Bone Turnover Markers in Patients With Nonalcoholic Fatty Liver Disease and/or Type 2 Diabetes During Oral Glucose and Isoglycemic Intravenous Glucose

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Context: Nonalcoholic fatty liver disease (NAFLD) is associated with type 2 diabetes (T2D) and *vice versa*, and both conditions are associated with an increased risk of fractures and altered bone turnover. Although patients with NAFLD typically suffer from decreased bone mineral density (BMD), T2D is associated with normal to high BMD. The pathophysiology is uncertain but may involve the gut–bone axis.

Objective: We investigated the influence of the gut on glucose-induced changes in plasma bone turnover markers in healthy controls and patients with T2D and/or biopsy-verified NAFLD.

Design: Cross-sectional cohort study.

Patients: Patients with NAFLD with normal glucose tolerance, patients with NAFLD and T2D, patients with T2D without liver disease, and healthy controls.

Interventions: Four-hour 50-g oral glucose tolerance test (OGTT) and an isoglycemic intravenous glucose infusion (IIGI).

Main Outcome Measures: Collagen type 1 C-telopeptide (CTX), osteocalcin, procollagen type 1 N-terminal propeptide (P1NP), and parathyroid hormone.

Results: Plasma glucose levels achieved during OGTTs were successfully matched on corresponding IIGI days. Patients with NAFLD and T2D exhibited similar CTX suppression during the two glucose challenges ($P = 0.46$) and pronounced suppression of P1NP during IIGI compared with OGTT. Conversely, remaining groups showed greater ($P < 0.05$) CTX suppression during OGTT and similar suppression of bone formation markers during IIGI and OGTT.

Conclusions: OGTT-induced CTX suppression seems to be impaired in patients with NAFLD and T2D, but preserved in patients with either NAFLD or T2D, suggesting that coexistence of T2D and NAFLD may affect gut–bone axis. (***J Clin Endocrinol Metab*** **103: 2042–2049, 2018)**

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Abbreviations: AUC, area under the curve; BMD, bone mineral density; BMI, body mass index; CTX, collagen type 1 C-telopeptide; GIP, glucose-dependent insulinotropic peptide; GLP-2, glucagon-like peptide; HOMA, homeostatic model assessment; IIGI, isoglycemic intravenous glucose infusion; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity scores; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; PTH, parathyroid hormone; T2D, type 2 diabetes.

Type 2 diabetes (T2D) is a growing global disease (affecting millions of people worldwide) with well-established complications including nephropathy, retinopathy, and neuropathy as well as macrovascular disease. In recent years, it has been established that bone health is compromised in T2D and that patients with T2D have increased risk of hip fractures (1–3) and a variety of other fractures (4, 5). Some of these fractures might be explained by accidents caused by complications of diabetes and the pharmacological interventions (*e.g.*, causing hypoglycemia), but adjusting for these confounders does not seem to eliminate the increased fracture risk (6). Interestingly, bone mineral density (BMD) is normal or higher than normal in patients with T2D (4), but in spite of this, patients with T2D have a higher fracture risk when compared with individuals without diabetes with the same BMD (7, 8). Bone turnover markers are lower in patients with T2D, indicating an altered bone metabolism (9), and bone strength, measured with *in vivo* indentation, is compromised in patients with T2D (10). Studies using high-resolution peripheral quantitative computed tomography have shown that T2D is associated with an increased cortical porosity (10), whereas the trabecular bone seems normal (11).

