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The relationship between bone turnover and insulin sensitivity and secretion: Cross-sectional and prospective data from the RISC cohort study☆

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ABSTRACT

Bone metabolism appears to influence insulin secretion and sensitivity, and insulin promotes bone formation in animals, but similar evidence in humans is limited. The objectives of this study are to explore if bone turnover markers were associated with insulin secretion and sensitivity and to determine if bone turnover markers predict changes in insulin secretion and sensitivity. The study population encompassed 576 non-diabetic adult men with normal glucose tolerance (NGT; n = 503) or impaired glucose regulation (IGR; n = 73). Baseline markers of bone resorption (CTX) and formation (P1NP) were determined in the fasting state and after a 2-h hyperinsulinaemic, euglycaemic clamp. An intravenous glucose tolerance test (IVGTT) and a 2-h oral glucose tolerance test (OGTT) were performed at baseline, and the OGTT was repeated after 3 years. There were no differences in bone turnover marker levels between NGT and IGR. CTX and P1NP levels decreased by 8.0% ($p < 0.001$) and 1.9% ($p < 0.01$) between baseline and steady-state during the clamp. Fasting plasma glucose was inversely associated with CTX and P1NP both before and after adjustment for recruitment centre, age, BMI, smoking and physical activity. However, baseline bone turnover markers were neither associated with insulin sensitivity (assessed using hyperinsulinaemic euglycaemic clamp and OGTT) nor with insulin secretion capacity (based on IVGTT and OGTT) at baseline or at follow-up. Although inverse associations between fasting glucose and markers of bone turnover were identified, this study cannot support an association between insulin secretion and sensitivity in healthy, non-diabetic men.

1. Introduction

Fracture risk is increased in people with type 2 diabetes (T2D) despite bone mass generally being normal or increased [1]. Clinical studies revealed decreased levels of circulating biochemical markers of bone formation and resorption in individuals with T2D [1] as well as lower bone formation and resorption and lower bone quality in bone biopsies in T2D [2]. The mechanisms behind these changes in bone turnover and the increased risk of fracture in T2D are not fully elucidated.

Inadequate secretion of insulin and insulin resistance are the cornerstones in the development of T2D. Insulin is considered bone anabolic due to stimulatory effects on osteoblast differentiation [3], and mice not expressing the insulin receptor in osteoblasts have low bone mass [4]. Insulin signaling in osteoblasts favours osteoclast bone resorption activity through secretion of osteoprotegerin and, subsequently, generation and release of the undercarboxylated form of osteocalcin, an osteoblast-secreted marker of bone formation, which may stimulate insulin secretion from the pancreatic β-cells [5]. Furthermore, hyperglycaemia impairs osteoblast activity and survival [6–8] and promotes adipogenic rather than osteogenic differentiation of adipose and muscle-derived stem cells [9]. Additionally, gain-and-loss-of-function models of insulin signaling in mice osteoblasts provide evidence that a high fat diet causes insulin resistance in bone, which lowers bone turnover and osteocalcin activity, causing higher bone volume and glucose intolerance in mice [10].

Thus, based on preclinical investigations, insulin levels and beta-cell function as well as insulin sensitivity would be expected to correlate

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with markers of bone formation in humans, but clinical data from non-diabetic individuals remain to be established. Integration of bone and glucose homeostasis in humans is supported by direct associations between total osteocalcin and estimates of insulin secretion and sensitivity based on oral and intravenous glucose tolerance tests (OGTT or IVGTT) [11–14] and inverse associations with plasma glucose in T2D [15], age-related increases in plasma glucose [13], and incidence of T2D [16–18]. Also, markers of bone resorption but not bone formation were inversely associated with the incidence of T2D [17]. However, other investigations have not provided support of associations between total osteocalcin and plasma glucose or incident diabetes [19–22]. Although the increased levels of fasting insulin usually observed in early stages of T2D could promote bone formation and subsequently bone resorption due to coupling of bone formation and resorption, possibly explaining the association between bone mineral density (BMD) and fasting insulin levels observed in some [23,24] but not all epidemiological studies of non-diabetic individuals [25], insulin resistance in bone cells may reduce bone formation and resorption, which are reported to be lower in patients with T2D [1,26]. Corroborating these reports, insulin sensitivity assessed by an IVGTT was inversely associated with BMD in a selected group of non-diabetic, generally obese men with heart disease [27], and homeostasis model assessment of insulin resistance (HOMA-IR) was positively associated with volumetric BMD in postmenopausal, non-diabetic women [28]. While these studies suggest that insulin resistance may increase bone mass, possibly due to lower bone turnover in T2D [26], it remains unknown if insulin sensitivity measured using the gold standard, i.e. the hyperinsulinaemic, euglycaemic clamp and insulin secretion assessed by OGGT or IVGTT, are associated with bone turnover in non-diabetic individuals.

