The Association between Osteocalcin, Adiposity and Bone health: The Rotterdam Study

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ABSTRACT

Osteocalcin, an indicator of osteoblast differentiation, is an emerging bone turnover marker. Recently, there has been a growing interest in the potential extra-skeletal effects of osteocalcin such as regulation of glucose and energy metabolism; important predictors of bone mineral density (BMD). Therefore, we aimed to investigate the association between osteocalcin, adiposity and BMD. We included 6,619 participants (55.8% female) from the Rotterdam Study a prospective population-based cohort with mean age of 62.6±9.1. All participants underwent Dual Energy X-Ray Absorptiometry (DXA) to measure femoral neck (FN) BMD. Incident fractures were identified and collected from medical registry data. Different anthropometric and DXA-derived parameters were used as proxies for adiposity. Serum osteocalcin was measured by electrochemiluminescence immunoassay (ECLIA). Linear regression was performed adjusted for different confounders to test the association between mean osteocalcin levels with BMD and adiposity. Next, we used a time-to-event approach to estimate hazard ratios of sustaining future fracture. Our results showed strong inverse associations between osteocalcin and FN-BMD (β: -0.026; 95%CIs: -0.029 to -0.022) and Body mass index (BMI) (β: -0.919; 95%CIs: -1.093 to -0.745) in both males and females. Osteocalcin was also inversely associated with fat mass, waist circumference, and other measurements of adiposity. Individuals with low BMI and high osteocalcin levels had significantly higher prevalence of osteopenia and osteoporosis (60.2% and 12.6%) compared to individuals with BMI and osteocalcin levels in the middle range (46.4% and 4.3%). In addition, higher levels of osteocalcin were associated with increased risk of wrist fractures (HR=1.30; 95%CIs: 1.13 to 1.49) but not hip fractures (HR: 0.95; 95%CIs: 0.82 to 1.10). In conclusion, the presence of low BMI and high osteocalcin is an indication of seriously declined bone health, corroborated by the high prevalence of osteoporosis and the subsequent risk of wrist fractures.

INTRODUCTION

For a long time osteocalcin was considered to be a bone-specific protein involved in the process of bone mineralization; emerging as a prominent serum marker of osteoblastic bone formation in clinical practice.[1] Although, the majority of the osteocalcin is absorbed by the bone hydroxyapatite, around 10-20% of the total amount is released into the circulation[2] exerting significant effects on fat and global energy metabolism.[3,4]

The inverse relationship between serum osteocalcin levels and bone mineral density (BMD) is well documented in postmenopausal women.[5,6,7,8,9] In addition, it has been suggested that higher levels of osteocalcin are a marker of hip fractures in elderly women.[10,11] In the past few years, there has been growing evidence of the extra-skeletal effects of osteocalcin, demonstrating its inverse associations with fat mass, lipid metabolism, body mass index (BMI) and other measurements of adiposity.[12,13,14,15,16] BMI, a proxy of adiposity, is a relevant risk factor for osteoporosis and fracture risk[17,18] whose mechanisms of action go beyond mechanical loadings.[19,20] The adipose tissue secretes various factors such as adiponectin, leptin and adipokines, which may affect osteoblast differentiation and bone formation.[21,22] High adiposity has been also associated with increased insulin resistance which can suppress bone turnover and subsequently decrease osteocalcin levels.[23] Hence, adiposity may be an important mediator of the relationship between osteocalcin and BMD.

To our knowledge, there are no studies that have specifically investigated the joint effect of osteocalcin and adiposity on bone health. In addition, limited evidence exists on the effect of osteocalcin on BMI, BMD, and fracture risk in men. Therefore, to further understand the complex relationship between osteocalcin, adiposity and bone health, we sought to study 1) the joint effect of osteocalcin and adiposity on bone and 2) whether adiposity is a mediator of the association of osteocalcin with BMD in a large population-based cohort of elderly people.

MATERIALS AND METHODS

Study population

The Rotterdam Study is an ongoing prospective population-based cohort that investigates occurrence, determinants, and consequences of diseases in an ageing population.[24] The first baseline measurements of Rotterdam Study started in 1990. After two expansions in 2000 and 2006, it comprised 14,926 participants aged 45 years and over by the end of 2008. Follow-up visits were held every 3-5 years. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus

MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport. The Rotterdam Study has been entered into the Netherlands National Trial Register (NNTR) and into the WHO International Clinical Trials Registry Platform (ICTRP) under shared catalogue number (NTR6831). All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

The present study included participants from the baseline examination of the third visit of the first cohort (RS-I-3, n=1,682), first visit of the second cohort (RS-II-1, n=1,881), the first visit of the third cohort (RSIII-1, n=3,112) and their consecutive follow-ups (RSI-4, RSII-2 and RSIII-2) (**Figure 1**).

