CLINICAL STUDY

Serum myostatin levels are negatively associated with abdominal aortic calcification in older men: the STRAMBO study

P Szulc*, L C Hofbauer[1,2,*], M Rauner[1,2], C Goettsch[1,2], R Chapurlat and M Schoppet[3]

INSERM UMR 1033, University of Lyon, F-69437 Lyon, France, [1]Division of Endocrinology, Diabetes, and Bone Diseases, Department of Medicine III, Dresden Technical University Medical Center, Fetscherstraße 74, D-01307 Dresden, Germany, [2]Center for Regenerative Therapies, D-01307 Dresden, Germany and [3]Department of Internal Medicine and Cardiology, Philipps University, D-35043 Marburg, Germany

(Correspondence should be addressed to L C Hofbauer at Division of Endocrinology, Diabetes, and Bone Diseases, Department of Medicine III, Dresden Technical University Medical Center; Email: lorenz.hofbauer@uniklinikum-dresden.de)

*(P Szulc and L C Hofbauer contributed equally to this work)

Abstract

Objective: To assess the association between abdominal aortic calcification (AAC) and serum levels of myostatin, a negative regulator of skeletal muscle mass, which has been implicated in the development of atherosclerotic lesions in mice.

Design and patients: We assessed AAC semiquantitatively from the lateral spine scans obtained using dual energy X-ray absorptiometry in 1071 men aged 20–87 years. Serum myostatin levels were measured by an immunoassay that detects all myostatin forms.

Results: Total myostatin serum levels did not differ between men with or without self-reported ischemic heart disease, hypertension, or diabetes mellitus. Total serum myostatin levels were higher in men with higher serum calcium levels and lower in men with higher serum concentrations of highly sensitive C-reactive protein. Men with AAC had lower myostatin levels compared with men without AAC. Prevalence of AAC (AAC score >0) was lower in the highest myostatin quartile compared with the three lower quartiles ($P<0.05$). After adjustment for confounders, odds of AAC (AAC score >0) were lower (OR$=0.62$; 95% confidence interval (95% CI), 0.45–0.85; $P<0.005$) for the fourth myostatin quartile vs the three lower quartiles combined. In the sub-analysis of 745 men aged ≥60 years, the results were similar: AAC prevalence was lower in the highest myostatin quartile compared with the three lower quartiles combined (OR$=0.54$; 95% CI, 0.38–0.78; $P<0.001$).

Conclusions: In older men, total myostatin serum levels are inversely correlated with AAC. Further studies are needed to investigate mechanisms underlying this association and to assess utility of myostatin as a cardiovascular marker.

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Introduction

Myostatin belongs to the transforming growth factor-β superfamily of growth and differentiation factors. Besides its role as a regulator of skeletal muscle homeostasis, myostatin may influence: glucose metabolism; insulin secretion, sensitivity, and resistance; lipid metabolism; adipogenesis; and fat accumulation (1, 2). Moreover, myostatin synthesis is increased by tobacco smoking and tumor necrosis factor-α (3, 4). As these factors are also cardiovascular risk factors, myostatin may be involved in the pathogenesis of cardiovascular diseases. However, limited studies have addressed this in atherosclerosis or chronic heart failure. For instance, inactivation of myostatin protects against the development of atherosclerosis in LDL receptor-deficient mice (5), and mechanical stress

increases myocardial myostatin expression (6). In patients with heart failure, serum myostatin concentrations were increased compared with healthy controls in some, but not all, studies (7, 8, 9).

The above factors also contribute to the development of ectopic calcification in soft tissues, e.g. Mönckeberg's medial sclerosis in large arteries (10). Mönckeberg's sclerosis is distinct from atherosclerosis and results in abdominal aortic calcification (AAC). Severe AAC is associated with higher cardiovascular morbidity and mortality and a higher risk of fracture (11, 12). In addition to the above factors, AAC and myostatin secretion may share other mechanisms. In myostatin deficient mice, mesenchymal stem cells (MSCs) undergo increased osteogenic differentiation (13). Interestingly, the number of circulating osteoprogenitor cells was higher in postmenopausal osteoporotic women with

AAC compared with women without AAC (14). Lower levels of 25-hydroxycholecalciferol (25OHD) were associated with severe and more progressive AAC (14, 15), as well as lower myostatin levels (16). Exogenous testosterone inhibited vascular calcification and increased circulating myostatin levels (17, 18). Furthermore, hemizygous deletion of the genes of type 3 and 5 collagens and of myostatin was associated with aortic dissection (19). However, the link between myostatin and AAC has not been studied. Therefore, we conducted a cross-sectional study of the relation between total serum myostatin levels and AAC as assessed on the lateral scans of the spine obtained using dual energy X-ray absorptiometry (DXA) in older men.

