo B ratio was still significantly and positively associated with lnOC level ($\beta = 0.199$, $t = 3.348$, $p < 0.01$, $R^2 = 0.171$), lnCTx level ($\beta = 0.238$, $t = 4.084$, $p < 0.001$, $R^2 = 0.202$) and lnPINP level ($\beta = 0.162$, $t = 2.741$, $p < 0.01$, $R^2 = 0.172$).

TABLE 2 | Relationships between the LDL-C/Apo B ratio and BTMs.

Variables	Total		Male		Female	
n	335		178		157	
	r	*p* value	*r*	*p* value	*r*	*p* value
lnOC	0.244	<0.001	0.193	0.010	0.286	<0.001
lnCTx	0.226	<0.001	0.202	0.007	0.228	0.004
lnPINP	0.211	<0.001	0.152	0.043	0.251	0.002

r Pearson's correlation coefficient.

TABLE 3 | Multiple linear regression models displayed independent associations of the LDL-C/Apo B ratio with BTMs levels.

Models	B (95% CI)	*β*	*t*	*p*	R^2 for model
lnOC					
Model 0	0.278 (0.158-0.397)	0.244	4.582	<0.001	0.059
Model 1	0.213 (0.091-0.335)	0.193	3.426	0.001	0.073
Model 2	0.212 (0.083-0.341)	0.192	3.238	0.001	0.107
Model 3	0.220 (0.091-0.350)	0.199	3.348	0.001	0.171
lnCTx					
Model 0	0.312 (0.167-0.457)	0.226	4.220	<0.001	0.051
Model 1	0.295 (0.146-0.445)	0.213	3.885	<0.001	0.127
Model 2	0.304 (0.148-0.460)	0.219	3.833	<0.001	0.175
Model 3	0.333 (0.172-0.493)	0.238	4.084	<0.001	0.202
lnPINP					
Model 0	0.241 (0.121-0.362)	0.211	3.933	<0.001	0.045
Model 1	0.178 (0.055-0.300)	0.158	2.860	0.005	0.109
Model 2	0.174 (0.045-0.304)	0.155	2.644	0.009	0.131
Model 3	0.184 (0.052-0.316)	0.162	2.741	0.007	0.172

Model 0, unadjusted model.
Model 1, adjusted for age, sex, diabetic duration, BMI, SBP, DBP, smoking history.
Model 2, additionally adjusted for antidiabetic treatments, antihypertensive treatments, statins medications.
Model 3, additionally adjusted for HbA1c, HOMA-IR_{CP}, eGFR.

DISCUSSION

In the present study, we investigated the association between the LDL-C/Apo B ratio with BTMs in patients with T2D. The main findings are as follows: first, with the increase of the LDL-C/Apo B ratio tertile, serum OC, CTx and PINP levels gradually increased; second, the LDL-C/Apo B ratio was significantly and positively related to serum OC, CTx and PINP levels; third, after adjusting for sex, age, diabetic duration and other clinical factors *via* multiple linear regression analysis, the LDL-C/Apo B ratio was still significantly and positively associated with serum OC, CTx and PINP level. Collectively, a decreased LDL-C/Apo B ratio is independently associated with low bone turnover in patients with T2D.

Existing guidelines recommend detecting BMD (T-score) *via* DXA for the initial diagnosis of osteoporosis, and osteoporosis is diagnosed based on a T-score lower than or equal to -2.5 at the spine or hip (19). However, the Rotterdam study showed an increased risk of fracture despite higher BMD in patients with T2D or impaired glucose tolerance (IGT) compared with those with normal glucose metabolism (20). A meta-analysis got similar results that with BMDs in patients with T2D were 4-5% higher than in healthy population (21). These results suggested that BMD should not be the only concern for bone health in patients with T2D. Manavalan et al. performed bone biopsies in patients with T2D and controls, and found significant reduced bone formation rate, osteoid surface, and osteoblast surface in T2D patients, suggesting the importance of bone turnover (22). Most studies demonstrated that bone turnover in patients with T2D was decreased (5, 23, 24). Hence, the present study aimed to explore the relationship between the LDL-C/Apo B ratio and BTMs in patients with T2D.

Risk factors for low bone turnover in patients with T2D include glucose control, use of hypoglycemic drugs, hyperlipidemia, advanced glycation end products (AGEs), bone microvascular disease and so on (25). Poor glycemic control is the initiating factor of the onset and progression of diabetic chronic complications, and a cross-sectional study of men with T2D revealed that a HbA1c greater than 7% was closely associated with decreased bone turnover (26). Reactive oxygen species (ROS) is an important mediator of diabetic chronic complications induced by hyperglycemia, hyperlipemia and AGEs (27). ROS can directly inhibit Wnt/β-catenin signaling pathway, which is critical for bone formation (28). ROS is also a key signaling molecule mediating inflammation, which induces the expression of Dickkopf-related protein 1 (DKK1) (29). Subsequently, DKK1 can bind to lipoprotein receptor-related protein 6 (LRP6) to form a complex contributing to the internalization of LRP6. LRP6, a

member of the LDLR gene family, is a co-receptor of Wnt, and the internalization of LRP6 can lead to the inhibition of Wnt/β-catenin signaling pathway (30). In addition, in hyperlipidemia rat model hyperlipidemia could inhibit dishevelled-2 expression and its phosphorylation, thus inhibiting Wnt/β-catenin signaling pathway (31). Consistently, the present study found that a low LDL-C/Apo B ratio was an independent contributor to low bone turnover in patients with T2D.

LDL-C is an important risk factor for atherosclerosis, and a study revealed that serum LDL-C was an independent risk factor for fragility fractures in postmenopausal women (32). Compared with other LDL-Cs, sd-LDL-C has delayed catabolism and increased oxidation susceptibility (33). In high-fat fed rats, oxidized LDL could inhibit bone formation by blocking osteoblast progenitor cells differentiation and inhibiting OC expression in marrow (34). Similarly, in our study the LDL-C/Apo B ratio was positively associated with serum OC and PINP levels. Additionally, we also observed a positive relationship between the LDL-C/Apo B ratio and CTx level. However, oxidative lipids could promote the differentiation of marrow preosteoclasts *in vitro*, so oxidized lipids might be positively related to serum CTx level (35). This discrepancy may be explained by other mechanisms of low bone turnover in T2D.

Bone microvascular disease is also a vital pathogenesis of decreased bone turnover in patients with T2D (13). As well as peripheral vessels, a histological study showed that intraosseous arterioles also occurred atherosclerosis plagues (36). Legs without atherosclerotic plaques had higher BMD than those with atherosclerotic plaque (37). In addition, the accumulation of oxidized lipoprotein particles was observed in the subcutaneous space surrounding blood vessels (38). In the present study, proportion of peripheral artery disease (PAD) increased from the first tertile to the third tertile of the LDL-C/Apo B ratio. These results highlighted the role of lipids in affecting bone turnover by inducing bone microvascular injury.

In this study, the LDL-C/Apo B ratio was significantly and positively correlated with BTMs levels even after adjusting for other covariates. In line with these results, a review suggested that dyslipidemia might aggravate atherosclerosis independently of serum LDL-C levels, possibly due to the presence of sd-LDL-C (39). Worse, uses of statin did not significantly reduce serum sd-LDL-C levels in patients with acute ischemic stroke (40). Similarly, the present study also observed that proportion of statin use was highest in the first tertile of the LDL-C/Apo B ratio. Therefore, adequate attention should be paid to the LDL-C/Apo B ratio in patients with T2D.

Although the present study observed an independent positive correlation between the LDL-C/Apo B ratio and BTMs, we failed to observe a correlation between the LDL-C/Apo B ratio and T-score. This was similar to other studies (26, 41), which might ascribe to the fact that BMD or T-score could not fully reflect bone alternations in T2D.

Several limitations of this study should be pointed out. First, the causal relationship between the LDL-C/Apo B ratio and bone turnover could not be concluded based on the present study, which was an observational cross-sectional study. Longitudinal and intervention studies are needed to address the limitation. Second, although we intended to present the trend of BTMs among the LDL-C/Apo B ratio tertiles in **Table 1**, grouping by the tertiles of LDL-C/Apo B ratio had no biological significance. In future studies, the association between the LDL-C/Apo B ratios and sd-LDL-C levels needs to be evaluated, and then grouping may be more meaningful than grouping directly by the tertiles of this ratio. Third, the LDL-C/Apo B ratio is a substitute index rather than the gold standard for evaluating sd-LDL-C, but the relationship between LDL-C/Apo B ratio and sd-LDL-C has been fully verified by multiple clinical studies. Fourth, high-resolution peripheral quantitative computed tomography (HRpQCT) may reflect bone microarchitectural properties better than DXA, so HRpQCT should be carried out simultaneously in future studies. Fifth, the generalization of this study was limited by the fact that all subjects enrolled in this study were Chinese.

In a word, the LDL-C/Apo B ratio was significantly and positively associated with BTMs in patients with T2D. In clinical practice, more attention should be paid to the patients with T2D whose LDL-C/Apo B ratio is relatively low for the purpose of maintaining bone health. This study is a hypothesis generating study, and we hypothesis that a predominance of sd-LDL-C in LDL-Cs may be an important risk factor for low bone turnover in patients with T2D by inducing oxidative stress and artery trauma. This hypothesis requires further follow-up and intervention studies to evaluate the effects of sd-LDL-C on bone turnover in patients with T2D, and to explore the mechanisms of sd-LDL-C inducing low bone turnover in animal and cell experiments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the medical research ethics committee of Second Affiliated Hospital of Nantong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Cf-L and W-sL participated in the design of the study, data collection, analysis of the data, and drafting of the manuscript. X-qW and J-bS conceived of the study, participated in its design and revised the manuscript. X-qG, H-yH, and L-yH participated in data collection. All authors contributed to the article and approved the submitted version.

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SGLT2 inhibitor treatment is not associated with an increased risk of osteoporotic fractures when compared to GLP-1 receptor agonists: A nationwide cohort study

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Background: Type 2 diabetes mellitus (T2D) is associated with an increased fracture risk. It is debated whether sodium-glucose cotransporter 2 (SGLT2) inhibitors influence fracture risk in T2D. We aimed to investigate the risk of major osteoporotic fractures (MOF) with SGLT2 inhibitors compared to glucagon-like peptide 1 (GLP-1) receptor agonists when used as add-on therapies to metformin.

Methods: We conducted a population-based cohort study using Danish national health registries. Diagnoses were obtained from discharge diagnosis codes (ICD-10 and ICD-8-system) from the Danish National Patient Registry, and all redeemed drug prescriptions were obtained from the Danish National Prescription Registry (ATC classification system). Subjects treated with metformin in combination with either SGLT2 inhibitors or GLP-1 receptor agonists were identified and enrolled from 2012 to 2018. Subjects were then propensity-score matched 1:1 based on age, sex, and index date. Major osteoporotic fractures (MOF) were defined as hip, vertebral, humerus, or forearm fractures. A Cox proportional hazards model was utilized to estimate hazard rate ratios (HR) for MOF, and survival curves were plotted using the Kaplan-Meier estimator.

Results: In total, 27,543 individuals treated with either combination were identified and included. After matching, 18,390 individuals were included in the main analysis (9,190 in each group). Median follow-up times were 355 [interquartile range (IQR) 126-780] and 372 [IQR 136-766] days in the SGLT2 inhibitor and GLP-1 receptor agonist group, respectively. We found a crude HR of 0.77 [95% CI 0.56-1.04] for MOF with SGLT2 inhibitors compared to GLP-1 receptor agonists. In the

fully adjusted model, we obtained an unaltered HR of 0.77 [95% CI 0.56-1.05]. Results were similar across subgroup- and sensitivity analyses.

Conclusion: These results suggest that SGLT2 inhibitors have no effect on fracture risk when compared to GLP-1 receptor agonists. This is in line with results from previous studies.

KEYWORDS

SGLT2, GLP-1, fracture, diabetes, bone, osteoporosis

Introduction

Type 2 diabetes mellitus (T2D) is associated with an increased fracture risk (1) despite normal or even elevated bone mineral density (BMD) levels and higher body mass index (BMI), both of which are protective factors against fracture (2–4).

In the last decades, multiple new glucose-lowering drugs have become available for the management of T2D (5). Sodium-glucose cotransporter 2 (SGLT2) inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists have recently been recommended for treatment of T2D in subjects with cardiovascular disease (6). In addition, SGLT2 inhibitors are recommended to prevent progression of chronic kidney disease (6). Consequently, the use of these agents is increasing and so is the need for information about potential side effects or impacts on other organs.

Knowledge about the impact of SGLT2 inhibitors and GLP-1 receptor agonists on bone health and fracture risk is limited. Studies have attempted to investigate the effects of various glucose-lowering drugs on fracture risk, although these are generally observational in nature and subject to confounding and insufficient follow-up durations (7). For SGLT2 inhibitors, a meta-analysis of randomized controlled trials (RCT) on canagliflozin reported a 32% increase in fracture risk compared to placebo or active treatment (8), and a propensity-score matched cohort study found an initial increase in fracture risk in new users of SGLT2 inhibitors compared to dipeptidyl peptidase 4 (DPP-4) inhibitors, although this effect was attenuated with longer treatment duration (9). However, most studies found neutral effects on fracture risk (10–12), including a pooled analysis of RCT data by Kohler et al. (13), a pooled analysis of RCTs by Jabbour et al. (14) and a network meta-analysis of RCTs by Tang et al. (15). GLP-1 receptor agonists have been shown to exhibit neutral effects on fracture risk in cohort studies (16, 17) and meta-analyses (18, 19), although the RCTs analyzed suffer from median follow-up durations of no more than two years (and down to 12 weeks). A recent network meta-analysis of 117 RCTs contained estimates of the risk ratios of four separate GLP-1 receptor agonists compared to four separate SGLT2 inhibitors; all but one of the 16 comparisons were statistically non-significant (20).

In the present study, we aimed to investigate fracture risk in patients using SGLT2 inhibitors versus patients using GLP-1 receptor agonists. We hypothesized no difference in fracture risk between people with T2D treated with either drug class.

Study design and methods

The STROBE guideline for reporting of observational studies was followed (STROBE checklist can be found in Supplemental Table S1) (21).

Study design and setting

We conducted a nationwide registry-based cohort study using data from the Danish national registries. We included all individuals who initiated a combination of metformin and SGLT2 inhibitor or GLP-1 receptor agonist treatment between January 1st 2012 and December 31st 2018. We chose to collect data from 2012 onwards as SGLT2 inhibitors became available in Denmark in 2012. Outcome information was collected by identifying all fracture-related diagnoses from index data onwards. Users of SGLT2 inhibitors were considered the exposure group, and controls (GLP-1 receptor agonist users) were matched 1:1 using propensity scores.

Data sources

All data were provided in anonymized form by Statistics Denmark (*Danmarks Statistik*, project identifier no. 703382). Statistics Denmark obtained data from national Danish registries. All Danish citizens are assigned a unique 10-digit personal identification number (PIN) stored in the Danish Civil Registration System, which contains high-fidelity

individual-level information on all residents in Denmark and Greenland (22). This PIN allows easy and unambiguous individual-level record linkage between different Danish registers (23, 24). The Danish Government provides full health care to all Danish citizens, including free access to hospitals and full or partial reimbursement of drug expenses. The Danish National Prescription Registry contains information on all prescription drugs sold in Denmark since 1995 according to the Anatomical Therapeutical Chemical (ATC) classification (25, 26). All diagnosis codes are stored in the Danish National Patient Registry, which covers all in- and outpatient contacts to the hospital (27) All physician-assigned discharge diagnoses are included, coded according to the *International Classification of Diseases, Eight Edition* (ICD-8) from 1977 until 1993 and according to ICD-10 from 1994 onwards.

All data on sex, date of birth, death, emigration, and socioeconomic factors were obtained from the Danish Civil Registration System.

Study population

The study population included subjects alive and residing in Denmark. A flowchart of the inclusion process is presented in Figure 1.

The criteria for inclusion were treatment with metformin in combination with either SGLT2 inhibitors or GLP-1 receptor agonists and no concurrent treatment with any other glucose-lowering drugs between January 1st 2012 and December 31st 2018.