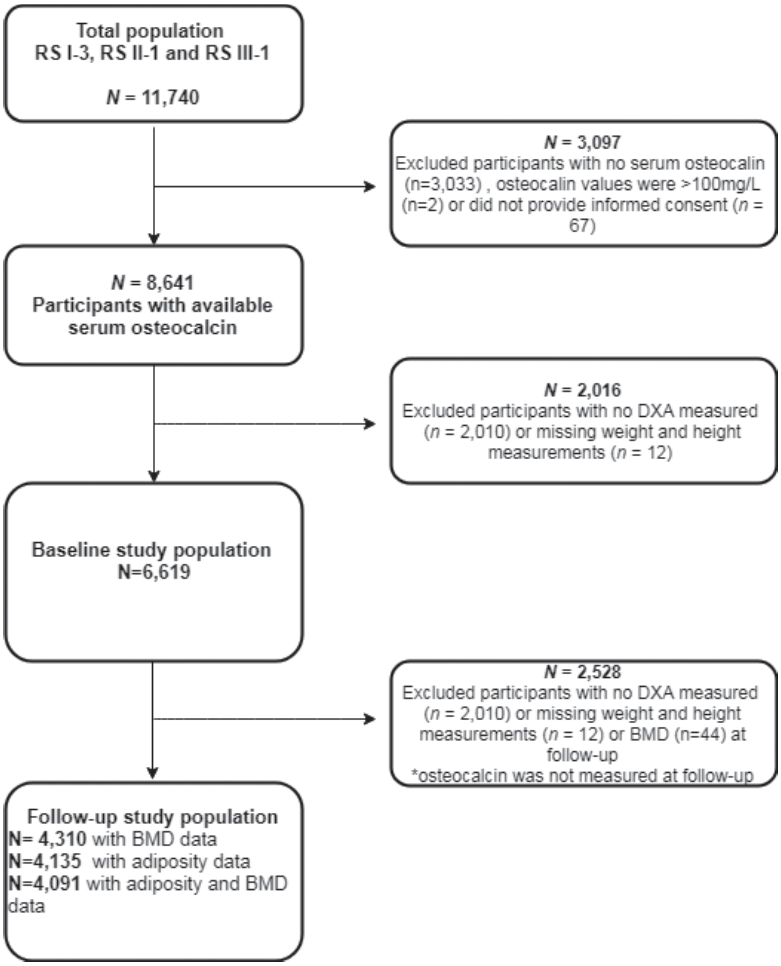

Figure 1 | Flow chart of the study population

Measurement of Total Serum Osteocalcin Levels

Total serum osteocalcin levels were measured at baseline by means of a N-MID osteocalcin electrochemiluminescence immunoassay (ELICA) kit (Roche Diagnostics, Mannheim, Germany). The monoclonal antibodies used in the test react specifically with epitopes on the N-MID-fragments and the N-terminal fragment, and detect the stable N-MID-fragment as well as the intact osteocalcin. The intact osteocalcin (amino acids 1-49) is unstable due to protease cleavage between amino acids 43 and 44. Therefore, the N-MID-fragment (amino acids 1-43) is considered more stable[25] and have been used as measure of total osteocalcin in clinical practice.

Assessment of Bone Mineral Density

Femoral neck BMD (FN-BMD) was measured by a Dual-energy X-ray absorptiometry (DXA) scanner. For RSIII-1 we used a Prodigy scanner (GE-Lunar Corp, Madison, WI, USA), and a DPX-L scanner (GE-Lunar Corp, Madison, WI, USA) was used for RSI-3 and RSII-1. In addition, we measured FN-BMD at the follow-up visits using a Prodigy scanner for RSI-4 and RSII-2 while an iDXA scanner for RSIII-2 was used. *Incidence fractures* were reported either by general practitioners (GPs) in the research area using digitalized medical records or through hospital discharge records. All events were verified by two trained research physicians which coded the events independently. Follow-up time was calculated as the time from baseline to first fracture, death or end of follow-up period (or loss to follow-up), whichever occurred first.[26]

Assessment of Adiposity

We derived several indexes of adiposity including BMI, waist circumference (WC), waist-to-hip ratio (WHR), total body fat mass and total body fat percentage. We also assessed gynoid and android fat mass as indices of fat mass distribution.

Height and body weight were measured at baseline with the participants standing without shoes and heavy outer garments. BMI was calculated as weight divided by height squared (kg/m^2). WC was measured using a tape measure at the level midway between the lower rib margin and the iliac crest with participants in standing position without heavy outer garments and with emptied pockets. Hip circumference was recorded as the maximum circumference over the buttocks. WHR was calculated as the ratio of the circumference of the waist over that of the hips. Body composition was measured by a DXA scanner (described above), in average five years after osteocalcin was measured. Fat mass of the total body and specific regions were analysed using enCORE software; deriving android and gynoid fat mass. Fat mass percentage (%) was calculated (i.e., fat mass as a percentage of total body weight).

Covariates

Information on medication use was derived from baseline questionnaires and included the use of vitamin supplementations, corticosteroids, drugs for bone diseases and antithrombotic drugs. Serum vitamin D and serum calcium were measured in blood samples using standard methods. Glucose was determined by automated enzymatic procedures in a fasting blood sample.[27] Type 2 diabetes (T2D) was defined as fasting plasma glucose >= 7mmol/l. The estimated glomerular filtration rate was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation in mL/min/1.732.[28] Total weekly duration of *physical activity* was assessed by an adapted version of the Zutphen Physical Activity Questionnaire[29] in the first and second cohort, and the LASA Physical Activity Questionnaire[30] in the third cohort. Metabolic equivalent of task (MET) values were assigned to all activities in the questionnaires and total physical activity per week was calculated.[31] Given the different type of questionnaires used, all the MET values were standardised prior analysis.

STATISTICAL ANALYSES

Phenotype Considerations

Prior analyses, we defined individuals with osteocalcin levels above 100 ng/mL as potential outliers and excluded them from the analyses (n=4). Next, given total serum osteocalcin did not followed approximately normal distribution we transformed it using an inverse normal transformation in order to model osteocalcin with a normal distribution.

Main Analyses

In the descriptive table of our study population we reported continuous variables as mean ± SD unless stated otherwise, and categorical variables were presented as percentages. We used linear regression models to examine if total serum osteocalcin levels were associated with BMD and different indices of adiposity measured at baseline and follow-up. We considered three models 1) *basic model* which was adjusted for sex, age and cohort effect; 2) *confounder model* which was additionally adjusted for physical activity, T2D, serum vitamin D, serum calcium, glomerular filtration rate, vitamin supplements, use of corticosteroid drugs, use of antithrombotic drugs and use of drugs for bone diseases and 3) *mediator model* which was adjusted additionally for BMI. Next, we preformed time to event analyses to assess the hazard risk of sustaining hip, wrist, or any non-vertebral fracture, and tested the all underlying assumptions. In order to observe the joint effect of osteocalcin and BMI on BMD we created sex and age specific tertiles of BMI and osteocalcin, resulting in 9 groups, where we compared the

prevalence of osteopenia and osteoporosis across the groups. Differences between the groups were tested using chi-square statistic. Finally, we preformed mediation analysis to test how much of the osteocalcin and BMD association is attributed to BMI using the *mediation* package in R.[32] We followed the same principle to estimate how much of the effect of osteocalcin on wrist fractures is attributed to BMD. All analyses were conducted using R (Version 3.5.1) .[33]

