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Plasma cystatin B association with abdominal aortic aneurysms and need for later surgical repair - a substudy from the VIVA trial

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Abstract

Objective—The development of abdominal aortic aneurysm (AAA) involves extensive extracellular matrix remodeling, leading to aortic wall weakening. This process is mediated by proteases including cysteinyl cathepsins. Cystatins are their endogenous inhibitors. This study tested whether plasma cystatin B levels in AAA patients differed from those of healthy controls.

Methods—Plasma samples from AAA and age-matched controls were selected from the Viborg Vascular (VIVA) screening trial for AAA. ELISA determined plasma cystatin B. T-test, logistic regression, Pearson's correlation, and Cox regression tested whether plasma cystatin B correlates with AAA size and growth rate, or serves as a marker for AAA.

Results—Plasma cystatin B levels were significantly higher in AAA patients than in controls (P<0.0001). Logistic regression analysis showed that cystatin B tertile at baseline associated with the presence of AAA before (odds ratio [OR]=1.656, P<0.0001) and after adjustment for PAD, COPD, and previous ischemic events (OR=1.526, P<0.0001). T-test showed significant association of cystatin B with peripheral aortic disease (PAD) at screening, hospital diagnosis of chronic obstructive pulmonary disease (COPD), previous atherosclerotic events, and use of low doses of aspirin. Pearson's correlation test showed positive and significant associations between cystatin B and AAA size (r=0.15, P<0.001). Cox regression test showed that plasma cystatin B tertile at baseline associated with later AAA surgical repair before (hazard ratio [HR]=1.387, P<0.0001) and after adjustment for PAD, COPD, previous ischemic event, and maximal infrarenal aortic diameter (HR=1.523, P<0.0001).

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Conclusion—In contrast to prior studies that showed that cystatin C associates negatively with AAA development, this study demonstrated a positive association of cystatin B with AAA sizes and associations of cystatin B tertile at baseline with AAA presence and need for later surgical repair. It is possible that these two cystatins inhibit cathepsin activity and participate in AAA with different mechanisms.

Keywords

cystatin B; cystatin C; abdominal aortic aneurysm; VIVA screening trial

INTRODUCTION

Cysteinyl cathepsins participate importantly in the pathogenesis of abdominal aortic aneurysm (AAA). Immunohistochemical analysis demonstrated elevated expression of cathepsins B, K, L, and S in macrophages, lymphocytes, luminal and microvessel endothelial cells (ECs), and smooth muscle cells (SMCs) in human AAA lesions.[1–4] Yet, their dominant endogenous inhibitor cystatin C is relatively deficient.[1,4] Human plasma cystatin C levels correlate negatively with AAA size and annual expansion rate,[5,6] and such association persists after adjusting for renal function, smoking, diastolic blood pressure, C-reactive protein, age, and AAA size.[6] In atherosclerosis-prone apolipoprotein E (ApoE)-deficient *Apoe*$^{-/-}$ mice, cystatin C-deficiency enhanced diet-induced atherosclerosis, associated thoracic and abdominal aorta dilation, and angiotensin-II perfusion-induced AAA.[7,8]

Similar to cystatin C, cystatin B (also called stefin B) together with cystatin A (or stefin A) is also a single-chain small molecule that forms complexes with cysteinyl cathepsins.[9] While cystatin A is expressed in immune follicular dendritic cells,[10] cystatin B is more widely expressed in the cytoplasma of most human cells. There is an increased expression of cystatin B in a variety of human malignant tumors, in lipopolysaccharide (LPS)-activated human blood monocytes, and in interferon-γ (IFN-γ)-induced mouse macrophages.[11,12] Cystatin B-deficiency in mice or loss-of-function mutation in humans results in neurological dysfunction as a form of epilepsy.[13,14] Relative to cystatin C, many fewer studies have been done to test the potential involvement of cystatin B in cardiovascular diseases (CVD). From 389 patients who developed coronary events and 409 age- and sex-matched controls from the Malmo Diet and Cancer cardiovascular cohort, plasma cathepsin L (CatL) and cystatin B levels were significantly higher in patients with coronary events, and associated with prevalent diabetes. The hazardous ratio (HR) for the incident of coronary events comparing the highest and lowest tertiles of cystatin B was 1.29 (95% CI: 1.01–1.57) after adjusting for age, sex, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride, body mass index (BMI), hypertension, and glucose, although such association becomes insignificant after additional adjustment for smoking.[15] This study contradicts to most of studies from cystatin C, which plays a protective role in CVD. It remains unknown whether the expression and activity of cystatin B also differ in AAA patients or differ from cystatin C.[1,4–8] Analysis of plasma cystatin B in AAA patients may help understand whether and how this inhibitor is involved in human AAA and design the next phase of studies.