Materials and methods

Participants

The Structure of the Aging Men's Bones (STRAMBO) study is a single center prospective cohort study on skeletal fragility and its determinants in men. The study is performed as a collaboration between Institut National de la Santé et de la Recherche Médicale and Mutuelle de Travailleurs de la Région Lyonnaise (MTRL) (20). It was approved by the Local Ethics Committee and is conducted in agreement with the Helsinki Declaration of 1975 and 1983. Letters inviting participation were sent to randomly selected men aged 20–87 years from the MTRL lists living in greater Lyon. During the recruitment in 2006–2008, 1169 volunteers replied to the invitation and provided written informed consent. All men able to give informed consent, to answer the questionnaire, and to participate in the exams were included. No specific exclusion criteria were applied. Both valid measurement of serum myostatin levels and DXA lateral scans were available and obtained in 1117 men. As we did not assess weight loss before recruitment, we could not employ the current definition of cachexia (21). Therefore, we defined cachexia *post hoc* as the coexistence of low BMI $< 20 \text{ kg/m}^2$ and of low relative appendicular skeletal muscle mass (RASM) $< 7.26 \text{ kg/m}^2$. We excluded men with cachexia ($n=12$) and men who took vitamin D supplements ($n=35$). The first analysis was performed in 1071 men aged 20–87 years; then, the analysis was repeated in 745 men aged 60 years and older.

Epidemiological questionnaire

All men responded to an interviewer-administered questionnaire. Men self-reported lifestyle factors: smoking (current smoker vs nonsmoker), alcohol intake (average amount of alcohol consumed weekly), and calcium intake (estimated by a food frequency questionnaire) (22). Comorbidities (ischemic heart disease, hypertension, diabetes mellitus, Parkinson's disease,

peripheral artery disease, and stroke) were self-reported, dichotomized (yes/no), and not further ascertained. Medications (statins, diuretics, vitamin K antagonists, and glucocorticoids) were self-reported, verified using medical prescriptions, whenever possible, and dichotomized (yes/no). Weight and height were measured using standard clinical equipment.

Serum measurements

Non-fasting serum was collected at 0100 h and stored at -80 °C. Total myostatin serum levels were measured by a commercially available competitive ELISA (Immundiagnostik AG, Bensheim, Germany) based on polyclonal antibodies raised against recombinant human myostatin in rabbit (23). The recombinant protein used for immunization is the full-length myostatin, including the propeptide and the C-terminal mature myostatin. The antibodies of the assay detect all subunits and the protein dimer as validated by western blot analysis and peptide mapping. There is no cross reactivity to GDF-11 or myoglobin. The free peptide in the sample binds to antibodies during a first incubation phase. The western blot analysis of the sera of Holstein cow (normal myostatin processing) and of Belgian Blue cow (premature stop codon of the myostatin gene) showed that our assay measures the 25 kDa dimer of active mature myostatin and a 50 kDa monomeric full-length promyostatin (16). The pre-incubated samples are transferred into microtiter wells coated with recombinant myostatin. The unbound antibodies in the pre-incubated samples bind to the immobilized antigen and are detected using a peroxidase-conjugated secondary antibody. Samples were measured using a microtiter plate reader at 450 nm against 620 nm as a reference. The detection limit is 0.273 mg/l. The intra- and interassay coefficients of variance were 10 and 15% respectively.

Serum levels of calcium, phosphate, and creatinine were measured using standard methods as described previously (24). 25OHD was measured with a RIA after acetonitrile extraction (DiaSorin, Stillwater, MN, USA) (24). Serum osteoprotegerin (OPG) was measured by ELISA (Biomedica, Vienna, Austria) (25). Fibroblast growth factor 23 (FGF23) was measured by ELISA (Biomedica, Vienna, Austria) as described previously (26). High-sensitivity C-reactive protein (hsCRP) was measured by an immunoturbidimetric assay (Cobas; Roche Diagnostics) (27). Testosterone was measured with a RIA after diethylether extraction; sex hormone-binding globulin was measured using an IRMA ([125]ISBP Coatria; Bio-Mérieux, Marcy l'Etoile, France) (28). Apparent free testosterone concentration (AFTC) was calculated as previously described (29).