We first identified persons treated with metformin and SGLT2 inhibitors (the exposure drug) and/or GLP-1 receptor

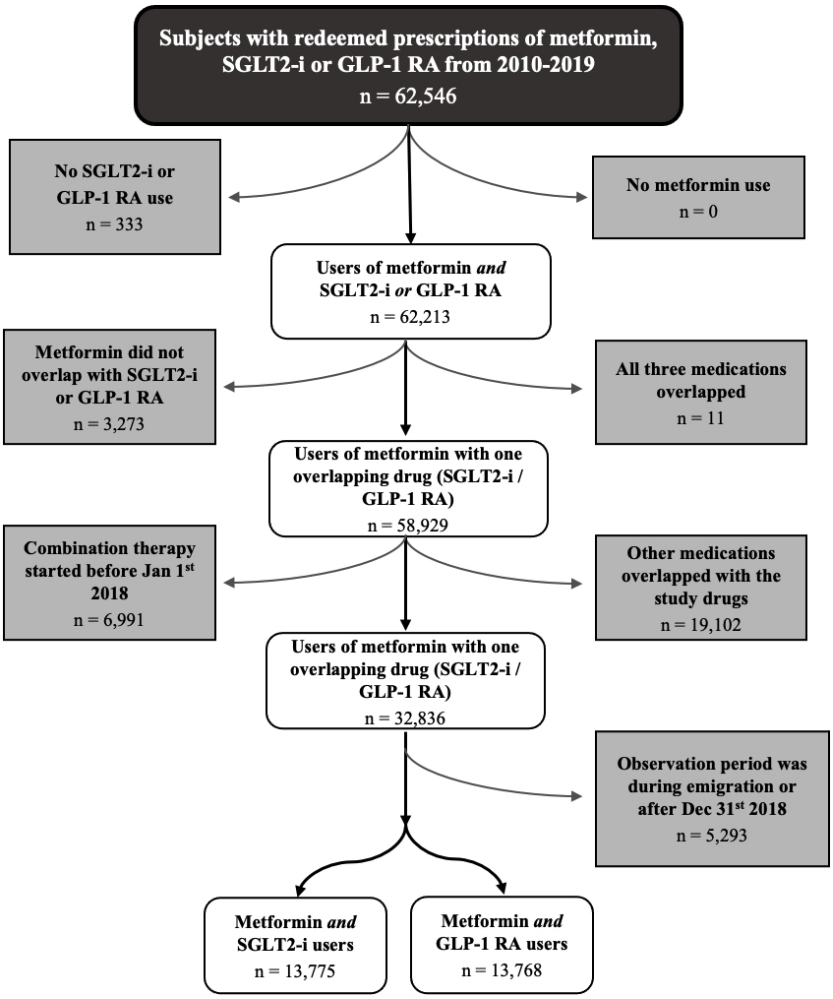

FIGURE 1
Flowchart of the process of in-/exclusion. SGLT2-i, Sodium-glucose co-transporter 2 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists.

agonists (the control drug) between January 1st 2010 and December 31st 2019. For each medication, we defined a start date (date of first redemption) and an end date (date of last redemption plus the number of daily doses redeemed on that date). We then excluded all individuals in which treatment with SGLT2 inhibitor and GLP-1 receptor agonist overlapped for the entire duration of treatment and those in which neither medication overlapped with metformin use. Remaining individuals were assigned to the exposure or control group based on which medication was first taken singularly in combination with metformin.

Then start and end dates were defined for each other class of glucose-lowering medication. Those who were already treated with an additional glucose-lowering drug (or several) at the beginning of combination therapy were included if (and when) the third medication was halted. *End of combination therapy* was defined as the day that treatment with metformin, the exposure drug, or the control drug ceased, or when another glucose-lowering medication was initiated. Glucose-lowering drugs were defined as any medications with ATC-codes beginning in "A10"; i.e., biguanides, SGLT2-i, GLP-1 RA, DPP-4 inhibitors, insulins, sulfonylureas, alpha-glucosidase inhibitors, glitazones, and repaglinide.

Finally, the cohort was limited to those in which *beginning of combination therapy* was on or after January 1st 2012.

Exposure

The National Prescription Registry contains data on redeemed drug prescriptions along with dates, doses and pack sizes. Each medication – including the exposure and control medications – was only considered used if an individual had redeemed at least three prescriptions in the period outlined above. Medications were identified using ATC codes (Supplemental Table S2).

From the National Prescription Registry, we obtained the Defined Daily Dose (DDD) variable, which is "the assumed average maintenance dose per day for a drug used for its main indication in adults", according to the World Health Organization Collaborating Centre for Drug Statistics Methodology (28). This date was added to the date of last prescription redemption to estimate a true end-of-treatment for each drug.

Of note, exposure to metformin, the exposure drug, and the control drug was in the main analysis assumed to be continuous between the dates of the first prescription redemption and end-of-treatment. To estimate the effects of pauses in these drugs, we calculated the cumulative dose (total number of DDDs) for each drug between the last prescription redeemed prior to or at index date until end of follow-up for each individual. We then assessed their compliance using the medication possession ratio (MPR); the ratio of the cumulative dose to the number of days in the same period. Individuals with an MPR < 0.5 were marked as having had a pause in the study period.

The follow-up period was defined as the time between the index date and *end of combination therapy*, emigration, death, or December 31st 2018, whichever came first.

Outcomes

The primary outcome in the study was incident major osteoporotic fractures (MOF). MOF were defined as any of the following fractures: Hip, vertebral, humerus, or forearm fracture. Fractures were identified by ICD-10 codes (Supplemental Table S3). Secondary analyses were performed to investigate separately the risks of any fracture, hip fracture, vertebral fracture, humerus fracture, and forearm fracture.

Covariates

Data on covariates were obtained using ICD-8 (1977–1993) and ICD-10 (1993–2018) codes (Supplemental Table S2), ATC codes (1995-2018) (Supplemental Table S3), or a combination of both (Supplemental Table S4). All covariates were assessed at baseline (index date) and did not vary over time.

Age at baseline was calculated from the index date and date of birth.

Debut of diabetes was estimated as first-ever prescription for glucose-lowering drug, and diabetes duration at baseline was calculated as the time from diabetes debut until index date.

Osteoporosis was defined as the presence of diagnosis codes for osteoporosis, previous/current treatment with antiosteoporotic medications and/or previous MOF; the variable was assigned three levels (2 = previous MOF, 1 = treatment/diagnosis, 0 = none).

Previous falls were identified from diagnosis codes related to falling.

Obesity (binary variable) was identified by diagnosis codes for obesity or previous use of weight-loss medications.

Alcohol abuse (binary variable) was defined as the presence of at least one diagnosis code related to alcohol consumption (e.g., intoxication, alcoholic liver disease, alcoholic cardiomyopathy, alcohol-related psychiatric illness etc.) or previous use of medication for alcohol abstinence.

As a proxy for smoking (binary variable), we used diagnosis codes related to lung diseases highly associated with tobacco exposure along with diagnosis codes for nicotine poisoning and psychiatric tobacco-related diagnoses. In addition, previous use of medications for the treatment of tobacco dependence and initiation of drugs for obstructive airway disease after the age of 40 were used as proxies for smoking. We expect this variable to represent heavy smoking.

Hypertension was defined by any diagnosis code for hypertension and/or ever use of an antihypertensive agent.

Hyperthyroidism was identified through diagnosis codes or treatment with any antithyroid medication.

Diabetic nephropathy, diabetic retinopathy, diabetic neuropathy, inflammatory bowel disease (IBD), kidney disease, chronic pancreatitis, visual impairment, hyperparathyroidism, and eating disorder/malabsorption were identified through diagnosis codes.

Previous insulin use and previous glucocorticoid use were identified through redeemed prescriptions.

The Charlson Comorbidity Index (CCI) was calculated based on other comorbidities. The CCI was modified to exclude kidney disease and late-diabetic complications, as these covariates were separately adjusted for in the statistical analyses.

Income, marital status and employment status (classified by Statistics Denmark according to the so-called *SOCIO13 classification*) were identified on the year preceding each individual's index year. Income (in Danish Kroner, DKK) was adjusted for inflation to a 2018 level according to the Consumer Price Index provided by Statistics Denmark and converted from DKK to Euros using an exchange rate of 7.4363 DKK/Euro..

Statistical analysis

Descriptive statistics

Descriptive statistics are presented as numbers and proportions (%), means and standard deviations (SD), or medians and interquartile ranges (IQR). Standardized mean differences (SMD) were also calculated for all baseline variables as recommended for propensity-score matched studies (29). Cohen suggested that SMD values above 0.2 be considered small, SMD values above 0.5 considered medium-sized, and SMD values above 0.8 considered large (29, 30).

Missing data

There were only missing data in the socioeconomic variables (marital status, income, and employment). Income was used as a covariate in the main analysis, and missing data were imputed beforehand. Missing data were assumed to be missing at random, and multivariate imputation by chained equations, a method of performing multiple imputations, was performed (31, 32). Ten imputations were produced, each of which ran for ten iterations. As the proportion of missing data was very low (0.2%), and the covariate (income) appeared to be balanced between groups and not alter the results of the survival analysis, it – and imputation – was omitted from all subgroup and sensitivity analyses.

Propensity-score matching

Due to imbalances in sex, age at baseline, and inclusion date (with GLP-1 receptor agonists having been introduced in Denmark approximately 5 years before SGLT2 inhibitors), we opted to match the two groups on propensity scores estimated from these variables. To produce these, we fitted a binomial logistic model to age, sex, and (a numeric value for) the inclusion date with treatment group as the dependent variable (33, 34). From the logistic regression, we predicted propensity scores for each individual in the main cohort.

To minimize bias, we matched subjects on the logit transformation of the propensity score using nearest-neighbor ("greedy") matching without replacement, using a caliper width equal to 0.2 x the SD of the transformed propensity scores (35, 36). As homogeneity of variances was violated (variance ratio of 2.5 between groups), the variance of the control group was used to set the caliper width.

For multiple imputed datasets, matching and statistical analysis were performed separately on each resultant dataset, and the statistical estimates were finally pooled.

After matching, balance in the matched variables was assessed by inspecting the distributions of propensity scores across groups and by calculating SMDs for each variable.

Multicollinearity

Multicollinearity was assessed using the Variance Inflation Factor (VIF) which yielded values no higher than 1.4 for any covariate. In addition, we examined Pearson's partial correlation coefficient for each pair of variables, and none revealed significant correlations.

Survival analysis

On a non-imputed matched dataset, the Kaplan-Meier Estimator was used to produce survival plots for all outcomes; a survival plot for MOF on a non-matched dataset was also produced (37).

For the primary analysis, we used the Cox proportional hazards model to estimate hazard rate ratios (HRs) for fracture between the exposure and the control groups. We estimated both crude and adjusted HRs for primary and secondary outcomes. The proportional hazards assumption was evaluated by examining the scaled Schoenfeld residuals of the Cox model and finding no trend with time for any variable (38). To account for pairing in the matched dataset, stratification by matched pairs or a robust variance estimator can be utilized (39, 40); as stratification may result in biased estimation of marginal hazard ratios, a robust variance estimator was used.

Finally, to also allow a non-multiplicative effect of SGLT2 inhibitors on fracture risk, we used Aalen's additive regression model to examine whether absolute rather than relative differences in hazard existed between the groups (41).

Sensitivity and subgroup analyses

We performed several sensitivity and subgroup analyses. For each subgroup, we performed matching anew using the previously computed propensity scores.

First, we split our cohort into males and females. Second, we performed an analysis excluding all who had pauses (MPR < 0.5) in their metformin or study drug (SGLT2 inhibitor or GLP-1 receptor agonist) during the study period. Third, we examined whether excluding individuals with kidney disease, previous pancreatitis, and previous falls would affect the results. Fourth, we examined whether excluding individuals with short follow-up time (less than 6 months) – who had not had enough time to manifest potential fractures – led to a difference in fracture risk. Fifth, due to previous studies hinting at possible drug-differential effects, we split the SGLT2 inhibitor group into specific drug groups based on which specific drug – canagliflozin, empagliflozin, or dapagliflozin – they had received the largest cumulative dose of during the study period. Ties were handled by allowing a person to appear in several of these subgroups; only three persons did so. Sixth, we examined the full cohort without matching. Seventh, we treated glucocorticoids as a reason for exclusion. Treatment with systemic glucocorticoids within the last year prior to inclusion was not allowed, and follow-up did not continue past initiation of systemic glucocorticoids. Lastly, we performed an analysis more similar to the "intention-to-treat" approach in clinical trials, in which we continued follow-up after changes in medication for an extra 2 years – or until death or emigration, whichever came first. This was to examine possible slow-emerging and/or long-lasting effects of the exposure on fracture risk.

Statistical software

All analyses were performed using R 4.1.0 (The R Core Team & The R Foundation for Statistical Computing, Vienna, Austria) in the integrated development environment (IDE) RStudio 1.4.1106 (RStudio, PBC, Boston, MA, USA). For imputation, the package "mice" (v 3.13.0) was used. Matching was performed using "MatchIt" (v. 4.2.0) and, for multiply imputed datasets, "MatchThem" (v. 1.0.0). Survival analyses – i.e., Cox model, Kaplan-Meier estimator, and Aalen's additive regression model – were performed using packages "Survival" (v. 2.1.11), "Survminer" (v. 0.4.9), and "Survey" (v. 4.0).

Results

Baseline characteristics

We identified 27,543 subjects treated with metformin in combination with either SGLT2 inhibitors (n = 13,775) or GLP-1 receptor agonists (n = 13,768). After propensity-score matching, a total of 18,380 (9,190 in each group) remained.

Matching was satisfactory, although due to the large effects of inclusion date and sex, the difference in age was not reduced.

Table 1 shows baseline characteristics of subjects in either group in both the full cohort and the matched cohort. Data from the matched cohort will be presented in short.

Follow-up time was balanced between the two groups with a median [IQR] of 355 [126–780] days in the SGLT2 inhibitor group and 373 [136–766] days in the control group. In total, we had 25,586 years of combined follow-up time.

Subjects in the SGLT2 inhibitor group were less likely to be female (38.5% vs. 40.0%) and were slightly older with mean (± SD) age of 61.1 (± 11.3) vs. 58.5 (± 12.0) years in the GLP-1 receptor agonist control group. Median [IQR] diabetes durations in the SGLT2 inhibitor group was 5.96 [2.80–9.35] years and, similarly, 5.91 [2.80–9.61] in the controls, and mean (± SD) CCI scores were 0.76 (± 1.19) and 0.79 (± 1.19) in the SGLT2 inhibitor and control group, respectively. Previous MOF were equally prevalent in both groups (10.0% vs. 10.6% in the SGLT2 inhibitor and control group, respectively).

Subjects in the control group had more complications of diabetes (25.3% vs. 18.4%), a lower occurrence pancreatitis (1.6% vs. 2.3%), and a higher prevalence of chronic kidney disease (4.3% vs. 2.4%), although all these effects sizes were below the minimum SMD threshold of 0.2. In addition, those in the control group were more likely to have a history of obesity (37.2% vs. 26.5%, SMD 0.232). In addition, the SGLT2 inhibitor group had a slightly larger fraction of subjects included in 2018, and a smaller fraction included in the years 2012, 2014, and 2015. The only covariates with SMDs above the threshold of 0.2 (for small differences) were age, obesity, and previous use of insulins, SGLT-2 inhibitors, DPP-IV inhibitors, and GLP-1 receptor agonists; with GLP-1 receptor agonists exhibiting by far the largest difference (SMD 0.865).

Socioeconomic variables were balanced between groups.

Risk of major osteoporotic fractures

Table 2 presents HRs for fractures in the matched cohort during the study period. A MOF occurred in 0.8% (n = 74) and 1.1% (n = 97) of SGLT2 inhibitor users and GLP-1 receptor agonist users, respectively. The Crude HR for MOF in the SGLT2 inhibitor group was 0.77 [0.57–1.04]. When adjusted for age and sex, this became statistically significant (HR 0.73 [0.54–0.99], although the effect was attenuated again in the fully adjusted model (HR 0.77 [0.56–1.05]). For each analysis in Table 2 and for the unmatched analysis of MOF, we also present Kaplan-Meier survival curves for crude illustrations (Figure 2), which similarly yielded non-significant results.

The Crude HR for any fracture was 0.87 [0.71–1.07], and the fully adjusted HR was 0.91 [0.74-1.12].

TABLE 1 Baseline Characteristics of Full and Matched Cohorts.

