Published in final edited form as:

Calcif Tissue Int. 2011 June ; 88(6): 503–510. doi:10.1007/s00223-011-9484-2.

INFLUENCE OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBP)-1 AND IGFBP-3 ON BONE HEALTH: RESULTS FROM THE EUROPEAN MALE AGEING STUDY (EMAS)

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Abstract

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DISCLOSURES: NONE

The aim of this study was to determine the influence of insulin-like growth factor binding proteins (IGFBP)-1 and IGFBP-3, and IGF-1 on calcaneal ultrasound parameters in middle-aged and elderly European men.

Men aged 40 to 79 years were recruited from population registers for participation in the European Male Ageing Study (EMAS). Subjects were invited by letter to complete a postal questionnaire and to attend for an interviewer-assisted questionnaire, quantitative ultrasound (QUS) of the calcaneus and a fasting blood sample from which serum levels of IGFBP-1, IGFBP-3, IGF-1, oestradiol (E_2) and SHBG were assayed. The questionnaires included the Physical Activity Scale for the Elderly (PASE) and questions about smoking and alcohol consumption. Estimated bone mineral density (eBMD) was derived as a function of the QUS parameters, speed of sound and broadband ultrasound attenuation. Height and weight were measured in all subjects.

3057 men, mean age 59.7 years (standard deviation [SD]=11.0) were included in the analysis. After adjusting for age, centre and BMI, higher levels of IGFBP-1 were associated with lower eBMD. Higher levels of both IGFBP-3 and IGF-1 were associated with higher eBMD. After further adjustment for PASE score, current smoking, alcohol consumption, free E_2 and SHBG, IGFBP-3 and IGF-1, though not IGFBP-1, remained significantly associated with eBMD.

IGFBP-1 was associated with bone health though the effect could be explained by other factors. IGFBP-3 and IGF-1 were independent determinants of bone health in middle aged and elderly European men.

Keywords

insulin-like growth factor binding protein 1; insulin-like growth factor binding protein 3; calcaneus quantitative ultrasound; population-based; men

INTRODUCTION

It is well established that the growth hormone (GH)- insulin-like growth factor – 1 (IGF-1) axis plays an important role in bone metabolism, including stimulating osteoblast function and inhibiting collagen matrix degradation [1]. Synthesised mainly in the liver, IGF-1 secretion is under the physiological control of growth hormone (GH). GH is secreted in a pulsatile fashion and IGF-1 is more widely used as an indicator of the axis function. The majority [2-8], but not all [9] previous studies have shown associations between serum IGF-1 levels and bone health in men as assessed using dual energy X-ray absorptiometry (DXA).

Less than one percent of the total serum IGF-1 is freely circulating, the remainder is bound to six high affinity insulin-like growth factor binding proteins (IGFBPs) [1,3,5,10,11], which play an important role in regulating IGF-1 activity and bio-availability, having both stimulatory and inhibitory effects. IGFBPs may also have IGF-independent effects [1,11]. The main circulating binding protein is IGFBP-3, which binds to IGF-1 forming a complex [1,3,10,11]. This complex stabilises the IGF-1 molecule, prolonging its half-life in the circulation, and regulating its availability to target tissues [12]. IGFBP-3 is considered to be the binding protein that best reflects growth hormone activity. Several studies have examined the association between IGFBP-3 and bone health in men though with somewhat inconsistent results [3,5,10,13,14].

The potential influence that IGFBP-1, another potent modulator of IGF-1 activity, may have on bone health has received far less attention, despite evidence from cell culture studies that IGFBP-1 may be present in osteoblasts [15]. There has only been a small number of cross-

sectional studies examining IGFBP-1 and bone health in men, which have yielded inconsistent results. This is possibly due to the cross-sectional study designs, small sample sizes, differences in study populations and the inadequate adjustment for potential confounders [3,10,16].