The aim of this study was to investigate the relationship between bone turnover using markers of bone resorption (CTX) and formation (P1NP), and insulin secretion and sensitivity assessed with the hyperinsulinaemic euglycaemic clamp and measures derived from intravenous and oral glucose tolerance tests in clinically healthy, non-diabetic men. Furthermore, we explored if bone turnover was associated with insulin secretion and 3-year changes in insulin secretion and sensitivity.

2. Materials and methods

The Relationship between Insulin Sensitivity and Cardiovascular Risk Study (RISC) is a prospective cohort study conducted at 19 European research centres across 14 European countries [29]. Baseline and 3-year follow-up data were included in the present study. In short, between 2002 and 2004, 1556 clinically healthy female and male volunteers aged 29–61 years were recruited from the local community. Individuals being treated for obesity, diabetes, hypertension or lipid disorders were excluded from participation. The exclusion criteria comprised recent weight change (>5 kg) or major surgery, chronic pulmonary and cardiovascular diseases, renal failure including renal transplant, seizure disorders including epilepsy, steroid treatment, and any diagnosis of cancer in the previous 5 years but not osteoporosis or treatment for osteoporosis. After physical examination, biochemical testing and a 75-g 2-h OGTT were performed. Individuals with increased fasting or 2-h glucose levels (≥7 and 11.1 mmol/L, respectively), increased blood pressure (≥140/90 mmHg) or increased lipids (triglyceride ≥4.6 mmol/l and total cholesterol ≥7.8 mmol/L) were excluded [29]. In order to limit the effects of factors known or anticipated to influence bone and glucose homeostasis such as menstrual cycle, only male participants of the RISC study were selected for the present investigation.

2.1. Anthropometrics and lifestyle

Body height was measured using a standard ruler (stadiometer) without shoes. Waist size was measured on bare skin at the smallest point between costal edges and the iliac crest. Body weight and fat free mass (FFM) were measured with participants in light clothes and in the fasting state using a Tanita bioimpedence TBF-300 body composition analyser (Tanita International, United Kingdom). Physical activity was registered by the 7-day International Physical Activity Questionnaire (IPAQ) and used to calculate metabolic equivalent energy expenditure per week. The level of physical activity was explored both as a continuous and a categorical measure as the study population was categorized in three groups based on their level of physical activity (inactive, minimally active and health enhancing physical activity). Smoking status was dichotomized according to whether the participant reported current use of tobacco products.

2.2. Assessment of glucose homeostasis

All participants underwent a 75-g OGTT after an overnight fast, with samples being collected at after 0, 30, 60, 90 and 120 min, at baseline and at 3 years. At baseline, hyperinsulinaemic euglycaemic clamp was performed within one week of the OGTT. During the clamp, insulin was infused at a rate of 240 pmol per min per square meter, and infusion of dextrose (20%) was modified at 5–10 min intervals in order to keep plasma glucose levels within 0.8 mmol/L of 4.5–5.5 mmol/L. To evaluate first phase insulin secretion, an intravenous glucose tolerance test (IVGTT) was performed after the clamp in a subset of the participants ($n = 438$ men). A weight-adjusted dose of glucose (0.3 g per kg bodyweight) was infused in one minute, and samples were subsequently collected after 2, 4, 6 and 8 min.

2.3. Biochemical tests

Blood samples were separated into serum and plasma and stored at −80 degrees until biochemical tests were performed. Samples were transferred on dry ice between sites and laboratories. Glucose was measured using the glucose oxidase technique (Cobas Integra, Roche) (within- and between assay coefficients of variation: 1.8% and 2.1%). Serum insulin and C-peptide were assessed using a two-sided time-resolved flouroimmunoassay (AutoDELFIA, Insulin Kit, Wallac Oy, Turku, Finland) based on monoclonal antibodies (Within and between assay coefficients of variation: Insulin (normal levels): 4.3% and 3.7%. C-peptide (normal levels): 5.3% and 2.6%). Serum Procollagen type I amino-terminal propeptide (PINP) and C-telopeptide of type I collagen (CTX-1) were measured by the chemiluminescence method in the fasting state and at steady-state of a euglycaemia during the clamp (IDS-iSYS. Within and between assay coefficients of variation (CV): PINP: 7% and 7%. CTX-1: 5% and 18%). Vitamin D was measured using direct competitive electrochemiluminescence immunoassay (COBAS 311, Roche).