Assessment of the AAC score

DXA was performed using the Hologic Discovery A device equipped with a rotatory C-arm (Hologic, Inc.,

Waltham, MA, USA). Lateral single-energy scans of the spine were obtained in the dorsal decubitus position (Fig. 1). The AAC score was assessed using a semiquantitative method (30). Calcifications in the abdominal aorta adjacent to the first four lumbar vertebrae were assessed for the posterior and anterior aortic walls using the midpoint of the intervertebral space above and below the vertebra as boundaries (eight segments). Individual severity scores for each segment (0–3) were added to yield AAC scores ranging from 0 to 24. Intra- and inter-rater reproducibility was assessed previously by two experienced readers for 76 scans (31). Intra- and inter-rater agreement (intraclass correlation coefficient) for AAC score (continuous scale) was 0.95 (95% confidence interval (95% CI), 0.91–0.99) and 0.90 (95% CI, 0.86–0.94) respectively. Intra- and inter-rater agreement (κ) for AAC score >0 vs 0 (a cut-point used in the current study) was $\kappa = 0.96$ (95% CI, 0.91–1.00) and $\kappa = 0.88$ (95% CI, 0.75–0.99) respectively.

Statistical analysis

Serum levels of total myostatin and hsCRP were log-transformed. Bivariate comparisons were performed using t-test (continuous variables) or χ^2-test (class variables). Serum myostatin (dependent variable) was compared across the classes of independent variables (AAC score >0 vs 0, comorbidities yes/no, quartiles of serum calcium or hsCRP) using the backward multivariable analysis of covariance. Variables with $P < 0.1$ were retained in the final model: age; BMI; intake of

Figure 1 Lateral scans of the first four vertebrae of the lumbar spine (L1–L4) obtained using the vertebral fracture assessment (VFA) software using the DXA technique on the Hologic Discovery A device. The left panel presents a scan obtained in a young adult man who has no aortic calcification (AAC score=0; A). The right panel presents a scan obtained in an older man with severe aortic calcification (AAC score=14; B). The dotted lines show the boundaries of the segment of the abdominal aorta which is assessed for the presence of the aortic calcification.

calcium, caffeine, and ethanol; smoking; Parkinson's disease; serum 25OHD; season; creatinine clearance; as well as serum hsCRP (except when used as the independent variable) and calcium (except when used as the independent variable).

Bivariate comparison between the presence of AAC and quartiles of myostatin levels was performed using the χ^2-test. The association between the presence of AAC (AAC score >0 vs 0) and the serum myostatin concentration (continuous, quartiles) was assessed by logistic regression adjusted for the confounders. Variables with P value <0.1 were retained in the final multivariable model (age, BMI, intake of calcium, caffeine and ethanol, smoking, diabetes mellitus, ischemic heart disease, hypertension, serum levels of calcium, and hsCRP). Testosterone and AFTC, 25OHD serum levels, creatinine clearance, the presence of Parkinson's disease, and concurrent medications were not significant ($P > 0.15$) and not retained in any of the above models.

Results

Description of the cohort

Table 1 presents the description of the cohort. Average age was 63 years and average weight was 79 kg. ACC score varied from 0 to 22 with highly skewed distribution (median, 0; interquartile range, 0–2). Five hundred and seventy-three men (53%) had an AAC score of 0, 251 (23%) had an AAC score of 1–2, 145 (14%) had an AAC score of 3–6, and 102 (10%) had an AAC score of >6 (examples for different AAC scores in Fig. 1).

Analysis of the potential confounders

Men who had AAC (AAC score >0) were older (71 vs 56 years; $P < 0.001$), lighter (78 vs 80 kg; $P < 0.001$), and shorter (168 vs 172 cm; $P < 0.001$). AAC was more prevalent in men with ischemic heart disease (73 vs 43%; $P < 0.001$), hypertension (64 vs 39%; $P < 0.001$), or diabetes mellitus (64 vs 45%; $P < 0.05$) compared with men without these characteristics. Men with AAC had higher OPG levels (median, 3.94 vs 3.26; $P < 0.001$), lower GFR (75.2 vs 83.6 ml/min; $P < 0.001$), and lower AFTC (244 vs 270 pmol/l; $P < 0.001$), as well as similar 25OHD (21 vs 22 ng/ml; $P < 0.41$) as compared with men without AAC.