The European Male Ageing Study (EMAS) is a large multicentre population based study of ageing in middle aged and elderly men which includes an extensive range of clinical, biochemical, health and lifestyle information. We used data from EMAS to examine the influence of IGFBP-1, IGFBP-3 and IGF-1 on bone health in men measured using quantitative ultrasound of the calcaneus. We looked also whether any of the observed associations could be explained by other factors.

METHODS

Subjects

The subjects included in this analysis were recruited for participation in EMAS. Detailed methods have been described previously [17]. Briefly, men were recruited from population based sampling frames in 8 centres: Florence (Italy), Leuven (Belgium), Lodz (Poland), Malmö (Sweden), Manchester (UK), Santiago de Compostela (Spain), Szeged (Hungary), Tartu (Estonia). Participating centres were selected to provide geographical and socio-economic diversity within Europe, and facilities to perform epidemiological surveys. Stratified random sampling was used with the aim of recruiting equal numbers of men in each of four 10-year age bands: 40-49 years, 50-59 years, 60-69 years, and 70 years and over. Subjects were invited by letter to complete a postal questionnaire and attend for an interviewer-assisted questionnaire, and assessment of physical performance. Subjects were contacted again usually within 4 weeks if they did not reply following a first letter. Ethical approval for the study was obtained in accordance with local institutional requirements in each centre. All subjects provided written informed consent.

Assessments

The postal questionnaire included questions concerning current smoking and alcohol consumption in the previous year (response set = every day / 5-6 days per week / 3-4 days per week / 1-2 days per week / less than once a week / not at all). There was a question also about prior fracture since the age of 25 years (response set = no / yes / don't know). The main study questionnaire included the physical activity scale for the elderly (PASE) [18], which is a measure of participation in activities (sitting, walking, light, moderate, or strenuous sport or recreational activity, strength or endurance exercises, light or heavy housework/gardening) in the past seven days with higher scores reflecting higher levels of activity. The questionnaire also included questions about medications. In addition, height and weight were measured in a standardised fashion.

Biochemical assessments

A single fasting morning (before 10.00h) venous blood sample was obtained from all subjects. Serum was separated immediately after phlebotomy and stored at -80 C. All samples were transported in frozen state to a single laboratory for measurement of IGF-1, IGFBP-1 & IGFBP-3 (Molecular Endocrinology, Faculty of Medicine, University of Santiago de Compostela, Spain) by quimioluminescence. Within- and between-assay coefficients of variation (CV) for IGF-1 were 7.4 and 2.9%, IGFBP-1 4.8 and 5.5% and IGFBP-3 7.2 and 4.2%. Detection limits of the respective assays were 20 ng/mL, 0.1 µg/mL and 0.5 µg/L for IGF-1, IGFBP-1 and IGFBP-3 respectively. IGFBP-1 was not measured in one EMAS centre. Serum was assayed for parathyroid hormone (PTH) using a chemiluminescence immunoassay (Nichols Advantage Bio-Intact PTH assay, Quest

Diagnostics, Madison, NJ, USA). Within- and between-assay CVs for PTH were 6% and 2.8%, respectively. The detection limit of the assay was 1.45 ng/mL.

Each serum sample was also assayed for testosterone (T), oestradiol (E₂) and sex-hormone binding globulin (SHBG). SHBG was measured by Modular E170 platform electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany) at a single laboratory (General Laboratory, Azienda Ospedaliero-Universitaria Careggi, Florence, Italy) as described previously [19]. T and E₂ were measured using gas chromatography-mass spectroscopy (GC-MS) as described before [20,21]. Free (non-SHBG-bound) T and E₂ levels were derived using the total hormone measurement and levels of SHBG and albumin using mass action equations and association constants [22,23].

