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1 Circulating levels of Dickkopf-1, Osteoprotegerin and sclerostin are higher in
2 old compared with young men and women and positively associated with
3 whole-body bone mineral density in older adults

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1

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4

5 **Summary**

6 Bone mineral density declines with increasing older age. We examined the levels of
7 circulating factors known to regulate bone **metabolism** in healthy young and older adults.

8 The circulating levels of dickkopf-1, osteocalcin, osteoprotegerin and sclerostin were
9 positively associated with WBMD in older adults, despite the average WBMD being lower
10 and circulating dickkopf-1, osteoprotegerin and sclerostin being higher in old than young.

11

1 **Abstract**

2 **Purpose:** To investigate the relationship between whole-body bone mineral density
3 (WBMD) and levels of circulating factors with known roles in bone remodelling during
4 'healthy' ageing.

5 **Methods:** WBMD and fasting plasma concentrations of dickkopf-1, fibroblast growth factor-
6 23, osteocalcin, osteoprotegerin, osteopontin and sclerostin were measured in 272 older
7 subjects (69 to 81 years; 52% female) and 171 younger subjects (18-30 years; 53% female).

8 **Results:** WBMD was lower in old than young. Circulating osteocalcin was lower in old
9 compared with young, while dickkopf-1, osteoprotegerin and sclerostin were higher in old
10 compared with young. These circulating factors were each positively associated with
11 WBMD in the older adults and the relationships remained after adjustment for covariates (r-
12 values ranging from 0.174 to 0.254, all $p < 0.01$). In multivariate regression, the body mass
13 index, circulating sclerostin and whole-body lean mass together accounted for 13.8% of the
14 variation with WBMD in the older adults. In young adults, dickkopf-1 and body mass index
15 together accounted for 7.7% of variation in WBMD.

16 **Conclusion:** Circulating levels of dickkopf-1, osteocalcin, osteoprotegerin and sclerostin are
17 positively associated with WBMD in community-dwelling older adults, despite the average
18 WBMD being lower and circulating dickkopf-1, osteoprotegerin and sclerostin being higher
19 in old than young.

20

21

1 **Introduction**

2 Progressive loss of bone mineral density (BMD) in older age leads to osteoporosis as the
3 balance of bone remodelling favours resorption of mineralised extracellular matrix over
4 formation. This common change is characterized by ‘micro-architectural’ deterioration of
5 bone tissue and increases the risk of fracture [1]. Circulating factors influencing bone
6 development have been implicated in the age-related changes to BMD. This includes
7 regulatory factors released from osteoblasts and osteocytes involved in bone formation and
8 from osteoclasts with bone resorption, which can enter the circulation where their
9 concentrations may be related to BMD in older age.

10 Some of the candidate circulating factors possibly related to BMD include osteoprotegerin
11 (OPG), which is expressed by osteocytes and osteoblasts and can reduce production of
12 osteoclasts by binding receptor activator of nuclear factor kappa-B ligand (RANKL) [2].
13 Osteocalcin (OC) is a major non-collagen protein of the bone matrix secreted by osteoblasts
14 for bone formation, but released from the matrix during bone resorption [3]. Dickkopf-1
15 (DKK1) [4] and sclerostin, released primarily by osteocytes [5], negatively regulate bone
16 formation and have emerged as therapeutic targets to tackle osteoporosis [6]. Fibroblast
17 growth factor 23 (FGF23) is produced by a variety of cell types, including osteoblasts and
18 osteocytes, and released into the circulation where it acts on the kidney to increase
19 excretion of phosphate and reduce production of 1-25 OH Vitamin D [7]. Osteopontin (OPN)
20 is an extracellular matrix protein released by osteoblasts, osteocytes and osteoclasts to
21 facilitate bone resorption [8].

22 It remains unclear how the combination of these circulating markers of bone turnover are
23 related to BMD in older age. Therefore, the purpose of this study was to compare plasma
24 concentrations of these markers between recreationally active, community dwelling older
25 adults and a reference group of young adults, and to examine the association of these with
26 whole-body bone mineral density (WBMD). It was hypothesised that older adults would
27 have higher circulating levels of factors related to bone resorption compared with young,
28 and higher circulating markers of bone resorption were expected to be associated with
29 lower BMD in old age.