Sensitivity Analyses

We examined whether the association between osteocalcin and BMD or BMI differed by sex and age at the time osteocalcin was measured. Therefore, we evaluated the statistical interaction by adding the product term of osteocalcin with age or sex. If there were significant interactions ($p<0.1$), the analyses were stratified accordingly. Next, for our main analyses we only had DXA-derived estimates of adiposity measured at follow-up. Nevertheless, for a subset of our population (N=1,962) we had measurements at both follow-up and baseline. Thus, we evaluated the association between osteocalcin and DXA-derived estimates of adiposity at baseline and follow-up in this subgroup. In addition, we estimated the correlations between the adiposity measures at baseline and follow-up. Finally, we re-analysed the mediation analysis with the mediator and outcome measured five years after the exposure to avoid possible bias due to reverse causation.

Missing values were less than 10%. To adjust for potential bias associated with missing data among confounders we used multiple imputation procedure (N=10 imputations with 50 iterations). Results after the multiple imputation procedure did not differ from the original data (data not shown).

RESULTS

Characteristics of the Study Population

Our study included 6,619 participants with mean age of 62.6 ±9.1 years from which 44.2% were men. Men were taller, heavier and with higher WC compared to women. On the other hand, women had higher total body fat mass and body fat percentage compared to men. BMD was lower in women that also presented with higher total serum osteocalcin levels compared to men. Finally, during a median follow-up of 7.9 years (inter-quartile range [IQR] 5.0-12.0 years) 14% of the participants experienced at least one fracture from which the majority occurred in women (**Table 1**).

Table 1 | Descriptive statistics of the study population.

Characteristics	Total (n=6,619)	Female (n=3,693)	Male (n=2,926)
Baseline			
Age, years	62.6±9.1	62.8±9.3	62.5±8.9
Height, cm^2	168.9±9.5	163.0± 6.4	176.5±7.1
Weight, kg	78.2±14.5	72.8±13.2	84.9±13.2
BMI, kg/m^2	27.3±4.2	27.4±4.6	27.2±3.6
Waist circumference (cm)	93.6±12.1	89.6±11.9	98.6±10.3
Waist-to-hip ratio	0.89±0.09	0.85±0.08	0.95±0.08
FN-BMD, g/cm^2	0.93±0.15	0.89±0.14	0.97±0.14
Osteocalcin, ng/mL	20.3(2.7-99.2)	22.3(4.9-94.3)	19.6(2.7-99.2)
CKD-EPI, $ml/min/1.73m^2$	81.83 ±15.76	81.51±15.80	82.23±15.71
Type 2 diabetes, n (%)	589 (8.9)	274 (7.4)	315 (10.8)
Serum vitamin D, nmol/L Calcium	58.23±27.77	56.06±27.36	60.97±28.04
Serum calcium, mmol/L	2.43±0.10	2.45 ±0.10	2.42 ±0.10
Physical activity,	0.03±0.97	0.21 ±0.95	-0.19 ±0.94
Vitamins, n (%)	1347 (20.4)	864 (23.4)	483 (16.5)
Corticosteroid drugs, n (%)	69 (1.0)	38 (1.0)	31 (1.1)
Bone drugs, n (%)	95 (1.4)	85 (2.3)	10 (0.3)
Antithrombotic drugs, n (%)	1081 (16.3)	455 (12.3)	626 (21.4)
Incident fractures			
Hip fractures, n (%)	179 (2.7)	123 (3.3)	53 (1.8)
Wrist fractures, n (%)	211 (3.2)	180 (4.8)	31 (1.1)
Any-type of fracture, n (%)	933 (14.0)	644 (14.6)	269 (9.1)
Follow-up			
Age, years	66.6±7.9	66.5±8.0	66.3±7.7
Height, cm^2	169.13±9.45	163.31±6.5	176.89±7.0
Weight, kg	78.77±14.5	73.11 ±12.9	86.31±13.0
BMI, kg/m2	27.46±4.2	27.4±4.5	27.6±3.6
Waist circumference (cm)	93.43±12.3	89.0±11.6	99.3±10.56
Waist-to-hip ratio	0.90±0.09	0.85±0.08	0.97 ±0.07
Total body fat mass. kg	27.76±9.0	29.14±9.2	25.9±8.23
Body fat percentage, %	0.35±0.08	39.0±10.0	29.0±6.0
Gynoid fat mass, kg	4.14±1.4	4.65±1.4	3.46±1.1
Android fat mass, kg	2.63±1.1	2.51±1.1	2.79±1.1
FN-BMD, g/cm^2	0.91±0.1	0.87±0.1	0.96±0.1

CKD-EPI= Estimated glomerular filtration rate. Footnote: At the second round there were in total 4,135 individuals with available anthropometric and body composition data and 4,320 with FN-BMD. Round 2 was done in average five years after round 1.

Osteocalcin Correlations with Age, Weight and BMD

Serum osteocalcin levels correlated positively with age in women but not in men (**Supplementary Figure 1A**). In addition, osteocalcin was negatively correlated with weight and BMD in both men and women (**Supplementary Figure 1B-C**).

Osteocalcin Association with Bone Health

Osteocalcin was inversely associated with FN-BMD (β: -0.026, 95%CI: -0.029 to -0.022) at baseline and at follow-up (β: -0.029, 95%CI: -0.033 to -0.025) (**Table 2**). Furthermore, osteocalcin was associated with increased risk of wrist fractures (HR: 1.34, 95%CI: 1.15 to 1.55 but not with hip or all non-vertebral fractures (**Table 3**). There were negligible differences between the basic and the confounder model , suggesting that the reported associations were not driven by the confounders. The results are based on the sex-combined analyses.

Table 2 | Association of osteocalcin with FN-BMD as dependent variable measured at baseline and follow-up overall and stratified by cohort.