Quantitative Heel Ultrasound (QUS)

QUS of the calcaneus was performed using the Sahara Clinical Sonometer (Hologic, Bedford, Massachusetts) using a standardized protocol. Each centre used the same machine model, and each calibrated daily with the physical phantom provided by the manufacturer. Outputs included broadband ultrasound attenuation (BUA), speed of sound (SOS) and a machine derived parameter: calcaneal bone mineral density (eBMD) in g/cm², $eBMD = 0.002592 \times (BUA + SOS) - 3.687$. To establish the short-term precision of the method in this population, duplicate measurements were performed in 20 randomly selected cohort members in one of the centres (Leuven, Belgium). The in vivo CVs were 2.8%, 0.3%, 3.4% for BUA, SOS and eBMD respectively. Detailed methods have been described previously [24].

Analysis

Subjects with complete QUS, IGFBP-3 and IGF-1 measurements were included in the analysis. Subjects taking insulin or on medications that may have influenced bone turnover (including glucocorticoids) were excluded from the analysis. Body mass index (BMI) was calculated as weight in kilograms divided by height in metres squared.

We looked initially at what factors (age, height, weight, lifestyle factors, hormones and QUS parameters) were associated, separately with IGFBP-1, IGFBP-3 and IGF-1 using linear regression with the latter as dependent variables. For IGFBP-1, the natural log was used as the distribution was skewed. The QUS parameters were transformed into 'z' scores for ease of interpretation.

We looked next at the association between the IGF parameters and QUS measures, again using linear regression though on this occasion with the QUS parameters as the dependent variables. We looked initially at the results after adjustment for age and centre and then after further adjustment for BMI. In a final model we used backwards stepwise regression including all the potential confounding factors (age, centre, BMI, PASE score, current smoking, alcohol consumption, free T, free E₂, SHBG and PTH), removing non-significant factors one at a time in stepwise fashion until only significant variables remained. In all these models we looked at the IGF parameters both as continuous measures and also after categorisation into quintiles. The results for all ultrasound parameters (SOS, BUA and eBMD) were similar and for ease of presentation we include here details of eBMD only. Statistical analysis was performed using STATA version 9.2 (<http://www.stata.com>).

RESULTS

Subjects

3057 subjects were included in the analysis. Characteristics of the subjects are shown in Table 1. Mean age was 59.7 (SD=11.0) years, mean height 173.7 (SD=7.3) cm, weight 83.4 (SD=13.8) kg and BMI 27.6 (SD=4.1) kg/m². Mean PASE score was 198.2 (SD=90.7). Twenty one percent of subjects reported that they currently smoke and 57% reported consuming alcohol at least one day per week. 26% reported a previous fracture. Mean BUA was 80.3 (SD=18.8) dB/MHz, SOS 1551.0 (SD=34.1) m/s, and eBMD 0.543 (SD=0.135) g/cm². Mean IGFBP-1 was 2.6 (SD=4.2) µg/l, IGFBP-3 was 4.4 (SD=1.0) µg/ml and IGF-1 was 133.4 (SD=42.9) ng/ml.

Factors associated with IGFBP-1, IGFBP-3 and IGF-1

(Log) IGFBP-1 significantly increased with age while both IGF-1 and IGFBP-3 decreased with age, see Table 2. (Log) IGFBP-1 was negatively associated with height, weight, BMI, PASE score, free testosterone and all three QUS parameters and positively associated with current smoking and SHBG and PTH levels. Infrequent alcohol consumption was associated with higher values, see table 2. IGFBP-3 was significantly positively associated with height, weight, PASE score, frequent alcohol consumption, free testosterone and all three QUS parameters and negatively associated with SHBG, PTH, infrequent alcohol consumption and previous fracture. IGF-1 was positively associated with height, PASE score, free testosterone and all three QUS parameters, and negatively associated with BMI, SHBG, PTH and previous fracture. Both regular and infrequent alcohol consumption (compared to consumption on 1-2 days per week) was associated with lower IGF-1.