30

1 **Materials and Methods**

2 **Study Design**

3 The cross-sectional European multi-centre MYOAGE cohort consists of relatively healthy
4 older men and women (aged 69 to 81 years) and young adults (aged 18-30 years) [9]. The
5 study was approved by ethics committees at each institute and written informed consent
6 was obtained from all participants. Participants were recruited by advertisement in
7 newspapers, the University of the Third Age and Association of Emerti. All measurements
8 were performed according to standard operating procedures that had been unified at the
9 study centres and data collection was ceased through December-March and July-August.
10 Volunteers were excluded if: dependent living, unable to walk a distance of 250 m, presence
11 of morbidity (such as neurologic disorders, metabolic diseases, rheumatic diseases, heart
12 failure, severe chronic obstructive pulmonary disease and hemocoagulative syndromes),
13 immobilization for one week during the last three months and orthopaedic surgery during
14 the last two years or still causing pain or functional limitations. The inclusion and exclusion
15 criteria were designed to ensure the selection of relatively healthy participants and to
16 minimize the confounding effect of comorbidity on sarcopenia [9] and we recorded the use
17 of bisphosphonates, calcium and vitamin D supplements. The present study included 443
18 participants (Leiden, The Netherlands (young; n=35, old; n=75); Jyvaskyla, Finland (young;
19 n=34, old; n=65); Tartu, Estonia (young; n=39, old; n=60), Paris, France (young; n=35, old;
20 n=30) and Manchester, UK (young; n=28, old; n=42)) with complete BMD and bloods results.

21

22 **Dual-energy X-ray absorptiometry**

23 A whole body scan was performed using DXA while the participants lay supine, as previously
24 reported [9] (The Netherlands: Hologic QDR 4500, version 12.4, Hologic Inc., Bedford, MA,
25 USA; Finland: Lunar Prodigy, version en-Core 9.30; Estonia: Lunar Prodigy Advanced, version
26 en-Core 10.51.006; France: Lunar Prodigy, version encore 12.30; United Kingdom: Lunar
27 Prodigy Advance, version enCore 10.50.086). A trained technician completed the daily
28 equipment calibration and the DXA scans according to local and manufacturers' quality
29 control procedures. Participants wore a light cotton garment to reduce effects of clothing
30 absorption on the scanning results. The whole-body lean mass, fat mass and the WBMD
31 were recorded after manual adjustment of the regions of interest carried out offline.

1

2 **Blood sample analysis**

3 Blood samples were collected from a vein in the forearm into vacutainer EDTA tubes in the
4 morning when participants were in a fasted state. Samples remained at room temperature
5 for 15-30 min and were then centrifuged for 15 min at 2,000 *g at 4° C. The plasma was
6 collected and stored at -80°C until analysis. Plasma concentrations of the selected analytes
7 were determined in the research laboratory in Manchester, UK, using multiplex
8 immunoassays (Millipore, Billerica, MA, USA). The manufacturer instructions were followed
9 and the magnetic bead panels quantified DKK1, OPG, OC, OPN, sclerostin and FGF23 using a
10 96-well plate after an overnight incubation. The sensitivity of each analyte was 1.4 (DKK1),
11 1.9 (OPG), 68.5 (OC), 37.7 (OPN), 31.1 (sclerostin) and 9.2 (FGF23) pg/mL. Samples were
12 processed using a Luminex 200 Bioanalyser and protein concentrations were estimated
13 using the xPONENT software (Luminex, v.3.1.871).

