Differential impact of glucose administered intravenously or orally on bone turnover markers in healthy male subjects.

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Abstract

Background: Patients with type-1 (T1D) and type-2 diabetes mellitus (T2D) have an increased risk of hip fracture. The underlying mechanisms may involve disturbances in the incretin hormones. Our aim was to clarify if glucose administration i.e. orally or intravenously differentially affects bone turnover markers in healthy males.

Methods: 12 healthy males were included in a cross-over study consisting of three tests following an 8 hour fast. First, an oral glucose tolerance test (OGTT) was performed. Subsequently, we carried out an isoglycemic intravenous glucose infusion (IIGI) that closely mimicked the glucose response curve to the oral glucose load. We analyzed blood samples for the bone turnover markers serum C-terminal telopeptide of type I collagen (s-CTX) and serum procollagen type I N propeptide (s-P1NP), as well as insulin, glucose, gastric inhibitory peptide (GIP), glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2). Finally, eight of the twelve participants underwent a control experiment where they fasted for three hours (Control).

Results: While OGTT induced a 50% reduction in s-CTX, only a ~30 % reduction was seen during the IIGI and the Control. Neither intervention influenced s-P1NP. The concentration of insulin was highest during the OGTT. However, insulin was also increased significantly during the IIGI compared to the Control. Plasma concentrations of GIP, GLP-1 and GLP-2 were higher under the OGTT than during the IIGI and Control. A linear regression indicated that peak p-GIP significantly predicts nadir s-CTX ($p=0.03$), and that peak p-GIP could explain 34% of the variability in nadir s-CTX (adjusted $R^2=0.34$).

Conclusion: This study indicates that glucose *per* se does not acutely affect bone turnover markers. However, gastrointestinal hormones, especially GIP, possibly in combination with hyperglycemia, may have an acute, uncoupling effect on bone turnover leading to a decrease in bone resorption but no change in bone formation.

Keywords: Oral glucose tolerance test (OGTT); isoglycemic intravenous glucose infusion (IIGI); bone turnover markers (BTMs); C-terminal telopeptide of type I collagen (s-CTX); procollagen type I N propeptide (s-P1NP); gastric inhibitory peptide (GIP); glucagon-like peptide-1 (GLP-1); glucagon-like peptide-2 (GLP-2)

Introduction

Type-1 (T1D) and type-2 diabetes mellitus (T2D) are associated with an increased risk of fractures (1). A meta-analysis demonstrated, that T1D is associated with an increased risk of hip fracture of 6.94 fold (CI: 3.25-14.78) and T2D with an increased risk of 1.38 fold (95% CI: 1.25-1.53) compared to persons without diabetes (1). Oddly, T2D subjects have higher bone mineral density (BMD) than healthy subjects (1). Furthermore, the lower BMD among T1D subjects cannot explain the magnitude of the bone fracture incidence in this patient group (1). The bone resorption marker C-terminal telopeptide of type I collagen (CTX) and the bone formation marker osteocalcin is decreased in patients with diabetes (2). Subjects with diabetes mellitus (DM) have higher blood glucose levels than patients without DM. Therefore, increased circulating blood glucose may affect bone turnover and bone turnover markers. It is, however, unknown whether glucose *per se* has a negative effect on bones leaving them more prone to fracture, or whether other mediators are involved.

Glucose in a test meal or as an oral glucose tolerance test (OGTT) may potentially influence bone turnover. Thus, it has been shown that OGTT induces a 50% reduction in s-CTX over 2 hours while an intravenous glucose tolerance test (IVGTT) induced less reduction in postmenopausal women (3). There was only a <10% reduction in s-CTX during fasting (3). The effect of an OGTT on s-CTX may be due to a direct effect of glucose, an effect of gastro-intestinal hormones or an additive or synergistic effect of glucose and gastro-intestinal hormones. The fact that an IVGTT induces less reduction in s-CTX than OGTT, indicates that gastrointestinal hormones may play an important role in the acute effect of glucose on bone resorption. However, the glucose infused during the IVGTT was given as a bolus infusion, so a difference in glucose curves between the OGTT and the IVGTT could be responsible for the differential effects (3). *Clowes et al.* highlighted the importance of gastro-intestinal hormones as a mediator of the effect of glucose on bone. This was performed with octreotide, a somatostatin-analogue that blocks the release of gastrointestinal hormones. They demonstrated that octreotide decreased the reduction in s-CTX in response to an OGTT (4).