Total myostatin levels did not differ between men who did or did not self-report ischemic heart disease, myocardial infarction, Parkinson's disease, history of stroke, hypertension, peripheral artery disease, or diabetes mellitus ($P > 0.30$ after adjustment for confounders). The analysis of other potential confounders (fat mass, smoking, and hormones) in the same cohort was described recently (16).
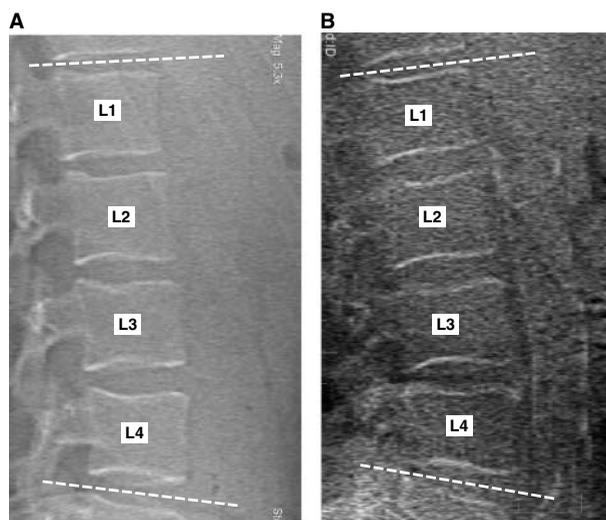
Table 1 Descriptive characteristics of the cohort ($n=1071$). Values are presented as mean$\pm$s.d.

Parameter	
Age (years)	63 ± 17
Weight (kg)	79 ± 11
Height (cm)	170 ± 7
BMI (kg/m^2)	27.2 ± 3.6
Current smokers[a]	117 (11%)
Coffee intake[b] (cups/week)	9 (6; 17)
Alcohol intake[b] (g/week)	69 (14; 192)
Calcium intake[b] (mg/day)	750 (600; 940)
Ischemic heart disease[a]	128 (12%)
Hypertension[a]	328 (30%)
Diabetes mellitus[a]	113 (10%)
Parkinson's disease[a]	14 (2%)
Statins[a]	304 (28%)
Diuretics[a]	79 (8%)
Vitamin K antagonists[a]	35 (3%)
Oral glucocorticoids[a]	10 (1%)
Abdominal aortic calcification score[b]	0 (0; 2)
Calcium (mmol/l)	2.37 ± 0.16
Phosphate (mmol/l)	1.07 ± 0.16
25-Hydroxycholecalciferol (ng/ml)	22 ± 10
Parathyroid hormone (pg/ml)	41 (32; 54)
Apparent free testosterone concentration (pmol/l)	258 ± 99
C-reactive protein[b] (mg/l)	1.46 (0.73; 2.88)
Myostatin[b] (mg/l)	29.7 (22.9; 38.4)
Osteoprotegerin[b] (pmol/l)	3.56 (2.84; 4.52)
Fibroblast growth factor 23[b] (RU/ml)	23.1 (17.3; 30.3)
Glomerular filtration rate (ml/min)	80 ± 18

[a]Number and percentage.
[b]Median (first quartile; third quartile).

Serum myostatin increased across quartiles of serum calcium ($P<0.001$ for trend; Fig. 2A). Serum myostatin was 2.8% lower in the fourth hsCRP quartile vs the three lower quartiles combined (0.22 s.d., $P<0.005$; Fig. 2B). By contrast, serum myostatin was not associated with serum levels of factors involved in vascular calcification, such as OPG, FGF23, phosphate, or the calcium$\times$phosphate product regardless of the statistical approach ($P>0.15$). Similar trends were found when the analyses were limited to 745 men aged 60 and older.