14

15 **Statistical analysis**

16 Participant descriptive characteristics (Table 1) were normally distributed and are presented
17 as mean \pm standard error of the mean (SEM). Comparisons between age and gender were
18 assessed using multivariate ANOVA. Relationships between body stature, BMI, total body
19 lean mass and supplement use (independent variables) with WBMD (dependent variable)
20 were assessed using bivariate Pearson's product moment correlation. Data for circulating
21 factors were not normally distributed and are presented as median (25th/75th) centiles. The
22 results were log-transformed and z-scores calculated by expressing each log-transformed
23 value as a standard deviation from the mean of the gender-matched young. Z-scores of
24 WBMD, lean mass and BMI were also calculated for use in subsequent correlation and
25 regression analysis. Spearman's rho partial correlations were performed to assess
26 relationships between the z-score WBMD with z-scores of circulating factors using two
27 models. The first model included adjustment for country of testing to account for any
28 systematic differences. The second accounted for the positive correlations we observed
29 between WBMD and BMI in men and women (r-values ranging from 0.210 – 0.387) and
30 WBMD and lean mass for men (r-values in men ranging from 0.268 – 0.357, and women
31 0.085 – 0.099) as well as health status and use of bisphosphonates, calcium or vitamin D

1 supplements. Thus, the second model included adjustments for: country of testing, z-score
2 of lean mass, z-score of BMI, self-reported health and supplement use. A stepwise multiple
3 linear regression using the self-reported health and supplement use as well as z-scores for
4 BMI, lean mass and circulating factors was then used to evaluate which combination of the
5 independent variables was associated with z-score WBMD (dependent variable) in older
6 adults and in young adults. Data was analysed using SPSS for Windows (v.21; IBM, USA) and
7 significance accepted as $p < 0.05$.

8

9 **Results**

10 Based on z-scores relative to gender-matched young, 26% of the older participants had
11 WBMD values between -1.5 to -2.49 below the mean for young and 10.6% were ≥ -2.5
12 below the mean of young. There was a significant age-by-gender interaction for WBMD z-
13 scores ($p < 0.0005$).

14 Table 2 shows concentrations of the circulating factors. Compared with young, older
15 participants had higher concentrations of DKK1, OPG and sclerostin. Concentrations of OC
16 were significantly lower in old compared with young. OPN and FGF23 did not differ
17 significantly between young and older participants although this was after removal of 37%
18 of FGF23 samples [similar proportions of young and old] that fell below the level of assay
19 detection. Compared with men, women had higher circulating concentrations of OPG, but
20 lower OPN and sclerostin. There were no significant differences between men and women
21 for DKK1, FGF23 and OC. Age x gender interactions were found for OC, OPG and sclerostin
22 (all $p < 0.05$): the difference between young and old in OC, OPG and sclerostin was greater for
23 men than it was for women.

24 Table 3 shows the associations between circulating bone regulatory factors and WBMD.
25 When using z-scores of all variables and including all participants, while adjusting for
26 country, WBMD was positively associated with DKK1. This association remained significant
27 after additionally adjusting for lean mass, BMI, self-reported health and supplement use. In
28 older participants only, DKK1, OC, OPG and sclerostin were positively associated with
29 WBMD after adjusting for country. This remained the case when additionally adjusting for
30 lean mass, BMI, self-reported health and supplement use. In younger participants only,

1 DKK1 was positively associated with WBMD after adjusting for country as well as when
2 additionally adjusting for lean mass, BMI, self-reported health and supplement use.
3 Stepwise multiple linear regression was performed including z-score WBMD as the
4 dependent variable and independent variables included: self-reported health, supplement
5 use and z-scores of the variables BMI and lean mass, as well as the z-scores derived from
6 log-transformed data for DKK1, FGF23, OC, OPG, OPN and sclerostin. Results in the young
7 showed DKK1 accounted for 5.1% of the variation in WBMD (adjusted $r^2=0.051$, $p=0.010$),
8 while DKK1 and BMI accounted for 7.7% of the variation in WBMD (adjusted $r^2=0.077$,
9 $p=0.005$). In the old, BMI alone accounted for 8.9% of the variation in WBMD (adjusted
10 $r^2=0.089$, $p<0.0005$); BMI and sclerostin together accounted for 12.0% of the variation in
11 WBMD (adjusted $r^2=0.120$, $p<0.0005$), while BMI, sclerostin and whole body lean mass
12 accounted for 13.8% of the variation in WBMD (adjusted $r^2=0.138$, $p<0.0005$).