Our aim was to investigate the acute effects of glucose on bone markers *per se* in healthy males. We examined the acute effects of an oral glucose tolerance test (OGTT), an isoglycemic

intravenous glucose infusion (IIGI) and a 3-hour fasting period on bone turnover markers and gastrointestinal hormones.

Methods

Registration and approval

The study was registered at ClinicalTrials.gov (NCT02213276). Approval was obtained from the Danish Data Protection Agency (2007-58-0010) and the Ethics Committee of the Central Denmark Region (1-16-02-377-13).

Study design and subjects

Twelve healthy Caucasian males, aged 20 to 50 years, were recruited by postings at Aarhus University and online at www.forsøgsperson.dk. Subjects attended the hospital for an oral information session and signing a consent form. The study was conducted as a crossover study with two experiments on two separate days at least one week apart. After completing both experiments, participants were asked for participation in a control experiment. Eight of the twelve participants were enrolled in the control experiment. The day before each experiment, participants were asked to refrain from exercise, smoking and taking vitamin supplements. A standard meal delivered by the clinic was to be ingested between 17 and 23 o'clock and participants were asked to fast (water allowed) from 23 o'clock until they arrived for the experiment the next morning. They were asked to arrive by car or bus to the clinic.

Oral glucose tolerance test

The first experiment consisted of an OGTT where participants drank a glucose solution consisting of 82.5 g of glucose monohydrate (equal to 75 g of D-glucose), 225 ml of water and 225 mg of benzoic acid. Upon arrival of the participant, a peripheral intravenous catheter was placed in a cubital vein. Blood samples were collected at -15, -10, 0, 15, 30, 60, 120 and 180 minutes from ingestion of the glucose which lasted 5 minutes. Plasma glucose was measured at -15, -10 minutes from ingestion and every 5 minutes for the first 2 hours and every 15 minutes for the last hour using

an Accu-Chek Inform II apparatus (Roche Diagnostics, Basel, Switzerland). The apparatus is calibrated by the Department of Clinical Biochemistry, Aarhus University Hospital.

Isoglycemic intravenous glucose infusion

The second experiment consisted of an IGII where a glucose solution of 20% D-glucose was infused in a cubital vein to mimic the plasma glucose curve obtained during the OGTT. Upon arrival, two peripheral intravenous catheters were placed in contralateral cubital veins; one was used for glucose solution infusion and one for collecting blood samples. Blood samples were collected and plasma glucose measured at the same time points as for the OGTT. Infusion rate for the glucose solution was adjusted according to the measured actual plasma glucose level, the goal for the following measurement (the plasma glucose level measured at the OGTT for the same time point) and the anticipated insulin secretion rate of the participant.

3-hour fasting control

Upon arrival, a peripheral intravenous catheter was placed in a cubital vein. This marked the start of the control experiment. Blood samples were collected after 0, 1, 2 and 3 hours. Plasma glucose was measured at the beginning of the experiment and after 1, 2 and 3 hours using the Accu-Chek Inform II apparatus.

Blood samples

Before the first experiment, a fasting blood sample was drawn and plasma levels of total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, HbA1c, TSH, PTH, vitamin D and ionized calcium were analyzed at the Department of Clinical Biochemistry at Aarhus University Hospital accredited according to ISO 15189. Blood samples for plasma analysis (insulin, GIP, GLP-1 and GLP-2) were centrifuged at 2000 x g for 15 minutes at 4°C immediately after they were taken. Samples for serum analysis (CTX and P1NP) were kept at room temperature for 30-60 minutes and were then centrifuged at 2000 x g for 10 minutes. After this, all samples were kept at -80°C until analysis.