Baseline	**Overall (n=6,619)**	**RSI-3 (n=1,682)**	**RSII-1 (n=1,881)**	**RSIII-1 (n=3,112)**
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Basic model	-0.033 (-0.036, -0.030)	-0.028 (-0.035, -0.022)	-0.031 (-0.038, -0.025)	-0.035 (-0.040, -0.031)
Confounder model	-0.036 (-0.039, -0.033)	-0.030 (-0.037, -0.024)	-0.033 (-0.040, -0.027)	-0.040 (-0.045, -0.036)
Mediator model	-0.029 (-0.032, -0.026)	-0.024 (-0.031, -0.018)	-0.025 (-0.031, -0.018)	-0.033 (-0.038, -0.029)
Follow-up	**Overall (n=4,310)**	**RSI-3 (n=1,162)**	**RSII-1 (n=773)**	**RSIII-1 (n=2,385)**
	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Basic model	-0.032 (-0.036, -0.028)	-0.028 (-0.035, -0.021)	-0.030 (-0.039, -0.021)	-0.035 (-0.040, -0.029)
Confounder model	-0.035 (-0.039, -0.031)	-0.031 (-0.038, -0.023)	-0.032 (-0.041, -0.022)	-0.038 (-0.044, -0.033)
Mediator model	-0.029 (-0.033, -0.025)	-0.025 (-0.032, -0.017)	-0.023 (-0.033, -0.014)	-0.031 (-0.037, -0.026)

The basic model was adjusted for age, sex, and cohort effect (for the overall analysis); **The confounder model** was additionally adjusted for physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases. **The mediator model** was additionally adjusted for BMI. β=effect estimate.

Table 3 | Association of osteocalcin with incident non-vertebral, hip or wrist fracture as outcome.

	Any-type of fracture (935 cases/ 5,740 controls)	Hip fracture (179 cases / 6,496 controls)	Wrist fracture (212 cases / 6,462 controls)
	HR (95%CI)	HR (95%CI)	HR (95%CI)
Basic model	1.01 (0.94-1.07)	0.96 (0.83-1.11)	1.30 (1.13-1.50)
Confounder model	1.04 (0.97-1.12)	0.98 (0.84-1.14)	1.34 (1.15-1.55)

The basic model was adjusted for age, sex and cohort effect. **The confounder model** was additionally adjusted for physical activity., BMI, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, and use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases.

Osteocalcin Association with Adiposity

Inverse associations were also observed between osteocalcin and both BMI (β: -0.919, 95%CI: -1.093 to -0.745) and WC (β: -2.323, 95%CI: -2.778 to -1.867) at baseline, and with follow-up measurement of BMI (β: -0.815, 95%CI: -0.997 to -0.634) and WC (β: -2.213, 95%CI: -2.676 to -1.750) (**Table 4**). Further, osteocalcin was inversely associated with total body fat mass (β: -1.1345, 95%CI: -1.715 to -0.975), body fat

Table 4 | Association of osteocalcin with different measurements of adiposity as dependent variable. Using anthropometric measurements of adiposity at the time osteocalcin was measured (1st round) and anthropometric measurements and DXA-derived parameters of adiposity five years after osteocalcin was measured (2nd round; n=4,135).

Adiposity Outcomes:	**Women (n=2,364)**		**Men (n=1,771)**	
Baseline	β	95%CI	β	95%CI
BMI (kg/m2)	-1.016	(-1.193, -0.838)	-0.735	(-0.922, -0.549)
Waist circumference (cm)	-2.493	(-2.959, -2.027)	-1.757	(-2.297, -1.217)
Waist-to-hip ratio	-0.013	(-0.016, -0.010)	-0.013	(-0.015, -0.008)
Follow-up				
BMI (kg/m2)	-0.915	(-1.099, -0.728)	-0.765	(-0.964, -0.566)
Waist circumference (cm)	-2.402	(-2.877, -1.927)	-1.943	(-2.531, -1.355)
Waist-to-hip ratio	-0.014	(-0.017, -0.011)	-0.013	(-0.017, -0.010)
Total body fat mass, kg	-1.499	(-1.877, -1.120)	-1.441	(-1.895, -0.993)
Body fat percentage, %	-0.010	(-0.013, -0.007)	-0.011	(-0.014, -0.008)
Gynoid fat mass, kg	-0.163	(-0.223, -0103)	-0.142	(-0.203, -0.081)
Android fat mass, kg	-0.196	(-0.239, -0.152)	-0.207	(-0.266, -0.147)

The models are adjusted for age, sex, cohort effect physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases. All analyses were performed in the individuals with available data at both baseline and follow-up (n=4,135). β=effect estimate. **Footnote:** for comparison purposes the analyses at baseline and follow-up were performed in the same group of individuals. Osteocalcin was measured only at baseline.

percentage (β: -0.009, 95%CI: -0.012 to -0.007), gynoid (β: -0.140, 95%CI: -0.119 to -0.081) and android (β: -0.179, 95%CI: -0.221 to -0.137) fat mass measured at follow-up (**Table 4**). The associations attenuated after adjustment for T2D but still remained significant. The results are based on the sex-combined analyses.

Joint Effect of Osteocalcin and BMI on Bone Health

Participants with low BMI and high osteocalcin levels had significantly higher prevalence of osteopenia and osteoporosis (60.2% and 12.4%) compared to individuals with BMI and osteocalcin levels (OC) in the middle range (46.5% and 4.1%). The prevalence of osteoporosis in the highest tertile of osteocalcin levels decreased significantly with the increase of BMI (12.4% [high OC-low BMI], 5.9% [high OC-middle BMI] and 3.1% [high OC-high BMI]). In the group of participants with low OC-high BMI the prevalence of osteoporosis was the lowest (1.2%) (**Figure 2**).