13

14

15 **Discussion**

16 The results of this study showed that circulating factors DKK1, OPG and sclerostin were each
17 higher in old compared with young, but positively associated with WBMD in older adults.
18 Circulating OC was lower in old compared with young and positively associated with WBMD.
19 In multivariate regression, BMI, circulating sclerostin and whole-body lean mass together
20 accounted for 13.8% of the variation with WBMD in the older adults. In young, DKK1 and
21 BMI together accounted for 7.7% of variation in WBMD.

22 **Circulating factors associated with whole-body BMD**

23 Five out of the six circulating factors differed in concentration between old and young (Table
24 2). Of those, DKK1, OC, OPG and sclerostin were identified from both partial correlation
25 models as associated with WBMD in older participants (Table 3).

26 Sclerostin and DKK1 are released primarily by osteocytes and inhibit bone formation by
27 blocking the osteoblast Wnt/ β -canenin signalling pathway [4, 10], with sclerostin and DKK1
28 also stimulating bone resorption through RANKL [11]. Down-regulation of sclerostin [6] and
29 DKK1 [4, 6] is associated with markedly increased bone formation. For these reasons, an

1 inverse association between circulating sclerostin and DKK1 with WBMD would be expected,
2 but is not entirely what was observed. In line with expectations, our results revealed, on
3 average, a 1.8 fold higher circulating sclerostin and approximately 1.2-fold higher DKK1 in
4 old compared with young, which is consistent with an inverse association between
5 sclerostin and BMD in older age [12] and with results from a small sample of 36 patients
6 showing an inverse association between DKK1 and lumbar and femur BMD [13]. However,
7 contrary to expectations, the circulating levels of sclerostin and DKK1 were positively
8 associated with WBMD in the older participants (Table 3). Similar positive associations
9 between circulating sclerostin with BMD and bone micro-architecture in old age has been
10 previously reported [14-17].

11 Similar to the findings for sclerostin and DKK1, a paradoxical relationship existed for OPG
12 and WBMD in older adults: we found higher circulating OPG in old compared with young
13 (Table 2), but circulating OPG was positively associated with WBMD (Table 3). OPG released
14 by osteocytes and osteoblasts promotes bone formation. It has been shown to protect
15 against generalised bone resorption by blocking TNF α in models of chronic inflammation
16 [18] and is considered to be a decoy receptor for RANKL to reduce osteoclast-driven bone
17 resorption [19]. There are conflicting reports about the direction of association between
18 circulating OPG and BMD. A study of postmenopausal women of mean age 62 years [20],
19 and a study of middle aged men [21] reported inverse relationships between BMD and OPG,
20 while others reported no relationship [22, 23]. Conversely, and in line with the results of the
21 present work, when adults in their eighth and ninth decades of life were included in the
22 sample population the relationship between OPG and BMD was positive [24, 25]. These
23 conflicting results cannot be explained by the differences between studies in skeletal site
24 examined. Conflicting results may be related to the differences in the age range of the study
25 samples and possible gender differences. Our results for OPG and sclerostin showed
26 significant age x gender interactions indicating that the differences between young and
27 older men were greater than those between young and older women (Table 2). It is already
28 known that sex hormones can regulate bone turnover and may interact with these
29 circulating factors [26].

30 It is not clear why circulating sclerostin, DKK1 and OPG were positively associated with
31 WBMD in older age, despite the conflicting overall trend for higher circulating levels and

1 lower WBMD in the old. One possibility is that the older, but healthy mature osteocytes
2 generally release higher absolute levels of sclerostin, DKK1 and OPG into the circulation [27]
3 [28]. For example, a positive correlation was found for circulating sclerostin with trabecular
4 density, number and thickness in older men [14, 27], suggesting the more advanced
5 trabecular resorption in osteoporotic bone leaves fewer mature osteocytes and thus, lower
6 sclerostin release than healthy older bone. However, analysis of bone biopsies showed
7 similar sclerostin mRNA levels in young and old despite higher circulating sclerostin levels in
8 the old [12] which indicates that the age-related differences in circulating sclerostin may not
9 be due to increased osteocyte sclerostin gene expression, although this does not necessarily
10 equal protein production [29].