Nonalcoholic fatty liver disease (NAFLD) is closely associated with obesity and T2D, and it is estimated that up to 70% of patients with T2D have NAFLD (12–14), whereas it is the case for ~30% of the general population (13, 15). NAFLD is considered a risk factor for T2D, although it is not fully elucidated how these two diseases are related in terms of causality (16). NAFLD also associates with impaired bone health, but the evidence is scarce, and controversy exists. A meta-analysis including 1276 participants found no difference in BMD between patients with NAFLD and healthy controls and attributed the lower BMD observed in some studies to be confounded by body mass index (BMI) and insulin resistance (17). Recently, three studies including a total of 9152 participants showed that BMD was decreased in men but not in women with NAFLD independently of BMI (18, 19) and insulin resistance (20). To our knowledge, the risk of fractures in patients with NAFLD has only been examined in one cross-sectional study including 7797 Chinese adults, which found the prevalence of osteoporotic fractures to be higher in patients with NAFLD (21).

Bone mass is maintained by a continuous resorption and formation of bone, and a disequilibrium of these processes will thus lead to a change in bone mass. Bone resorption is known to have a diurnal variation, which is mainly driven by food intake, whereas bone formation seems to be relatively stable throughout the day (22–24).

When nutrients enter the intestines, gut hormones are released to the blood. Two of these gut hormones, glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-2 (GLP-2), have been proposed as mediators that suppress bone resorption in the postabsorptive state (25–27). Chailurkit *et al.* (28) found a reduced suppression of bone resorption after administration of oral glucose in postmenopausal women with T2D. Although the mechanism was not clear, defects in gut hormone signaling may be the cause. By applying an isoglycemic clamp technique, we will try to differentiate the impact of gut hormones from glucose excursions. Furthermore, as both patients with NAFLD and T2D have impaired bone health, and the role of NAFLD in T2D and *vice versa* on bone health has not been investigated, we will take NAFLD into account. Therefore, we investigated the influence of the gut on glucose-induced changes in plasma bone turnover markers in four groups: (1) patients with NAFLD and normal glucose tolerance (NGT), (2) patients with both NAFLD and T2D, (3) patients with T2D without NAFLD, and (4) healthy controls with NGT.

Materials and Methods

Trial design

A detailed description of the study design and metabolic parameters were provided previously (29). In short, healthy individuals and patients with biopsy-verified liver disease and/or T2D were evaluated: (1) patients with NAFLD and NGT, (2) patients with NAFLD and T2D, (3) patients with T2D without NAFLD, and (4) healthy controls. NGT was ensured using a 2-hour 75-g oral glucose tolerance test (OGTT). Patients taking medication known to affect bone turnover were excluded (topiramate, warfarin, and bisphosphonates), whereas we did not exclude patients taking eltroxin, calcium, and vitamin D supplements. Participants were examined at a screening visit for eligibility, and on two separate experimental days, a 50-g 4-hour OGTT and 4-hour isoglycemic intravenous glucose infusion (IIGI), respectively, were performed. Before both experimental days, participants were instructed to avoid strenuous exercise the day before and start fasting the night before. Participants met in the morning of the experimental day and were placed in recumbent position, and cannulas were inserted in a cubital vein for drawing blood. The ipsilateral hand was placed in a heating pad to arterialize blood. On both days, blood was drawn regularly from 15 minutes before ingestion or infusion of glucose until 4 hours after. For the IIGI, an additional cannula was inserted in a cubital vein of the contralateral arm for the infusion of 20% (weight-to-volume ratio) glucose. The glucose solution was infused at a variable rate to achieve the same plasma glucose levels as obtained in the OGTT (29). The study was approved by the Scientific-Ethical Committee of the Capital Region of Denmark (registration number H-16045041), notified to the Danish Data Protection Agency (reference number 2012-58-0004), and registered at ClinicalTrials.gov (NCT01492283). The study conformed to the latest revision of the Declaration of Helsinki.

Laboratory analyses

Plasma collagen type 1 C-telopeptide (CTX) was measured using the IDS-iSYS CTX (Crosslaps®) assay (Immunodiagnostic Systems, Tyne & Wear, United Kingdom). Plasma

procollagen type 1 N-terminal propeptide (P1NP) was measured using the IDS-iSYS intact P1NP assay (Immunodiagnostic Systems). Plasma osteocalcin was measured using the N-MID Osteocalcin assay (Immunodiagnostic Systems). Plasma parathyroid hormone (PTH) was measured using the IDS-iSYS Intact PTH assay. All assays were carried out on a dedicated automated analyzer, iSYS (Immunodiagnostic Systems), according to the manufacturer's instructions. All assays are chemiluminescence immunoassays.