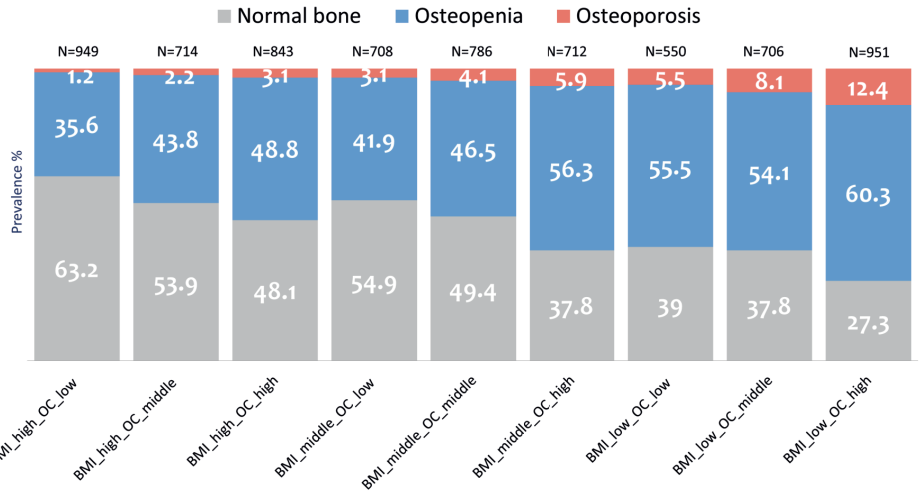

Figure 2 | Shows the prevalence of osteopenia (blue) and osteoporosis (red) according to BMI and OC sex and age adjusted tertiles

Mediation Analysis

The mediation analyses showed that significant proportion of the associations between osteocalcin and BMD was mediated by BMI. All three conditions for the mediation analysis were fulfilled. First, as we presented above, osteocalcin was significantly associated with FN-BMD and wrist fracture (Condition 1 fulfilled). Second, osteocalcin was associated with BMI (Condition 2 fulfilled). Third, BMI was associated with FN-BMD (Condition 3 fulfilled) (**Supplementary Table 1**). We showed that the total effect of osteocalcin on FN-BMD is -0.033 (95%CI: -0.036 to -0.030) while the direct effect

controlling for BMI is -0.026 (95%CI: -0.029 to -0.0022); meaning that around 23% of the association between osteocalcin and FN-BMD was mediated through BMI (**Figure 3a**). Next, we tested also how much of the association between osteocalcin and wrist fracture was medicated by BMD. We observed that only 25% of the effect of osteocalcin on wrist fractures is medicated by BMD. (**Figure 3b**).

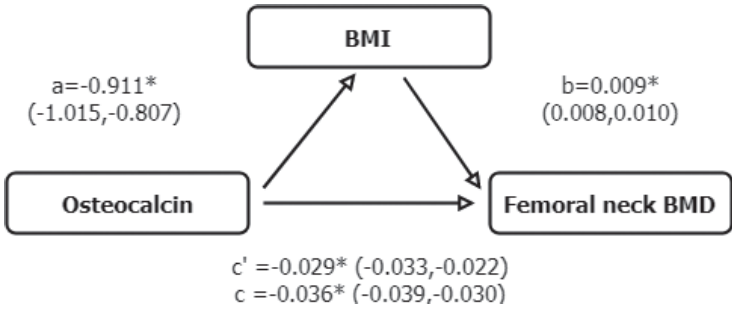

Figure 3a | Mediation analysis on the relationship between osteocalcin and femoral neck BMD. The total effect of osteocalcin on femoral neck BMD is -0.036 (path c). This effect can be mediated by BMI. The effect of osteocalcin on BMI is -0.11 (path a); the effect of BMI on femoral neck BMD, controlling for osteocalcin is 0.009 (path b); The direct effect of osteocalcin on femoral neck BMD, controlling for BMI, is -0.029 (path c'); The mediating effect of osteocalcin on femoral neck BMD through BMI is -0.007 (23%) (path a*b). Total sample size for 6,619 (baseline). * indicates significant association.

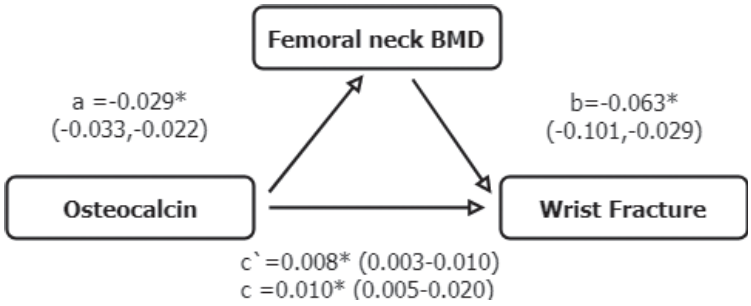

Figure 3b | Mediation analysis on the relationship between osteocalcin and wrist fracture. The total effect of osteocalcin on Wrist fractures is 0.010 (path c). This effect can be mediated by femoral neck BMD. The effect of osteocalcin on femoral neck BMD is -0.029 (path a); the effect of femoral neck BMD on wrist fracture, controlling for osteocalcin is -0.063 (path b); The direct effect of osteocalcin on Wrist fracture, controlling for femoral neck BMD, is -0.008 (path c'); The mediating effect of osteocalcin on Wrist fracture through femoral neck BMD is 0.002 (20%) (path a*b). Total sample size for 6,619 (baseline). * indicates significant association

Sensitivity Analyses

There was significant interaction (P<0.05) between osteocalcin levels and sex for the BMD and BMI analyses. Although, the association with BMD was stronger in women

compared to men (-0.030 [95%CI: -0.034, -0.026] and -0.025 [95%CI: -0.031, -0.020]) the estimates were comparable (**Supplementary Table 2**). On the other hand, the sex differences were more pronounced for the adiposity outcomes, thus, the main results were presented stratified by sex (**Figure 4**). We also observed significant interaction between osteocalcin levels and age ($p_{interaction}$= <0.01) for both BMI and BMD. After stratifying our analyses for age, we observed similar associations between osteocalcin and BMD for all age categories, however, the effect estimates were stronger among the eldest individuals (**Supplementary Table 3**). On the other hand, in elderly people, the effect of osteocalcin on BMI was less strong compared to the younger individuals (**Supplementary Table 4**). Next, in the sub-sample (n=1,962) where body composition was measured twice (baseline and follow-up visit) the association between osteocalcin and the different measures of adiposity was significant at both visits with a slight attenuation overtime for the majority of the measures (**Supplementary Table 5**). There was high correlation between the adiposity measures at both visits (**Supplementary Figure 2**). Finally, as a sensitivity step, we reanalysed the mediation analyses with the mediator and outcome measured five years after the exposure and we observed similar findings to the reported in the main analyses (**Supplementary Table 6**). Moreover, all measures of adiposity also showed significant mediating proportion which was less strong than BMI (**Supplementary Table 6**).