2.4. Insulin sensitivity and beta-cell function

Based on the OGTT, participants were classified into two groups, individuals with normal glucose tolerance (NGT) or impaired glucose regulation (IGR), which included individuals with impaired fasting glycaemia (6.1–6.9 mmol/l), impaired glucose tolerance (2-h-OGTT glucose levels between 7.8 and 11.0 mmol/l) and a combination of both. Insulin sensitivity was calculated as the ratio of the average glucose infusion rate during the last 40 min of the 2-h clamp (adjusted for fat-free mass), M, and mean insulin levels during the same time interval (M/I). At both baseline and the 3-year follow-up, insulin sensitivity was assessed using plasma glucose and insulin levels measured during the 2-h-OGTT at baseline and follow-up. We used the oral glucose insulin sensitivity index normalized to lean body mass optimized for the RISC-study (OGIS-RISC), which has been shown to correlate with insulin sensitivity assessed by clamp [30]. Insulin resistance was also assessed using homeostatic model assessment (Fasting plasma glucose – fasting plasma insulin divided by 22.5).

Beta-cell function was assessed as the total insulin secretion during the OGTT (ISR_{tot}) and beta-cell glucose sensitivity (GluSens), which reflects the response to changes in glucose levels. Furthermore, the ratio between the incremental insulin secretions assessed using C-peptide and plasma glucose concentration in the first 8 min after the intravenous glucose bolus were used to determine the acute insulin response (AIR) during IVGTT.

3. Statistics

Data are presented as percentages, and as mean (±standard deviation) or median (interquartile range) as appropriate. First, the study population was stratified into two groups according to the glycaemic status, NGT *or* IGR. Second, differences in baseline anthropometrics as well as measurements of fasting and insulin-stimulated levels of bone turnover markers and glucose homeostasis between individuals with NGT or IGR were investigated using Student's *t*-test, Mann-Whitney's test and Chi-square tests. Third, the associations between bone turnover markers and measures of insulin sensitivity and secretion were assessed using Lowess-plots and regression analyses. For the regression analyses, three different regression models were used: 1) adjusted for recruitment centre 2) further adjustment for age and BMI 3) further adjustment for smoking and physical activity (measured as a continuous variable) for the entire study population, and then for the normal glucose tolerance and the impaired glucose regulation groups. Skewed variables were log transformed prior to performing the regression analyses, which were performed with the available data and without imputations as data were assumed to be missing at random. These regression analyses were performed for the complete study population as no interaction between glycaemic statuses was observed. The regression models were repeated with adjustment for 25-OHD levels (continuous measure) or after restriction of the analyses to participants with normal levels of 25-OHD as well as used to test if baseline bone turnover markers predicted changes in fasting plasma glucose, 2-h glucose and measures of insulin sensitivity and secretion. Fourth, changes in bone turnover markers during the clamp were evaluated using Student's *t*-test (paired data). Significance was accepted at $p < 0.05$.

Calculations were performed using STATA, v. 14 (StataCorp, College Station, Tx, US).

4. Results

4.1. Baseline anthropometrics and life style factors

Characteristics of the study population are presented in Table 1. None of the participants were treated with corticosteroids or antiresorptive drugs. Among the 576 non-diabetic men, 503 (87%) had NGT and 73 IGR. Age, body weight and BMI were higher in individuals with IGR (Table 1). Neither physical activity nor tobacco use differed between individuals with NGT and IGR (Table 1).

4.2. Glucose homeostasis

Insulin sensitivity assessed from the OGTT (OGIS-RISC) or from the hyperinsulinaemic euglycaemic clamp (M/I) was higher and HOMA-IR lower in individuals with NGT (Table 2). Participants with NGT had lower ISR_{tot} and higher GluSens during the OGTT than those with IGR (Table 2), while no difference in AIR during the IVGTT was observed between these groups.

The OGTT was repeated in 468 of the 576 (73%) participants after three years. Fasting plasma glucose (+0.2 mmol/L, $p < 0.001$) and fasting serum insulin (+2 pmol/L, $p < 0.01$) were slightly increased after 3-years follow-up. Moreover, ISR_{tot} was increased (+2 pmol/L, $p < 0.001$), insulin sensitivity assessed by OGIS-RISC was reduced (−0.3 μmol/min/Kg_{FFM}, $p < 0.01$), and HOMA-IR was increased (+0.6, $p < 0.001$) after three years (Table 2). Significant changes in these outcomes at follow-up were observed only in individuals with NGT, possibly due to lower number of individuals with IGR (Table 2).

Table 1
Basic anthropometrics, life style factors and bone turnover markers in men. The EGIR-RISC Study.