All samples were analyzed using one single batch of each assay. Assay performance was verified using the manufacturers' control specimens. The intermediary precisions expressed as coefficients of variation for CTX were 5.3% (at CTX concentration 213 ng/L), 3.4% (869 ng/L), and 3.5% (2113 ng/L) for iSYS. For P1NP, the intermediary precisions were 5.4% (19.0 μg/L), 6.5% (48.5 μg/L), and 6.1% (122 μg/L) for iSYS (30). For osteocalcin, the intermediary precisions were 3.0% (8.73 μg/L), 3.6% (27.6 μg/L), and 3.5% (68.7 μg/L).

Serum 25(OH) vitamin D concentrations were measured using Cobas e411 (Roche Diagnostics, Mannheim, Germany) with intermediary precisions of 8.8% (43 nmol/L) and 6.2% (84 nmol/L).

Calculations and statistical analysis

All results are presented as medians and interquartile range (quartiles 1 to 3). Percent change from baseline was calculated for each patient using the fasting value on the respective experimental day. Area under the curve (AUC) was calculated using the trapezoidal rule. Fasting values of CTX, osteocalcin and P1NP were calculated as a mean of the baseline samples from the two experimental days for each patient. Differences between groups were tested with the Mann-Whitney *U* test, and within-group comparisons were made using the Wilcoxon signed-rank test. The steady- state insulin resistance was estimated as homeostatic model assessment (HOMA) of insulin resistance using the HOMA2 calculator (31). No adjustment for multiple testing was made. *P* values <0.05 were considered to indicate statistical significance.

Results

Metabolic parameters

Detailed results on plasma glucose, insulin, C-peptide, incretin hormones, and gastric emptying were reported previously (29). Participants were overweight to obese and middle-aged (characteristics listed in Table 1). In summary, controls exhibited a higher incretin effect compared with the remaining three groups, and fasting hyperglucagonemia was seen in patients with NAFLD with and without diabetes, whereas fasting glucagon levels were lower (but similar during OGTT and IIGI) in patients with T2D and no liver disease as well as in healthy controls. All groups had similar GLP-1 and GIP responses to OGTT (29). Most patients with liver disease had simple steatosis, whereas two [NAFLD: one (12.5%); and NAFLD and T2D: one (12.5%)] were characterized as patients with nonalcoholic steatohepatitis. NAFLD activity scores (NAS) ranged from two to five.

Fasting values of bone turnover markers

Fasting values of bone turnover markers are listed in Table 2. CTX levels were lower in patients with NAFLD and T2D compared with healthy controls ($P = 0.034$). P1NP and osteocalcin were lower in both groups of patients with T2D compared with healthy controls [osteocalcin: $P < 0.001$; P1NP: $P = 0.0016$ (vs NALFD and T2D) and $P = 0.036$ (vs T2D)]. P1NP and osteocalcin were lower in patients with NAFLD and T2D vs patients with NAFLD and NGT (P1NP: $P = 0.003$; osteocalcin: $P = 0.015$). No differences were found between groups for fasting levels of PTH and vitamin D.