Association between AAC score and serum myostatin levels

In bivariate analysis, men who had AAC (AAC score >0) had lower total myostatin levels than men who did not have (medians, 29.0 vs 30.3 mg/l; Kruskal–Wallis test; $P<0.05$). However, the difference became non-significant after adjustment for confounders ($P=0.33$). Distribution of the AAC score >0 across the myostatin quartiles was as follows: 50, 49, 51, 39%; $P<0.05$. AAC prevalence was lower in the highest quartile compared with the three lower quartiles combined ($P<0.005$). After adjustment for confounders (the above plus diabetes mellitus, ischemic heart disease, and hypertension), odds of AAC >0 decreased with

increasing myostatin levels slightly but not significantly (OR$=0.88$/s.d.; 95% CI, 0.76–1.02). In the multi-variable analysis of AAC across the quartiles of myostatin levels, odds of AAC were not different for
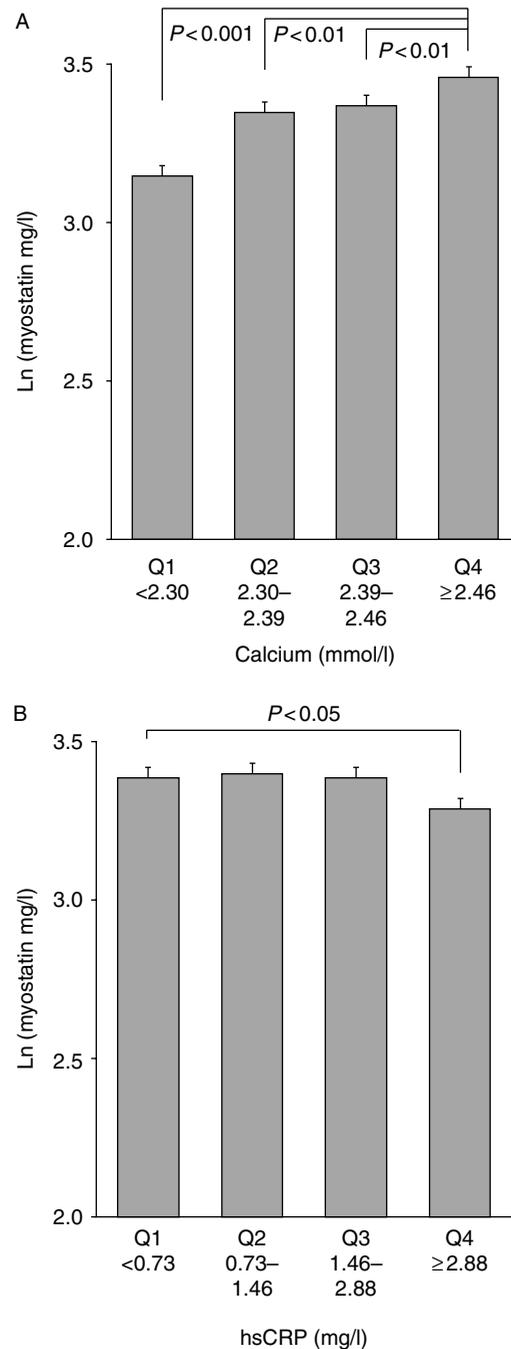

Figure 2 Average log-transformed serum total myostatin concentrations according to the quartiles of serum calcium level (A) and of serum high-sensitivity C-reactive protein (hsCRP) level (B). Data presented as multi-adjusted mean$\pm$s.e.m. Multivariable models adjusted for age, BMI, intake of calcium, caffeine and ethanol, smoking, Parkinson's disease, serum 25-hydroxycholecalciferol (25OHD), season, creatinine clearance, as well as serum hsCRP (for serum calcium), and serum calcium (for hsCRP).

the second and third quartile compared with the lowest myostatin quartile (OR=0.97; 95% CI, 0.66–1.43 and OR=1.06; 95% CI, 0.71–1.56 respectively; Fig. 3A). By contrast, odds of AAC prevalence were lower for the upper myostatin quartile (OR=0.62 vs the lowest quartile; 95% CI, 0.42–0.92; $P<0.005$). Odds of AAC prevalence were lower for the fourth myostatin quartile vs the three lower quartiles combined (OR=0.62; 95% CI, 0.45–0.85; $P<0.005$). AFTC and 25OHD were not significant and not retained in the final model regardless of how they were introduced (continuous, various thresholds).