	All n = 576	NGT n = 503	IGR n = 73	p-Value (comparison of glycaemic groups)
Anthropometrics				
Age (years)	42 (36–49)	42 (36–49)	47 (39–51)	0.028
BMI (kg/m^2)	26.2 (3.5)	26.1 (3.4)	27.9 (3.4)	<0.001
Smokers (%)	28%	28%	32%	0.717

Physical activity	n = 503	n = 482	n = 69	
Physical activity				
1: inactive	1: 21%	20%	26%	
2: minimally active	2: 42%	42%	42%	
3: health enhancing activities	3: 37%	38%	32%	<0.001
Data available in 551 individuals				
METS per week	2300 (960–4668)	2253 (990–4746)	2475 (735–4158)	0.465

Vitamin D levels	n = 445	n = 397	n = 48	
Vitamin D (ng/dL)	21.1 (10.2)	21.3 (10.2)	20.0 (10.0)	0.413
Vitamin D deficiency % 25-OHD <20 ng/dL	47%	46%	58%	0.109
Bone turnover markers				
Fasting serum CTX (μg/L)	466 (172)	468 (170)	461 (194)	0.719
Fasting serum P1NP (μg/L)	49 (17)	49 (17)	48 (18)	0.629
Insulin-stimulated serum CTX (μg/L)	428 (157)§§§	430 (157)§§§	418 (156)	0.546
Insulin-stimulated serum P1NP (μg/L)	48 (17)§§	48 (17)§§	47 (17)	0.541

Comparison of bone turnover markers at baseline and at 3-years: §p < 0.05, §§p < 0.01 and §§§p < 0.001.

4.3. Bone turnover markers

No differences in fasting levels of CTX and P1NP were observed between individuals with NGT and IGR (Table 1). After 120 min of insulin stimulation (during the clamp), CTX and P1NP levels had decreased by 8.0% ($p < 0.001$) and 1.9% ($p < 0.01$), respectively (Fig. 1). Decreases in both CTX and P1NP during insulin stimulation were observed in individuals with NGT, whereas only CTX decreased significantly in individuals with IGR (Table 1). Vitamin D was measured in 77% (n = 451) of the participants. Vitamin D deficiency defined as a serum 25-OHD below 20 ng/dl was observed in 47% (n = 211) of the study population, and the prevalence of vitamin D deficiency was comparable in individuals with NGT and IGR (Table 1).

4.4. Relationship between bone turnover and glucose homeostasis

Both CTX and P1NP were inversely associated with fasting plasma glucose in the study population before and after adjustment for potential confounders, but these relations were not reflected in an association between bone turnover markers and fasting levels of serum insulin (Table 3).

The relationships between bone turnover markers and measures of insulin sensitivity (OGIS-RISC and Log-M/I) as well estimates of insulin secretion during IVGTT (AIR) and OGTT (ISR_{tot} and GluSens) were first explored using Lowess plots (Supplementary material, a–f) but none of these plots pointed towards associations.

Table 2
Glucose homeostasis at baseline and at 3-years of follow-up in men. The EGIR-RISC Study.

	All n = 576	NGT n = 503	IGR n = 73	p-Value (comparison of glycaemic groups)
Glucose homeostasis at baseline				
Fasting plasma glucose (mmol/L)	5.2 (0.5)	5.2 (0.5)	5.7 (0.6)	<0.001
120 min plasma glucose (mmol/L)	5.7 (1.4)	5.4 (1.1)	7.6 (1.5)	<0.001
Fasting serum insulin (pmol/L)	32 (22–47)	31 (21–45)	47 (33–67)	<0.001
120 min serum insulin (pmol/L)	131 (75–230)	122 (68–210)	286 (160–509)	<0.001
Total insulin secretion (OGTT)	41 (15)	40 (14)	50 (17)	<0.001
GluSens (pmol/min/m^2 mM)	102.3 (74.2–139.1)	107 (78–142)	78 (57–102)	<0.001
OGIS-RISC (μmol/min/kg FFM)	10.2 (1.9)	10.4 (1.8)	8.3 (1.4)	<0.001
HOMA-IR	1.4 (0.8–1.9)	1.4 (0.8–1.8)	2.1 (1.3–2.7)	<0.001
M/I (μmol/kg FFM/min/nmol/L)	111.9 (81.4–151.5)	116 (85–156)	86 (62–126)	<0.001
	n = 438	n = 380	n = 58	
AIR (pmol/L) (IVGTT)	790 (486–1133)	802 (501–1152)	701 (414–1002)	0.112
Glucose homeostasis at follow-up				
	n = 461	n = 413	n = 48	
Fasting plasma glucose (mmol/L)	5.4 (0.6)§§§	5.3 (0.6)§§§	5.6 (0.6)	<0.001
Fasting serum insulin (pmol/L)	33 (24–49)§§	33 (23–47)§§§	39 (27–59)	0.093
Total insulin secretion (OGTT)	43 (15)§§§	42 (15)§§§	46 (18)	0.179
HOMA-IR	1.6 (0.9–2.0)§§§	1.6 (0.9–1.9)§§§	1.9 (1.1–2.5)	0.027
OGIS-RISC (μmol/min/kg FFM)	9.9 (2.1)§§	10.1 (2.1)§§§	9.0 (1.9)	0.001
GluSens (pmol/min/m^2 mM)	107 (78–140)	109 (85–149)	74 (59–87)	<0.001

Comparison of glucose homeostasis at baseline and at 3-years: $^{§}p < 0.05$, $^{§§}p < 0.01$ and $^{§§§}p < 0.001$.