Plasma analysis

Plasma insulin was measured by ELISA using a DAKO insulin kit (Code: K6219; Dako, Glostrup, Denmark). All samples were extracted in a final concentration of 70% ethanol before GIP and GLP-

1 measurements and 75% before GLP-2 measurements. Total GIP was measured using a radioimmunoassay with a C-terminally directed antibody (code no. 80867), which reacts fully with intact GIP and N-terminally truncated forms as described by Lindgren et al.(5). The standard was human GIP (Bachem, cat no. H-5645) and the tracer was ^{125}I-labeled human GIP (Perkin Elmer, cat no. Nex402). Total GLP-1 was measured as described by Ørskov et al. (6) using a radioimmunoassay (antibody code no 89390) specific for the C-terminal of the GLP-1 molecule and reacting equally with intact GLP-1 and the primary (N-terminally truncated) metabolite. Intact GLP-2 was measured using a radioimmunoassay originally described by Hartmann et al. (7). The antiserum (code no. 92160) is directed against the N-terminus of GLP-2 and therefore measures only fully processed GLP-2 of intestinal origin. For standards, we used recombinant human GLP-2 and the tracer was ^{125}I-labeled rat GLP-2 with an Asp33 -> Tyr33 substitution. Sensitivity for all the radioimmunoassays was below 5 pmol/l, and intra assay coefficient of variation below 10 %.

Bone turnover markers

The International Osteoporosis Foundation, and the International Federation of Clinical Chemistry and Laboratory Medicine recommend that serum procollagen type I N propeptide (s-P1NP), and serum C-terminal telopeptide of type I collagen (s-CTX) are used as reference bone turnover markers in clinical studies (8). Serum CTX and P1NP were measured by immunometric sandwich assays using the COBAS 6000 E (Roche Diagnostics, Basel, Switzerland). The coefficient of variation for the analyses are 5% and 3.7%, respectively. Analyses were carried out at the Department of Clinical Biochemistry at Aarhus University Hospital.

Statistical analysis

Statistical analysis was carried out using the STATA 13 package (StataCorp, College Station, Texas, USA). Repeated measures ANOVA was performed to compare levels of glucose, bone turnover markers, insulin and gastrointestinal hormones between OGTT, IIGI and the 3- hour fasting control. Normality of data was checked via qq-plots. The compound symmetry assumption was checked by examining the pooled within-subject covariance. We performed three conservative F-tests to ensure validity of results. We calculated nadir of s-CTX and peak of gastrointestinal hormones as percentage of baseline. Area under the curve (AUC) was calculated using trapeze-sum for all parameters. Using a simple linear regression model, we then examined the association between the nadir s-CTX and peak of the gastrointestinal hormones.

Results

Subject characteristics

Table 1 presents clinical characteristics of the healthy men included. All participants had HbA1c levels below the limit for diagnosing diabetes (48 mmol/mol).

Plasma glucose response curves

Figure 1 presents plasma glucose response curves during the OGTT and IIGI. To evaluate whether the curves between OGTT and IIGI were similar a repeated measures ANOVA and F-tests were performed and showed no difference between the glucose response curves overall (p=0.98) nor between glucose response curves-over-time (p=1.00).

Bone turnover markers

Figure 2a presents the s-CTX levels during the three interventions, presented as percentage change from baseline. There was a significantly larger decrease in S-CTX during the OGTT compared to both the IIGI and the control experiment, with a nadir of -51% (95% CI: -56;-45) at two hours. During the IIGI and control experiment, the s-CTX decreased to -31% (95% CI: -37; -26) and -28 % (95% CI: -35; -21), respectively at three hours. Repeated measures ANOVA for all three interventions showed that the response curve-over-time interaction was significant, as well as time and intervention on their own. All interactions had p-values below 0.001. The s-CTX drop at the IIGI and the control experiments were not different from each other, as the response curve-over-time and intervention interactions were insignificant (p=0.25).

Figure 2b presents s-P1NP levels during the three interventions, presented as percentage change from baseline. The repeated measures ANOVA showed no individual effect of intervention or interaction between time and intervention for s-P1NP.

Gastrointestinal hormones

We found significantly higher insulin and gastrointestinal hormone responses to OGTT compared to IIGI and the control (figure 3).

The peak insulin concentration after OGTT and IIGI was seen after 30 minutes, corresponding to 274 pM (95% CI: 243; 304) and 111 pM (95% CI: 80; 142), respectively. As expected there was no increase in insulin concentration during control. Repeated measures ANOVA showed a significantly higher concentration of insulin during the IIGI compared to the fasting control (p=0.03) and a significantly higher insulin concentration during the OGTT compared to the IIGI (p=0.002).