In this study, we measured plasma cystatin B levels from 551 men with AAA and 198 age-matched healthy controls from the Viborg Vascular (VIVA) screening trial for AAA[16–18] and tested whether plasma cystatin B associates with AAA size, growth rate, presence of AAA, or need for later surgical repair of this fatal human aortic disease.

MATERIALS AND METHODS

The VIVA trial background

The VIVA screening trial is a randomized, clinically controlled study designed to evaluate the benefits of vascular screening and modern vascular prophylaxis in a population of 50,000 men aged 65–74 years.[16] A combined screening program for AAA, peripheral artery disease (PAD), and hypertension was offered at local hospitals. Participants with positive test results were offered secondary prophylaxis and/or referred to their general practitioner. The program set-up included decentralized screening by three mobile teams at 14 venues. Enrollment started October 2008 and ended January 2011. Diagnostic criteria included an aortic diameter of at least 30 mm for AAA, ankle brachial blood pressure index (ABI) below 0.9 or above 1.4 for PAD, and blood pressure above 160/100 mmHg for hypertension.[17] Chronic obstructive pulmonary disease (COPD) was defined by hospital-recorded diagnosis from the national patient registry, and familial disposition to AAA was defined as a first-degree sibling with AAA. AAA sizes greater than 50 mm were referred for vascular surgical evaluation, and those below were invited annually to a control scan to check for expansion. After one year, cases with PAD were also invited for surveillance to check for ABI and optimize preventive actions. Controls were chosen as consecutive, healthy, and age-matched male attenders. Plasma and DNA samples were taken from cases with AAA, PAD, and 200 healthy controls at baseline for biobanking. The nationwide registry-based follow-ups were performed after 5 years.[18] The trial is approved by the scientific ethical committee of the Mid region of Denmark (M20080018) and registered in the Clinical trials register (NCT00662480). The Brigham and Women's Hospital Human Research Committee approved the use of de-coded patient information (2010P001930/BWH).

Designs and outcome measures

A case-control design using cases of AAA and healthy controls was used to evaluate cystatin B association with AAA. In addition, a prospective cohort design was used to investigate the potential association between cystatin B levels and annual aneurysm growth rate and need for surgical repair.

Patients

Overall, 18,749 men (uptake 74.7%) attended the screening. An AAA was diagnosed in 615 cases (3.3% [95% CI: 3.0% to 3.6%]), PAD in 2,043 (10.9% [95% CI: 10.5% to 11.0%]), and potential undiagnosed hypertension in 1,969 (10.5% [95% CI: 10.0% to 10.9%]).[17] Lipid-lowering and/or antiplatelet treatment was initiated among 34.8% of all participants. Five-year follow-up data were available concerning all outcome variables.[18]

Plasma cystatin B measurement

Plasma samples from 551 AAA patients and 198 age-matched controls were used.[5,19,20] Plasma cystatin B levels were determined blindly using the human cystatin B ELISA Kit from MyBioSource (Cat# MBS761966, San Diego, CA), according to the manufacturer's instructions. This kit was based on a sandwich enzyme-linked, immune-sorbent assay technology. The detection range was 0.156–10 ng/ml. Plasma samples were diluted by 1:200 (i.e. 5 (μl of sample into 995 (μl of sample/standard dilution buffer). The standard, plasma sample, and biotin-conjugated detection antibody were added to each well subsequently. The absorbance was read in a microplate reader at a 450-nm wavelength, and the cystatin B concentrations were calculated based on the standard curve.