	Full Cohort		Matched Cohort		
	SGLT2-i group	**GLP-1 RA group**	**SGLT2-i group**	**GLP-1 RA group**	**SMD**
	13,775	13,768	9,190	9,190	
Sex (female), n (%)	4,934 (35.8%)	5,840 (42.4%)	3,540 (38.5%)	3,680 (40.0%)	0.031
Age (years), mean (±SD)	60.0 (±11.4)	57.4 (±12.1)	61.1 (±11.3)	58.5 (±12.0)	**0.218**
Follow-up (days), median [IQR]	334 [139–662]	497 [185–1,077]	355 [126–779.8]	372 [136.2–766]	0.011
Inclusion Year, n (%)					0.179
2012	4 (0.0%)	2,482 (18.0%)	4 (0.0%)	61 (0.7%)	
2013	394 (2.9%)	1,841 (13.4%)	394 (4.3%)	329 (3.6%)	
2014	664 (4.8%)	1,544 (11.2%)	664 (7.2%)	957 (10.4%)	
2015	1179 (8.6%)	1,767 (12.8%)	1,156 (12.6%)	1,709 (18.6%)	
2016	2,494 (18.1%)	1,776 (12.9%)	1,823 (19.8%)	1,776 (19.3%)	
2017	3,780 (27.4%)	1,885 (13.7%)	1,916 (20.8%)	1,885 (20.5%)	
2018	5,260 (38.2%)	2,473 (18.0%)	3,233 (35.2%)	2,473 (26.9%)	
Diabetes Duration (years), median [IQR]	5.80 [2.62–9.14]	5.56 [2.57–9.20]	5.96 [2.80–9.35]	5.91 [2.80–9.61]	0.024
Charlson Comorbidity Index, mean (±SD)	0.73 (±1.17)	0.72 (±1.14)	0.76 (±1.19)	0.79 (±1.19)	0.022
Complications of diabetes, n (%)	2,472 (17.9%)	3,557 (25.8%)	1,687 (18.4%)	2,325 (25.3%)	0.169
Diabetic Neuropathy	385 (3.8%)	563 (4.1%)	268 (2.9%)	378 (4.1%)	0.065
Diabetic Nephropathy	213 (1.5%)	450 (3.3%)	141 (1.5%)	319 (3.5%)	0.124
Diabetic Retinopathy	709 (5.1%)	915 (6.6%)	498 (5.4%)	558 (6.1%)	0.028
Other	1,642 (11.9%)	2,477 (18.0%)	1,116 (12.1%)	1,631 (17.7%)	0.158
Osteoporosis, n (%)					0.030
No history	12,126 (88.0%)	12,167 (88.4%)	8,090 (88.0%)	8,073 (87.8%)	
Diagnosed / Treated	273 (2.0%)	212 (1.5%)	183 (2.0%)	146 (1.6%)	
Previous MOF	1,376 (10.0%)	1,389 (10.1%)	917 (10.0%)	971 (10.6%)	
Risk factors for falls, n (%)					
Hypoglycemic episodes	94 (0.7%)	115 (0.8%)	66 (0.7%)	86 (0.9%)	0.024
Previous Falls	516 (3.7%)	575 (4.2%)	353 (3.8%)	405 (4.4%)	0.028
Visual Impairment	185 (1.3%)	153 (1.1%)	131 (1.4%)	106 (1.2%)	0.024
Any pancreatitis, n (%)	313 (2.3%)	226 (1.6%)	211 (2.3%)	145 (1.6%)	0.052
Acute Pancreatitis	267 (1.9%)	210 (1.5%)	181 (2.0%)	133 (1.4%)	0.040
Chronic Pancreatitis	97 (0.7%)	38 (0.3%)	72 (0.8%)	24 (0.3%)	0.073
Glucose-Lowering Drugs, n (%)					
Metformin	13,561 (98.4%)	13,527 (98.2%)	9,069 (98.7%)	9,025 (98.2%)	0.039
SGLT2 inhibitors	1,782 (12.9%)	493 (3.6%)	1,205 (13.1%)	483 (5.3%)	**0.275**
GLP-1 receptor agonists	261 (1.9%)	4,447 (32.3%)	178 (1.9%)	2,904 (31.6%)	**0.865**
DDP4 inhibitors	2,347 (17.0%)	3,336 (24.2%)	1,612 (17.5%)	2,408 (26.2%)	**0.211**
Insulin, any	890 (6.5%)	1,772 (12.9%)	582 (6.3%)	1,220 (13.3%)	**0.235**
Sulfonylureas	3,572 (25.9%)	5,030 (36.5%)	2,557 (27.8%)	3,066 (33.4%)	0.120
Alpha-glucosidase inhibitors	32 (0.2%)	92 (0.7%)	24 (0.3%)	63 (0.7%)	0.062
Glitazones	284 (2.1%)	525 (3.8%)	218 (2.4%)	269 (2.9%)	0.035
Repaglinide	125 (0.9%)	185 (1.3%)	87 (0.9%)	104 (1.1%)	0.018
Hypertension, n (%)	10,818 (78.5%)	11,080 (80.5%)	7,327 (79.7%)	7,461 (81.2%)	0.037
Chronic Kidney Disease, n (%)	321 (2.3%)	499 (3.6%)	218 (2.4%)	399 (4.3%)	0.110
Liver Disease, n (%)	433 (3.1%)	409 (3.0%)	289 (3.1%)	294 (3.2%)	0.003
Mild	390 (2.8%)	382 (2.8%)	259 (2.8%)	278 (3.0%)	0.012

(Continued)

TABLE 1 Continued

	Full Cohort		Matched Cohort		
	SGLT2-i group	GLP-1 RA group	SGLT2-i group	GLP-1 RA group	SMD
Moderate to severe	84 (0.6%)	64 (0.5%)	54 (0.6%)	44 (0.5%)	0.015
Hyperparathyroidism, n (%)	54 (0.4%)	82 (0.6%)	42 (0.5%)	62 (0.7%)	0.029
Hyperthyroidism, n (%)	364 (2.6%)	386 (2.8%)	271 (2.9%)	248 (2.7%)	0.015
Hypogonadism, n (%)	24 (0.2%)	39 (0.3%)	15 (0.2%)	32 (0.3%)	0.037
Eating disorder or malabsorption, n (%)	98 (0.7%)	83 (0.6%)	66 (0.7%)	62 (0.7%)	0.004
Venous thromboembolism, n (%)	1,014 (7.4%)	1,144 (8.3%)	723 (7.9%)	792 (8.6%)	0.027
Inflammatory bowel disease, n (%)	450 (3.3%)	480 (3.5%)	311 (3.4%)	346 (3.8%)	0.021
Osteoarthritis, n (%)	2,261 (16.4%)	2,445 (17.8%)	1,614 (17.6%)	1,745 (19.0%)	0.037
Dementia, n (%)	808 (5.9%)	801 (5.8%)	560 (6.1%)	588 (6.4%)	0.013
Alcohol abuse, n (%)	1,012 (7.3%)	1,000 (7.3%)	678 (7.4%)	680 (7.4%)	0.001
Smoking, n (%)	4,266 (31.0%)	4,627 (33.6%)	2,921 (31.8%)	3,190 (34.7%)	0.062
Obesity, n (%)	3,509 (25.5%)	5,373 (39.0%)	2,434 (26.5%)	3,420 (37.2%)	**0.232**
Other medications, n (%)					
Statins	11,214 (81.4%)	11,136 (80.9%)	7,551 (82.2%)	7,479 (81.4%)	0.020
Thiazides	5,080 (36.9%)	5,889 (42.8%)	3,551 (38.6%)	3,973 (43.2%)	0.093
Loop Diuretics	2,655 (19.3%)	3,530 (25.6%)	1,925 (20.9%)	2,416 (26.3%)	0.126
Potassium-sparing diuretics	1,428 (10.4%)	1,716 (12.5%)	1,003 (10.9%)	1,193 (13.0%)	0.064
Antipsychotic drugs	1,730 (12.6%)	1,770 (12.9%)	1,125 (12.2%)	1,152 (12.5%)	0.009
Antiepileptic drugs	2,003 (14.5%)	2,231 (16.2%)	1,329 (14.5%)	1,596 (17.4%)	0.079
Antiarrhythmic drugs	214 (1.6%)	235 (1.7%)	147 (1.6%)	177 (1.9%)	0.025
Hypnotics	3,876 (28.1%)	4,158 (30.2%)	2,680 (29.2%)	2,818 (30,7%)	0.033
Antidepressants	4,691 (34.1%)	5,320 (38.6%)	3,123 (34.0%)	3,559 (38.7%)	0.099
Anxiolytics	3,645 (26.5%)	3,996 (29.0%)	2,501 (27.2%)	2,644 (28.8%)	0.035
Opioids	7,799 (56.6%)	8,199 (59.6%)	5,246 (57.1%)	5,561 (61.5%)	0.090
NSAID	12,144 (88.2%)	12,344 (89.7%)	8,138 (88.6%)	8,289 (90.2%)	0.053
Sex hormones	3,425 (24.9%)	4,333 (31.5%)	2,447 (26.6%)	2,792 (30.4%)	0.083
Antacids	7,378 (53.6%)	7,498 (54.5%)	5,014 (54.6%)	5,204 (56.5%)	0.042
Glucocorticoids	4,597 (33.4%)	4,736 (34.4%)	3,153 (34.3%)	3,259 (35.5%)	0.024
Income (euros), median [IQR]	34,109 [24,590–50,254]	34,885 [25,307–50,504]	*33,100 [24,233–48,944]*	*34,800 [25,188–50,482]*	*0.022*
Income quintiles, n (%)					*0.048*
1st	2,876 (20.9%)	2,622 (19.0%)	*1,972 (21.5%)*	*1,792 (19.5%)*	
2nd	2,697 (19.6%)	2,802 (20.4%)	*1,910 (20.8%)*	*1,796 (19.5%)*	
3rd	2,724 (19.8%)	2,774 (20.1%)	*1,823 (19.8%)*	*1,901 (20.7%)*	
4th	2,696 (19.6%)	2,803 (20.4%)	*1,774 (19.3%)*	*1,856 (20.2%)*	
5th	2,755 (20.0%)	2,744 (19.9%)	*1,698 (18.5%)*	*1,827 (19.9%)*	
Missing Data	27 (0.2%)	23 (0.2%)	*13 (0.1%)*	*18 (0.2%)*	
Marital Status, n (%)					0.073
Unmarried	2,501 (18.2%)	2,723 (19.8%)	1,530 (16.6%)	1,785 (19.4%)	
Married / Registered Partnership	7,920 (57.5%)	7,831 (56.9%)	5,356 (58.3%)	5,166 (56.2%)	
Divorced / Annulled Partnership	2,265 (16.4%)	2,264 (16.4%)	1,492 (16.2%)	1,559 (17.0%)	
Widowed	1,035 (7.5%)	899 (6.5%)	783 (8.5%)	641 (7.0%)	
Missing Data	54 (0.4%)	51 (0.4%)	29 (0.3%)	39 (0.4%)	
SOCIO13 group, n (%)					0.088
Working	6,039 (43.8%)	6,235 (45.3%)	3,799 (41.3%)	4,041 (44.0%)	
Unemployed	1,186 (8.5%)	1,249 (9.1%)	704 (7.7%)	816 (8.9%)	
Retired	6,182 (44.9%)	5,879 (42.7%)	4,469 (48.6%)	4,066 (44.2%)	

(Continued)

TABLE 1 Continued

	Full Cohort		Matched Cohort		
	SGLT2-i group	GLP-1 RA group	SGLT2-i group	GLP-1 RA group	SMD
Student	40 (0.3%)	112 (0.8%)	23 (0.3%)	72 (0.8%)	
Other	301 (2.2%)	270 (2.0%)	182 (2.0%)	177 (1.9%)	
Missing Data	54 (0.4%)	51 (0.4%)	13 (0.1%)	18 (0.2%)	

Alle data are presented as n (%), mean (±SD), or median [IQR]. SGLT2-i, sodium-glucose cotransporter 2 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists; SMD, standardized mean difference. SMDs above 0.2 are highlighted with bold font. Data on income in the matched cohort (italicized) are presented without imputations.

Examining HRs for each specific type of MOF yielded generally similar results. The crude HR for hip fracture was 0.87 [0.47–1.61], which was unaltered in the fully adjusted model (HR 0.87 [0.45–1.67]). The crude HR for vertebral fractures was 0.94 [0.45–1.95] with negligible change after full adjustment (HR 0.86 [0.40–1.88]). For forearm, the crude HR was 1.00 [0.63–1.60] and the fully adjusted HR 1.14 [0.70–1.86]. In contrast, the analysis of humerus fractures indicated a protective effect with an adjusted HR of 0.35 [0.18–0.70]. However, there were very few events for each subtype of fracture, making interpretation difficult.

Subgroup and sensitivity analyses

Various subgroup and sensitivity analyses yielded similarly non-significant results (Table 3).

Effects were similar between males and females. When excluding those with pauses in medication or those with chronic kidney disease, previous pancreatitis and previous falls did not alter the results, either. When excluding subjects with follow-up times less than 6 months, 12,916 individuals remained. In this group, we found an unadjusted HR of 0.73 [0.53–1.01] which was similarly to the main analysis significant upon adjusting for age and sex but once again attenuated in the fully adjusted model (HR 0.77 [0.55–1.07]).

Dividing the SGLT2 inhibitor group into subgroups based on which specific drug yielded three groups; canagliflozin, empagliflozin, and dapagliflozin. Neither empagliflozin nor dapagliflozin showed effects different from the main results. Only 302 individuals were in the canagliflozin group, and although an unadjusted HR of 0.42 [0.11–1.53] was found, this result was based on a mere total of three fractures.

Examining the full (unmatched) cohort yielded similar results (unadjusted HR 0.84 [0.66–1.07] and fully adjusted HR 0.78 [0.59–1.03]).

Defining recent or ongoing glucocorticoid use as an exclusion criterion did not impact the results (adjusted HR 0.73 [0.52–1.03]).

In addition, performing an "intention-to-treat" analysis yielded an adjusted HR of 0.94 [0.72–1.21], slightly closer to a fully neutral effect.

Finally, we performed an entirely separate test of MOF hazard on the matched cohort using the Aalen's additive

TABLE 2 Hazard Ratios (HR) for various fracture types in the matched cohort.

Fracture	Fractures, n (%)	Unadjusted (HR [95% CI])	Age, Sex-HR [95% CI]	Full Model-HR [95% CI]
MOF	SGLT2-i: 74 (0.8) GLP-1 RA: 97 (1.1)	0.77 [0.57 – 1.04]	**0.73 [0.54 – 0.99]**	Model 1: 0.77 [0.56 – 1.05]
Any	SGLT2-i: 174 (1.9) GLP-1 RA: 201 (2.2)	0.87 [0.71 – 1.07]	0.86 [0.70 – 1.05]	Model 1: 0.91 [0.74 – 1.12]
Hip	SGLT2-i: 19 (0.2) GLP-1 RA: 22 (0.2)	0.87 [0.47 – 1.61]	0.80 [0.43 – 1.49]	Model 2: 0.87 [0.45 – 1.67]
Vertebral	SGLT2-i: 14 (0.2) GLP-1 RA: 15 (0.2)	0.94 [0.45 – 1.95]	0.88 [0.43 – 1.83]	Model 2: 0.86 [0.40 – 1.88]
Humerus	SGLT2-i: 11 (0.1) GLP-1 RA: 29 (0.3)	**0.38 [0.20 – 0.76]**	**0.36 [0.18 – 0.71]**	Model 2: **0.35 [0.18 – 0.70]**
Forearm	SGLT2-i: 35 (0.4) GLP-1 RA: 35 (0.4)	1.00 [0.63 – 1.60]	1.00 [0.62 – 1.59]	Model 2: 1.14 [0.70 – 1.86]

HR, Hazard Ratio; MOF, major osteoporotic fracture; SGLT2-i, sodium-glucose cotransporter 2 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists; Bold font: the HR was significantly different from 1.00.
Full model 1: Adjusted for sex, age, inclusion date, diabetes duration, Charlson Comorbidity Index, diabetic nephropathy, diabetic retinopathy, diabetic neuropathy, previous falls, inflammatory bowel disease, previous insulin use, previous glucocorticoid use, osteoporosis (including prevalent MOF), hypertension, kidney disease, alcohol, smoking, obesity, income, chronic pancreatitis, visual impairment, hyperthyroidism, hyperparathyroidism, eating disorder/malabsorption.
Full model 2: Corresponding to Model 1 but excluding chronic pancreatitis, diabetic neuropathy, visual impairment, hyperthyroidism, hyperparathyroidism and eating disorder/malabsorption as covariates.