DISCUSSION

In this large population-based study (N=6,619), we found strong inverse associations between osteocalcin, BMD, and several distinct measures of adiposity such as BMI and fat mass. While higher serum osteocalcin levels were associated with increased risk of wrist fracture, there was no association of osteocalcin with hip or all non-vertebral fractures. We also showed that the effect of osteocalcin on BMD was partly explained by BMI (23%). Finally, individuals with high serum osteocalcin levels and low BMI may have significant deterioration of bone mass corroborated by the high prevalence of osteopenia or osteoporosis in this group.

To our knowledge, this is the first study to evaluate the joint effects of osteocalcin and adiposity on bone health. First, we found osteocalcin and BMI to be independently associated with BMD what is in line with previous reports.[8,9,13,16] Next, we observed that the prevalence of osteopenia and osteoporosis was highest in the group of people with low BMI and high serum osteocalcin levels and lowest in the opposite case (i.e., high BMI and low osteocalcin). The BMI and osteocalcin tertiles were adjusted for age and sex, thus, the differences in prevalence were not influenced by these variables. Weight dependent-loading of the bone is pivotal for the accrual

and maintaining of bone mass and strength throughout the life.[34] Therefore, decrease in weight (i.e. low BMI) leads to a reduced mechanical stress on the bone which may have detrimental effects on BMD. This is supported by our findings of high prevalence of osteoporosis in the lowest BMI group. Interestingly, the prevalence of osteoporosis in the low BMI group increased with the increase of serum osteocalcin levels. This, can simply be result of the increased bone-turnover. However, it has been postulated that osteocalcin may also act directly on the bone by inhibiting bone mineralization or indirectly by regulating global energy metabolism. Whether osteocalcin has a direct effect on bone mineral density and leads to osteoporosis or the high levels in osteoporotic individuals are only result of increased bone-turnover remains unclear. Nevertheless, the presence of low BMI and high osteocalcin levels may be an indication of deteriorated bone health.

Fractures are the most clinically relevant endpoint of osteoporosis. Therefore, it is of interest to identify other potential predictors of fracture risk beside BMD. Previously, osteocalcin has been shown to be a good predictor of hip fractures in elderly women.[11,35] This is in contrast with our findings, where we observed no effect of osteocalcin on hip fractures in both men and women. Several factors can explain these discrepancies. First, the study by Szulc et al,[35] was performed in elderly institutionalized women with relative small sample size, thus the results cannot be directly compared. Second, our population was on average 10 years younger in comparison with the other studies. As hip fractures are most common in the elderly (75+), in our population they are yet to occur. Third, there was a big difference in the follow-up time with previous studies having an average follow-up of 2-3 years while ours was longer (8.9 years) with a maximum of 15 years of follow-up. This raises the possibility that osteocalcin is only a good predictor of short-term fractures but not long-term ones. Finally, Vergnaud et al[11] found that only undercarboxylated osteocalcin but not total osteocalcin is associated with hip fractures, which is in line with our findings using total osteocalcin. Noteworthy, we did observe an association between total serum osteocalcin levels and wrist fractures. To the extent of our knowledge, this is the first study to evaluate the association between osteocalcin and wrist fractures. We observed a 20% increase in wrist fracture risk per unit increase in total serum osteocalcin in both men and women. Using mediation analysis, we showed that only 20% of the effect of osteocalcin on wrist fracture can be attributed to BMD while the remaining 80% can be results of a direct effect of osteocalcin or other pathways, not included in the analysis, on wrist fracture. The underlying mechanisms for this relationship are still unknown, but may be through both impaired bone formation[36] and bone resorption.[37] Recently, it has been postulated that osteocalcin may also enhance bone toughness by acting as bridge between the matrix and mineral fractions.[38] Thus, the increased bone-turnover coupled with impaired γ-carboxylation of osteocalcin in

elderly people can lead to abnormal bone mineralization which may affect the material properties of the bone and increase the risk of fracture. As the wrists are less mechanically loaded compared to the hip, they are more fragile and likely to fracture earlier in the life course, as shown in our previous effort.[26] However, these findings need to be validated by other large-scale studies, followed by studies to elucidate the underlying causal pathways.

As aforementioned, numerous observational studies have shown the negative association between osteocalcin levels and BMD.[8,9,39,40] Notably, most of the past research have been done in post-menopausal women whereas data on men have traditionally been scarce. Here, we confirmed the findings in women and increase the evidence in men. Moreover, we observed an increase in the effect size of 10-20% in elderly people which is in line with current evidence.[41] The age dependent effect of osteocalcin on BMD can be result of the increased bone remodelling in the eldest population leading to increase in total serum osteocalcin levels[42]. Also, it has been shown that the vitamin K-dependent carboxylation of osteocalcin to reduce with age; indicating higher levels of undercarboxylated osteocalcin in the circulation, what may influence the bone metabolism in return.

Besides being associated with BMD, osteocalcin was also negatively associated with BMI, WC and different measures of fat mass; in line with other observational studies.[13,16,43,44] The increase in fat mass was initially observed in osteocalcin deficient mice, which highlighted the endocrine role of the bone, in particular, its extra-skeletal effects.[45] Large body of evidence have implicated osteocalcin in glucose, fat and overall energy metabolism.[46,47,48] It has been shown that infusion of osteocalcin in wild mice reduced obesity,[49] what has been corroborated in human studies showing that increases in undercarboxylated osteocalcin are associated with decreases in body weight and fat.[50] On the other hand, the excess of fat mass exhibit dual and opposite effects on bone metabolism which will depend on the type of fat mass accumulation and the circulating factors; this can affect osteocalcin levels in both directions. Recently, it has been also shown that the adipose tissue may produce osteocalcin on its own.[51] This just adds up to the molecular complexity of the bone-fat interaction. Unfortunately, we were not able to test the potential bidirectional effects between osteocalcin and fat mass in our population as we did not have follow-up data on osteocalcin. Finally, we showed that around 23% of the effect of osteocalcin on BMD is mediated by BMI.