Statistical analyses

Binary responses were coded 0=no and 1= yes. Normality was tested using Shapiro-Wilks test, q-q plots, and histograms. Mean is given with standard deviation (SD), and differences between cases and controls in exposures of interest were assessed by student's t-test. Pearsson's correlation coefficient was used to study the association between cystatin B and maximal aortic diameter, lowest ankle brachial index, and aneurysmal growth rate. AAA growth rates were calculated based upon the ultrasound scans from the baseline and annual follow-up visits using individual linear regression between sizes and time. Logistic regression analysis was used to identify whether the tertiles of circulating cystatin B levels was independently associated with AAA or PAD compared to the controls adjusted for potential confounders identified in the univariate analyses by a p-value below 0.10. Multiple linear regression analyses was used to identify whether the exposure of interest was independently associated with maximal aortic diameter adjusted for potential confounders identified in the univariate analyses by a p-value below 0.10. Finally, we constructed a multivariate Cox proportional hazard model to study whether the tertile level of cystatin B at baseline associated with needing surgical repair or death. The model was adjusted for potential confounders identified in the univariate analyses by a p-value below 0.10. The residuals of the regression analyses were plotted in histograms and q-q plots to test for normal distribution. The statistical tests were defined in priori. SPSS 21.0 and STATA/SE 13.1 for windows were used as statistical package.

Power calculation

When the sampling occurred, the hypothesis about an association between cystatin B and AAA had not yet been formulated. However, based upon our previous study,[17] we assumed if the mean of cystatin B is 0.83 and SD is 0.103,[21] the smallest detectable difference between AAA and controls is ±0.037 corresponding to below 5% of the mean at 1% significance level and 99% power, suggesting a very robust study concerning the comparison between controls and AAA. We could not find any reasonable assumptions to perform a power calculation concerning aneurysmal growth rates and need for later AAA repair.

RESULTS

VIVA screening trial background

From the 18,749 men who attended the VIVA screening trial, including 615 men with AAA, the proportion of patients with familiar disposition to AAA, current smoking, hypertension, previous stroke, previous acute myocardial infarction (AMI), PAD by screening, previous atherosclerotic events, use of low-dose aspirin, and use of statin were all significantly higher in patients with AAA than those without AAA. The proportions of having hospital-diagnosed COPD and use of glucocorticoid were also higher in AAA patients than in those without AAA. As expected, the proportions of patients with diabetes mellitus were significantly lower in AAA patients than in those without AAA (Table 1).

Association of plasma cystatin B tertile at baseline with the presence of AAA

Crude logistic regression analysis showed that the presence of AAA increased by 65.6% per tertile of plasma cystatin B at baseline (unadjusted odds ratio [OR]=1.656 [95% C.I.: 1.341; 2.045], $P<0.001$). After adjusting for PAD at screening, outpatient or hospital-recorded COPD, and previous schemic event, the presence of AAA still increased by 52.6% per tertile of plasma cystatin B at baseline (adjusted OR=1.526 [95% C.I.: 1.224; 1.901], $P<0.001$) (Table 2).

Plasma cystatin B level associates with AAA size

The plasma cystatin B levels were significantly higher in patients with AAA than those in age-matched controls (251.6±90.2 ng/mL *vs.* 220.3±64.7 ng/mL, mean±SD, P=2.49E-8) (Figure 1A). Among the dichotomous clinical characteristics, a t-test showed that plasma cystatin B levels did not associate with familiar disposition to AAA, current smoking, hypertension, previous stroke, previous AMI, use of statin, and use of glucocorticoid. Yet, plasma cystatin B levels associated significantly with PAD by screening, hospital-diagnosed COPD, previous atherosclerotic events, and use of low dose aspirin (Table 3). From the continuous clinical variables, Pearson's correlation test showed that plasma cystatin B associated with only maximal infrarenal aortic diameter (r=0.15, $P<0.001$) (Figure 1B), but not with age, BMI, systolic and diastolic blood pressures, lowest ABI, and AAA growth rate (Table 3).