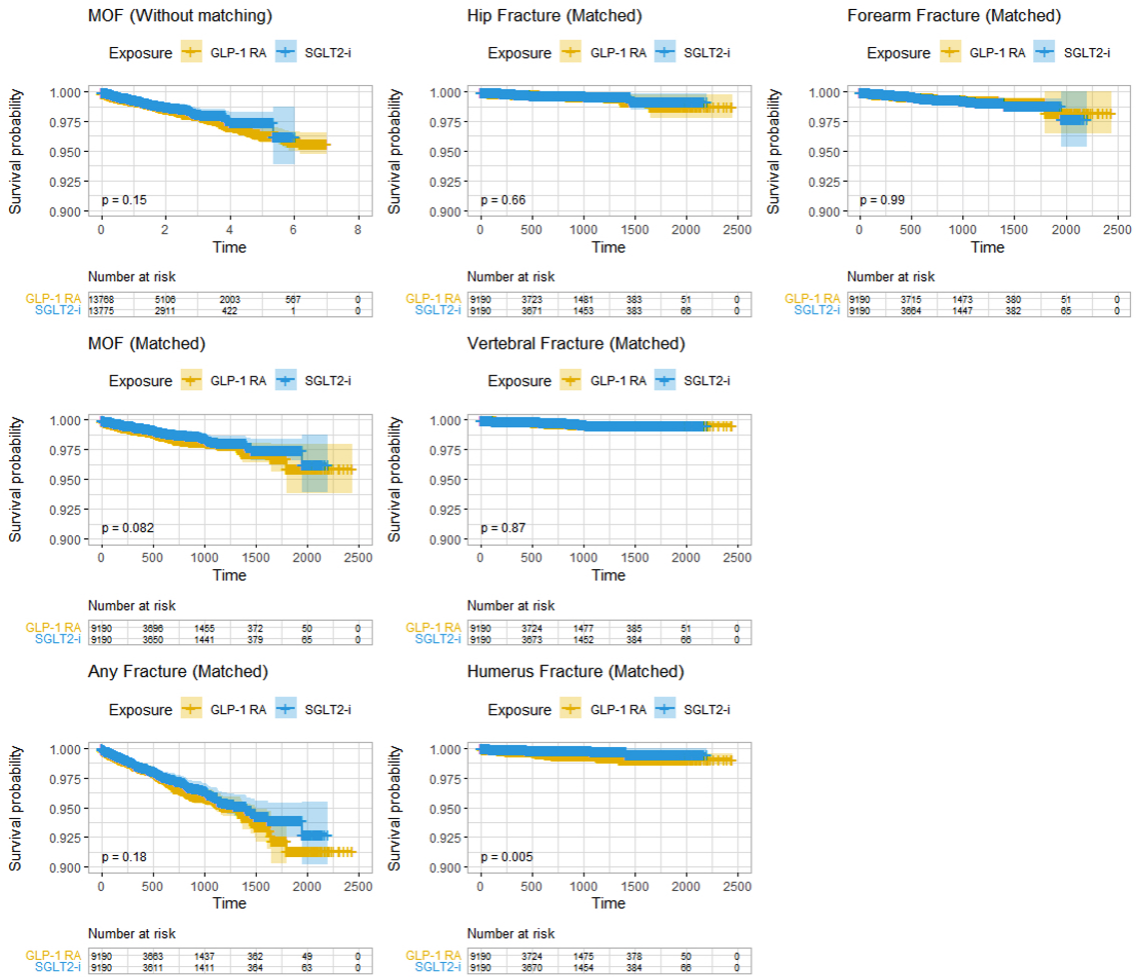

FIGURE 2
Kaplan-Meier Survival Curves of fracture. Survival curves are presented with *number-at-risk* tables. Time in days on the x-axes. Note, the y-axes go from 0.90 to 1.00. MOF, Major osteoporotic fracture; GLP-1 RA, Glucagon-like peptide-1 receptor agonists; SGLT2-i, sodium-glucose cotransporter 2 inhibitors.

regression model (Figure 3). This test revealed no time-varying effects of the exposure/control drugs with a slope of -0.0058 (p = 0.08).

As a final measure, we analyzed deaths in the two groups to examine whether an imbalance in these may have influenced the results, as competing risks were not formally accounted for in the main analyses. In the SGLT2 inhibitor group, 59 (0.6%) deaths occurred with a median [IQR] time-to-event of 286 [124–828] days, whereas the GLP-1 receptor agonist group experienced 84 (0.9%) deaths with a median time-to-event of 188 [54–670] days. Indeed, the crude HR for death (with MOF as a censoring event) in the SGLT2 inhibitor group with the GLP-1 receptor agonist group as reference was 0.70 [0.51–0.98]. When adjusted for age and sex, this became 0.65 [0.47–0.91] and when fully adjusted 0.81 [0.58–1.12].

Discussion

Summary of findings

In the present study, we found that the risk of MOF was similar between treatment with GLP-1 receptor agonist and SGLT2 inhibitors as add-on therapies to metformin. Whereas some other research has indicated bone protective effects of GLP-1 receptor agonists and bone detrimental effects of SGLT2 inhibitors (perhaps particularly canagliflozin), our results showed a small, non-significant trend toward fewer fractures with SGLT2 inhibitors.

We found no drug-differential effects but were unfortunately unable – due to small sample size – to evaluate the risk with canagliflozin.

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TABLE 3 Hazard Ratios for MOF in subgroup and sensitivity analyses.

Analysis	n =	Fractures, n (%)	Unadjusted (HR [95% CI])	Age, (Sex)-HR [95% CI]	Full Model-HR [95% CI]
Males	SGLT2-i: 5,377	30 (0.6)	0.75 [0.47 – 1.20]	0.74 [0.46 – 1.19]	Model 2
	GLP-1 RA: 5,377	38 (0.7)			0.80 [0.50 – 1.29]
Females	SGLT2-i: 3,795	50 (1.3)	0.87 [0.60 – 1.26]	0.80 [0.55 – 1.16]	Model 2
	GLP-1 RA: 3,795	63 (1.7)			0.83 [0.56 – 1.22]
No Pause	SGLT2-i: 7,432	65 (0.9)	0.84 [0.60 – 1.17]	0.79 [0.56 – 1.10]	Model 2
	GLP-1 RA: 7,432	78 (1.0)			0.84 [0.60 – 1.18]
No CKD etc.	SGLT2-i: 8,309	60 (0.7)	0.73 [0.52 – 1.02]	0.73 [0.52 – 1.02]	Model 2
	GLP-1 RA: 8,309	82 (1.0)			0.80 [0.56 – 1.13]
6+ months follow-up	SGLT2-i: 6,458	63 (1.0)	0.73 [0.53 – 1.01]	**0.72 [0.52 – 0.99]**	Model 2
	GLP-1 RA: 6,458	89 (1.4)			0.77 [0.55 – 1.07]
Canagliflozin	SGLT2-i: 302	1 (0.3)	0.42 [0.11 – 1.53]	0.42 [0.10 – 1.69]	N/A
	GLP-1 RA: 302	2 (0.7)			
Empagliflozin	SGLT2-i: 6,893	49 (0.7)	0.78 [0.54 – 1.13]	0.77 [0.53 – 1.12]	Model 2
	GLP-1 RA: 6,893	65 (0.9)			0.80 [0.55 – 1.17]
Dapagliflozin	SGLT2-i: 5,772	48 (0.8)	0.70 [0.48 – 1.02]	0.70 [0.48 – 1.02]	Model 2
	GLP-1 RA: 5,772	60 (1.0)			0.81 [0.55 – 1.19]
Full cohort (unmatched)	SGLT2-i: 13,775	105 (0.8)	0.84 [0.66 – 1.07]	0.82 [0.64 – 1.05]	Model 1
	GLP-1 RA: 13,768	189 (1.4)			0.78 [0.59 – 1.03]
Glucocorticoid as exclusion	SGLT2-i: 8,464	62 (0.7)	0.74 [0.54 – 1.03]	**0.70 [0.50 – 0.97]**	Model 1
	GLP-1 RA: 8,464	84 (1.0)			0.73 [0.52 – 1.03]
Intention-to-treat analysis	SGLT2-i: 9,190	116 (1.3)	0.95 [0.74 – 1.22]	0.87 [0.68 – 1.12]	Model 1
	GLP-1 RA: 9,190	135 (1.5)			0.94 [0.72 – 1.21]
Age: <65	SGLT2-i: 6,088	37 (0.6)	0.73 [0.48 – 1.12]	0.72 [0.47 – 1.10]	Model 2
	GLP-1 RA: 6,088	50 (0.8)			0.81 [0.51 – 1.28]
Age: 65–74	SGLT2-i: 2,401	26 (1.1)	0.89 [0.53 – 1.49]	0.87 [0.52 – 1.47]	Model 2
	GLP-1 RA: 2,401	31 (1.3)			1.02 [0.59 – 1.77]
Age: ≥ 75	SGLT2-i: 670	11 (1.6)	0.60 [0.29 – 1.23]	0.54 [0.27 – 1.09]	N/A
	GLP-1 RA: 670	19 (2.8)			

HR, Hazard Ratio; MOF, major osteoporotic fracture; SGLT2-i, sodium-glucose cotransporter 2 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists; Bold font = the HR was significantly different from 1.00.
"No pause": excluded those with pauses in metformin, SGLT2 inhibitor or GLP-1 receptor agonist during the study period. "No CKD etc.": Excluded those with chronic kidney disease, previous falls and previous chronic pancreatitis. "6+ months follow-up": Excluding all with follow-up times less than 183 days.
Full model 1: Adjusted for sex, age, inclusion date, diabetes duration, Charlson Comorbidity Index, diabetic nephropathy, diabetic retinopathy, diabetic neuropathy, previous falls, inflammatory bowel disease, previous insulin use, previous glucocorticoid use, osteoporosis (including prevalent MOF), hypertension, kidney disease, alcohol, smoking, obesity, chronic pancreatitis, visual impairment, hyperthyroidism, hyperparathyroidism, eating disorder/malabsorption.
Full model 2: Corresponding to Model 1 but excluding chronic pancreatitis, diabetic neuropathy, visual impairment, hyperthyroidism, hyperparathyroidism and eating disorder/malabsorption as covariates.
N/A means "Not applicable.

Examining specific fracture sites revealed no difference between SGLT2 inhibitors and GLP-1 receptor agonists in the cases of hip, forearm, and vertebral fractures. Only in the case of humerus fractures did our results reveal a statistically significant effect. However, this secondary analysis was based on only 40 fractures in total, and our study has not taken multiple testing into account, which means that significance is to be expected at some level, even if not clinically meaningful. Indeed, the authors are not aware of a mechanism whereby the drugs would have a protective effect on the humerus but not on other bone tissue.

In our sensitivity analysis in which subjects were followed for up to an additional two years, we found HRs closer to 1.00 than in the main analysis. This suggests that there are no long-term detrimental effects on bone by either drug after discontinuation, switch, or addition of other glucose-lowering drugs.

As increased fall risk may be a contributor to the fracture risk in diabetes (42), we attempted to compensate for this by performing a subgroup analysis without those with previous diagnosis codes pertaining to falls. In addition, we adjusted for covariates related to falls, diabetic neuropathy, diabetic retinopathy, and visual impairment.

We speculated whether differential mortality in the two groups may have influenced the results, and found a difference, albeit relatively small and non-significant when

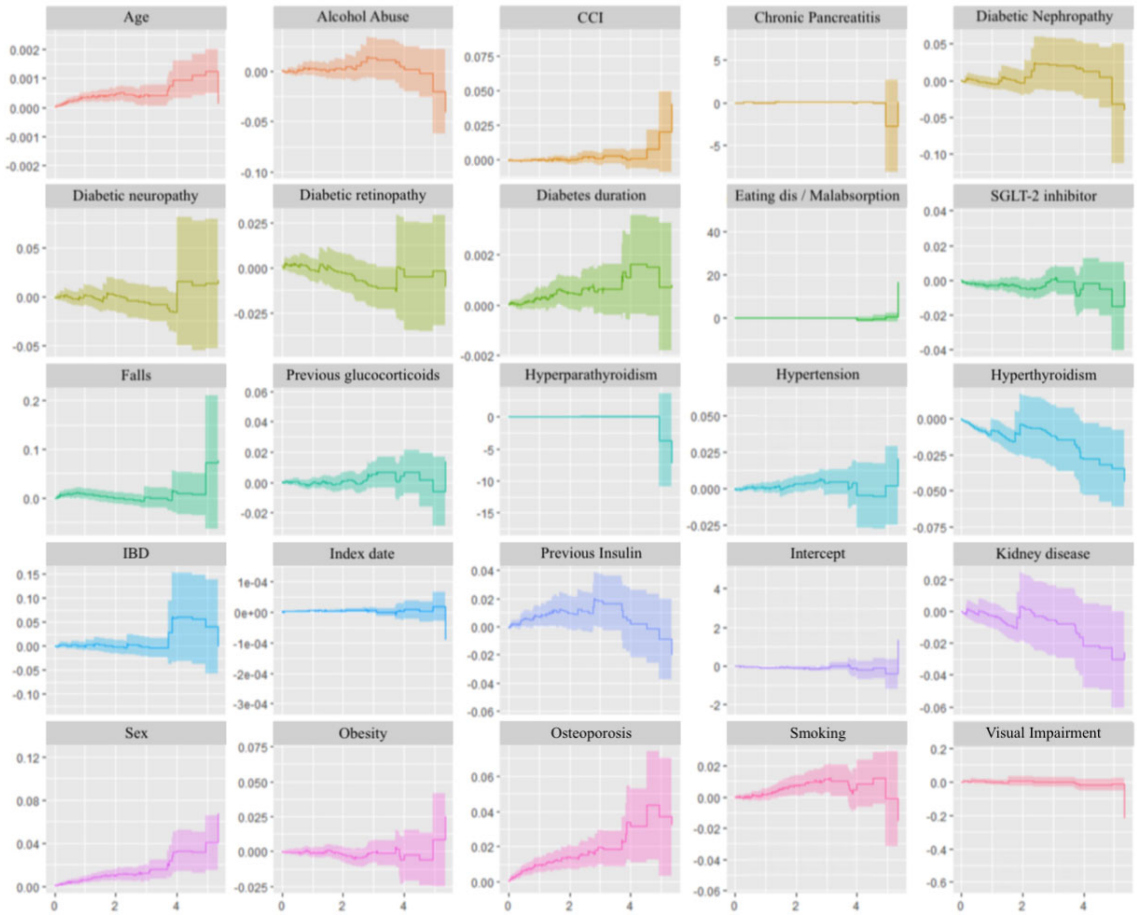

FIGURE 3
Aalen's Additive Regression Plots. Plots of the time-varying additive hazards plotted against time (years) on the x-axis for covariates used in Aalen's Additive Regression Model. CCI, Charlson Comorbidity Index; Eating dis, eating disorder; IBD, inflammatory bowel disease. This regression model assumes that the risks attributable to each risk factor are additive (producing hazard rate differences) rather than multiplicative (hazard rate ratios). Each plot shows the cumulative hazard associated with a given covariate at each time point – the slopes at any point in time represent hazard rates, and positive slopes correspond to increased risk, whereas negative slopes correspond to reduced risk. As all effects are allowed to be time-varying, a covariate may at one timepoint increase risk and a reduce risk at another timepoint. The intercept term represents a baseline hazard; i.e., the hazard when the contributions from all covariates (including exposure) are zero.

fully adjusted. A higher mortality in the GLP-1 receptor agonist group would mean an overestimation of fracture hazard in this group. Therefore, the true hazard ratio may be slightly closer to 1, but as deaths were so rare, it is unlikely that any such bias will have produced our results if the true hazard ratio were above 1.

Previous research

SGLT2 inhibitors became available in Denmark in 2012 as a treatment for T2D. Most observational (10–12) and (13–15) clinical studies have found neutral effects on fracture risk with SGLT2 inhibitors, although one meta-analysis of RCTs with long follow-up found increased fracture risk in canagliflozin treatment (8). For GLP-1 receptor agonists, observational studies and meta-analyses of RCTs on fracture risk have found mostly neutral effects (16–20, 43), although one meta-analysis found reduced risk of fractures (44).

Most studies, however, are limited by short follow-up durations (7). Furthermore, interpretation of the body of observational research is generally made difficult in the context of glucose-lowering drugs by the heterogeneity inherent in the variety of study designs, particularly the choice of many different comparators. In contrast, it is rarely feasible to perform clinical studies on the timescales required for proper evaluation of such long-term outcomes as osteoporotic fractures.

Strengths and limitations

This cohort study was performed on a nationwide level with individual-level data on all prescription medications and diagnosis codes along with a variety of socioeconomic factors. This allows access to high-fidelity information on treatments and comorbidities in the whole period in which SGLT2 inhibitors have been marketed in Denmark with limited missing data using an unbiased study population, providing results that are highly generalizable to populations at a wide range of ages that are comparable to the Danish population.

The use of GLP-1 receptor agonists as a comparator provided a highly comparable control group, particularly as both drugs were used in the setting of sole add-on medication to metformin. As both drugs have equal priority in the management of T2D, we expect very limited confounding by indication to appear in this study. However, GLP-1 receptor agonists may in many cases be preferred for subjects with obesity, and although we attempted to adjust for this, we did not have direct measurements of BMI.

Propensity-score matching is a method of mimicking some of the characteristics of a randomized controlled trial (34); i.e., the propensity score is a balancing score which guarantees the same distribution of observed baseline characteristics between two groups if subjects have the same propensity score. The caliper width was set according to previous studies on minimizing bias with propensity-score matching (35), and we obtained a fairly balanced matching, although the age distribution was not balanced out.

Furthermore, the matching process resulted in the discarding of a large number of subjects; the cohort reduced from 27,543 to 18,380 individuals. Hence, a sensitivity analysis was performed on the full cohort to examine whether any bias was introduced or efficiency lost in the matching process.

In addition, this study performed a variety of subgroup and sensitivity analyses, almost all of which point towards no difference in fracture risk between the two treatments. This robustness of the results supports the conclusion of neutral effects on fracture with SGLT2 inhibitor treatment compared to GLP-1 receptor agonist treatment in this population.