During the OGTT, GIP, GLP-1 and GLP-2 concentrations rose to maximally 45 pM (95% CI: 42; 50), 43 pM (95% CI: 40; 46) and 49 pM (95% CI: 44; 53) respectively, within 30 minutes. There was no change in GIP concentration during the the IIGI or control experiment, and no difference between the two, measured by repeated measures ANOVA (p=0.25). A linear regression indicated that peak p-GIP statistically significantly predicts nadir s-CTX (p=0.03), and that peak p-GIP can explain 34% of the variability in nadir s-CTX (adjusted R^2=0.34). For every percent of baseline peak p-GIP increased, the nadir of s-CTX fell 0.024%. There was no linear association between the peak of insulin, GLP-1 or GLP-2 and the nadir of s-CTX.

Discussion

In this study, we have demonstrated that although glucose levels were similar during the OGTT and IIGI, differential response of bone turnover markers was observed. This indicates that glucose levels *per se* do not influence bone turnover, but rather that bone formation may be influenced by gastrointestinal hormones.

OGTT induced a more pronounced decline in the bone resorption marker CTX than IIGI. No significant effects of glucose were seen on the bone formation marker P1NP, although there was a small, but insignificant, decline during the OGTT. This indicates an uncoupling of bone turnover upon ingestion of glucose. Thus bone resorption is decreased while bone formation remains almost unchanged allowing an anabolic effect with incorporation of calcium, phosphate in the mineralized matrix, and protein in the organic matrix of bone.

We found a correlation between the decline in CTX and GIP whereas no association was detected with insulin, GLP-1 or GLP-2. Interestingly, Tsukiyama et al. found that the number of osteoclasts, was significantly increased in mice lacking the GIP receptor (9). In vitro GIP dose dependently reduced osteoclast formation and resorption (10). *Nissen et al.* also suggested that GIP may be a mediator of the postprandial change in bone turnover in humans (11). The authors found that the combination of hyperglycemia and GIP administration lowered s-CTX more than both GIP administration and hyperglycemia individually (11). GIP receptors are present in osteoblasts and GIP has been shown to increase collagen type 1 synthesis and alkaline phosphatase activity in isolated osteoblasts (12,13). In mice experiments, *Xie et al*. found that knocking out the GIP receptor induced a low bone mass phenotype (14) while mice with moderate overexpression of GIP had an increased bone mass phenotype (13). The GIP receptor is also present in osteoclasts and GIP has been shown to inhibit the resorptive activity of mature osteoclasts (15). Therefore, a difference in GIP levels between the OGTT and the IIGI may explain the observed difference in s-CTX. However, *Henriksen et al*. did not find that GIP bolus infusions influenced the s-CTX levels in postmenopausal women (16). Our study group consisted solely of men and differences in gender may explain some of the difference in GIP response in other studies. As somatostatin inhibits the release of gastrointestinal hormones and insulin, this may explain why administration of somatostatin abrogated the effects of the OGTT on bone turnover markers in the study by Clowes et al. (4).

Since there was a similar decrease in s-CTX following the IIGI and the fasting control, despite the difference in insulin concentrations, this suggests that insulin alone does not have a clear acute effect on bone resorption. *Basu et al.* examined the acute effect of low (~150 pmol/liter), intermediate (~350 pmol/liter) and high (~700 pmol/liter) plasma insulin levels on bone metabolism (17). Using a glucose clamp combined with somatostatin infusion as well as replacement infusions of growth hormone and glucagon, they were able to disentangle the isolated effect of insulin. Similarly to our results they found no differences in bone metabolic bone parameters at the different insulin levels. Interestingly, they found that higher insulin sensitivity was associated with decreasing s-CTX levels. This indicates that insulin potentially has longer-term effects on bone turnover, but is in line with our results i.e. that insulin itself does not mediate the acute effect of glucose ingestion on bone turnover markers.

A subcutaneous bolus injection of GLP-1 does not result in a reduction in the s-CTX level within 90 minutes (16). However, it was demonstrated that treatment with a GLP-1 analogue for 52

weeks increased markers of bone formation and prevented weight-loss induced bone-loss as measured by bone mineral content (18). Studies indicate that GLP-1 receptors are present in osteoblast precursor cells (19), while receptors have not been found in mature osteoblasts or osteoclasts (12,13). In light of the presence of GLP-1 receptors on early osteoblast stages, GLP-1 may affect the differentiation of osteoblasts, but there is no evidence showing that GLP-1 has acute effects on bone turnover. Therefore, it seems unlikely that GLP-1 is responsible for the acute effects on s-CTX following an OGTT.