A strength of this study is the large population-based settings involving 6,619 individuals, enhancing the extrapolation of the findings to other older adults. While the vast majority of previous research have been focused largely on post-menopausal women our study included both men and women. Thus, significantly contributing to the literature regarding the effects of osteocalcin on BMD and fracture risk in men.

Moreover, this is the first study to investigate the association of osteocalcin with wrist fractures and until now the study with the longest follow-up of up to 15 years (7.9 years on average) to evaluate hip, wrist and all non-vertebral fracture risk. However, several limitations of our study need to be noted. First of all, the study was performed using a cross-sectional design with only one measurement of total serum osteocalcin. Therefore, we were not able to evaluate whether changes in osteocalcin levels have an impact on bone and fat mass changes, neither we were able to test for possible bidirectional effects and discard the possibility of reverse association. Although we had two measurements of BMD, we could not properly evaluate the effect of osteocalcin on BMD change, as a result of the software incompatibility between the first and follow-up DXA measurement. Next, the DXA derived body composition parameters were assessed five years after the measurement of osteocalcin. Total osteocalcin levels and body composition may have changed over this time period. However, there was no major variation of BMI and/or body composition among our population during this time period. We observed a strong correlation ($r>0.90$) between the anthropometric measurements of adiposity (BMI and WC) at the time osteocalcin was measured and five years after. Similar correlations were observed for the body composition parameters in a subset of individuals (N=1,962) with two available DXA measurements. In the same group, we also observed that the effect of osteocalcin on fat mass measurements was stable across visits. Therefore, if body composition was measured at the same time osteocalcin was measured, we would have observed similar findings as with the follow-up assessment. Although we made attempt to adjust for all possible confounders, we did not have available data on vitamin K supplementation or dietary vitamin K intake; which can significantly influence the levels of serum undercarboxylated osteocalcin levels. We were only able to account for overall vitamin supplementation. Next, we only measured total serum osteocalcin, while undercarboxylated osteocalcin or carboxylated osteocalcin were not measured. Animal studies have shown that undercarboxylated osteocalcin is the active form of osteocalcin;[45,49] however, these findings should be validated in humans as studies have provided conflicting results.[52] Finally, our findings can't be generalized to younger populations and or different ethnicities.

In conclusion, high osteocalcin levels and low BMI appear to play an important role in the decrease of bone mineral density, consequently leading to developing of osteoporosis and increased fracture.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1 | Association of BMI with FN-BMD as dependent variable stratified by cohort.

	Overall (n=6,619)	RSI-3 (n=1,682)	RSII-1 (n=1,881)	RSIII-1 (n=3,112)
β	0.008	0.009	0.010	0.007
95%CI	(0.007,0.009)	(0.007,0.011)	(0.009,0.012)	(0.006,0.008)

The model is adjusted for age, sex, cohort effect, osteocalcin, physical activity and T2D

Supplementary Table 2 | Association of osteocalcin with FN-BMD as dependent variable stratified by sex.

	Women	Men
β	-0.030	-0.025
95%CI	(-0.034, -0.026)	(-0.031, -0.020)

The model is adjusted for age, sex, cohort effect, physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases.

Supplementary Table 3 | Association of osteocalcin with FN-BMD as dependent variable stratified by age.

Age	<55 (n=1,240)	55-59 (n=1,667)	60-64 (n=1,525)	65-69 (n=767)	70-74 (n=624)	75-79 (n=476)	80-85 (n=226)	>85 (n=94)
β	-0.030	-0.034	-0.033	-0.040	-0.042	-0.036	-0.047	-0.054
95% CI	(-0.037, -0.022)	(-0.041, -0.028)	(-0.040, -0.025)	(-0.050, -0.031)	(-0.052, -0.032)	(-0.048, -0.023)	(-0.064, 0.031)	(-0.082, -0.025)

The model is adjusted for age, sex, cohort effect, physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases.

Supplementary Table 4 | Association of osteocalcin with BMI as dependent variable stratified by age.

Age	<55 (n=1,240)	55-59 (n=1,667)	60-64 (n=1,525)	65-69 (n=767)	70-74 (n=624)	75-79 (n=476)	80-85 (n=226)	>85 (n=94)
β	-0.830	-0.913	-1.077	-0.806	-0.869	-0.787	-0.109	0.411
95% CI	(-1.107, -0.554)	(-1.126, -0.699)	(-1.288, -0.866)	(-1.090, -0.522)	(-1.163, -0.574)	(-1.105, -0.470)	(-0.603, 0.385)	(-0.436, 1.257)

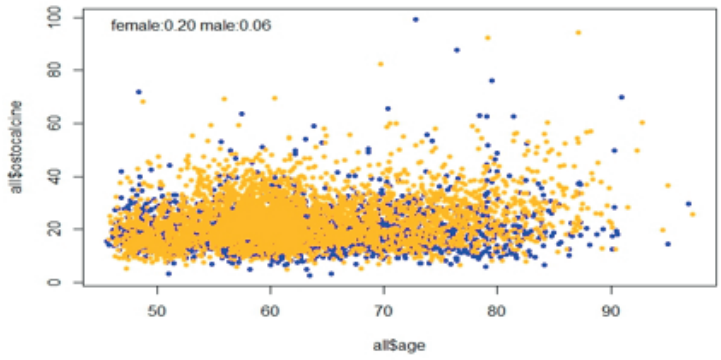
The model is adjusted for age, sex, cohort effect, physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases.

Supplementary Table 5 | Association of osteocalcin with different measurements of adiposity as dependent variables in a subset of the population with measurements available at the two rounds (n=2,038).