Postprandial suppression of CTX

OGTT-induced changes of CTX are illustrated in Fig. 1. During the OGTT, patients with T2D (without NAFLD) had an attenuated postprandial suppression of CTX compared with healthy controls, and patients with NAFLD and T2D had an attenuated CTX suppression compared with patients with NAFLD and NGT. Differences in total AUC of percent change from baseline between the OGTT and IIGI are summarized in Fig. 2. Within-group comparison showed that patients with NAFLD and T2D had similar CTX suppression on OGTT and IIGI ($P = 0.46$), whereas the remaining groups had a

Table 1. Participant Characteristics

	NAFLD + NGT	NAFLD + T2D	T2D	Healthy Controls	*P* Value
N	8	8	8	9	
Age, y	55.5 (39.5–62.3)	65.0 (60.3, 65.8)	58.5 (50.5–66.8)	54.0 (49.0–64.0)	0.54
Sex, n (%) (men)	5 (62.5)	4 (50.0)	4 (50.0)	5 (55.6)	0.95
BMI, kg/m^2	30.0 (26.4–34.8)	30.0 (28.0–31.5)	27.7 (25.8–28.0)	28.3 (28.0–29.0)	0.33
HbA_{1c}, mmol/mol	33.0 (32.0–37.0)	50.0 (40.5–53.3)	43.0 (39.0–47.8)	36.0 (34.0–37.0)	0.001
FPG, mM	6.1 (5.3–6.5)	8.6 (6.9–9.2)	8.0 (6.6–8.9)	5.5 (5.1–5.6)	<0.001
Fasting insulin, pM	164 (108–220)	156 (121–191)	78.2 (52.0–94.9)	76.3 (52.3–97.3)	0.001
Fasting C-peptide, pM	970 (745–1102)	1027 (725–1079)	628 (380–720)	462 (394–492)	0.003
Total vitamin D, nM	67 (50–77)	55 (27–71)	77 (70–99)	60 (48–77)	0.42
HOMA2-IR	3.1 (2.1, 4.2)	3.3 (2.6–3.8)	1.6 (1.1–2.1)	1.5 (1.0–1.8)	0.001
NAS	3 (2 to 3)	4 (3 to 4)	—	—	0.21

Data are presented as medians (interquartile range) unless otherwise noted.

Abbreviations: FPG, fasting plasma glucose; HbA_{1c}, glycated hemoglobin; HOMA2-IR, HOMA of insulin resistance.

Table 2. Fasting Levels of Bone Turnover Markers

	NAFLD + NGT	NAFLD + T2D	T2D	Healthy Controls
CTX, ng/L	300 (170–410)	150 (130–220)[a]	280 (250–330)	290 (290–540)
Osteocalcin, μg/L	17.4 (14.3–21.6)	12.1 (9.5–14.4)[a]	14.4 (13.7–15.7)[a]	22.4 (18.9–24.3)
P1NP, μg/L	43.8 (36.2–59.8)	33.7 (29.2–34.1)[a]	32.9 (29.0–45.7)[a]	45.9 (40.9–55.5)

Data are presented as medians (interquartile range).

[a] $P < 0.05$ vs. healthy controls.

more pronounced CTX suppression during OGTT compared with IIGI (healthy controls: $P = 0.004$; NAFLD: $P = 0.016$; T2D: $P = 0.04$). Patients with NAFLD and T2D had a reduced or absent OGTT-induced CTX suppression compared with healthy controls ($P = 0.021$) and patients with NAFLD ($P = 0.038$) (Fig. 2).

Postprandial changes of bone formation markers

Changes of osteocalcin and P1NP during OGTT and IIGI, respectively, are illustrated in Fig. 1. No differences in suppression of osteocalcin and P1NP during OGTT were found between groups. Within-group comparison showed that patients with NAFLD and T2D had a more pronounced P1NP suppression on IIGI compared with OGTT ($P = 0.016$), whereas no differences were found for the remaining groups or for osteocalcin. Delta values between days are summarized for osteocalcin and P1NP in Fig. 2. No differences were observed for osteocalcin between groups, but patients with NAFLD and T2D had a more pronounced suppression of P1NP on IIGI vs OGTT compared with each of the other groups ($P < 0.016$).

PTH

PTH concentrations during OGTT and IIGI are illustrated in Fig. 3. No differences for PTH were found between OGTT and IIGI in any of the groups, and the groups had similar total AUCs of PTH during the OGTT and IIGI.