As this was an observational study, residual confounding cannot be ruled out. Particularly, we were unable to account for diet and exercise, both of which might be associated with the exposure (as obesity may influence the choice of glucose-lowering drug) and with the outcome. Lack of access to lab results and other clinical information meant that data on glycemic control, BMD, BMI, and other markers of significance to bone health were not available to be adjusted for. As such, we did not have information on vitamin D status or vitamin D supplementation prior to or during the study period, which poses a limitation to the study. However, although vitamin D status is causally connected to the outcome of the study, we do not expect a causal relationship between baseline vitamin D status and choice of SGLT2-i vs. GLP-1 RA treatment; therefore, any association between vitamin D status and the choice of exposure drug is expectedly governed by underlying common causes, which we expect to have been adjusted for *via* the other covariates. In addition, of the covariates we did include in the model, some were crude proxy-variables, e.g., obesity, smoking, and alcohol consumption. Similarly, data on falls and other risk factors for fracture were limited, as the utility of diagnosis codes to identify such factors is limited.

The relatively recent introduction of SGLT2 inhibitors in 2012 and the unavailability of outcome data after 2018 meant relatively short follow-up periods in the study. As fractures are in part a result of poor bone health, and changes in bone structure appear slowly, it is not certain that a differential effect on fracture risk would manifest during the study period. However, in the matched cohort, a full 9,153 individuals had at least one year of follow-up time, with 4,961 of those having more than two years.

Arguably the most important limitation of this study is the relatively small number of fractures (171 MOF in total in the main analysis), which is linked to the relatively short follow-up period. However, as all HRs found were below 1.00 (and the upper bounds of the confidence intervals close to 1.00), it is unlikely that a harmful effect of SGLT2 inhibitors has been overlooked, whereas a slight protective effect cannot be ruled out entirely.

Conclusion

Overall, the results indicate no effect on fracture risk with SGTL2 inhibitor treatment when compared to GLP-1 receptor agonist treatment. The study is in line with previous research and supports the continued use of both drugs in the management of T2D in patients at risk of (osteoporotic) fracture.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Access to the utilized registries can be applied for at Statistics Denmark by any authorized research institution. Requests to access these datasets should be directed to forskningsservice@dst.dk

Author contributions

All authors contributed to the article according to the ICJME requirements for co-authorship. All authors critically revised the paper for intellectual content and approved the submitted versions and the final version of the manuscript. ZKA, RV, and JS-L designed the study. ZKA, RV, JS-L and PV had access to all data used in the study. ZKA performed data management and statistical analyses with assistance from all co-authors. ZKA and RV interpreted the data and wrote the

manuscript. JSL, PV and SG made ongoing critical revisions of study design and data interpretation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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The risk of major osteoporotic fractures with GLP-1 receptor agonists when compared to DPP-4 inhibitors: A Danish nationwide cohort study

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Background: Type 2 diabetes mellitus (T2D) is associated with an increased fracture risk. There is little evidence for the effects of glucagon-like peptide 1 receptor agonists (GLP-1RA) on fracture risk in T2D. We aimed to investigate the risk of major osteoporotic fractures (MOF) for treatment with GLP-1RA compared to dipeptidyl peptidase 4 inhibitors (DPP-4i) as add-on therapies to metformin.

Methods: We conducted a population-based cohort study using Danish national health registries. Diagnoses were obtained from discharge diagnosis codes (ICD-10 and ICD-8-system) from the Danish National Patient Registry, and all redeemed drug prescriptions were obtained from the Danish National Prescription Registry (ATC classification system). Subjects treated with metformin in combination with either GLP-1RA or DPP-4i were enrolled from 2007 to 2018. Subjects were propensity-score matched 1:1 based on age, sex, and index date. MOF were defined as hip, vertebral, humerus, or forearm fractures. A Cox proportional hazards model was utilized to estimate hazard rate ratios (HR) for MOF, and survival curves were plotted using the Kaplan-Meier estimator. In addition, Aalen's Additive Hazards model was applied to examine additive rather than relative hazard effects while allowing time-varying effects.

Results: In total, 42,816 individuals treated with either combination were identified and included. After matching, 32,266 individuals were included in the main analysis (16,133 in each group). Median follow-up times were 642 days and 529 days in the GLP-1RA and DPP-4i group, respectively. We found a crude HR of 0.89 [0.76–1.05] for MOF with GLP-1RA compared to DPP-4i. In the fully adjusted model, we obtained an unaltered HR of 0.86 [0.73–1.03]. For the case of hip fracture, we found a crude HR of 0.68 [0.49–0.96] and a similar adjusted

HR. Fracture risk was lower in the GLP-1RA group when examining higher daily doses of the medications, when allowing follow-up to continue after medication change, and when examining hip fractures, specifically. Additional subgroup- and sensitivity analyses yielded results similar to the main analysis.

Conclusion: In our primary analysis, we did not observe a significantly different risk of MOF between treatment with GLP-1RA and DPP-4i. We conclude that GLP-1RA are safe in terms of fracture.

KEYWORDS

GLP-1, DPP-4, fracture, diabetes, bone, osteoporosis, antidiabetic, glucose-lowering drugs

Introduction

Although bone mineral density (BMD) is normal or even elevated in individuals with type 2 diabetes mellitus (T2D), T2D has been associated with an increased fracture risk (1). In addition to increased BMD, individuals with T2D tend to have a higher body mass index (BMI) than controls, which is believed to be protective against fractures (2–4).

Glucagon-like peptide-1 receptor agonists (GLP-1 RA) and dipeptidyl peptidase 4 inhibitors (DPP-4i) were both introduced in Denmark in 2007, and new drugs in the classes are continually being introduced (5). GLP-1 RAs have recently been recommended for treatment of T2D in subjects with cardiovascular disease (6) and are also used for weight loss (7). Consequently, the use of these agents is increasing, creating a need for information on potential effects on other organs such as bone.

Knowledge about the impact of GLP-1 RAs on bone health and fracture risk is limited. Studies attempting to investigate the effects of various glucose-lowering drugs on fracture risk are often subject to confounding and insufficient follow-up durations (8). Cohort studies (9, 10) and meta-analyses (11, 12) have reported GLP-1 RAs to be associated with neutral effects on fracture risk. One meta-analysis, however, found a reduced fracture risk with GLP-1 RAs (13). However, the RCTs analyzed suffer from use of different comparators and short follow-up durations (median durations between 12 weeks and 2 years), and any beneficial effects on fracture rates on such short time-scales may be due to a lower risk of falling rather than improved bone quality. A recent network meta-analysis of 117 RCTs contained estimates of the risk ratios of six separate GLP-1 RAs compared to seven separate DPP-4 inhibitors; findings were neutral except all comparisons against trelagliptin and the comparison of semaglutide to saxagliptin, all of which showed protective effects of the GLP-1 RAs in question (14). All comparisons of GLP-1 RAs to placebo in the network meta-analysis similarly revealed neutral effects except for albiglutide which showed a significant protective effect.

For DPP-4is, most studies reported no association with fracture risk (15–25). However, a few studies did find DPP-4is to be associated with a reduced risk of fractures compared to non-DPP-4i use (26, 27) or compared to glitazones (20).

In the present study, we aimed to investigate fracture risk in individuals using GLP-1 RAs versus individuals using DPP-4is. We hypothesized that there is no difference in fracture risk between the two drug classes.

Study design and methods

The STROBE guideline for reporting of observational studies was followed (STROBE checklist can be found in Supplemental Table S1) (28).

Study design and setting

We conducted a nationwide registry-based cohort study using data from the Danish national registries. We included all individuals who initiated a combination of metformin and GLP-1 RA or metformin and DPP-4i treatment between January 1st 2007 and December 31st 2018. As subjects were included when either treatment combination was initiated, any previous use of metformin, GLP-1 RA or DPP-4is alone or in combination with any other glucose-lowering drug was allowed. We chose to collect data from 2007 onwards as both GLP-1 RAs and DPP-4is became available in Denmark in 2007. Outcome information was collected by identifying all fracture-related diagnoses from

index data onwards. Users of GLP-1 RAs were considered the exposure group, and controls (DPP-4i users) were matched 1:1 using propensity scores.

Data sources

All data were provided in anonymized form by Statistics Denmark (*Danmarks Statistik*, project identifier no. 703382). Statistics Denmark obtained data from national Danish registries. All Danish citizens are assigned a unique 10-digit personal identification number (PIN) stored in the Danish Civil Registration System, which contains high-fidelity individual-level information on all residents in Denmark and Greenland (29). This PIN allows easy and unambiguous individual-level record linkage between different Danish registers (30, 31). The Danish Government provides full health care to all Danish citizens, including free access to hospitals and full or partial reimbursement of drug expenses. The Danish National Prescription Registry contains information on all prescription drugs sold in Denmark since 1995 according to the Anatomical Therapeutical Chemical (ATC) classification (32, 33). All diagnosis codes are stored in the Danish National Patient Registry, which covers all in- and outpatient contacts to the hospital (34). All physician-assigned discharge diagnoses are included, coded according to the *International Classification of Diseases, Eight Edition* (ICD-8) from 1977 until 1993 and according to ICD-10 from 1994 onwards.

All data on sex, date of birth, death, emigration, and socioeconomic factors were obtained from the Danish Civil Registration System.

Study population

The study population included subjects residing in Denmark. A flowchart of the inclusion process is presented in Figure 1.

We first identified persons treated with metformin and GLP-1 RAs (the exposure drug) and/or DPP-4is (the control drug) between January 1st 2005 and December 31st 2019. These dates were set outside the study period to ensure that follow-up wasn't initiated inappropriately late or terminated early simply due to natural intervals between redemptions (e.g., an individual with a prescription redemption in Jan 2019 mistakenly has follow-up terminated in early December 2018). For each medication, we defined a start date (date of first redemption) and an end date (date of last redemption plus the number of daily doses redeemed on that date). We then excluded all individuals in which treatment with GLP-1 RAs and DPP-4is overlapped for the entire duration of treatment and those in which neither medication overlapped with metformin use. Remaining individuals were assigned to the exposure or control group based on which medication was first taken singularly in combination with metformin.

Then start and end dates were defined for each other class of glucose-lowering medication. Those who were already treated with an additional glucose-lowering drug (or several) at the beginning of combination therapy were included if (and when) the additional medication was halted and the individual thus received only a combination of metformin and GLP-1 RA or metformin and DPP-4i treatment. *End of combination therapy* was defined as the day that treatment with metformin, the exposure drug, or the control drug ceased or when another glucose-lowering drug was initiated. Glucose-lowering drugs were defined as any drugs with an ATC code beginning in "A10"; i.e., insulins and analogues, biguanides, sulfonylureas, alpha-glucosidase inhibitors, thiazolidinediones, DPP-4is, GLP-1 RAs, sodium-glucose co-transporter 2 inhibitors, and repaglinide.

Finally, the cohort was limited to those in which *beginning of combination therapy* was between January 1st 2007 and Dec 31st 2018.

Exposure

The National Prescription Registry contains data on redeemed drug prescriptions along with dates, doses and pack sizes. Each medication–including the exposure and control medications–was only considered used if an individual had redeemed at least three prescriptions in the period outlined above. Medications were identified using ATC codes (Supplemental Table S2).

From the National Prescription Registry, we obtained the Defined Daily Doses (DDD) variable, which is based on "the assumed average maintenance dose per day for a drug used for its main indication in adults", according to the World Health Organization Collaborating Centre for Drug Statistics Methodology (35). The resultant number of days was added to the date of last prescription redemption to estimate a true end-of-treatment for each drug.

Of note, exposure to metformin, the exposure drug, and the control drug was assumed to be continuous between initiation and end-of-treatment. To estimate the effects of pauses in these drugs, we calculated the cumulative dose (total number of DDDs) for each drug between the last prescription redeemed prior to or at index date until end of follow-up for each individual. We then assessed pauses using the medication possession ratio (MPR); the ratio of the cumulative number of daily doses to the number of days in the same period. To remove the effects of pauses in medication or low average medication dose, several thresholds for MPR were used: MPR $\geq$ 0.5, MPR $\geq$ 0.75, and MPR $\geq$ 0.95. Lower thresholds likely exclude individuals without pauses in medication, whereas higher thresholds more likely relate to the actual dosage that

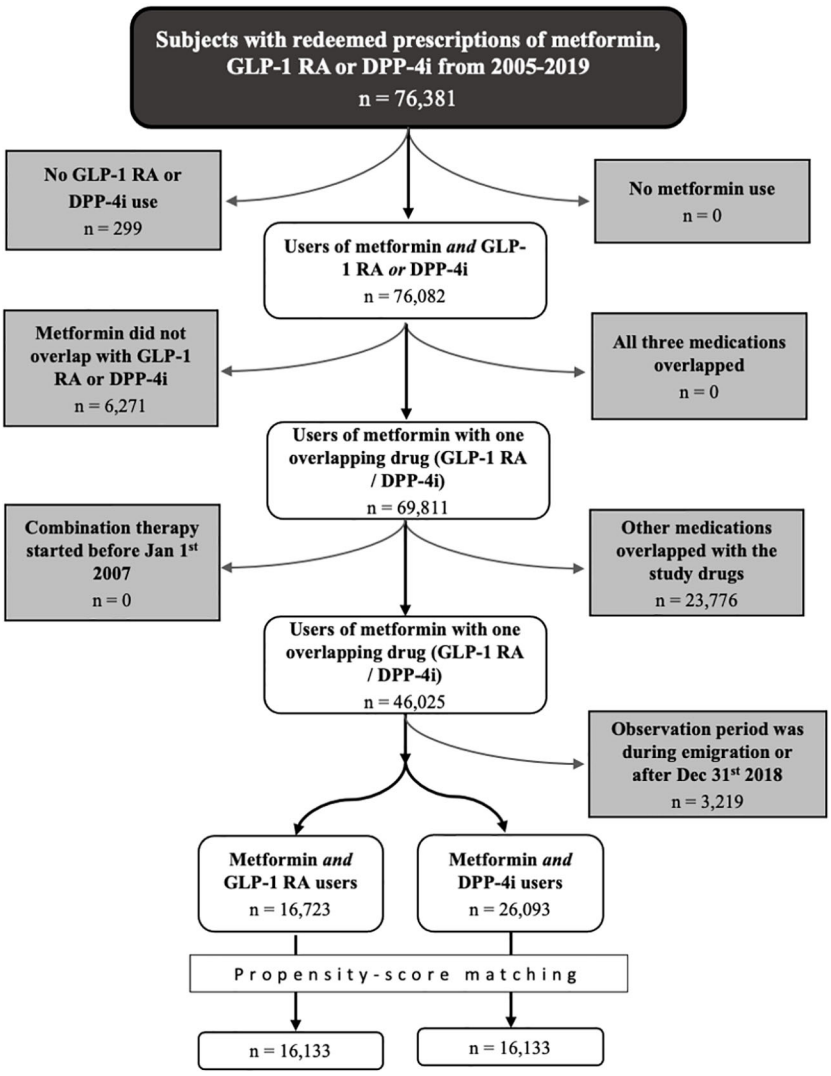

FIGURE 1
Flowchart of the process of in-/exclusion. DPP-4i, dipeptidyl peptidase 4 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists.

individuals receive (i.e., those with no pauses but receiving low-to-intermediate doses are excluded with these thresholds).

The follow-up period was defined as the time between the index date and *end of combination therapy*, emigration, death, or December 31st 2018, whichever came first.

Outcomes

The primary outcome was incident major osteoporotic fracture (MOF). MOF were defined as any of the following fractures: Hip, vertebral, humerus, or forearm fracture. Fractures were identified by ICD-10 codes (Supplemental Table S3). The risks of any fracture, hip fracture, vertebral fracture, humerus fracture, and forearm fracture were estimated in secondary analyses.

Covariates

Data on covariates were obtained using ICD-8 (1977–1993) and ICD-10 (1993–2018) codes (Supplemental Table S2), ATC codes (1995–2018) (Supplemental Table S3), or a combination of both (Supplemental Table S4). All covariates were assessed at baseline (index date) and did not vary over time.

Age at baseline was calculated from the index date and date of birth. Debut of diabetes was estimated as first-ever prescription for glucose-lowering drug, and diabetes duration

at baseline was calculated as the time between diabetes debut until index date.

Osteoporosis was defined as the presence of diagnosis codes for osteoporosis, previous/current treatment with antiosteoporotic medications and/or previous MOF; the variable was assigned three levels (2 = previous MOF, 1 = treatment/diagnosis, 0 = none).

As a proxy for heavy smoking (binary variable), we used diagnosis codes related to lung diseases highly associated with tobacco exposure along with diagnosis codes related to nicotine or tobacco, previous use of medications for the treatment of tobacco dependence, and initiation of drugs for obstructive airway disease after the age of 40.

Obesity, alcohol consumption and hypertension (binary variables) were defined by any diagnosis codes related to the conditions in question and/or ever use of medications for their treatment.

Late-diabetic complications, inflammatory bowel disease (IBD), kidney disease, and previous falls (binary variables) were identified through diagnosis codes.

The Charlson Comorbidity Index (CCI, numeric variable) was calculated based on other comorbidities. The CCI was modified to exclude kidney disease and late-diabetic complications, as these covariates were separately adjusted for in the statistical analyses.

Previous insulin use and previous glucocorticoid use were identified through redeemed prescriptions (binary variables).