Association of plasma cystatin B tertile at baseline with the need for late surgical repair of AAA

Crude Cox regression analysis showed that after a median follow-up of 4.85 years, the need for surgical repair of AAA increased by 38.7% per tertile of plasma cystatin B at baseline (unadjusted hazard ratio [HR]=1.387 [95% C.I.: 1.157, 1.662], $P<0.001$). After adjusting for outpatient or hospital-recorded COPD, previous schemic event, PAD at screening, and maximal infrarenal aortic diameter, the need for surgical repair of AAA increaed by 52.3% per tertile of plasma cystatin B at baseline (adjusted HR=1.523 [95% C.I.: 1.251, 1.854], $P<0.001$) (Figure 2 and Table 4).

DISCUSSION

The presented case-control and cohort studies demonstrated that the circulating level of cystatin B and its tertile at baseline associated with the presence and natural history of AAA (AAA size change and need for later repair during follow up). Selection bias seems unlikely, as the study group included attendees in a population-based screening trial with a relative high attendance rate (74.7%).[18] Information bias seems also unlikely, as the ultrasound-based measurement of aorta was performed by a standardised and validated method showing high precision,[22] and information of the need for later AAA repair was based upon nationwide registry data, where all AAA repair procedures were recorded due to law and reimbursement.[23] Consequently, all had follow-up without missing data. The analyses were adjusted for known potential risk factors for AAA and the progression of AAA, yet by nature there will always be a risk of residual confounding. These conditions and the fact that the analyses were performed with a power of 90% at a 5% significance level make the novel finding of cystatin B as associated with AAA quite robust, but replication and mechanistic studies are needed for a final conclusion. Observations from this study differ from what were reported regarding cystatin C in AAA.[1,4–6] Using the same samples, we reported negative correlations between plasma cystatin C with AAA size and with AAA growth rate.[5,6] AAA growth has never been linear. Because the plasma samples were collected at registration, it is always possible that the correlation between plasma cystatin B and AAA size may change over time during the course of AAA growth and rupture. Plasma cystatin B levels in patients with fast-growing AAA may differ from those with relatively slow-growing or stablized AAA at follow-up years, even their measurments at registration may be comparable. Yet, our median follow-up of 4.85 years has not allowed us to see such differences.

A direct role of cystatin B in AAA or in other CVD has never been tested. Only limited information remains available regarding cystatin B in CVD. Plasma cystatin B levels have correlated with coronary events[15] and body adiposity.[24] In a community-based cohort of 170 8–11 year-old children, plasma cystatin B correlated positively with total body fat, abdominal fat, and pulse pressure, but negatively with maximal oxygen uptake, all of which are surrogate markers for CVD.[24] However, plasma cystatin B has been broadly tested in cancer patients with mixed conclusions depending on the type of cancer. In patients with epithelial ovarian cancer,[25–27] the bladder cancer transitional cell carcinoma,[28] and laryngeal carcinoma,[29] tissue cystatin B was overexpressed and correlated with tumor burden and disease recurrence. In patients with colorectal cancer, plasma cystatin B level correlated with survival. Patients with high levels of cystatin B had high risk of death.[30] Yet, in human lung cancer[31] and squamous cell carcinoma of the head and neck,[32] tissue cystatin B correlated inversely with tumor burden, although it showed no influence on the probability of patient survival. One early study showed relatively higher prevalence of breast, colon, gastric, lung, lymphoma, prostate, renal, and urinary cancers in 298 patients with AAA than in 151 patients with atherosclerotic occlusive disease ($P<0.014$).[33] A more recent study showed that patients with AAA had increased risk of developing lung and other cancers.[34] There is no report showing that patients with one type of cancer a had a higher risk of AAA than those with other type of cancers. Therefore, it remains uncertain whether cystatin B links the risk between cancers and AAA.