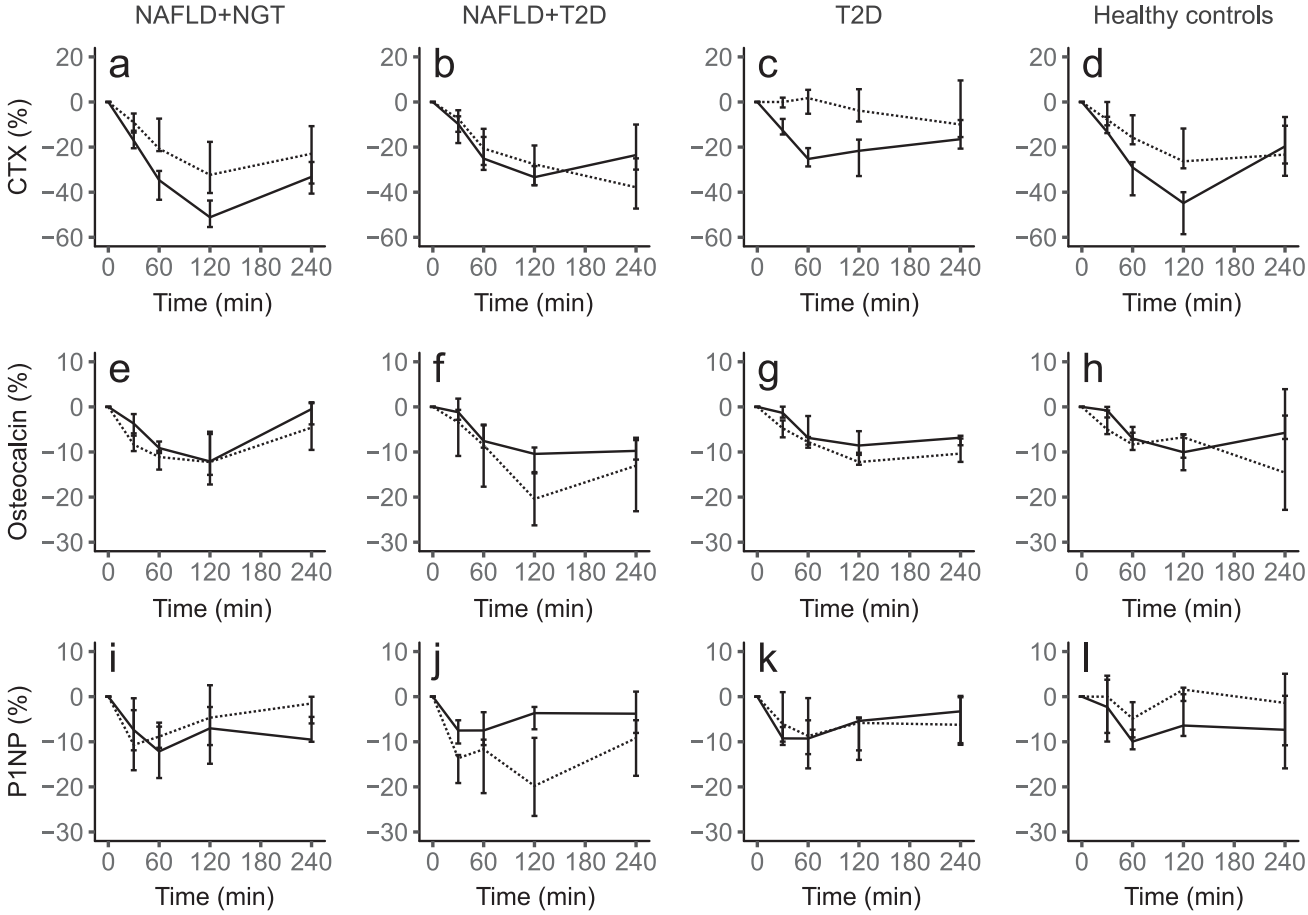

Figure 1. Percentage change of bone turnover markers during OGTT (solid line) and IIGI (dotted line) in (a, e, and i) patients with NAFLD + NGT, (b, f, and j) patients with NAFLD + T2D, (c, g, and k) patients with T2D, and (d, h, and l) healthy controls. Data are medians and interquartile ranges.