Income (numeric variable) along with marital status and employment status (categorical variables; the latter classified by Statistics Denmark according to the so-called *SOCIO13 classification*) were identified on the year preceding each individual's index year. Income (in Danish Kroner, DKK) was adjusted for inflation to a 2018 level according to the Consumer Price Index provided by Statistics Denmark and converted from DKK to Euros using an exchange rate of 7.4363 DKK/Euro.

Statistical analysis

Descriptive statistics

Descriptive statistics are presented as numbers and proportions (%), means and standard deviations (SD), or medians and interquartile ranges (IQR). In the case of CCI, median and 10^{th}-90^{th} percentile were presented rather than median and IQR, as we expected a large majority of all subjects to have CCI values of 0 or 1. Standardized mean differences (SMD) were also calculated for all baseline variables as recommended for propensity-score matched studies (36). Cohen suggested that SMD values above 0.2 be considered small, SMD values above 0.5 considered medium-sized, and SMD values above 0.8 considered large (36, 37).

Missing data

There were only missing data in the socioeconomic variables (marital status, income, and employment). Income was used as a covariate in the main analysis, and missing data were imputed beforehand. Missing data were assumed to be missing at random, and multiple imputation was performed by multivariate imputation using chained equations (38, 39). Ten imputations were produced, each of which ran for ten iterations. As the proportion of missing data was very low (0.3%), and the covariate (income) appeared to be balanced between groups and not alter the results of the survival analysis, it was omitted from all subgroup and sensitivity analyses.

Propensity-score matching

Due to imbalances in sex, age at baseline, and inclusion date, we matched the two groups on propensity scores estimated from these variables. A binomial logistic model was fitted to age, sex, and inclusion date using treatment group as the dependent variable (40, 41) and propensity scores were predicted for each individual in the main cohort.

We matched subjects 1:1 on the logit transformation of the propensity score by nearest-neighbor ("greedy") matching without replacement, using a caliper width equal to 0.2 x the (pooled) SD of the transformed propensity scores (42, 43).

For multiple imputed datasets, matching and statistical analyses were performed separately on each resultant dataset, and the statistical estimates were finally pooled.

For subgroups, matching was done using the previously computed propensity scores. In the subgroups examining specific GLP-1 RAs, *k*:1 matching was performed, with *k* being the highest possible number up to 10 which allowed every individual in the exposure group to be matched to *k* controls within the set calipers.

After matching, balance in the matched variables was assessed by inspecting the distributions of propensity scores across groups and by calculating SMDs for each matching variable.

Multicollinearity

Multicollinearity was assessed using the Variance Inflation Factor (VIF) which yielded values no higher than 1.4 for any covariate. In addition, we examined Pearson's partial correlation coefficient for each pair of variables, and none revealed significant correlations.

Survival analysis

On a non-imputed matched dataset, the Kaplan-Meier Estimator was used to produce survival plots for all fracture

types; a survival plot for MOF on a non-matched dataset was also produced (44). For each subgroup and sensitivity analysis, Kaplan-Meier curves for MOF were also produced.

For the primary analysis, we used the Cox proportional hazards model to estimate hazard rate ratios (HRs) for fracture between the exposure and the control groups. We estimated both crude and adjusted HRs for primary and secondary outcomes. The proportional hazards assumption was evaluated by examining the scaled Schoenfeld residuals of each variable (45). In the fully adjusted model, the covariate osteoporosis was found to violate the proportional hazards assumption and was therefore used as a stratification variable rather than included in the adjustment model. To account for pairing in the matched dataset, a robust variance estimator was used (46, 47).

Finally, to examine a possible additive effect of GLP-1 RAs on fracture risk, we used Aalen's additive hazards regression model; that is, to examine whether absolute rather than relative differences in hazard could be found (48). In short, Aalen's additive hazards model produces a plot for each included covariate, depicting how the given covariate affects the absolute hazard of the outcome at all timepoints; i.e., all effects are allowed to be time-varying. The plot for the intercept corresponds to the baseline hazard that an individual would experience if effects from all covariates and exposure were set to zero.

Sensitivity and subgroup analyses

Several sensitivity and subgroup analyses were performed.

First, we examined males and females separately. Second, we performed sensitivity analyses excluding those with low MPR (selected thresholds are described previously in the section Exposure) in either metformin or study drug (GLP-1 RA or DPP-4i) during the study period. Third, we examined a cohort excluding individuals with kidney disease, previous pancreatitis, or previous falls. Fourth, we performed a sensitivity analyses excluding individuals with follow-up times less than 6 months. Fifth, we split the GLP-1 RA group into specific drug groups–liraglutide, semaglutide, exenatide, dulaglutide, and lixisenatide–based on the drug of which they had received the largest cumulative dose during the study period; ties were handled by allowing any person to appear in several of these subgroups, and only three persons did so. Sixth, we performed the main analysis in the full cohort without prior matching. Seventh, we performed a sensitivity analysis excluding individuals treated with systemic glucocorticoids within the last year prior to inclusion, while not allowing follow-up to continue past initiation of systemic glucocorticoid treatment. Lastly, we performed an analysis analogous to the "intention-to-treat" approach in clinical trials; we continued follow-up after changes in medication for an extra 2 years – or until death or emigration, whichever came first. The sensitivity and subgroup analyses were performed on matched groups unless stated otherwise.

Statistical software

All analyses were performed using R 4.1.0 (The R Core Team & The R Foundation for Statistical Computing, Vienna, Austria) in the integrated development environment (IDE) RStudio 1.4.1106 (RStudio, PBC, Boston, MA, USA). For imputation, the package "mice" (v 3.13.0) was used. Matching was performed using "MatchIt" (v. 4.2.0) and, for multiply imputed datasets, "MatchThem" (v. 1.0.0). Survival analyses–i.e., Cox model, Kaplan-Meier estimator, and Aalen's additive hazards regression–were performed using packages "Survival" (v. 2.1.11), "Survminer" (v. 0.4.9), and Survey (v. 4.0).

Results

Baseline characteristics

We identified 42,816 subjects treated with metformin in combination with either GLP-1 RAs (n = 16,723) or DPP-4is (n = 26,093). After propensity-score matching, a total of 32,266 (16,133 in each group) remained.

Table 1 shows baseline characteristics of subjects in either group in both the full cohort and the matched cohort. The most noticeable differences between the unmatched GLP-1 RA group and the DPP-4i group were sex (43.1% vs. 40.3% females, respectively), age (mean 56.6 vs. 63.6 years, respectively), income (median 35,458 vs. 30,459 euros, respectively), and employment status (59.0% vs. 41.1% retired, respectively). Upon matching, these differences were highly attenuated, and matching was satisfactory. Data from the matched cohort will be presented in short in the following.

Median [IQR] follow-up times in the two groups were of 642 [223–1,414] days in the GLP-1 RA group and 529 [207–1,131] days in the DPP-4i group. In total, we had 75,848 years of combined follow-up time.

Sex was balanced between the groups with 42.3% females in the GLP-1 RA group vs. 41.3% in the DPP-4i group. The GLP-1 RA group had a mean (± SD) age of 57.5 (± 11.3) vs. 57.9 (± 11.0) years in the DPP-4i group. Median [IQR] diabetes duration was longer in the GLP-1 RA group with 4.95 [2.15–8.55] years compared to 3.80 [1.33–7.03] years in the DPP-4i group. CCI scores were balanced with medians [10^{th}-90^{th} percentile] of 0 [0–2] in both groups. Previous MOF were equally prevalent (9.6%) in both groups.

Subjects in the GLP-1 RA group had more complications of diabetes (26.0% vs. 18.2%) and a higher occurrence of hypertension (80.9% vs. 75.4%) compared to the DPP-4i group, although these differences were below the minimum SMD

TABLE 1 Baseline characteristics of full and matched cohorts.

	Full Cohort		Matched Cohort		
	GLP-1 RA group	DPP-4i group	GLP-1 RA group	DPP-4i group	SMD
n =	16,723	26,093	16,133	16,133	
Sex (female), n (%)	7,210 (43.1%)	10,510 (40.3%)	6,827 (42.3%)	6,660 (41.3%)	0.021
Age (years), mean (±SD)	56.6 (±12.0)	63.6 (±12.4)	57.5 (±11.3)	57.9 (±11.0)	0.034
Follow-up time (days), median [IQR]	637 [222–1,403]	519 [196–1,133]	642 [223–1,414]	529 [207–1,131]	0.157
Inclusion Year, n (%)					**0.290**
2007	23 (0.1%)	712 (2.7%)	22 (0.1%)	428 (2.7%)	
2008	171 (1.0%)	1,639 (6.3%)	160 (1.0%)	1,035 (6.4%)	
2009	439 (2.6%)	1,207 (4.6%)	421 (2.6%)	777 (4.8%)	
2010	2,026 (12.1%)	1,752 (6.7%)	1,986 (12.3%)	1,130 (7.0%)	
2011	2,397 (14.3%)	2,074 (7.9%)	2,313 (14.3%)	1,276 (7.9%)	
2012	2,107 (12.6%)	2,047 (7.8%)	2,045 (12.7%)	1,204 (7.5%)	
2013	1,544 (9.2%)	2,270(8.7%)	1,488 (9.2%)	1,416 (8.8%)	
2014	1,290 (7.7%)	2,598 (10.0%)	1,232 (7.6%)	1,585 (9.8%)	
2015	1,446 (8.6%)	2,887 (11.1%)	1,384 (8.6%)	1,846 (11.4%)	
2016	1,457 (8.7%)	3,128 (12.0%)	1,394 (8.6%)	1,841 (11.4%)	
2017	1,607 (9.6%)	2,986 (11.4%)	1,559 (9.7%)	1,824 (11.3%)	
2018	2,216 (13.3%)	2,793 (10.7%)	2,129 (13.2%)	1,771 (11.0%)	
Diabetes Duration (years), median [IQR]	4.84 [2.07–8.44]	4.51 [1.71–5.54]	4.95 [2.15–8.55]	3.80 [1.33–7.03]	**0.240**
Charlson Comorbidity Index, mean (±SD)	0.69 (±1.12]	0.92 (±1.34)	0.70 (±1.13)	0.73 (±1.20)	0.023
Charlson Comorbidity Index, n (%)					0.024
Score 0	10,066 (60.2%)	13,947 (53.5%)	9,624 (59.7%)	9,655 (59.8%)	
Score 1	3,779 (22.6%)	5,873 (22.5%)	3,675 (22.8%)	3,539 (21.9%)	
Score 2	1,742 (10.4%)	3429 (13.1%)	1,708 (10.6%)	1,687 (10.5%)	
Score 3	683 (4.1%)	1,555 (6.0%)	678 (4.2%)	731 (4.5%)	
Score ≥4	450 (2.7%)	1,289 (4.9%)	448 (2.8%)	521 (3.2%)	
Complications of diabetes, n (%)	4,275 (25.6%)	5,545 (21.3%)	4,188 (26.0%)	2,936 (18.2%)	0.188
Diabetic Neuropathy	694 (4.2%)	884 (3.4%)	690 (4.3%)	423 (2.6%)	0.091
Diabetic Nephropathy	499 (3.0%)	787 (3.0%)	489 (3.0%)	393 (2.4%)	0.036
Diabetic Retinopathy	1,192 (7.1%)	1,375 (5.3%)	1,160 (7.2%)	784 (4.9%)	0.098
Other	2,968 (17.7%)	3,787 (14.5%)	2,912 (18.1%)	1,947 (12.1%)	0.168
Osteoporosis, n (%)					0.031
No history	14,851 (88.8%)	22,504 (86.2%)	14,338 (88.9%)	14,272 (88.5%)	
Diagnosed / Treated	244 (1.5%)	687 (2.6%)	242 (1.5%)	307 (1.9%)	
Previous MOF	1,628 (9.7%)	2,902 (11.1%)	1,553 (9.6%)	1,554 (9.6%)	
Risk factors for falls, n (%)					
Hypoglycemic episodes	145 (0.9%)	368 (1.4%)	136 (0.8%)	150 (0.9%)	0.009
Previous Falls	669 (4.0%)	1080 (4.1%)	645 (4.0%)	584 (3.6%)	0.020
Visual Impairment	180 (1.1%)	442 (1.7%)	178 (1.1%)	188 (1.2%)	0.006
Any pancreatitis, n (%)	289 (1.7%)	514 (2.0%)	281 (1.7%)	306 (1.9%)	0.012
Acute Pancreatitis	261 (1.0%)	450 (1.7%)	253 (1.6%)	276 (1.7%)	0.011
Chronic Pancreatitis	63 (0.4%)	158 (0.6%)	61 (0.4%)	93 (0.6%)	0.029
Glucose-lowering drug use (prior to study period), n (%)					
Metformin	16,377 (97.9%)	25,340 (97.1%)	15,807 (98.0%)	15,664 (97.1%)	0.057
SGLT2 inhibitors	694 (4.2%)	380 (1.5%)	673 (4.2%)	239 (1.5%)	0.163
GLP-1 receptor agonists	4,540 (27.1%)	178 (0.7%)	4,463 (27.7%)	124 (0.8%)	**0.835**
DDP-4 inhibitors	1,157 (6.9%)	4,242 (16.3%)	1,131 (7.0%)	2,222 (13.8%)	**0.223**
Insulin, any	2,256 (13.5%)	1,261 (4.8%)	2,156 (13.4%)	793 (4.9%)	**0.296**

(Continued)

TABLE 1 Continued

	Full Cohort		Matched Cohort		
	GLP-1 RA group	DPP-4i group	GLP-1 RA group	DPP-4i group	SMD
Sulfonylureas	6,277 (37.5%)	8,248 (31.6%)	6,194 (38.4%)	4,279 (26.5%)	**0.256**
Alpha-glucosidase inhibitors	112 (0.7%)	136 (0.5%)	111 (0.7%)	61 (0.4%)	0.043
Glitazones	807 (4.8%)	1,033 (4.0%)	797 (4.9%)	572 (3.5%)	0.069
Repaglinide	337 (2.0%)	404 (1.5%)	336 (2.1%)	199 (1.2%)	0.067
Hypertension, n (%)	13,303 (79.5%)	21,046 (80.7%)	13,054 (80.9%)	12,168 (75.4%)	0.133
Chronic Kidney Disease, n (%)	545 (3.3%)	1,263 (4.8%)	533 (3.3%)	599 (3.7%)	0.022
Liver Disease, n (%)	488 (2.9%)	762 (2.9%)	472 (2.9%)	494 (3.1%)	0.008
Mild	447 (2.7%)	664 (2.5%)	431 (2.7%)	438 (2.7%)	0.003
Moderate to severe	84 (0.5%)	175 (0.7%)	84 (0.5%)	105 (0.7%)	0.017
Hyperparathyroidism, n (%)	84 (0.5%)	149 (0.6%)	84 (0.5%)	82 (0.5%)	0.002
Hyperthyroidism, n (%)	453 (2.7%)	897 (3.4%)	443 (2.7%)	442 (2.7%)	0
Hypogonadism, n (%)	36 (0.2%)	41 (0.2%)	36 (0.2%)	31 (0.2%)	0.007
Eating disorder or malabsorption, n (%)	82 (0.5%)	230 (0.9%)	72 (0.4%)	116 (0.7%)	0.036
Venous thromboembolism, n (%)	1,419 (8.5%)	2,316 (8.9%)	1,403 (8.7%)	1,258 (7.8%)	0.033
Inflammatory bowel disease, n (%)	532 (3.2%)	900 (3.4%)	505 (3.1%)	541 (3.4%)	0.013
Osteoarthritis, n (%)	2,804 (16.8%)	4,518 (17.3%)	2,785 (17.3%)	2,261 (14.0%)	0.090
Dementia, n (%)	931 (5.6%)	1,813 (6.9%)	888 (5.5%)	973 (6.0%)	0.023
Alcohol, n (%)	1,178 (7.0%)	1,862 (7.1%)	1,153 (7.1%)	1,251 (7.8%)	0.023
Smoking, n (%)	5,572 (33.3%)	8.699 (33.3%)	5,519 (34.2%)	4,933 (30.6%)	0.078
Obesity, n (%)	6,929 (41.4%)	6,058 (23.2%)	6,708 (41.6%)	4,284 (26.6%)	**0.321**
Other medications (prior to study period), n (%)					
Statins	13,229 (79.1%)	20,664 (79.2%)	12,959 (80.3%)	12,385 (76.8%)	0.087
Thiazides	7,306 (43.7%)	11,756 (45.1%)	7,219 (44.7%)	6,241 (38.7%)	0.123
Loop Diuretics	4,294 (25.7%)	7,019 (26.9%)	4,245 (26.3%)	3,344 (20.7%)	0.132
Potassium-saving diuretics	2,100 (12.6%)	3,480 (13.3%)	2,071 (12.8%)	1,731 (10.7%)	0.065
Antipsychotics drugs	2,160 (12.9%)	3,240 (12.4%)	2,047 (12.7%)	2,221 (13.8%)	0.032
Antiepileptics drugs	2,485 (14.9%)	3,546 (13.6%)	2,388 (14.8%)	2,255 (14.0%)	0.023
Antiarrhythmic drugs	299 (1.8%)	541 (2.1%)	297 (1.8%)	218 (1.4%)	0.039
Hypnotics	5,091 (30.4%)	7,892 (30.2%)	4,965 (30.8%)	4,578 (28.4%)	0.053
Antidepressants	6,431 (38.5%)	8,740 (33.5%)	6,177 (38.3%)	5,665 (35.1%)	0.066
Anxiolytics	4,914 (29.4%)	7,739 (29.7%)	4,797 (29.7%)	4,591 (28.5%)	0.028
Opioids	9,651 (57.7%)	14,437 (55.3%)	9,400 (58.3%)	8,582 (53.2%)	0.102
NSAID	14,911 (89.2%)	22,448 (86.0%)	14,430 (89.4%)	13,921 (86.3%)	0.097
Sex hormones	5,297 (31.7%)	6,612 (25.3%)	4,950 (30.7%)	4,417 (27.4%)	0.073
Antacids	8,794 (52.6%)	13,710 (52.5%)	8,251 (52.8%)	8,147 (50.5%)	0.046
Glucocorticoids	5,560 (33.2%)	8607 (33.0%)	5,436 (33.7%)	4,977 (30.8%)	0.061
Income (euros), median [IQR]	35,458 [25,456–51,287]	30,459 [23,026–44,975]	*35,613 [25,512–51,563]*	*34,162 [25,067–49,448]*	*0.038*
Income quintiles, n (%)					*0.066*
1st	2,772 (16.6%)	5,779 (22.1%)	*2,631 (16.3%)*	*2,772 (17.2%)*	
2nd	2,931 (17.5%)	5,607 (21.5%)	*2,850 (17.7%)*	*2,980 (18.5%)*	
3rd	3,255 (19.5%)	5,291 (20.3%)	*3,114 (19.3%)*	*3,284 (20.4%)*	
4th	3,683 (22.0%)	4,869 (18.7%)	*3,537 (21.9%)*	*3,526 (21.9%)*	
5th	4,056 (24.3%)	4,497 (17.2%)	*3,977 (24.7%)*	*3,526 (21.9%)*	
Missing Data	26 (0.2%)	50 (0.2%)	*24 (0.1 %)*	*45 (0.3%)*	
Marital Status, n (%)					0.027
Unmarried	3,274 (19.6%)	3,822 (14.8%)	2,935 (18.2%)	3,097 (19.2%)	