11 Lower circulating OC was found in old compared with young (Table 2) and, consistent with
12 this, circulating OC levels were positively correlated with WBMD in the old (Table 3). OC
13 released by osteoblasts plays a role in bone formation, so the positive correlation with
14 WBMD may be expected. However, others suggest that higher circulating OC indicates
15 greater rates of bone resorption because fragments or whole OC protein is released into the
16 circulation during bone resorption [3]. A previous study of young and middle-aged women
17 suggested that circulating levels peaked soon after menopause and dropped thereafter,
18 although levels were higher in those with osteoporosis than those without [30].

19 Interestingly, our results also showed a positive association between DKK1 and WBMD in
20 the young adults from univariate and multivariate analyses. This association may be a
21 reflection of the numbers of mature osteocytes or related to total bone mass, but more
22 work is needed to confirm. One previous study of children and adolescents did not find any
23 association between circulating DKK1 and BMD, but the young included in that study of
24 youths were in stages of rapid developmental growth, which could present different results
25 from the steady- state of young adults [31].

26

27 **Strengths and limitations**

28 The MYOAGE study included young and older participants relatively free from lifestyle-
29 related comorbidities for their age and the results are therefore indicative of age-related
30 effects. Nevertheless, the associations identified in this cross-sectional study cannot be
31 interpreted as causal relationships despite the clear roles for the selected circulating

1 markers in bone remodelling. The results for FGF23 showed no significant age- or gender-
2 differences, nor correlations with WBMD, but a large proportion of the results were below
3 the level of assay sensitivity, so firm conclusions cannot be drawn for this analyte. We have
4 measured the circulating levels of markers, which may be influenced by release from non-
5 bone cells, so it is not possible to determine the originating cell type. It is possible that
6 altered renal function can affect the levels of the circulating factors, but markers of renal
7 function was not included in the present study due to limitation of plasma sample quantity.
8 A phantom was not used to calibrate the DXA scanners across sites and we did not adjust
9 the results to derive “standardised” DXA values, as others have done for hip and femur sites
10 [32]. Instead, all study centres followed the local quality control procedures, including use of
11 phantoms and daily calibration and the results were adjusted for country of testing to
12 account for possible systematic differences.

13 Future studies should determine the reasons for the positive relationship between
14 circulating sclerostin, DKK1 and OPG with BMD in older adults, despite the old having on
15 average higher circulating levels of these factors and lower WBMD.

16 **Conclusion**

17 Sclerostin, DKK1, OPG and OC were each positively associated with WBMD in older adults,
18 despite the average WBMD being lower and circulating DKK1, OPG and sclerostin being
19 higher in old than young. Multiple linear regression identified BMI, circulating sclerostin and
20 whole-body lean mass as explaining approximately 14% of all variation on WBMD amongst
21 older adults.

22

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25 Medical Research Council (MR/K025252/1).

26

1 **TABLES**

2

3 **Table 1. Participant descriptive characteristics.**

	Old		Young		p-value	
	Men (n=129)	Women (n=143)	Men (n=82)	Women (n=89)	Age	Gender
Age (years)	74.6±0.3	74.0±0.3	23.6±0.3	23.2±0.3	<.0005	
Height (m)	1.74±0.01	1.61±0.01	1.81±0.01	1.67±0.01	<.0005	<.0005
Body mass (kg)	78.8±1.0	65.1±0.8	75.4±1.2	62.4±1.0	<.0005	.018
BMI (kg/m ²)	25.8±0.3	25.2±0.3	23.1±0.3	22.4±0.3	.017	<.0005
Body fat (kg)	20.1±0.7	22.7±0.6	12.9±0.7	18.8±0.7	<.0005	<.0005
Lean mass (kg)	55.9±0.6	40.2±0.5	59.9±0.9	41.4±0.6	<.0005	<.0005
Body fat (%)	25.5±0.6	34.6±0.6	16.6±0.7	29.6±0.7	<.0005	<.0005
Lean mass (%)	71.9±0.6	63.0±0.6	79.8±0.7	67.2±0.7	<.0005	<.0005
WBMD (g/cm ²)	1.19±0.01	1.04±0.01	1.25±0.01	1.15±0.01	<.0005	.001
WBMD (z-score)	-0.63±0.10	-1.47±0.11	0.00±0.11	0.00±0.11	<.0005	<.0005