Association between IGF parameters and QUS

After adjustment for age and centre, higher levels of IGFBP-1 were associated with lower eBMD, see table 3. Compared to those in the lowest quintile of IGFBP-1, those in the highest quintile had a lower eBMD (β co-efficient = -0.040 g/cm²). This association was slightly attenuated but remained statistically significant after further adjustment for BMI. The association became non-significant, however, after additional adjustment for other factors that remained significantly associated with eBMD in a backwards stepwise regression which included PASE score, current smoking, alcohol consumption, free E₂ and SHBG in addition to age, BMI and centre, see table 3.

Higher levels of IGFBP-3 were associated with a higher eBMD after adjustment for age and centre. Compared to those in the lowest quintile of IGFBP-3, those in the highest quintile had a higher eBMD (β co-efficient = 0.024 g/cm²). This association appeared to be broadly linear and persisted after further adjustment for BMI and other confounders (the same factors remained in the model as for IGFBP-1) though the strength of the association was attenuated, see Table 3.

Similarly, after adjusting for age and centre, higher levels of IGF-1 were, as expected, associated with a higher eBMD. Compared to those in the lowest quintile of IGF-1, those in the highest quintile had a higher eBMD (β co-efficient = 0.036 g/cm²). This association appeared to be linear and persisted after further adjustment for BMI. Additional adjustment for the other confounding factors attenuated the strength of the association though the results remained statistically significant, see Table 3.

DISCUSSION

In this large population based study of middle aged and elderly European men, higher levels of IGFBP-1 were associated with lower estimated calcaneal BMD, however, the association could be explained by other factors. In contrast, higher levels of IGFBP-3 and IGF-1 were independently associated with higher eBMD.

IGFBP-1 has received little attention in a population setting. Our observation of an increase in IGFBP-1 with age is in keeping with one previous finding [16]. In contrast, the decline in serum IGF-1 levels with age has been demonstrated in most studies [3-5,7-9,11,16,25,26]. Our observation of a decrease in serum IGFBP-3 levels with age is similar to previous reports [3,5,7]. These changes are compatible with the well-described age-related fall in circulating growth hormone [1].

There are few studies examining the influence of IGFBP-1 on bone health in men. We found that higher levels of IGFBP-1 were significantly associated with lower QUS parameters after adjustment for age, centre and BMI; however, this relationship became non-significant after further adjustment for other confounding factors such as lifestyle, sex steroids, SHBG and PTH levels. Jehle (1998) reported that IGFBP-1 was negatively correlated to hip BMD_a in Type 1 diabetes patients, but the association became non-significant after further adjustment for other confounding factors [10]. Investigators from the Rancho Bernardo Study reported a relationship between IGFBP-1 and BMD_a in 633 community dwelling men which disappeared after adjustment for age, BMI and other confounders [16]. Similarly, the study of 55 Swedish men by Gillberg et al also found the effects of IGFBP-1 on bone to be explained by age and body weight [3]. Our observation, in a much larger group of men, of a BMI-independent association could in part be due to our greater statistical power to detect weaker associations. The association with IGFBP-1 was weaker than that observed for IGF-1 and IGFBP-3 and disappeared once a number of factors were added to the statistical model. This is consistent with the notion that IGFBP-1 has less influence on IGF-1 activity than IGFBP-3. There is also less IGFBP-1 in the circulation than BP-3. In terms of potential mechanisms, IGFBP-1 mRNA and protein expression has been observed in osteoblasts [15]. IGFBP-1 appears to inhibit collagen gene expression by blocking IGF-1 access to its receptor, particularly when there is an excess of IGFBP in relation to IGF-1 [27].

We observed that higher levels of the most common binding protein, IGFBP-3, was associated with higher ultrasound measurements, a result which persisted after adjustment for a range of confounding factors. This would be in keeping with the role of the binding protein to enhance the anabolic effect of IGF-1 on bone by prolonging its half life in serum [12]. Our finding is in keeping with some [3] though not all published reports [5,10,13]. The reasons behind the discrepancy in reports is unclear, though may be due to differences in sample size, the nature of the populations studied and inadequate adjustment for confounders.