Several major cathepsins, including cathepsins S, K, L, and C play essential roles in experimental AAA.[35] Increased cystatin B in AAA subjects might implicate a protective mechanism to minimize the damages from these cathepsins. Yet we do not know the source or the consequence of increased plasma cystatin B in AAA patients. It is possible that increased plasma cystatin B represents a mechanism of aortic tissue protection. Besides extracellular matrix protein degradation, cathepsins target multiple subcellular compartments. This hypothesis is supported by the finding that upon LPS stimulation cystatin B targets the mitochondria in macrophages. Macrophages from cystatin B-deficient mice showed increased amounts of caspase-1 and −11 for IL-1β processing due to increased mitochondrial membrane destabilization and superoxide generation.[36] Therefore, high levels of cystatin B in AAA patient plasma and possibly in AAA lesions may reduce IL-1β processing, thereby controlling lesion and systemic inflammation. Such speculation requires detailed studies at the molecular and cellular levels.

Together, this study reported an inverse association of plasma cystatin B with AAA size, and also demonstrated the associations between cystatin B tertile at baseline with the presence of human AAA and the need for future surgical repair. As we have recently summarized, cathepsins may play different roles in various subcellular compartments, such as lysosomes/endosomes, extracellular milieu, cell membrane, and nucleus.[35] Cystatins C and cystatin B may target cathepsins at different locations, thereby exerting different activities towards the development of AAA. All these hypotheses can be important topics in general cathepsin biology and AAA pathobiology, and merit future investigation.

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What this paper adds

Prior studies demonstrated cystatin C-deficiency in human abdominal aortic aneurysm (AAA) lesions and negative correlations between plasma cystatin C and human AAA size or growth rate. Both cystatin C and cystatin B are endogenous inhibitors of cysteinyl cathepsins, but this study revealed a positive association of plasma cystatin B with AAA size. This study also revealed associations of plasma cystatin B tertile level at baseline with AAA presence and the need for later surgical repair. Different associations of cystatin C and cystatin B with AAA size suggest their possible mechanistic differences in regulating cathepsin activity during the development of human AAA.

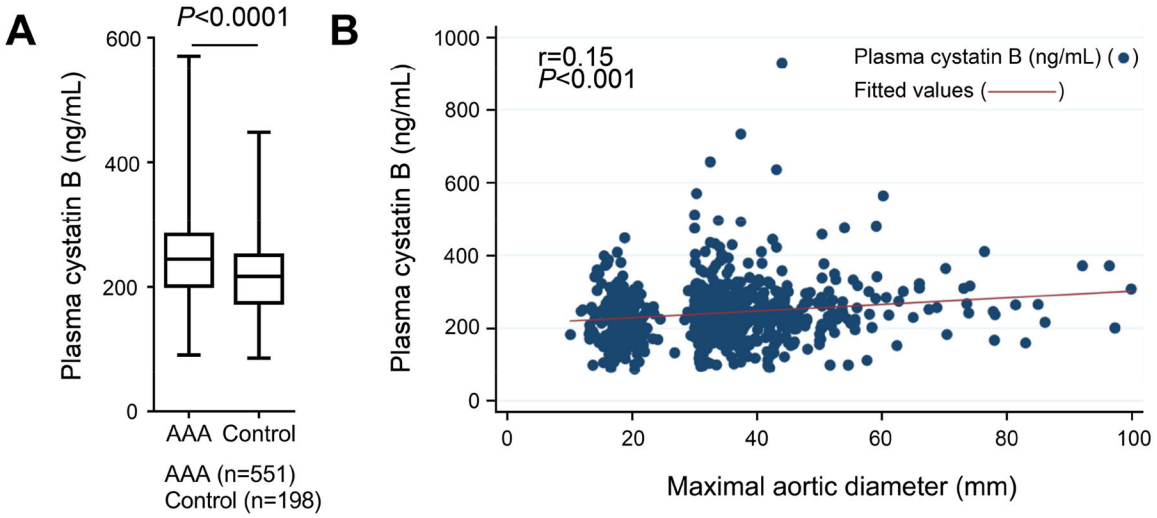

Figure 1.