In the analysis limited to 745 men aged ≥ 60 years, total myostatin levels were lower in men with AAC score >0 compared with men without AAC (30.8 vs 28.5 mg/l; $P<0.005$). The difference remained significant after adjustment for confounders ($P<0.05$).
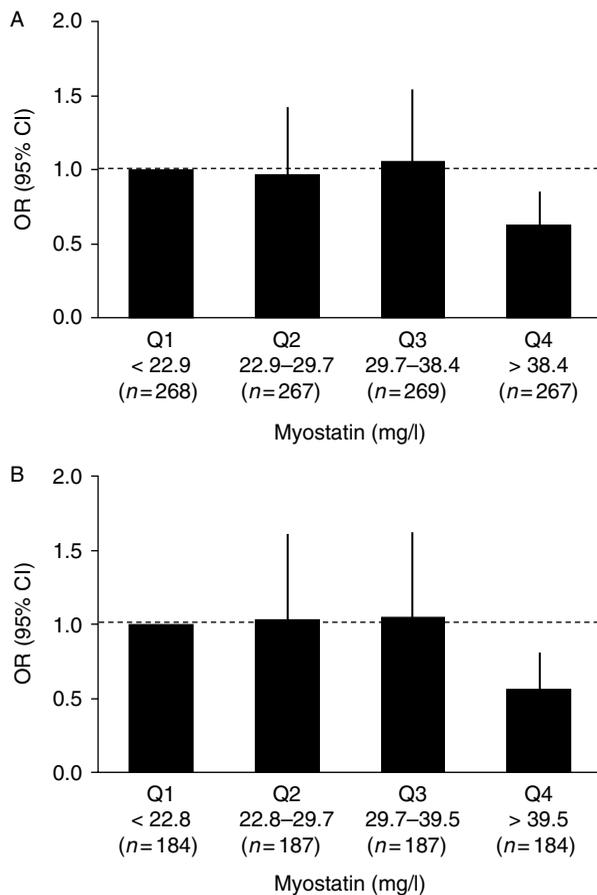

Figure 3 Multi-adjusted odds (OR) and 95% confidence interval (95% CI) for the presence of the abdominal aortic calcification (AAC score >0) according to the quartiles of serum total myostatin levels (reference, lowest quartile). Multivariate model adjusted for age; BMI; intake of calcium, caffeine, and ethanol; smoking, Parkinson's disease; diabetes mellitus; ischemic heart disease; hypertension; serum levels of calcium, 25-hydroxycholecalciferol (25OHD), and high-sensitivity C-reactive protein (hsCRP); season; and creatinine clearance. (A) entire cohort ($n=1071$), and (B) sub-cohort of men aged 60 years and older ($n=745$).

Distribution of the AAC score >0 across the myostatin quartiles was as follows: 62, 63, 66, and 46% ($P=0.001$). Multi-adjusted logistic regression provided the results similar to those found in the entire cohort. Odds of AAC >0 decreased with increasing myostatin levels (OR=0.81/s.d.; 95% CI, 0.69–0.95; $P<0.01$). Odds of AAC were not different for the second and third quartiles compared with the lowest myostatin quartile (OR=1.03; 95% CI, 0.67–1.57 and OR=1.05; 95% CI, 0.68–1.62 respectively; Fig. 3B). By contrast, odds of AAC prevalence were lower for the upper myostatin quartile (OR=0.56 vs the lowest quartile; 95% CI, 0.36–0.86; $P<0.001$). Odds of AAC prevalence were lower for the fourth myostatin quartile vs the three lower quartiles combined (OR=0.54; 95% CI, 0.38–0.78; $P<0.001$).

Discussion

Vascular calcification mimics bone remodeling at sites of the body normally not ossifying. Mineralization of the vasculature is associated with an excess risk of cardiovascular events due to an increase in arterial stiffness and pulse wave velocity (32, 33). Different factors including a mineral imbalance promote vascular calcification, whereas endogenous local or circulating calcification inhibitors suppress vascular calcification by blocking the deposition of minerals in the vascular wall or by the transformation of vascular smooth muscle cells (VSMC) to osteochondrogenic cells (34).

We used an immunoassay which does not distinguish between the active C-terminal dimer of myostatin and the N-terminal propeptide. Therefore, total myostatin serum levels were higher than those found using immunoassays specific for the active C-terminal dimer (18, 35). In our study, total myostatin levels correlated negatively with fat mass, mainly central fat mass, but not with lean mass or grip strength (16). Thus, the levels that we found may reflect synthesis and secretion rate of myostatin, and not its biological activity.