In our study higher levels of circulating IGF-1 was, as expected, associated with higher estimated calcaneal BMD an association which remained significant after adjustment for age, centre, BMI, PASE score, current smoking, alcohol consumption, free E₂ and SHBG. In a study of 218 healthy individuals aged 55-80 from the Rotterdam Study, of which 103 were men, a weak positive association was observed between IGF-1 and BMD_a at the lumbar spine and proximal femur in the men [4]. Similarly, in a Greek study of 363 healthy men, mean age 51±8.7 years, a positive association was observed between BMD_a and IGF-1 at the lumbar spine, femoral neck and the trochanter [5]. The results also accord with data from human intervention studies where IGF-1 is linked with bone turnover in women [28,29]. Others [2,3,6,7] support our findings, though there are discrepant reports. As part of the

Framingham Heart Study, Langlois (1998) reported a positive association between IGF-1 and BMD_a in women though not men [30]. Similarly, a cross-sectional study of elderly men and women from the Rancho-Bernardo study reported a significant association between IGF-1 and BMD_a in women only. None of the other confounders measured, including BMI, smoking, alcohol intake, physical activity, and diuretic use could explain this gender difference [9]. The reason for the apparent lack of association in men in these studies was unclear.

We observed no association between IGFBP-1 and self reported history of fracture. However, we did observe a significant association between self reported history of fracture and both IGFBP-3 and IGF-1 though the association with IGFBP-3 became non-significant after adjustment for age, BMI and centre. The only prospective study to date examining IGF-1, its binding proteins and incident fracture came from the Study of Osteoporotic Fracture (SOF) group, who found that women in the lowest quartile of IGF-1 had a 60% greater risk of hip and incident vertebral fracture. IGFBP-3 was not associated with an increased risk of fracture in SOF [31]. Further prospective studies are needed, particularly in men, to assess the impact of the GH-IGF axis on fracture risk.

This is the largest population based study to date examining the effects of IGFBP-1 and also IGFBP-3 and IGF-1 on bone health in ageing men. The study used standardised methods to assess bone health, the IGF axis and other confounding factors. However, there are some limitations which need to be considered when interpreting the results. The overall response rate for participation was 45%. Although below 50%, this is consistent with other large-scale European [32] and US studies [33]. Levels of IGF parameters may have differed in those who did and did not take part and so absolute levels of these parameters need to be interpreted with caution. However, any such selection bias should not influence the association between IGF parameters and QUS measurements, which was based on an internal comparison of those who took part in the study. Given the cross-sectional design it is not possible to determine the temporal nature of any of the associations for which prospective data is needed. Finally our study was based on assessment of middle aged and elderly European men and generalisation beyond this population should be made with caution.

In conclusion, in middle aged and older European men, IGF-1 and IGFBP-3 were independently associated with heel ultrasound parameters. IGFBP-1 was also associated with these parameters though the effect could be explained by other factors.

Acknowledgments

The European Male Ageing Study (EMAS) is funded by the Commission of the European Communities Fifth Framework Programme "Quality of Life and Management of Living Resources" Grant QLK6-CT-2001-00258 and supported by funding from the Arthritis Research UK. The Principal Investigator of EMAS is Professor Frederick Wu, MD; Dept of Endocrinology, Manchester Royal Infirmary, UK. The authors wish to thank the men who participated in the eight countries, the research/nursing staff in the eight centres: C Pott, Manchester, E Wouters, Leuven, M Nilsson, Malmö, M del Mar Fernandez, Santiago de Compostela, M Jedrzejowska, Lodz, H-M Tabo, Tartu, A Heredi, Szeged for their data collection and C Moseley, Manchester for data entry and project coordination. Dr Vanderschueren is a senior clinical investigator supported by the Clinical Research Fund of the University Hospitals Leuven, Belgium. Dr. Boonen is a senior clinical investigator of the Fund for Scientific Research-Flanders, Belgium (F.W.O.-Vlaanderen). Dr. Boonen is holder of the Leuven University Chair in Metabolic Bone Diseases.