It was seen, that 14-day subcutaneous GLP-2 administration of both 1.6 and 3.2 mg GLP-2 resulted in an acute and sustained reduction of the s-CTX level compared to saline (20). S-P1NP levels were unaffected by the intervention, mimicking the uncoupling effect on bone turnover seen with food intake. Four-month treatment with GLP-2 increases hip BMD in post-menopausal women with low BMD, supporting the hypothesis that GLP-2 induces a decrease in bone resorption without suppressing bone formation, thereby changing the bone turnover process in favor of bone formation. GLP-2 receptors are present in early osteoblast stages (19). GLP-2 may at least in part explain the difference in bone markers between an OGTT and an IIGI. However, our study found no association between the peak s-GLP-2 and the nadir of s-CTX in a linear regression model.

Our results corroborate previous studies on bone turnover (3,21), and support important roles of glucose and GIP on bone turnover. Interestingly, the size of the suppression of s-CTX following the IIGI did not differ from the 28% decrease observed in fasting controls. Neither intervention affected bone formation, as portrayed by s-P1NP. An increased circulating insulin concentration was found following OGTT compared to IIGI and Control. The insulin concentration was also significantly higher following the IIGI compared Control. The s-GIP, -GLP-1 and -GLP-2 responses showed a similar pattern, with a 125-300% increase in their concentration following the OGTT within 30 minutes, which was significantly higher than the IIGI and Control. The results were more inconsistent comparing IIGI to Control, with no difference in s-GIP, a small increase in s-GLP-1 and a small decrease in s-GLP-2.

Although our study was designed to examine the effect of glucose on bone markers in healthy subjects, the area of research interest originated from the finding of decreased bone quality in the diabetic state. As discussed previously, gastrointestinal factors seem to influence bone turnover. Thus, it appears that GLP-1 as well as insulin have long-term effects and GLP-2 and GIP have more acute effects. Apparently, the secretion and action of the incretin hormones are impaired in type-2

diabetes (22). GIP seems to lose its insulinotropic actions, even at supraphysiological doses, whereas GLP-1 retains its insulinotropic actions, although some of its potency is lost (23,24). To our knowledge, there are no studies examining whether the secretion of GLP-2 is disrupted in patients with diabetes. Lopes et al. found that postmenopausal women with T2D were unable to suppress their bone resorption to the same extent as healthy controls, following a mixed meal tolerance test (25). It supports the theory that the actions of gastrointestinal hormones are altered in T2D and may exert negative effects on the bone quality. Viljakainen et al. report lower circulating levels of bone turnover markers, including s-CTX and s-P1NP, in young obese adults compared to gender- and age-matched nonobese controls (26). During OGTT, s-CTX and s-P1NP responded similarly between the groups. Levels of gastro-intestinal hormones were not measured in this study. Hormonal factors, such as insulin and leptin, may play a major role in the observed difference in bone turnover markers, however increased food intake in obesity and subsequent release of gastrointestinal hormones may also play a role in the prolonged overall suppression of bone turnover in obesity presented in Viljakainen et al.'s study.

The strengths of this study are the successful matching of glucose curves between the oral and intravenous intervention and the standardized settings of the experiment. Limitations to the study include the moderate number of study subjects and the short duration of the study. In spite of the attempts to standardize the settings of the experiment, there may be varying environmental and hormonal differences between the subjects that can influence the results.

Conclusion

Gastrointestinal hormones – especially GIP - possibly in combination with hyperglycemia may have an acute, uncoupling effect on bone turnover resulting in reduced bone resorption but no change in bone formation.

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Figures and tables

Table 1 Characteristics of the healthy male subjects (n=12)

	Mean	*Range*
Age (years)	30.6	25–49
Systolic blood pressure (mmHg)	133	119-155
Diastolic blood pressure (mmHg)	80	70-96
Height (m)	1.83	1.74-1.94
Weight (kg)	81.0	69.5-95.0
BMI (kg/m^2)	24.2	21.0-28.8
Total cholesterol (mmol/l)	4.0	2.9-5.9
HDL-cholesterol (mmol/l)	1.5	1.2-1.8
LDL-cholesterol (mmol/l)	2.1	1.2-3.7
Triglyceride (mmol/l)	1.0	0.6-2.2
HbA1c (mmol/mol)	33	29-39
TSH (10^-3 IE/l)	2.2	0.77-4.86
PTH (pmol/l)	4.4	3.0-6.9
Vitamin D (nmol/l)	59.2	42-75
Ionized calcium (mmol/l)	1.24	1.17-1.28
s-CTX (µg/l)	0.63	0.23-0.93
s-P1NP (µg/l)	73.5	31.8-138.5

Figure 1: Plasma glucose response curves during the oral glucose tolerance test (OGTT) and the intravenous isoglycemic glucose infusion (IIGI) in the healthy male subjects (n=12). The error bars present 95%-confidence intervals.