Nevertheless, insulin sensitivity determined by OGIS-RISC was directly associated with both bone turnover markers in the complete study population. However, the association disappeared after inclusion of age and BMI in the regression models (Table 3). Neither of the bone turnover markers was associated with hyperinsulinaemic euglycaemic clamp-based measurements of insulin sensitivity (M/I) in unadjusted models. Insulin resistance assessed using the HOMA-IR was inversely associated with both CTX and P1NP in unadjusted analyses and remained inversely associated with P1NP but not CTX in the fully adjusted models. While insulin secretion during the IVGTT (AIR) was not associated with bone turnover markers either before or after adjustments, ISR_{tot} during the OGTT was inversely associated with levels of P1NP in unadjusted but not adjusted models (Table 3).

As vitamin D deficiency could interfere with the assessment of a relationship between bone turnover markers and glucose homeostasis, regression analyses were repeated with adjustments for vitamin D levels (as a continuous variable) and in participants that were vitamin D replete (25-OHD ≥ 20 ng/dL), however, neither of the results of the regression analyses changed noticeably after these adjustments (data not shown).

4.5. Bone turnover markers and changes in insulin sensitivity and secretion

Baseline levels of neither P1NP nor CTX were associated with increases in fasting plasma glucose levels during follow-up (Table 4). Baseline levels of CTX and P1NP were not associated with changes in fasting serum insulin levels, ISR_{tot}, OGIS-RISC, or HOMA-IR during follow-up (Table 4).

5. Discussion

The primary aim of this study was to investigate if bone turnover and insulin secretion and sensitivity are integrated in humans as expected, based on previous animal studies. We expected that levels of bone formation and resorption markers would be directly associated with insulin

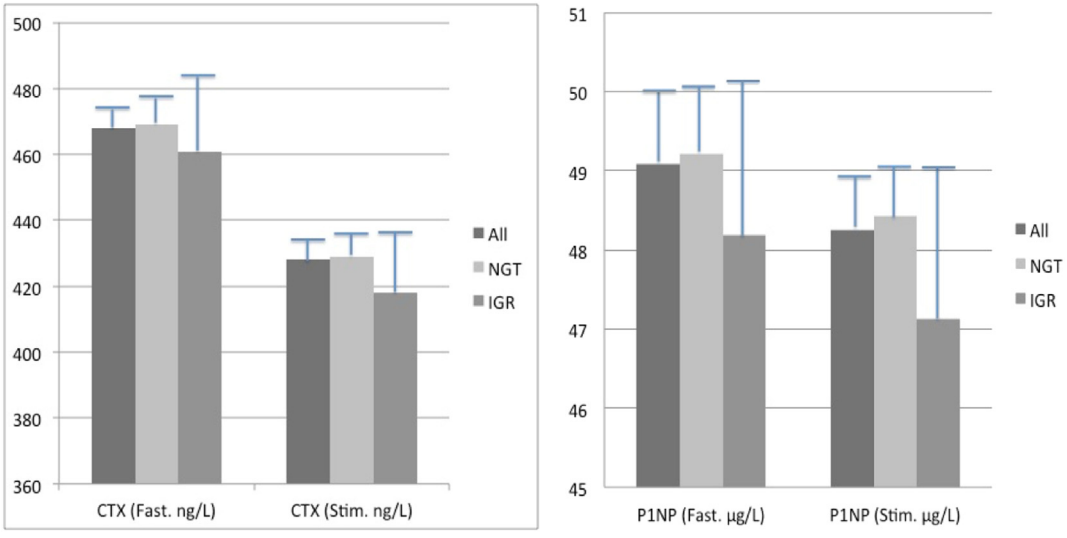

Fig. 1. Bone turnover markers measured in the fasting state and after insulin stimulation (120 min) during a hyperinsulinaemic euglycaemic clamp in 567 non-diabetic adult men (ALL) with normal glucose tolerance (NGT; n = 503) or impaired glucose regulation (IGR; n = 73). Data are mean (standard deviation). $^{§§§}p < 0.001$ and $^{§§}p < 0.01$ versus fasting.

Table 3
Regression coefficients (95% confidence intervals) between bone turnover markers and measures of glucose homeostasis at baseline, according to glucose tolerance status. B The EGIR-RISC Study.