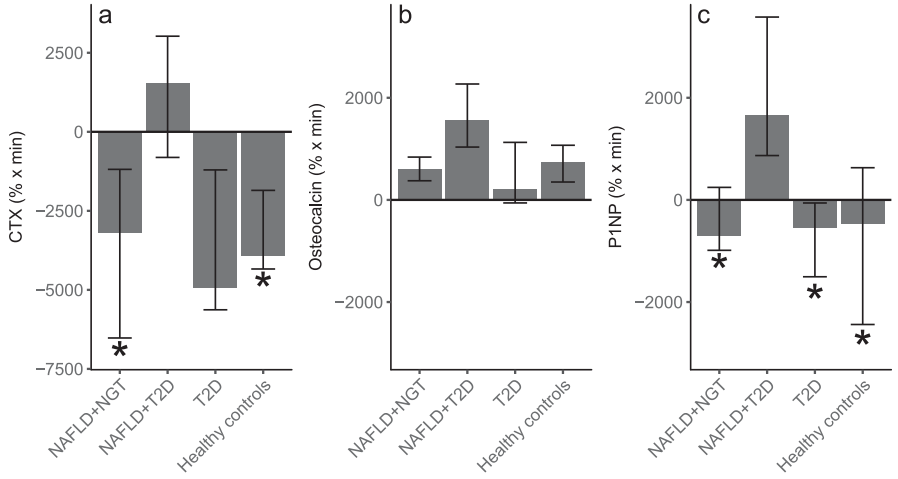

Figure 2. Differences in AUC between OGTT and IIGI ($AUC_{IIGI} - AUC_{OGTT}$) for bone turnover markers in patients with NAFLD + NGT, patients with NAFLD + T2D, patients with T2D, and healthy controls. (a) CTX, (b) osteocalcin, and (c) P1NP. AUCs are calculated based on percent change from baseline. Negative values indicate pronounced suppression during OGTT. Data are medians and interquartile ranges. $*P < 0.05$ vs. NAFLD + T2D.

Discussion

We show that oral vs intravenous administration of glucose suppresses circulating levels of the bone resorption marker CTX, whereas markers of bone formation did not differ between the two glucose stimuli. This was observed in healthy controls and patients with either T2D or NAFLD and NGT. In contrast, our patients with NAFLD and T2D exhibited similar CTX suppression during oral and intravenous administration of glucose and a pronounced suppression of bone formation markers during the intravenous glucose challenge compared with the OGTT.

Limitations of our study are the small number of participants in each group, which raises the risk of type II errors, and the inclusion of multiple groups, increasing the risk of type I errors. An important strength is that the presence (or absence in patients with T2D) of NAFLD was verified by liver biopsy, which ensures reliable categorization of patients. As we did not have a continued fasting day as control, we cannot tell whether the observed suppression of CTX on the IIGI is caused by glucose infusion or diurnal variation, but another study did not find any considerable difference between fasting and intravenous glucose infusion on CTX in healthy young males (24). Also, we do not have data on the physical activity of the participants, which may have influenced bone turnover.