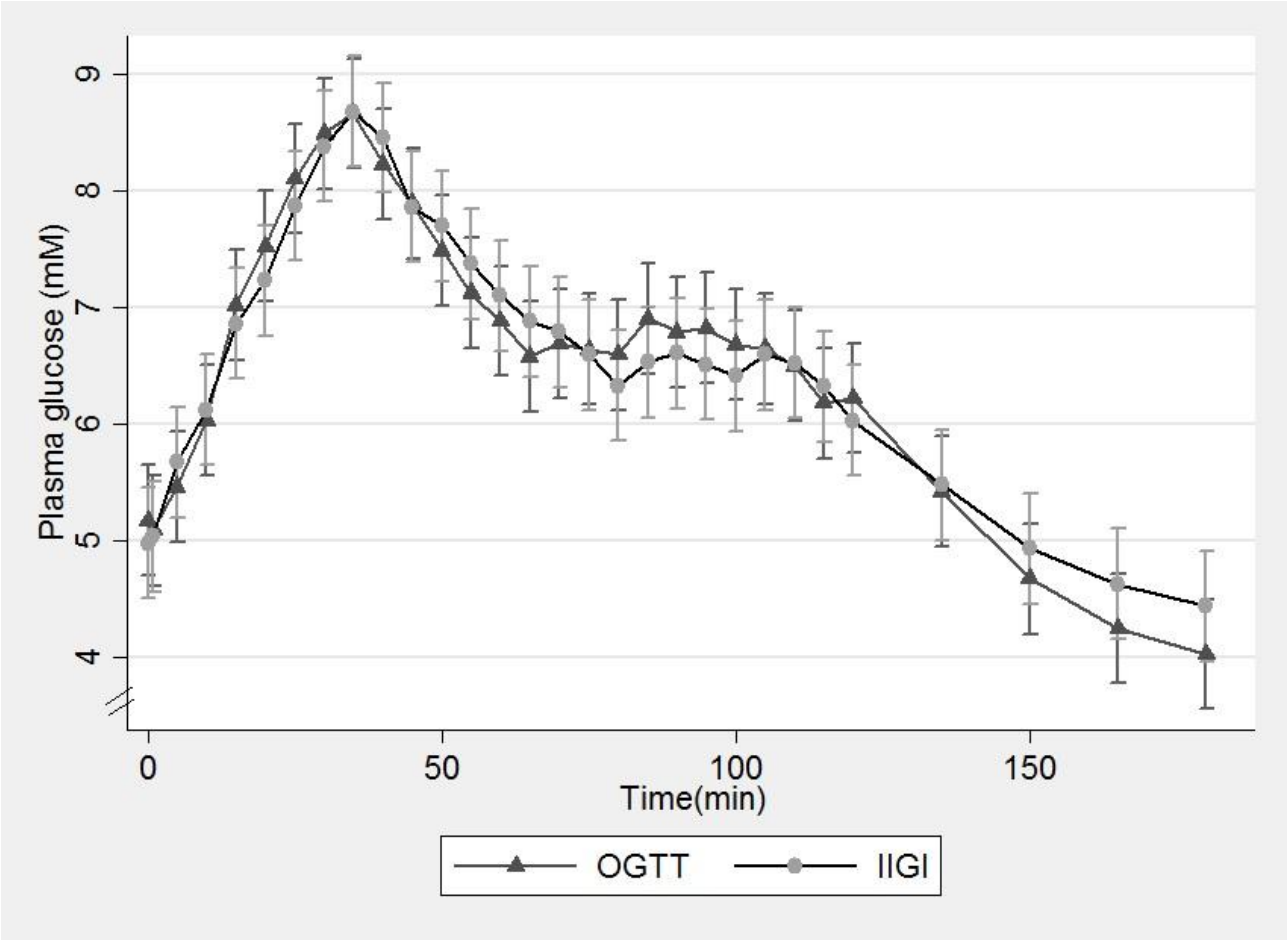


Figure 2: a) Serum CTX and b) P1NP response curves from the 12 healthy males by oral glucose tolerance test (OGTT), intravenous isoglycemic glucose infusion (IGII) and during fasting (Control). The error bars present 95%-confidence intervals.