	CTX (ng/L)		P1NP (ng/L)	
	All	p-Value	All	p-Value
Adjusted for centre				
Fasting plasma glucose (mmol/L)	−53.7 (−81.9; −25.4)	<0.001	−5.1 (−7.9; −2.3)	<0.001
Fasting serum insulin (pmol/L)	−0.4 (−1.1; 0.3)	0.280	−0.1 (−0.1; 0.0)	0.082
Log AIR (pmol/L)	15.7 (−6.5; 37.8)	0.165	1.1 (−1.1; 3.2)	0.326
Total insulin secretion (OGTT)	−0.2 (−1.3; 0.7)	0.570	−0.1 (−0.2; −0.1)	0.017
Log GluSens (pmol/min/m^2 mM)	16.8 (−8.6; 42.2)	0.195	0.7 (−1.8; 3.3)	0.571
Log M/I (μmol/kg FFM/min/nmol/L)	2.7 (−24.9; 30.4)	0.845	1.4 (−1.3; 4.2)	0.306
OGIS-RISC (μmol/min/kg FFM)	12.4 (4.9; 19.9)	0.001	1.0 (0.3; 1.8)	0.007
Log HOMA-IR	−36.3 (−61.3; −11.3)	0.004	−3.6 (−6.1; −1.1)	0.005
Adjusted for age, BMI and centre				
Fasting plasma glucose (mmol/L)	−36.9 (−65.5; −8.3)	0.011	−3.2 (−6.0; −0.4)	0.028
Fasting serum insulin (pmol/L)	0.5 (−0.3; 1.3)	0.198	0.0 (−0.1; 0.1)	0.702
Log AIR (pmol/L)	17.9 (−4.2; 40.1)	0.112	0.9 (−1.2; 3.1)	0.400
Total insulin secretion (OGTT)	0.7 (−0.3; 1.7)	0.180	−0.0 (−0.1; 0.1)	0.524
Log GluSens (pmol/min/m^2 mM)	12.2 (−12.7; 37.1)	0.335	0.2 (−2.3; 2.6)	0.889
Log M/I (μmol/kg FFM/min/nmol/L)	−36.1 (−66.6; −5.6)	0.020	−1.9 (−4.9; 1.2)	0.225
OGIS-RISC (μmol/min/kg FFM)	3.9 (−4.9; 12.6)	0.384	0.1 (−0.8; 1.0)	0.824
Log HOMA-IR	−9.4 (−37.8; 19.1)	0.518	−1.0 (−3.9; 1.8)	0.474
Adjusted for age, BMI, smoking, physical activity and centre				
Fasting plasma glucose (mmol/L)	−49.1 (−86.3; −11.9)	0.010	−5.8 (−9.7; −1.9)	0.004
Fasting serum insulin (pmol/L)	−0.1 (−1.2; 1.0)	0.801	−0.1 (−2.9; 2.8)	0.955
Log AIR (pmol/L)	11.6 (−16.1; 39.3)	0.410	−0.1 (−0.2; 0.1)	0.327
Total insulin secretion (OGTT)	0.2 (−0.2; 1.6)	0.817	−0.1 (−0.2; 0.1)	0.220
Log GluSens (pmol/min/m^2 mM)	5.7 (−26.5; 37.9)	0.729	−0.7 (−4.1; 2.7)	0.674
Log M/I (μmol/kg FFM/min/nmol/L)	−27.5 (−65.7; 10.7)	0.158	−1.7 (−5.8; 2.3)	0.392
OGIS-RISC (μmol/min/kg FFM)	5.2 (−5.6; 16.1)	0.345	0.4 (−0.8; 1.5)	0.506
Log HOMA-IR	−31.2 (−69.0; 6.5)	0.104	−4.1 (−8.1; −0.1)	0.043

secretion, but neither of the bone turnover markers investigated were associated with insulin secretion capacity assessed using the IVGTT and the OGTT. Equally, we did not observe any relation between bone turnover markers and insulin sensitivity assessed using OGTT and the gold-standard method, i.e. hyperinsulinaemic euglycaemic clamp. Therefore, these results demonstrate that bone turnover assessed using bone turnover markers and peripheral insulin sensitivity and insulin secretion are unrelated in healthy, non-diabetic men.

Our observations are not in complete alignment with previous investigations. An inverse association between bone formation assessed using P1NP and insulin sensitivity determined using an IVGTT and a direct association between P1NP and insulin resistance based on HOMA-IR was observed in 63 men and women with NGT or IFG [31]. While we detected an inverse relation between P1NP and insulin sensitivity based on hyperinsulinaemic euglycaemic clamp in individuals with IGR, the association was only present after adjustments. These differences in observations may be explained by skeletal muscle or hepatic insulin resistance that leads to fasting hyperinsulinaemia, which promotes bone formation, or insulin resistance in bone, that impairs bone turnover. Furthermore, an inverse association between insulin resistance determined using HOMA-IR and bone resorption (CTX) but not formation (P1NP) was detected in 2955 elderly men, but neither of these bone turnover markers was associated with insulin resistance after adjustment for age, BMI and comorbidities [17]. Further, P1NP was directly associated with insulin resistance

Table 4
Change in per cent from baseline of measures of glucose homeostasis and regression coefficients (95% confidence intervals) between baseline bone turnover marker levels and delta values of measures of glucose homeostasis. The EGIR-RISC Study.