Increased plasma cystatin B level in human AAA patients correlates with AAA sizes. **A**. ELISA revealed higher plasma cystatin B level in AAA patients (n=551) than that in age-matched controls (n=198). Data are mean±SD, *P*=2.49E-8. **B**. Pearson's correlation analysis of plasma cystatin B *versus* maximal aortic diameters in mm.

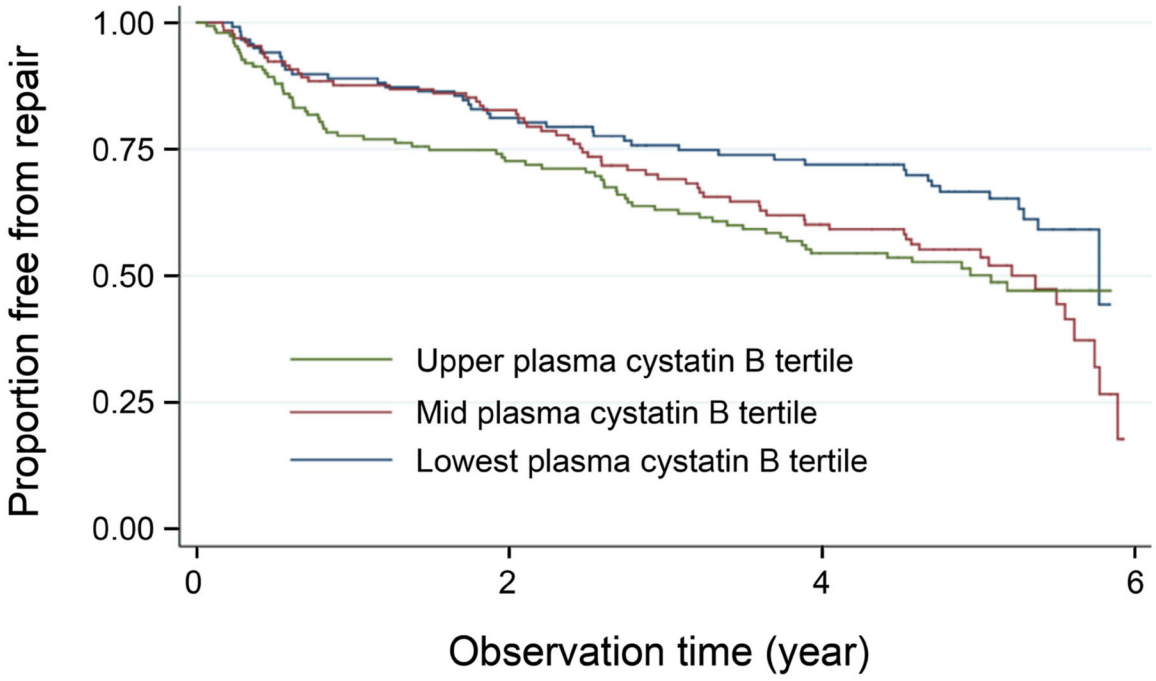

Figure 2.

Cox regression analyses of cystatin B tertiles for proportion of free from late surgical repairs of AAA over the follow-up years. Crude HR=1.387 [95% C.I.: 1.157, 1.662], $P<0.001$. Adjusted HR=1.523 [95% C.I.: 1.251, 1.854], $P<0.001$.

Table 1.

Expected differences in clinical characteristics between AAA patients and controls.