In this study, older men with AAC had lower serum myostatin levels, whereas higher myostatin levels were associated with lower AAC prevalence. Our cross-sectional study cannot explain the mechanism underlying this association, but the inverse association between serum myostatin and AAC may reflect the effect of factors regulating AAC and/or counter-regulatory mechanisms. For instance, smoking is associated with higher risk of AAC and higher myostatin expression (3, 36). Higher fat mass and lower lean mass are associated with higher risk of AAC (37). Intriguingly, myostatin is a strong regulator of muscle mass and fat accumulation (2). High extracellular calcium levels promote VSMC calcification and are present in atherosclerotic lesions (38), and we show here that myostatin levels positively correlated with serum calcium. In addition, high fat mass,

especially central fat mass, was associated with lower myostatin concentration and higher AAC severity (16, 39). However, the association between AAC and myostatin levels remained significant after adjustment for smoking, fat mass, and serum calcium levels. In contrast, myostatin was negatively associated with hsCRP, a parameter directly correlated with the risk for coronary artery disease and arterial stiffness (40). However, mice with double myostatin and LDL receptor knockout had fewer atherosclerotic lesions (5). It suggests that inactivation of myostatin rather protects against atherogenesis; however, the mechanisms involved in atherogenesis are different from those responsible for the calcification in large arteries.

As mentioned above, low 25OHD levels were previously associated with greater AAC severity and lower myostatin levels, whereas testosterone administration inhibited vascular calcification and increased circulating myostatin (14, 15, 17, 18). In this study, the association between AAC and myostatin remained significant even after adjustment for 25OHD and testosterone. AAC are more frequent in diabetic patients; however, the investigated association remained significant after adjustment for diabetes. Moreover, data on myostatin secretion in experimental diabetes are discordant, whereas a recent clinical study showed that diabetic patients had an increased expression of the myostatin gene, but plasma myostatin concentrations similar to nondiabetic controls (41, 42, 43).

Biomarkers may reveal novel pathways linked to vascular calcification and can provide additional information to conventional risk factors. Lower levels of calcification inhibitors (fetuin-A, matrix GLA protein) were observed in vascular diseases, whereas OPG levels were elevated (44, 45, 46). So far, myostatin has not been implicated in mineral homeostasis crucial for the pathophysiology of vascular calcification. However, bone marrow-derived MSCs obtained from myostatin-null mice exhibited increased osteogenic differentiation compared with cells from wild-type mice (13), and myostatin expression increased transiently after fracture (47). Myostatin treatment reduced type II collagen synthesis and expression of Sox9 mRNA, a transcription factor for chondrocyte differentiation (48). Thus, it is tempting to speculate that myostatin may promote the differentiation of MSC or MSC-derived cells into osteoblastic cells, an important step in the initiation of AAC.

From our data, it is speculative to attribute that myostatin has a calcification inhibitory or promoting role. The same cytokine networks involved in the pathophysiological development of AAC may also impinge on circulating myostatin, without a causal but rather a passive relationship. The fact that inactivation of myostatin has been reported to protect against atherosclerosis may be due to the fact that atherosclerosis (as seen in myostatin knock-out mice) is pathophysiologically distinct from vascular calcification, which we measured in our study. Alternatively, lowering myostatin may be an attempt of the vascular system to downregulate the expression of procalcific factors, or AAC formation itself is a stimulus for downregulating myostatin as a feedback mechanism to limit vascular disease. Myostatin secretion may also decrease after the development of AAC. AAC may be associated with calcification in other large arteries leading to lower blood flow and hypoxia in muscles. Indeed, chronic restriction of blood flow resulted in a drop in the myostatin content in rats (49). However, myostatin upregulation was associated with the skeletal muscle response to hypoxia-mimicking agents *in vitro* (50).

Our study has potential limitations. One limitation is inherent to the cross-sectional design of a study. Moreover, we have not assessed inhibitors of myostatin such as follistatin. The binding of myostatin to plasma proteins, which interferes with measurement of circulating myostatin could, in principle, be prevented by acidification (18, 51). However, our antiserum loses affinity at acidic pH – a phenomenon that has also been reported by Lakshman *et al.* for most of their myostatin antibodies (16, 18). Finally, the immunoassay that we used measures all the myostatin forms and does not exclusively reflect the expression of active myostatin in tissues.

In conclusion, men with higher total myostatin levels have lower AAC prevalence independently of potential confounders. Further studies are needed to validate these findings in other cohorts. In addition, the mechanisms underlying this inverse association and its potential clinical implictions require further investigation.

Declaration of interest

Funding

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