	Δ Fasting plasma glucose (mmol/L)	Δ Fasting serum insulin (pmol/L)	Δ Total insulin secretion (OGTT)	Δ GluSens (pmol/min/m^2 mM)	Δ OGIS (μmol/min/kg FFM)	Δ HOMA-IR
	n = 468	n = 439	n = 415	n = 415	n = 398	n = 437
% change from baseline	+2.9%	+8.4%	+6.8%	+1.5%	−3.0%	+11.2%
Bone turnover markers						
CTX (μg/L)[#]	0.002 (−0.001; 0.001)	0.001 (−0.011; 0.011)	−0.003 (−0.009; 0.004)	−0.012 (−0.051; 0.027)	−0.001 (−0.001; 0.001)	0.001 (−0.001; 0.001)
P1NP (μg/L)[#]	−0.001 (−0.004; 0.002)	−0.022 (−0.126; 0.823)	−0.012 (−0.076; 0.053)	0.122 (−0.267; 0.511)	0.005 (−0.004; 0.014)	−0.001 (−0.006; 0.003)
Adjusted for centre, age, BMI, smoker and physical activity						
CTX (μg/L)[##]	0.001 (0.000; 0.001)[*]	−0.003 (−0.015; 0.009)	−0.001 (−0.008; 0.005)	−0.001 (−0.050; 0.031)	−0.001 (−0.001; 0.001)	−0.001 (−0.001; 0.001)
P1NP (μg/L)[##]	0.001 (−0.003; 0.003)[**]	−0.038 (−0.150; 0.075)	−0.004 (−0.072; 0.064)	0.197 (−0.199; 0.593)	0.003 (−0.006; 0.013)	−0.001 (−0.006; 0.003)

[#] Adjusted for recruitment centre.
[##] Adjusted for centre, age, BMI, smoker, IPAQMETS.
[*] $p < 0.05$.
[**] $p < 0.01$.

assessed on the basis of HOMA-IR in 1010 Swedish men aged 70–81 years prior to but not after adjustment for osteocalcin levels, suggesting that osteocalcin but not P1NP is independently associated with glucose homeostasis [32]. In addition, urinary NTX, a bone resorption marker, was not associated with fasting plasma glucose, whereas the level of osteocalcin was inversely associated with fasting plasma glucose in 380 elderly men [13]. Jointly, these previous investigations do not consistently support an association between BMTs such as P1NP and CTX and glucose homeostasis. In light of the results of the present investigation, which relies on measurements of insulin sensitivity assessed on the basis of both OGTT and hyperinsulinaemic euglycaemic clamp, evidence of an independent relationship between bone turnover markers and insulin sensitivity or indeed secretion is limited.

By contrast, levels of osteocalcin appear to represent more than just bone formation. Accordingly, undercarboxylated osteocalcin and total osteocalcin are inversely associated with fasting plasma glucose and insulin resistance and directly associated with insulin sensitivity in humans investigated using OGTT and hyperinsulinaemic euglycaemic clamp [13,15,32,33,35]. These results emphasize that undercarboxylated osteocalcin and osteocalcin may reflect functions beyond that of bone remodeling. Furthermore, osteocalcin and undercarboxylated osteocalcin may predict changes in glucose levels or development of T2D according to some [13,16,17,36] but not all prospective investigations [20–22]. Osteocalcin was not measured in the present investigation as osteocalcin is sensitivity to degradation, prompting blood samples for osteocalcin measurements to be chilled at 4 degrees Celsius immediately after being collected, which was not part of the protocol in our investigation [37]. Furthermore, assays that measure bioactive osteocalcin, i.e. osteocalcin decarboxylated specifically at residue Glu17, are unavailable in humans [38]. The potential integration of bone and glucose homeostasis in humans needs to be assessed using other biochemical markers that may not portray the physiological effects of alterations in levels of bioactive osteocalcin during bone remodeling. Rather than assessing osteocalcin, we investigated the ability of commonly used bone turnover markers to predict alterations in glucose homeostasis during a short follow-up period. In keeping with the cross-sectional data, bone turnover markers were not associated with changes in insulin sensitivity or insulin secretion after 3-years follow-up; suggesting that bone turnover per se and glucose homeostasis are unrelated in healthy men.

The majority of previous clinical investigations have reported lower levels of bone turnover markers in individuals with type 1 or 2 diabetes than in controls [26]. Therefore, in order to investigate the relationship between bone turnover markers and glucose, insulin sensitivity and secretion independently of overt hyperglycaemia, patients with any type of diabetes were excluded from the present study. Although most of the study participants were glucose tolerant, a considerable number of participants had an abnormal glucose regulation. However, bone turnover markers were similar in those classified as either NGT or IGR suggesting that they may remain normal at least until development of overt hyperglycaemia.