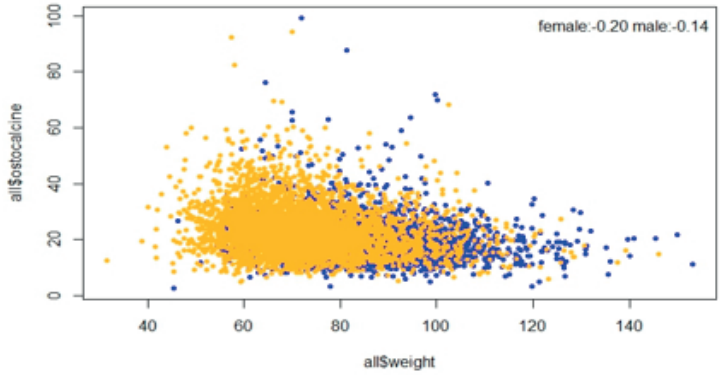
Adiposity Outcomes:	**Baseline**		**Follow-up**	
	β	95%CI	β	95%CI
Total body fat mass, kg	-1.537	(-1.949, -1.124)	-1.368	(-1.773, -0.962)
Body fat percentage,%	-0.977	(-0.014, -0.007)	-0.008	(-0.011, -0.005)
Gynoid fat mass, kg	-0.151	(-0.215, -0.087)	-0.136	(-0.197, -0.075)
Android fat mass, kg	-0.205	(-0.251, -0.159)	-0.207	(-0.258, -0.156)

The model is adjusted for age, sex, cohort effect, physical activity, T2D, serum calcium, serum vitamin D, glomerular filtration rate, vitamin supplements, use of corticosteroids drugs, use of antithrombotic drugs and use of drugs for bone diseases

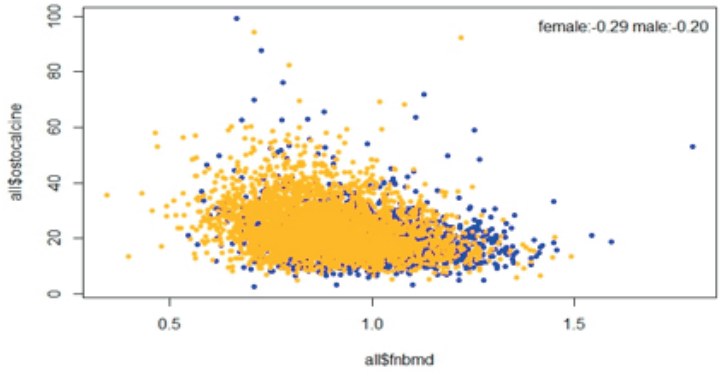
A)



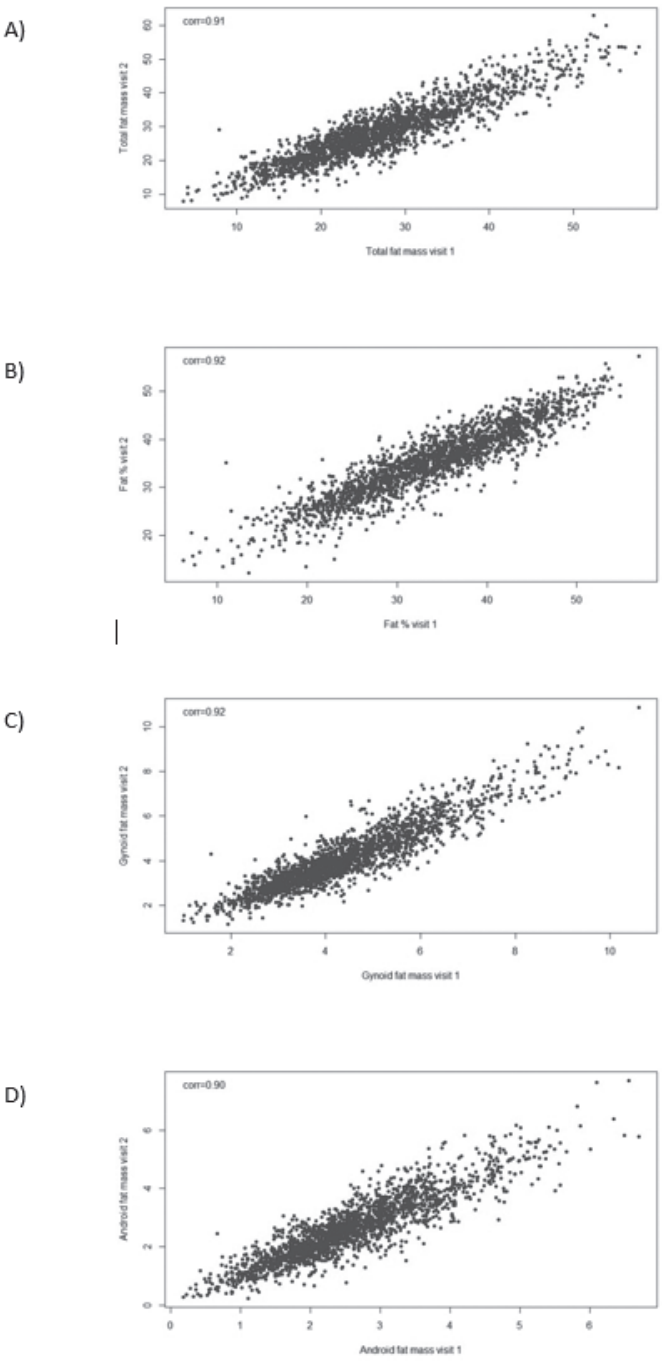
B)



C)



Supplementary Figure 1 | Scatter plot of osteocaline versus A) age , B) weight and C) femoral neck BMD, Female yellow ; Male blue



Supplementary Figure 2 | Scater plot of the correlation between body composition at visit 1 and visit 2 A) Total body fat mass, B) Body fat percentage, C) Gynoid fat mass and D) Android fat mass for subsample of the population where measurments at the both visit were done (n=1,962).