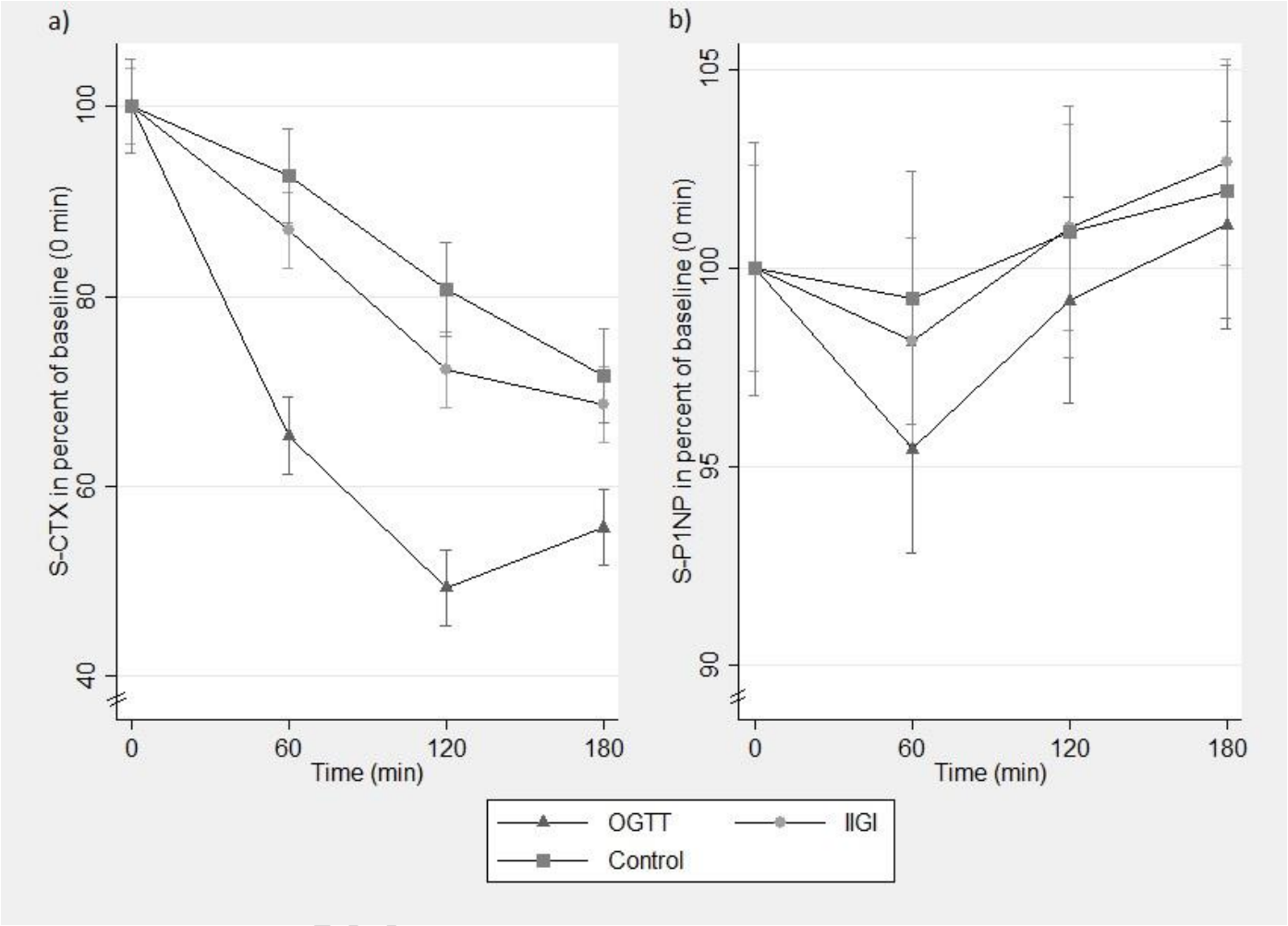
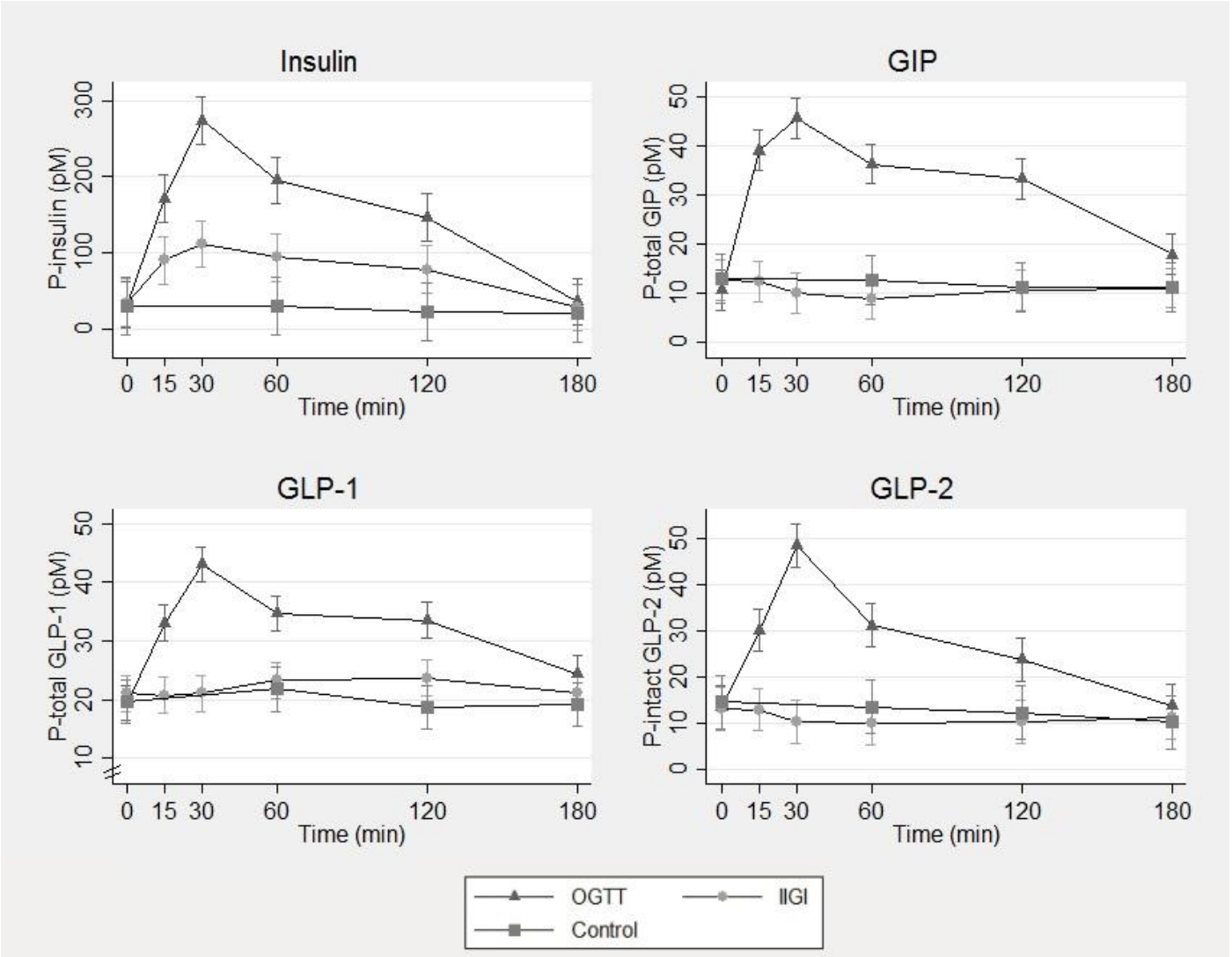


Figure 3: Gastrointestinal hormone response curves for the 12 healthy males during oral glucose tolerance test (OGTT), intravenous isoglycemic glucose infusion (IGII) and fasting (Control). The error bars present 95%-confidence intervals.



Highlights

- Oral glucose tolerance test and isoglycemic intravenous glucose infusion were performed
- No difference was observed between the blood glucose curves during the interventions
- OGTT induced a 50% reduction in the bone resorption marker s-CTX
- A ~30 % reduction was seen during IIGI and control
- P-GIP explained 34% of the variability in nadir s-CTX
- Gastrointestinal hormones may acutely affect bone turnover markers