Feeding mediates daily fluctuations in bone resorption, but the underlying mechanisms are uncertain. Plasma glucose excursions, gut hormones, and insulin are possible mediators. In our study, we obtained isoglycemia during the oral and intravenous glucose challenges and observed effects of gut stimulation on bone turnover independently of plasma glucose levels. As expected, an amplification of insulin secretion was seen during oral vs intravenous glucose administration in spite of isoglycemia. This is known as the incretin effect and is caused by secretion of the insulinotropic incretin hormones GIP and GLP-1 (32). This could indicate that the observed differences in CTX suppression in our study could be explained by an immediate inhibitory effect of insulin on bone resorption. In contrast, the effect of insulin on bone resorption has been studied using hyperinsulinemic-euglycemic clamps, and no inhibitory effect of intravenous insulin was observed after 2 hours of infusion (33–35). These studies were under normoglycemic conditions with plasma glucose at 5 mM,

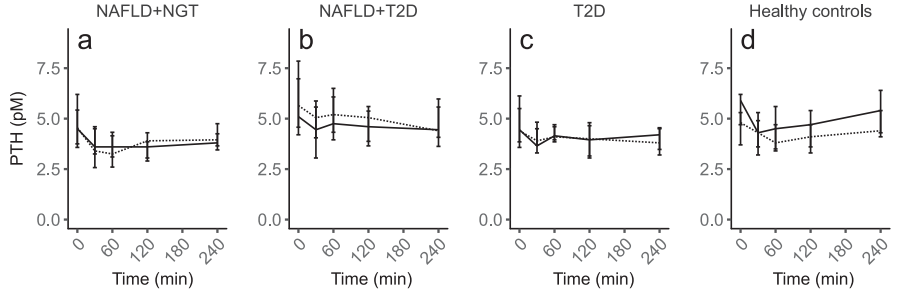

Figure 3. PTH during OGTT (solid line) and IIGI (dotted line) in (a) patients with NAFLD + NGT, (b) patients with NAFLD + T2D, (c) patients with T2D, and (d) healthy controls. Data are medians and interquartile ranges.

but the fasting plasma glucose for patients with T2D was higher, and plasma glucose in our study reaches postprandial levels at which the combination of insulin and glucose may act differently on bone turnover.

Intravenous glucose administration in itself may suppress bone resorption. In a study reported by Nissen *et al.* (25), CTX were more suppressed after a 90-minute hyperglycemic clamp at 12 mM compared with euglycemic clamping at 5 mM. In addition, Bjarnason *et al.* (22) found that an intravenous glucose tolerance test suppressed CTX from baseline (fasting) levels, but less than an OGTT. However, plasma glucose levels were not reported and might have differed between the two glucose administration forms. As intravenous infusion of glucose does not stimulate secretion of gut hormones, it is likely that increases in plasma glucose concentrations and/or glucose-stimulated insulin secretion *per se* are capable of suppressing bone resorption. Nevertheless, a recent study comparing the responses of bone turnover markers in healthy individuals during OGTT, IIGI, and fasting did not find any substantial differences in suppression of CTX between intravenous glucose administration and a control setting with 3 hours of fasting, but found a difference between OGTT and IIGI comparable to our finding in healthy individuals (24).

Clowes *et al.* (27) showed that octreotide, a somatostatin analog, which inhibits secretion of gut and pancreatic hormones, abolished OGTT-induced CTX suppression, but this finding should be interpreted with caution, as octreotide alone, without glucose, increased CTX concentrations compared with placebo.

Gut hormones *per se* have been shown to suppress bone resorption. Nissen *et al.* (25) showed that infusion of GIP in healthy subjects caused suppression of CTX at fasting plasma glucose levels, and clamp-induced hyperglycemia amplified the suppression even further. Another study with bolus injection of a pharmacological dose of GIP in middle-aged, overweight subjects did not show a considerable suppression of CTX (26), but the study was limited by a short duration (48 minutes). Also, bone strength is decreased in GIP receptor–deficient mice models (36, 37), and a common genetic variant in the GIP receptor gene leading to decreased receptor activity is associated with decreased BMD and a 50% increased fracture risk in postmenopausal women (38).