Clinical characteristics	No AAA		AAA		P
	N*	Proportion	N*	Proportion	
Familial disposition to AAA	18074	0.024	615	0.037	0.045
Current smoking	18042	0.020	615	0.413	0.000
Diabetes mellitus	18041	0.109	615	0.110	0.905
Hypertension	18001	0.421	615	0.547	0.000
Previous stroke	18074	0.024	615	0.037	0.045
Previous acute myocardial infarction	18074	0.025	615	0.063	0.000
PAD by screening	18041	0.104	613	0.271	0.004
COPD	18074	0.024	615	0.042	0.059
Previous atherosclerotic event	18074	0.127	615	0.225	0.000
Use of low dose aspirin	17599	0.323	613	0.507	0.000
Use of statins	17599	0.350	613	0.541	0.000
Use of glucocorticoid	16961	0.062	613	0.080	0.077

*Some patients (No AAA and AAA) had missing information for the listed clinical characteristics.

Table 2.

Crude and adjusted logistic regression analyses of cystatin B tertile at baseline and associations with the presence of AAA.

Unadjusted	**Odds Ratio**	**Std. Err.**	**P Value**	**95% C.I.**	
Cystatin B tertiles	1.656	0.178	0.000	1.341	2.045
Constant	1.439	0.181	0.004	1.125	1.841

Adjusted	**Odds Ratio**	**Std. Err.**	**P Value**	**95% C.I.**	
Cystatin B tertiles	1.526	0.171	0.000	1.224	1.901
PAD at screening	7.066	2.686	0.000	3.354	14.885
Outpatient or hospital-recorded COPD	3.603	2.753	0.093	.806	16.111
Previous ischemic event	2.046	0.549	0.008	1.208	3.463
Constant	1.066	0.146	0.642	.815	1.393

Table 3.

Association between plasma cystatin B and dichotomous clinical characteristics (t-test) and continuous clinical variables (Pearson's correlation).

Dichotomous clinical characteristics	No		Yes		P
	N	Mean (SD)	N	Mean SD	
Familial disposition to AAA	617	241.8 (85.7)	37	234.8 (61.4)	0.645
Current smoking	469	238.0 (83.2)	228	248.3 (86.3)	0.236
Diabetes mellitus	579	240.4 (85.1)	77	250.6 (79.0)	0.227
Hypertension	329	236.5 (80.1)	324	247.3 (88.5)	0.219
Previous stroke	637	241.2 (85.2)	20	253.6 (52.0)	0.175
Previous acute myocardial infarction	625	242.1 (85.2)	32	232.0 (65.8)	0.837
PAD at screening	528	237.5 (84.3)	123	256.8 (82.5)	0.004
COPD	636	240.7 (84.3)	21	267.9 (83.5)	0.059
Previous atherosclerotic event	536	239.0 (83.6)	121	253.3 (87.2)	0.049
Use of low dose aspirin	392	233.0 (70.1)	260	253.9 (101.6)	0.031
Use of statins	345	236.5 (76.4)	306	246.9 (93.0)	0.300
Use of glucocorticoid	589	239.1 (80.5)	52	252.5 (76.5)	0.157

Continuous clinical variables	Age	BMI	Systolic blood pressure	Diastolic blood pressure	Aorta size	Lowest ABI	AAA growth rate
R	0.06	0.01	−0.02	−0.02	0.15	−0.12	−0.02
P-value	0.12	0.84	0.99	0.99	<0.001	0.10	0.11

Table 4.

Crude and adjusted Cox regression analyses of cystatin B tertile at baseline and associations with the need for later surgical repair of AAA.

Unadjusted	Hazard Ratio	Std. Err.	P value	95% C.I.	
Cystatin B tertiles	1.387	0.128	0.000	1.157	1.662

Adjusted	Hazard Ratio	Std. Err.	P value	95% C.I.	
Cystatin B tertiles	1.523	0.153	0.000	1.251	1.854
Outpatient or hospital-recorded COPD	1.455	0.504	0.279	0.738	2.868
Previous ischemic event	0.951	0.191	0.802	0.642	1.409
PAD at screening	0.723	0.141	0.097	0.493	1.061
Maximal infrarenal aortic diameter	1.082	0.005	0.000	1.071	1.092