(Continued)

TABLE 1 Continued

	Full Cohort		Matched Cohort		
	GLP-1 RA group	DPP-4i group	GLP-1 RA group	DPP-4i group	SMD
Married / Registered Partnership	9,568 (57.2%)	14,867 (57.0%)	9,365 (58.0%)	9,304 (57.7%)	
Divorced / Annulled Partnership	2,756 (16.5%)	4,002 (15.3%)	2,711 (16.8%)	2,550 (15.8%)	
Widowed	1,054 (6.3%)	3,309 (12.7%)	1,054 (6.5%)	1,105 (6.8%)	
Missing Data	71 (0.4%)	93 (0.4%)	68 (0.4%)	77 (0.5%)	
Employment status, n (%)					0.033
Working	7,882 (47.1%)	8,800 (33.7%)	7,588 (47.0%)	7,395 (45.8%)	
Unemployed	1,462 (8.7%)	1,380 (5.3%)	1,322 (8.2%)	1,249 (7.7%)	
Retired	6,878 (41.1%)	15,406 (59.0%)	6,795 (42.1%)	7,052 (43.7%)	
Student	131 (0.8%)	58 (0.2%)	78 (0.5%)	57 (0.4%)	
Other	344 (2.1%)	399 (1.5%)	326 (2.0%)	335 (2.1%)	
Missing Data	26 (0.2%)	50 (0.2%)	24 (0.1%)	45 (0.3%)	

Alle data are presented as n (%); mean (±SD); or median [IQR]. DPP-4i, dipeptidyl peptidase 4 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists; SGLT2, sodium-glucose co-transporter 2; SMD, standardized mean difference; MOF, major osteoporotic fractures; NSAID, non-steroid anti-inflammatory drugs. SMDs above 0.2 are highlighted with bold font. Data on income in the matched cohort (italicized) are presented without imputations.

threshold of 0.2. In addition, those in the GLP-1 RA group were more likely to have a history of obesity (41.6% vs. 26.6%, SMD 0.321) and had a slightly larger fraction of subjects included in the years 2010-2012 and 2018 and a smaller fraction included in the years 2007-2009 compared to the DPP-4i group. The only covariates with SMDs above the minimum threshold of 0.2 were inclusion year, diabetes duration, obesity, and previous use of DPP-4is, GLP-1 RAs, insulins, and sulfonylureas; with previous use of GLP-1 RAs exhibiting an SMD of 0.835. In short, GLP-1 RA users had longer diabetes duration, higher prevalence of obesity, and higher prevalence of previous use of insulins and sulfonylureas than those in the DPP-4i group.

Socioeconomic variables were balanced between groups.

Risk of major osteoporotic fractures

Table 2 presents HRs for fractures in the matched cohort during the study period. A MOF occurred in 1.8% (n = 286) and 1.7% (n = 274) of GLP-1 RA users and DPP-4i users, respectively. The Crude HR for MOF with GLP-1 RAs compared to DPP-4is was 0.89 [0.76–1.05]. When adjusted for age and sex, this did not change (HR 0.91 [0.77–1.07]), nor did the fully adjusted model alter the result (HR 0.86 [0.73–1.03]). For each analysis in Table 2 and for the unmatched analysis of MOF, we also present Kaplan-Meier survival curves for crude illustrations (Figure 2), which yielded non-significant results in all analyses of the matched cohort.

TABLE 2 Hazard Ratios (HR) for various fracture types in the matched cohort.

Fracture	Fractures, n (%)	Unadjusted (HR [95% CI])	Age & sex (HR [95% CI])	Full model (HR [95% CI])
MOF	GLP-1 RA: 286 (1.8) DPP-4i: 274 (1.7)	0.89 [0.76 – 1.06]	0.91 [0.77 – 1.07]	0.86 [0.73 – 1.03]
Any	GLP-1 RA: 647 (4.0) DPP-4i: 552 (3.4)	1.01 [0.90 – 1.13]	1.01 [0.90 – 1.13]	0.97 [0.86 – 1.09]
Hip	GLP-1 RA: 61 (0.4) DPP-4i: 75 (0.5)	**0.68 [0.49 – 0.96]**	0.71 [0.51 – 1.00]	**0.65 [0.46 – 0.93]**
Vertebral	GLP-1 RA: 40 (0.2) DPP-4i: 49 (0.3)	0.70 [0.46 – 1.07]	0.72 [0.47 – 1.10]	0.71 [0.46 – 1.11]
Humerus	GLP-1 RA: 89 (0.6) DPP-4i: 84 (0.5)	0.92 [0.68 – 1.24]	0.93 [0.69 – 1.26]	0.91 [0.66 – 1.25]
Forearm	GLP-1 RA: 116 (0.7) DPP-4i: 88 (0.5)	1.12 [0.85 – 1.47]	1.10 [0.84 – 1.46]	1.06 [0.79 – 1.41]

DPP-4i, dipeptidyl peptidase 4 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists; HR, Hazard Ratio; MOF, major osteoporotic fracture; Bold font: the HR was significantly different from 1.00.
Full model: Adjusted for sex, age, inclusion date, diabetes duration, Charlson Comorbidity Index, any diabetic complication, previous falls, inflammatory bowel disease, ever insulin use, ever glucocorticoid use, hypertension, kidney disease, alcohol, smoking, obesity and income, and stratified by osteoporosis.

We found similar results when estimating HRs for hip, vertebral, and humerus fractures, although only hip fractures yielded a significant protective effect of GLP-1 RAs; the crude HR for hip fracture with GLP-1 RAs compared to DPP-4is was 0.68 [0.49–0.96], which was unaltered in the fully adjusted model (HR 0.65 [0.46–0.93]). The crude HR for vertebral fractures was 0.70 [0.46–1.07] with no change after full adjustment (HR 0.71 [0.46–1.11]) when comparing GLP-1 RAs with DPP-4is. For the humerus, the crude HR was 0.92 [0.68–1.24], and the adjusted HR was 0.91 [0.66–1.25] when comparing GLP-1 RAs with DPP-4is. Estimates for any fracture and for forearm fractures were neutral; for forearm fracture the crude HR was 1.12 [0.85–1.47] and the fully adjusted HR 1.06 [0.79–1.41], and for any fracture the crude HR was 1.01 [0.90–1.13], and the fully adjusted HR was 0.97 [0.86-1.09] when comparing GLP-1 RAs with DPP-4is.

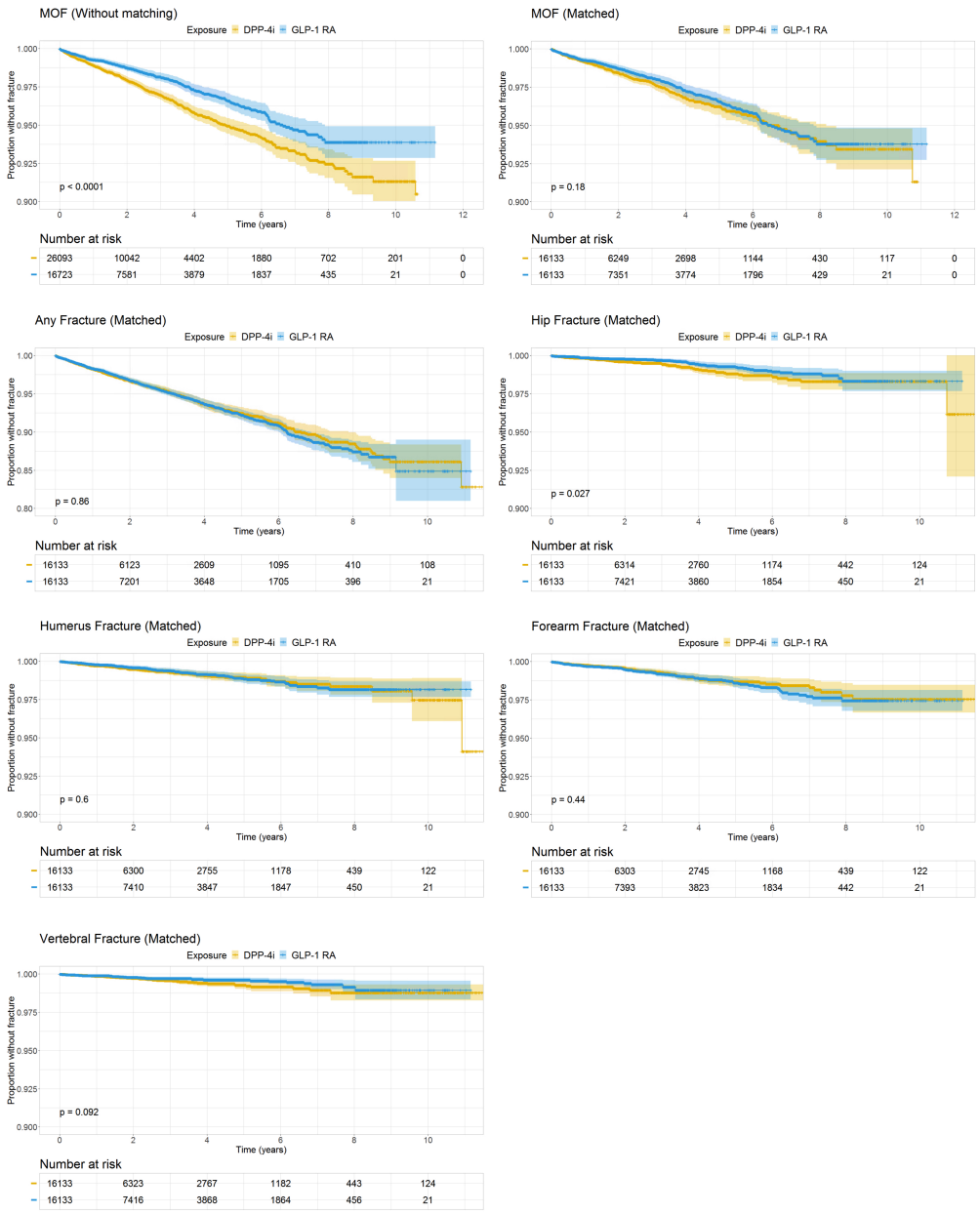

FIGURE 2
Kaplan-Meier Survival Curves of fracture. Survival curves are presented with *number-at-risk* tables. Time in years on the x-axes. Note, the y-axes go from 0.80 or 0.90 to 1.00. DPP-4i, dipeptidyl peptidase 4 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists; MOF, Major osteoporotic fracture.

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Subgroup and sensitivity analyses

Subgroup and sensitivity analyses are presented in Table 3. For the various analyses, Kaplan-Meier curves are presented in Supplemental Figure S1.

Effects between groups were similar between males and females. No changes in effect sizes were observed when excluding individuals with chronic kidney disease, previous pancreatitis and previous falls. When examining different thresholds for MPR, a clear trend was apparent with larger

TABLE 3 Hazard Ratios for MOF in subgroup and sensitivity analyses.

Analysis	n =	Fractures, n (%)	Unadjusted (HR [95% CI])	Age & sex (HR [95% CI])	Full model (HR [95% CI])
Males	GLP-1 RA: 9,409	103 (1.1)	0.90 [0.68 – 1.18]	0.90 [0.69 – 1.18]	0.85 [0.64 – 1.12]
	DPP-4i: 9,409	101 (1.1)			
Females	GLP-1 RA: 6,622	184 (2.8)	0.96 [0.78 – 1.19]	1.00 [0.80 – 1.23]	0.97 [0.77 – 1.22]
	DPP-4i: 6,622	156 (2.4)			
MPR ≥ 0.5	GLP-1 RA: 12,897	222 (1.7)	0.90 [0.75 – 1.09]	0.92 [0.76 – 1.11]	0.87 [0.71 – 1.06]
	DPP-4i: 12,897	211 (1.6)			
MPR ≥ 0.75	GLP-1 RA: 9,590	152 (1.6)	**0.80 [0.64–0.998]**	0.81 [0.64 – 1.01]	**0.75 [0.60 – 0.95]**
	DPP-4i: 9,590	163 (1.7)			
MPR ≥ 0.95	GLP-1 RA: 6,195	83 (1.3)	**0.72 [0.54 – 0.97]**	**0.73 [0.54 – 0.96]**	**0.62 [0.46 – 0.84]**
	DPP-4i: 6,195	99 (1.6)			
No CKD etc.	GLP-1 RA: 14,726	251 (1.7)	0.88 [0.74 – 1.05]	0.90 [0.75 – 1.07]	0.85 [0.70 – 1.02]
	DPP-4i: 14,726	244 (1.7)			
6+ months follow-up	GLP-1 RA: 12,695	275 (2.2)	0.86 [0.73 – 1.02]	0.88 [0.74 – 1.04]	0.84 [0.71 – 1.00]
	DPP-4i: 12,695	274 (2.2)			
Liraglutide	GLP-1 RA: 14,961	280 (1.9)	0.92 [0.77 – 1.09]	0.93 [0.79 – 1.11]	0.89 [0.75 – 1.06]
	DPP-4i: 14,961	249 (1.7)			
Semaglutide	GLP-1 RA: 615	1 (0.2)	0.81 [0.11 – 5.98]	N/A	N/A
	DPP-4i: 4,305	71 (1.6)			
Exenatide	GLP-1 RA: 435	3 (0.7)	0.42 [0.13 – 1.34]	N/A	N/A
	DPP-4i: 3,480	52 (1.5)			
Dulaglutide	GLP-1 RA: 325	3 (0.9)	1.25 [0.32 – 4.91]	N/A	N/A
	DPP-4i: 975	13 (1.3)			
Lixisenatide	GLP-1 RA: 15	0 (0)	N/A	N/A	N/A
	DPP-4i: 150	2 (1.3)			
Full cohort (unmatched)	GLP-1 RA: 16,723	290 (1.7)	**0.67 [0.58 – 0.78]**	0.91 [0.78 – 1.05]	0.87 [0.74 – 1.02]
	DPP-4i: 26,093	578 (2.2)			
Glucocorticoid as exclusion	GLP-1 RA: 14,635	242 (1.7)	0.93 [0.78 – 1.12]	0.95 [0.79 – 1.14]	0.89 [0.74 – 1.08]
	DPP-4i: 14,635	219 (1.5)			
Intention-to-treat analysis	GLP-1 RA: 16,133	410 (2.5)	0.89 [0.78 – 1.02]	0.90 [0.79 – 1.03]	**0.85 [0.74 – 0.98]**
	DPP-4i: 16,133	425 (2.6)			

DPP-4i, dipeptidyl peptidase 4 inhibitor; GLP-1 RA, glucagon-like peptide-1 receptor agonists; HR, Hazard Ratio; MOF, major osteoporotic fracture; MPR, medication possession rate; N/A, not available. Bold font = the HR was significantly different from 1.00.
"No pause": excluded those with pauses in metformin, SGLT2 inhibitor or GLP-1 receptor agonist during the study period. "No CKD etc.": Excluded those with chronic kidney disease, previous falls and previous chronic pancreatitis. "6+ months follow-up": Excluding all with follow-up times less than 183 days.
Full model: Adjusted for sex, age, inclusion date, diabetes duration, Charlson Comorbidity Index, any diabetic complications, previous falls, inflammatory bowel disease, ever insulin use, ever glucocorticoid use, hypertension, kidney disease, alcohol, smoking and obesity, and stratified by osteoporosis.

difference in fracture risk for increasing MPR thresholds between the GLP-1 RA group and the DPP-4i group. At MPR ≥ 0.5, the adjusted HR was quite similar to that in the main analysis (HR 0.87 [0.71-1.06]), but this became lower at MPR ≥ 0.75 (HR 0.75 [0.60 – 0.95]) and lower yet at MPR ≥ 0.95 (HR 0.62 [0.46 – 0.84])

When excluding subjects with follow-up times shorter than 6 months, 25,390 individuals remained, and the unadjusted HR for MOF was found to be 0.86 [0.73–1.02] for GLP-1 RAs compared to DPP-4is. The fully adjusted model yielded a similar HR of 0.84 [0.71–1.00].