Investigation of the impact of long-term fasting hyperinsulinaemia without concurrent hyperglycaemia in human poses obvious difficulties. The acute effect of insulin on bone turnover has been investigated in a limited number of clinical studies by using hyperinsulinaemic euglycaemic clamps that involved different rates and durations of insulin infusion, i.e. 40 mU/m^2 per min, 80 mU/m^2 per min or 0.5 mU/kg total body weight per min. Exposure to these levels of insulin had no apparent imminent effect on bone turnover assessed using bone turnover markers [39–41]. By contrast, a reduced bone resorption has been reported in hyperinsulinaemic euglycaemic clamps that were either of extended duration (40 mU/m^2 per min; 4 h), and hyperinsulinaemic hypoglycaemic clamps (80 mU/m^2 per min; BG reduced to 2.5 nM) [39,41]. Importantly, these investigations included relatively small study populations that differed with regard to age, body composition and presence of T2D. Notably, the design of the clamp applied in the study by Basu et al. [40] that included 7 individuals with T2D and 7 controls relied on a different study design as secretion of pancreatic hormones during the clamp was prevented by infusion of somatostatin, growth hormone and glucagon, and insulin was infused at three different rates in order to imitate the physiological range of insulin. While the investigators detected an association between levels of CTX and insulin sensitivity, after adjustment for BMI, the relationship was no longer significant, indicating that bone turnover and insulin sensitivity assessed during the clamp with control for potentially confounding secretions of pancreatic hormones known to influence glucose homeostasis and bone metabolism are distinct. However, while bone turnover markers seemingly remain fairly unchanged by insulin at physiological levels during a euglycaemic clamp, Clowes et al. [41] observed a substantial reduction in bone turnover markers during a hypoglycaemic clamp performed using similar levels of insulin, possibly explained according to the investigators by inhibitory effects of hypoglycaemia on bone cells, hormones counteracting hyperglycaemia or reductions in PTH levels observed during the hypoglycaemia.

We observed decreases in the level of CTX and minor reductions in P1NP levels during a 2-h hyperinsulinaemic euglycaemic clamp, which by design was similar to some of the studies that reported steady levels of bone turnover markers during insulin exposure. This disagreement may be explained by several factors including differences in the characteristics of the study population as the present investigation included substantially more individuals and was restricted to non-diabetic men, which may have reduced the variation, allowing us to identify alterations in bone turnover markers levels during insulin exposure. The overall effect of insulin on bone cell activity remains uncertain [42]. The decrease in P1NP and CTX during the clamp could be explained by several factors, including reduced levels of hormones known to promote bone activity such as IGF-1 or IGF-1 binding proteins, alterations in clearance of the bone turnover markers or inhibitory effects on osteoclasts activity, which leads to decreased osteoblast activity. CTX did not decrease in participants with IGR, but similar numerical changes in CTX levels were observed before and after insulin-stimulation, indicating that the discrepancy in CTX levels in the response to insulin stimulation between NGT and IGR could be explained by lower power. Importantly, hyperinsulinaemic euglycaemic clamps relies on physiological insulin levels that are sustained beyond that observed in normal physiology; therefore, these changes in bone turnover markers may not represent the effects of physiological levels of insulin on bone turnover. Furthermore, the observed reductions in bone turnover markers are markedly smaller than those observed during consumption of food [43], suggesting that the overall acute effect of intravenously administered insulin on bone turnover is minor.

Several factors need consideration when the results of the present investigation are to be interpreted. We cannot exclude the possibility that the relationships between bone turnover markers and insulin secretion and sensitivity differ in individuals with manifest diabetes or in women. Furthermore, although adjustment for 25-OHD levels or restriction of the investigations to those that were vitamin D replete appeared not to change the results, it could be argued that the relationship between bone turnover markers and glucose homeostasis may vary between those with low and normal levels of vitamin D. Because BMD was not measured, we were unable to assess if the relationship between bone turnover markers and glucose homeostasis differed between individuals with high or low bone mass. Finally, although the size of the study population provided sufficient power to investigate the main aims, i.e. the relationships between bone turnover markers and insulin sensitivity and secretion, several comparisons without simultaneous adjustment for multiple testing were performed, which invariably increases the risk of spurious findings.

6. Conclusions

The present investigation showed that levels of commonly used biochemical markers of bone formation and resorption are not associated

with insulin sensitivity and secretion assessed by use of different tests including hyperinsulinaemic euglycaemic clamp, IVGTT and OGTT in a very large study population of carefully characterized, non-diabetic men. Additionally, we did not observe any relationship between baseline bone turnover markers and 3-year changes in insulin sensitivity and secretion. Jointly, these results do not support the notion that bone turnover and glucose homeostasis are integrated in adult men. When possible, it needs to be investigated if levels of bioactive osteocalcin are associated with glucose homeostasis in adult non-diabetic individuals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2017.12.029.

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