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Table 1

Subject characteristics

Variable	N=3057
	Mean (SD)
Age at interview (years)	59.7 (11.0)
Height (cm)	173.7 (7.3)
Weight (Kg)	83.4 (13.8)
Body mass index (kg/m ²)	27.6 (4.1)
PASE score (0-1100)	198.2 (90.7)
Broadband ultrasound attenuation (dB/MHz)	80.3 (18.8)
Speed of sound (m/s)	1551.0 (34.1)
Estimated bone mineral density (g/cm ²)	0.543 (0.135)
Insulin-like growth factor binding protein 1 (µg/L)	2.6 (4.2)
Insulin-like growth factor binding protein 3 (µg/mL)	4.4 (1.0)
Insulin-like growth factor 1 (ng/mL)	133.4 (42.9)
Free testosterone (pmol/L)	290.6 (88.3)
Free oestradiol (pmol/L)	1.3 (0.4)
Sex hormone binding globulin (nmol/L)	42.6 (19.3)
Parathyroid hormone (ng/mL)	28.8 (13.9)
	%
Currently smoke (yes vs no)	21.2
Alcohol consumption 1day / week	56.5
Previous fracture since age 25 (yes vs no)	25.9

Table 2

Factors associated with IGFBP-1, IGFBP-3 and IGF-1

	Log(IGFBP-1)	IGFBP-3 (µg/mL)	IGF-1 (ng/mL)
	β co-efficient ^I (95% CI)		
Age (years)	0.040 (0.035, 0.046)*	-0.032 (-0.035, -0.029)*	-1.177 (-1.308, -1.045)*
Height (cm)	-0.027 (-0.036, -0.018)*	0.021 (0.016, 0.026)*	1.094 (0.888, 1.299)*
Weight (Kg)	-0.036 (-0.041, -0.032)*	0.008 (0.005, 0.011)*	0.107 (-0.003, 0.218)
BMI (Kg/m ²)	-0.109 (-0.125, -0.094)*	0.009 (0.000, 0.018)	-0.667 (-1.042, -0.292)*
PASE score (0-1100)	-0.001 (-0.002, -0.001)*	0.002 (0.001, 0.002)*	0.064 (0.047, 0.081)*
Current smoker (yes vs no)	0.203 (0.047, 0.359)*	-0.002 (-0.092, 0.088)	-1.824 (-5.557, 1.910)
Alcohol consumption (dys/wk)			
None	0.466 (0.250, 0.683)*	-0.348 (-0.469, -0.227)*	-12.132 (-17.169, -7.096)*
< 1	0.312 (0.118, 0.507)*	-0.147 (-0.253, -0.041)*	-4.462 (-8.852, -0.073)*
1-2	Referent	Referent	Referent
3-4	-0.153 (-0.394, 0.088)	0.138 (0.009, 0.267)*	-0.482 (-5.857, 4.892)
5-6	0.058 (-0.232, 0.349)	0.038 (-0.123, 0.200)	-6.804 (-13.523, -0.084)*
Every day	0.116 (-0.101, 0.333)	0.122 (0.001, 0.243)*	-15.342 (-20.376, -10.309)*
Free testosterone (pmol/L)	-0.001 (-0.002, -0.000)*	0.001 (0.000, 0.001)*	0.081 (0.064, 0.098)*
Free oestradiol (pmol/L)	-0.089 (-0.242, 0.064)	-0.065 (-0.150, 0.019)	0.856 (-2.662, 4.374)
SHBG (nmol/L)	0.028 (0.025, 0.031)*	-0.020 (-0.022, -0.018)*	-0.606 (-0.682, -0.530)*
Parathyroid hormone (ng/mL)	0.009 (0.004, 0.014)*	-0.004 (-0.007, -0.002)*	-0.266 (-0.376, -0.157)*
BUA (per SD)	-0.189 (-0.252, -0.126)*	0.082 (0.045, 0.119)*	3.301 (1.783, 4.819)*
SOS (per SD)	-0.196 (-0.259, -0.133)*	0.121 (0.085, 0.158)*	5.391 (3.881, 6.902)*
eBMD (per SD)	-0.201 (-0.265, -0.138)*	0.110 (0.074, 0.147)*	4.840 (3.327, 6.353)*
Previous fracture since age 25	0.078 (-0.069, 0.224)	-0.098 (-0.182, -0.014)*	-5.714 (-9.183, -2.244)*