Intravenous infusions as well as subcutaneous injections of GLP-2 cause acute suppression of bone resorption in healthy individuals (26, 39), and once-daily injections of GLP-2 administered to postmenopausal women for 4 months demonstrated an increase in BMD (40). Increases in insulin are not likely to explain the suppression of bone resorption because the insulinotropic properties of GIP is limited at fasting glucose levels (41, 42), and GLP-2 has no effect on insulin secretion (43), although insulin may still potentiate the suppression.

We found that T2D attenuated the OGTT-induced suppression of bone resorption. Lower fasting levels of CTX and attenuated suppression of bone resorption have been reported in patients with T2D after an OGTT (28) and a mixed meal (44), respectively, when compared with healthy controls. Interestingly, we found that the patients with T2D and NAFLD may have a disturbed gut-mediated suppression of bone resorption (missing potentiation of the suppression when gut is stimulated by orally administrated glucose), whereas the patients with T2D seem to have an intact gut-mediated suppression of bone resorption, although their suppression seemed attenuated on OGTT as well as IIGI. The postprandial GIP and GLP-1 concentrations were similar in all four groups (29); thus, differences in plasma levels of the incretin hormones are unlikely to explain the attenuated/missing gut-mediated effects in the patients with NAFLD and T2D. Genetic variants of the GIP receptor are associated with a notable small increased risk of T2D and decreased insulin secretion during OGTT (45, 46), and an explanation of the attenuated response regarding CTX could be osteoclast resistance to GIP or GLP-2 (*e.g.*, disturbed signal transduction or gene variants) that is related to the coexistence of NAFLD and T2D. In patients with NAFLD and T2D, we also observed a slightly pronounced suppression of P1NP during IIGI compared with OGTT, which was different from the other groups (which had no difference between days for bone formation).

It is uncertain whether this attenuated postprandial response of bone resorption is a cause or consequence of a disturbed bone metabolism, and it is unknown whether the observed abnormal postprandial responses are associated with altered bone structure and health. In addition, most patients with liver disease had NAFLD in early stages (with NAS ≤ 5), and thus, our results do not apply for patients with advanced NAFLD, including severe fibrosis and cirrhosis.

In conclusion, we find that coexistence of NAFLD and T2D seems to be associated with disturbed regulation of postprandial bone turnover. To our knowledge, the impact of NAFLD on bone health in patients with T2D has not previously been investigated, and our findings suggest that NAFLD is a comorbidity that could explain variation in studies regarding bone health in T2D. Furthermore, we show that suppression of bone resorption (measured as CTX) is more pronounced when glucose is administrated orally vs intravenously (isoglycemic conditions) in healthy individuals, which supports the role of gut hormones as mediators of postprandial suppression of bone resorption acting either directly or perhaps via gut-mediated increases in insulin secretion.

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Author Contributions: H.M. performed the statistical analyses, researched the data, and wrote the first draft of the manuscript. A.E.J. contributed to the study design and recruitment of participants, performed screening and experiments, and reviewed and edited the manuscript. N.R.J. analyzed plasma for bone turnover markers, contributed to the interpretation of the results, and reviewed and edited the manuscript. L.L.G. contributed to recruitment of participants and reviewed and edited the manuscript. F.K.K. and T.V. contributed to the study design and interpretation of the results and reviewed and edited the manuscript. T.V. and H.M. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Disclosure Summary: L.L.G. has served on scientific advisory panels for Novo Nordisk and as a consultant to Intercept Pharmaceuticals, AbbVie, Merck Sharp & Dohme/Merck, and Norgine. F.K.K. has received lecture fees from, participated in advisory boards of, received unrestricted grants from, and/or consulted for Amgen, AstraZeneca, Boehringer Ingelheim, Eli Lilly and Company, Merck Sharp & Dohme/Merck, Novo Nordisk, Sanofi, and Zealand Pharma. T.V. has received lecture fees from, participated in advisory boards of, received unrestricted grants from and/or consulted for Amgen, AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly and Company, Merck Sharp & Dohme/Merck, Novo Nordisk, and Sanofi. The remaining authors have nothing to disclose.

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