4

5 Values are mean ± SEM. WBMD: whole-body bone mineral density.

6

1 **Table 2. Circulating markers of bone remodelling in old and young, men and women.**

	Old		Young		p-value		
	Men	Women	Men	Women	Age	Gender	Age x Gender
DKK1 (pg.mL ⁻¹)	577.0 ± 352-804	575.3 ± 346-864	420.6± 290-627	494.3 ± 284-703	<.0005	.942	.843
FGF23 (pg.mL ⁻¹)	113.5 ± 72-274 (n=75)	103.0 ± 64-211 (n=87)	122.9.7 ± 87-195 (n=54)	141.7 ± 94-225 (n=60)	.792	.316	.700
OC (pg.mL ⁻¹)	14160.5 ± 9911-18708	16065.4 ± 11073-19933	17581.1 ± 13304-21223	16733.9 ± 12013-20715	<.0005	.880	.036
OPG (pg.mL ⁻¹)	319.2 ± 229-419	306.9 ± 257-392	159.4 ± 114-193	208.5 ± 160-260	<.0005	<.0005	<.0005
OPN (pg.mL ⁻¹)	26590.1 ± 17094-38028	21350.1 ± 13971-31255	24822.5 ± 16928-35662	20877.5 ± 15937-27777	.700	.009	.184
Sclerostin (pg.mL ⁻¹)	5690.3 ± 4348-7556	4147.6 ± 3349-5159	3016.1 ± 2079-3932	2366.0 ± 1923-3134	<.0005	<.0005	.034

2

3 Values are median ± 25th – 75th percentiles. For FGF23, the *n* is less than those given in Table 1 due to some
 4 samples having values that were below the level of detection. The *n* for all other analytes is the same as shown
 5 in Table 1.

6

7

1 **Table 3. Associations between circulating bone regulatory factors and whole body bone mineral density.**

Correlation with z-score WBMD	all participants combined		Old		Young	
	1	2	1	2	1	2
Adjustment models						
DKK1	r=.107 p=.026	r=.129 p=.008	r=.167 p=.007	r=.174 p=.005	r=.263 p=.001	r=.282 p<.0005
FGF-23	r=.067 p=.274	r=.051 p=.406	r=-.095 p=.235	r=-.079 p=.330	r=-.086 p=.370	r=-.130 p=.182
OC	r=-.124 p=.010	r=-.083 p=.088	r=.150 p=.015	r=.187 p=.003	r=-.023 p=.767	r=-.008 p=.916
OPG	r=-.096 p=.047	r=-.039 p=.419	r=.209 p=.001	r=.254 p<.0005	r=.081 p=.297	r=.055 p=.484
OPN	r=-.005 p=.918	r=-.001 p=.980	r=.055 p=.370	r=.073 p=.245	r=-.120 p=.124	r=-.122 p=.120
Sclerostin	r=-.091 p=.059	r=-.075 p=.126	r=.241 p<.0005	r=.240 p<.0005	r=.129 p=.096	r=.135 p=.086

2

3 Data are shown as spearman's rho. The circulating bone regulatory factors were log-transformed and their z-
 4 scores calculated. The p value indicates the level of significance after statistical analysis. Results were adjusted
 5 for 1) country; 2) country, z-score lean mass, z-score BMI, self-reported health and supplement use. Significant
 6 relationships are highlighted using bold text.

7

8

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