^I unadjusted bivariable analysis

* p<0.05

Table 3

Influence of IGFBP-1, IGFBP-3 and IGF-1 on (QUS) estimated BMD

	Estimated BMD (g/cm ²)		
	β coefficient ^a	β coefficient ^b	β coefficient ^c
IGFBP-1 (μ g/L)	-0.003 (-0.004, -0.002)*	-0.002 (-0.003, -0.000)*	-0.001 (-0.002, 0.001)
IGFBP-1 quintiles (μ g/L):			
1: < 0.41	Referent	Referent	Referent
2: 0.41 – 1.03	-0.009 (-0.025, 0.008)	-0.005 (-0.022, 0.011)	-0.008 (-0.025, 0.009)
3: 1.04 – 1.91	-0.026 (-0.043, -0.009)*	-0.018 (-0.034, -0.001)*	-0.017 (-0.034, 0.000)
4: 1.92 – 3.54	-0.015 (-0.031, 0.002)	-0.003 (-0.020, 0.015)	0.000 (-0.017, 0.018)
5: >3.54	-0.040 (-0.057, -0.023)*	-0.021 (-0.039, -0.003)*	-0.014 (-0.033, 0.005)
IGFBP-3 (μ g/mL)	0.010 (0.005, 0.015)*	0.009 (0.004, 0.014)*	0.007 (0.002, 0.012)*
IGFBP-3 quintiles (μ g/mL):			
1: <3.61	Referent	Referent	Referent
2: 3.61 – 4.20	0.005 (-0.010, 0.020)	0.007 (-0.008, 0.022)	0.005 (-0.011, 0.020)
3: 4.21 – 4.70	0.016 (0.001, 0.031)*	0.018 (0.003, 0.033)*	0.016 (0.001, 0.032)*
4: 4.71 – 5.30	0.030 (0.014, 0.045)*	0.029 (0.013, 0.044)*	0.023 (0.007, 0.039)*
5: > 5.30	0.024 (0.008, 0.040)*	0.021 (0.005, 0.037)*	0.016 (-0.001, 0.033)
IGF-1 (per 10ng/mL)	0.003 (0.002, 0.004)*	0.003 (0.002, 0.004)*	0.002 (0.001, 0.004)*
IGF-1 quintiles (ng/mL):			
1: <98.2	Referent	Referent	Referent
2: 98.2 – 118.6	0.010 (-0.005, 0.025)	0.013 (-0.002, 0.027)	0.004 (-0.011, 0.020)
3: 118.7 – 138.1	0.014 (-0.001, 0.028)	0.016 (0.001, 0.031)*	0.008 (-0.007, 0.024)
4: 138.2 – 166.2	0.022 (0.007, 0.037)*	0.026 (0.011, 0.041)*	0.020 (0.004, 0.035)*
5: >166.2	0.036 (0.020, 0.051)*	0.040 (0.025, 0.055)*	0.029 (0.012, 0.045)*

^a adjusted for age and centre^b adjusted for age, centre and BMI^c adjusted for age, centre, BMI, and other factors that remained significant after backwards stepwise regression: PASE score, current smoking, alcohol consumption, free oestradiol and SHBG

* p<0.05