Dividing the GLP-1 RA group into subgroups based on the specific drug yielded five groups; liraglutide, semaglutide, exenatide, dulaglutide, and lixisenatide. However, liraglutide users comprised the far majority of GLP-1 RA users (92%), and no other subgroup had sufficient fracture rates to allow reasonable estimation of HRs.

Examining the full (unmatched) cohort for MOF risk yielded a significant protective effect in the GLP-1 RA group (unadjusted HR 0.67 [0.58–0.78]) compared to the DPP-4i group. The same effect can be seen in the Kaplan-Meier plot of MOF in the unmatched cohort ($p < 0.0001$). However, this effect was attenuated in the fully adjusted model to entirely resemble the matched analyses (HR 0.87 [0.74–1.02]).

The results were not altered when defining recent or ongoing glucocorticoid use as an exclusion criterion (adjusted HR for MOF 0.89 [0.74–1.08]). When performing an "intention-to-treat" analysis, the HR for MOF was found to be 0.89 [0.78–1.02] with GLP-1 RAs compared to DPP-4is, although this became significant in the fully adjusted model (HR 0.85 [0.74 – 0.98]).

Using Aalen's additive hazards regression model, we attempted to model the effects of the drugs on fracture in an entirely different way (Figure 3). This test revealed a near-significant protective effect of the GLP-1 RAs compared to DPP-4is with a slope of -0.0042 ($p = 0.051$). However, this slope only reflects a linear approximation to the time-varying effect of the analysis. Assessing the plot, the excess hazard was initially negative (significantly so), but temporarily increased towards zero after around four to six years of exposure, after which it declined once more; this is consistent with a protective effect of GLP-1 RAs on both short and long time-scales.

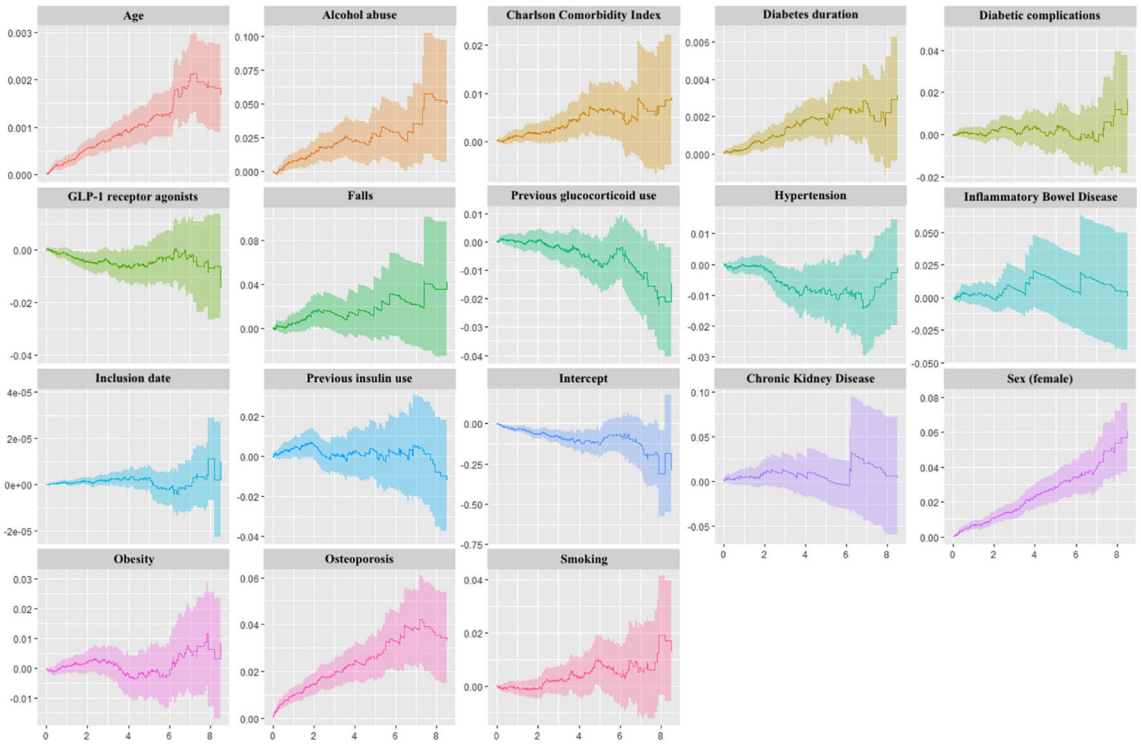

FIGURE 3
Aalen's Additive Regression Plots. Plots of the time-varying additive hazards plotted against time (years) on the x-axes for covariates used in Aalen's Additive Regression Model. This regression model assumes additive risks (producing hazard rate differences) rather than multiplicative risks (producing hazard rate ratios) for each covariate. The plots contain the cumulative hazards attributable to each covariate, and the slope at any point on the plot corresponds to a hazard rate; positive slopes represent increased risks, and negative slopes represent reduced risks. Note that effects may be time-varying, and the slopes can therefore be positive at one timepoint and negative at another timepoint. The intercept term represents a baseline hazard (the hazard of an individual for whom exposure and all covariate values are zero).

Performing the Aalen's additive hazards model on the *intention-to-treat* analysis revealed a continuing downward slope for GLP-1 RAs and a less pronounced attenuation on intermediate time-scales (slope -0.0038, p = 0.015).

As a final measure, we analyzed deaths in the two groups in order to assess potential bias induced by an imbalance in these. In the GLP-1 RA group, 190 (1.2%) deaths occurred with a median [IQR] time-to-event of 647 [188–1,407] days, whereas the DPP-4i group experienced 175 (1.1%) deaths with a median time-to-event of 549 [230–1099] days. Indeed, the crude HR for death (with MOF as a censoring event) in the GLP-1 RA group with the DPP-4i group as reference was 0.94 [0.77–1.15]. When adjusted for age and sex, this became 0.96 [0.78–1.18] and when fully adjusted 0.78 [0.64–0.98].

In addition to the estimates of treatment on fracture risk, we have presented all covariate estimates from the main analysis of MOF in Supplemental Table S5. Please note that these are merely associations as they appear in the given model and do not represent effects that may be interpreted in any causal manner.

Discussion

Summary of findings

In the present study, we found that the risk of MOF was slightly lower, albeit not significantly, in those treated with GLP-1 RA compared to those treated with DPP-4is as add-on therapies to metformin. HRs were generally on the order of magnitude of 0.85-0.90; i.e., a 10-15% lower risk of fractures with GLP-1 RAs. These results were similar across various analyses, which will be summarized in the following.

Examining specific fracture sites revealed non-significantly reduced risk of fractures of the humerus and of the spine with GLP-1 RAs compared to DPP-4is. Interestingly, however, in the case of hip fractures, we found a statistically significant effect of GLP-1 RAs compared to DPP-4i with risk reductions of as much as 30-35%. Risks of any fracture and of forearm fracture were similar between the groups.

When estimating HRs for MOF in the full unmatched cohort, the unadjusted analysis yielded a highly significant difference between the groups. However, this higher risk in the unmatched DPP-4i group appeared to be confounded by age, as the unmatched DPP-4i group was on average 7 years older than the GLP-1 RA group; indeed, the effect was attenuated in the adjusted analyses to resemble the results of the main analysis.

In our "intention-to-treat" sensitivity analysis with an additional two years of follow-up, we found an unadjusted HR very similar to the main analysis, although this became a significant protective effect in the fully adjusted analysis. Since changes in bone tissue manifest as fractures with a long delay, this may hint at more pronounced slow-acting effects on bone of the two drugs, although imbalances in confounding factors between the groups may also arise as time passes.

Similarly, when increasing the minimum thresholds for average daily dose received in the analysis of MOF, differences between the two groups became larger and increasingly significant. Although this study was not designed to examine a dose-response relationship between the exposure and the outcome, this finding may indicate a dose-dependent effect. This lends credence to a causal interpretation of the associations discussed above.

As increased fall risk may be a contributor to the fracture risk in diabetes (49), we performed a subgroup analysis excluding those with known previous falls. In addition, we adjusted for covariates related to falls, diabetic neuropathy, diabetic retinopathy, and visual impairment.

In order to rule out differential mortality as a source of bias in our study, we estimated HRs of death in the two groups and found a negligible difference, although this became significant in the fully adjusted model. A lower mortality in the GLP-1 RA group would expectedly lead to an underestimation of the fracture risk in that group, thereby exaggerating a protective effect of GLP-1 RAs. However, due to the small number of deaths, we believe that the magnitude of such an effect must be negligible.

Previous research

Observational studies and meta-analyses of RCTs on fracture risk with GLP-1 RAs have found mostly neutral effects (9–12, 14, 50), although one meta-analysis found reduced risk of fractures (13). Similarly, studies on DPP-4is have found neutral effects on fracture risk (23–25). Most studies, however, are limited by short follow-up durations (8). Furthermore, research on glucose-lowering drugs and fracture risk is subject to much heterogeneity between studies, particularly due to the many different choices of comparators. Performing randomized controlled clinical trials on the timescales required for long-term outcomes as osteoporotic fractures is often not feasible in a general population not otherwise at high risk of fractures.

However, studies on markers of bone health point towards direct beneficial effects of GLP-1 RAs on bone. Two randomized controlled trials demonstrated reduced bone loss during weight loss with GLP-1 RA compared to placebo (51, 52). Indeed, osteoblastic cell lines express GLP-1 receptors (53), and GLP-1 receptor knockout mice exhibited increased bone resorption and cortical osteopenia (54).

Strengths and limitations

This cohort study was performed using data from Danish nationwide registries. These contain individual-level data on all prescription medications and diagnosis codes along with socioeconomic factors. This provides high-fidelity information on diseases and treatments in the whole period in which GLP-1 RAs and DPP-4is have been marketed in Denmark, allowing an

unbiased study population with very little missing data, and providing results which are highly generalizable to other similar populations.

The use of DPP-4is as a comparator provided a highly comparable control group, particularly as both drugs were used in the setting of sole add-on medication to metformin, and both drugs have similar priority in the management of T2D. However, GLP-1 RAs are often preferred for T2D subjects with obesity or cardiovascular disease, providing a potential for confounding by indication. Although we attempted to adjust for this, we did not have direct measurements of BMI. In addition, a large proportion of the GLP-1 RA group (13.8%) had received DPP-4is before baseline, whereas only 0.8% of the DPP-4i group had received GLP-1 RAs prior. This indicates that DPP-4i treatment is in some cases attempted before switch to GLP-1 RAs, as the cost of DPP-4is is lower, and GLP-1 RAs (during the period in which this study was conducted) required injections. The price difference between the drugs was reflected in the income gap between the two groups, which was however diminished with matching. The tendency for some individuals to have received DPP-4is before switching to GLP-1 RAs may account for the longer diabetes duration and the slightly higher prevalence of diabetic complications and hypertension in the GLP-1 RA group.

Propensity-score matching is a method of mimicking some of the characteristics of a randomized controlled trial (41, 42), and it provided us with fairly balanced matching. However, matching resulted in the discarding of many subjects; the cohort reduced from 42,816 to 32,266 individuals. To examine whether this introduced any bias or resulted in the loss of efficiency, a sensitivity analysis was performed on the full cohort.

In addition, a variety of subgroup and sensitivity analyses confirmed the finding from the main analysis. This supports the conclusion of neutral or slightly reduced risk of fracture with GLP-1 RA treatment compared to DPP-4i treatment in this population.

Residual confounding in an observational study cannot be ruled out. Particularly, we were unable to account for diet and exercise, both of which may serve as confounders. Lack of access to lab results and other clinical information prevented adjustment for variables such as BMD, BMI, and glycemic control (e.g., HbA1c). In addition, some covariates such as smoking and alcohol consumption were crudely estimated through diagnosis codes and previous medications. Similarly, the utility of diagnosis codes to identify falls and other risk factors for fracture is limited, and therefore differential fall patterns between the two groups may still be a cause of residual confounding. However, those treated with GLP-1 RAs appear to have higher prevalence of late-diabetic complications and previous SU and insulin use. These factors indicate that the GLP-1 RA group is more severely affected by diabetes compared to the DPP-4i group, and the GLP-1 RA group may thus be subject to residual confounding associated with higher fracture risk; this would in turn lead to over-estimation of fracture risk in those receiving GLP-1 RAs. As a consequence, the true HR would potentially be more in favor of GLP-1 RAs than the HRs observed in this study.

As changes in bone structure take time to manifest as fractures, median follow-up times less than 2 years may not be sufficient to fully assess the effects of these drugs. However, in the matched cohort, a full 13,767 individuals had more than two years of follow-up time, with nearly half of those (n = 6,650) having more than four years.

Conclusion

In our primary analysis, the risk of MOF was not significantly different between users of GLP-1 RA and DPP-4i. However, in a secondary analysis, users of GLP-1 RA exhibited a significantly lower risk of hip fracture and a lower risk of MOF compared to DPP-4i users when allowing follow-up to continue after medication change. In addition, when examining higher doses of treatment, the difference in MOF risk between the two groups became increasingly larger (with increasing statistical significance) with higher dose thresholds. In contrast, the remaining analyses of MOF revealed fracture risks that are comparable between DPP-4i users and GLP-1 RA users. The results of this study are in line with previous research and support the continued use of GLP-1 RAs in the management of T2D in patients at risk of fracture.

Data availability statement

The datasets presented in this article are not readily available because. All eligible research organizations can apply for access to data at Statistics Denmark. Requests to access the datasets should be directed to https://www.dst.dk/da/TilSalg/Forskningsservice/Dataadgang.

Author contributions

All authors contributed to the article according to the ICJME requirements for co-authorship. All authors critically revised the paper for intellectual content and approved the submitted versions and the final version of the manuscript. ZKA, RV, and JS-L designed the study. ZKA, RV, JS-L and PV had access to all data used in the study. ZKA performed data management and statistical analyses with assistance from all co-authors. ZKA interpreted the data and wrote the manuscript. JS-L, RV, PV, RF-N and SG made ongoing critical revisions of study design and data interpretation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.882998/full#supplementary-material

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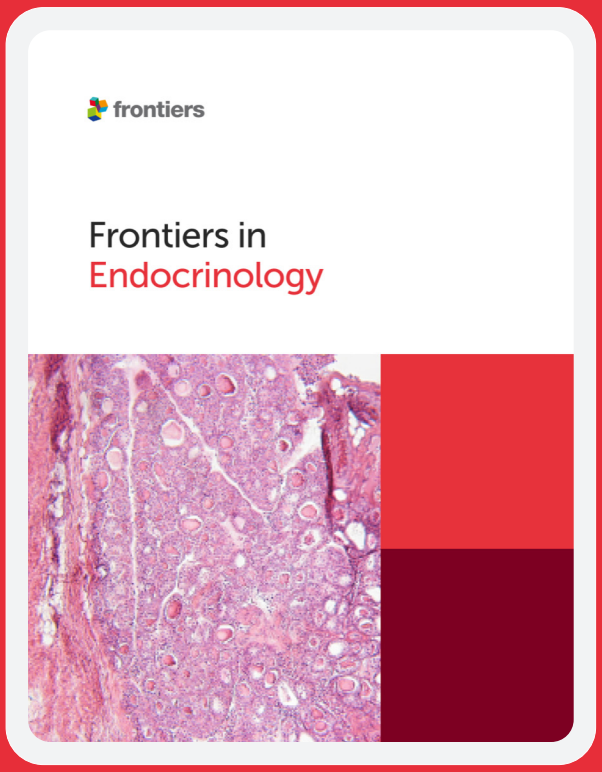

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