RESEARCH ARTICLE

Inflammatory markers associated with abdominal aortic aneurysm

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ABSTRACT. ***Purpose:*** **Inflammation with leukocytic infiltration, degradation of extracellular matrix (ECM), and depletion of vascular smooth muscle cells (VSMC) are pathological hallmarks of abdominal aortic aneurysm (AAA). The aim of this study was to further evaluate relationships between AAA and inflammatory biomarkers, interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), endothelin-1 (ET-1) and soluble urokinase-type plasminogen activator receptor (suPAR), by comparing levels in 65-year-old men with and without AAA at ultrasound screening. We also evaluated whether any biomarker can independently predict AAA at screening, and clarified potential correlations between aortic diameter and blood levels of these biomarkers.** ***Results:*** **There were significant ($p \leq 0.05$) differences between subjects with and without AAA for the following variables: p-leukocyte count (TLC) ($p<0.001$), p-homocysteine ($p<0.001$), p-TNF-α ($p = 0.023$), p-IL-6 ($p<0.001$), p-ET-1 ($p = 0.002$), p-suPAR ($p<0.001$), ankle brachial index (ABI) ($p<0.001$), plasma (p)-creatinine ($p = 0.049$), p-total cholesterol ($p<0.001$), p-high density lipoprotein (HDL) ($p<0.001$) and low density lipoprotein (LDL) cholesterol ($p = 0.001$), smoking habits ($p<0.001$), and use of antihypertensive ($p<0.001$) and lipid-lowering ($p = 0.001$) drugs. When the above variables were stepwise excluded in a logistic regression model, only p-IL-6 ($p = 0.002$), p-homocysteine ($p = 0.015$), p-HDL ($p = 0.004$), ABI in the right ($p = 0.005$) and left ($p = 0.094$) leg, smoking habits ($p = 0.003$), and antihypertensive drug use ($p = 0.045$), differed between groups. Significant correlations with aortic diameter existed for p-TNF-α ($p = 0.028$), p-IL-6 ($p<0.001$), p-ET-1 ($p = 0.002$) and p-suPAR ($p<0.001$) in the entire study population, and for p-TNF-α ($p = 0.023$), p-ET-1 ($p = 0.009$) and p-suPAR ($p = 0.001$) among men with AAA.** ***Conclusions:*** **Several inflammatory biomarkers were significantly elevated and correlated with aortic diameter among 65-year old men with AAA at ultrasound screening. IL-6, homocysteine and use of antihypertensive medication remained elevated in the logistic regression model, together with known risk markers for AAA such as smoking and signs of atherosclerosis.**

Key words: abdominal aortic aneurysm, biomarkers, IL-6, TNF-alpha, ET-1, SuPAR

BACKGROUND

The pathological hallmarks of abdominal aortic aneurysm (AAA, defined as a permanent, focal widening of the abdominal aortic diameter of ≥30 mm [1]) are, inflammation with leukocytic infiltration, degradation of extracellular matrix (ECM), and depletion of vascular smooth muscle cells (VSMC).

During the last two decades, research on the pathogenesis of AAA has been focused on abnormal inflammation and immune reactions [2]. Several studies have reported a correlation between increased levels of inflammatory mediators, such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α, and AAA formation and expansion [2-4]. IL-6 and TNF-α are involved in the accumulation of immunoglobulins and neovascularisation of the aneurysm wall [3]. Higher serum levels of IL-6 and TNF-α have been found in patients with AAA compared to both healthy controls and patients with coronary heart disease [3]. Another biomarker proposed to be associated with AAA diameter is the endothelial-derived vasoconstrictor peptide endothelin (ET)-1 [4]. ET-1 has a growth promoting effect on the vessel wall, and serum levels are elevated in patients during aneurysm rupture or dissection [4]. ET-1 also potentiates production of IL-6 and stimulates muscle cell mitogenesis [5]. However, the importance of ET-1 for AAA growth has not yet been fully clarified [6]. A novel biomarker associated with AAA development is soluble urokinase-type plasminogen activator receptor (suPAR) [5], formed when the inflammatory mediator glycosylphosphatidylinositol phospholipase D (GPI-PLD) cleaves the anchor of the urokinase plasminogen activator receptor (uPAR) [5]. SuPAR is a marker for low grade inflammation and is involved in tissue remodelling [5]. It is also associated with elevated risks for cardiovascular disease, cancer and type 2 diabetes mellitus [5].

The risk of AAA rupture correlates with aortic diameter [7-9], and rupture is often the first symptom of an AAA [7]. As rupture is associated with a mortality rate of 75%, in Sweden, 65-year-old men are subjected to screening for AAA with ultrasonography. Screening-detected AAA are kept under ultrasound surveillance at regular intervals if not

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immediately repaired [10]. If an AAA is ≥55 mm, shows extensive growth, or is symptomatic, the patient is referred for surgical intervention [1, 10]. Aneurysm growth is exponential, but with great variability between individuals [7]. Identification of biomarkers related to aortic diameter and AAA growth might help identify patients in need of extra surveillance or earlier surgical intervention [2]. A biomarker associated with AAA might be used as a complement to ultrasound in screening. In addition, biomarkers linked to aneurysm diameter might help identify patients with a higher risk of aneurysm expansion, rapid growth and rupture. At present however, no biomarkers have sufficiently high sensitivity and specificity to be used in clinical practice [11].

OBJECTIVE

The primary aim of this study was to further evaluate relationships between AAA and inflammatory biomarkers, IL-6, TNF-α, ET-1 and suPAR in 65-year-old men with and without AAA. Secondary aims were to evaluate whether any biomarkers could independently predict AAA, and to investigate potential correlations between aortic diameter and blood levels of these biomarkers.

MATERIALS AND METHODS

Study design

A cross-sectional study.

Materials

All men who are over 65 and from the city of Malmö and 15 neighbouring municipalities are invited to AAA ultrasound screening with at the Department of Vascular Diseases, Skåne University Hospital, Malmö. Over the period 2010-2014, 200(1.6%) of 12714 men examined were found to have AAA, out of whom 116 (58%) accepted to undergo physical examination, blood sampling, and medical history reporting. As a control group, we invited 239 randomly-selected, screened 65-year-old men with normal aortic diameter.

Methods

The following variables were analysed: aortic diameter, systolic (S), and diastolic (D) blood pressure (BP), ankle brachial index (ABI) in both legs, use of antihypertensive and lipid-lowering drugs, plasma (p)-creatinine, p-total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, p-triglycerides (TG), p-haemoglobin (Hb), p-total leukocyte count (TLC), p-platelet count (PC), p-glucose, p-homocysteine, p-ET-1, p-TNF-α, p-IL-6, p-suPAR and smoking habits. Furthermore, smoking habits were categorised into three groups; current, previous, and never smokers.

Ultrasound examinations were performed by biomedical scientists and registered nurses using the LOGICe (General Electric Healthcare Inc, Chalfont S. Giles, UK). The maximal infrarenal anteroposterior diameter of the aorta was evaluated, and an AAA was defined as an aortic diameter of ≥30 mm, using the leading-edge to leading-edge (LELE) technique. In seven cases (1%) where ultrasound was not conclusive, subjects were referred to a conventional computer tomography (CT) scan without contrast.

Blood samples were centrifuged at 4°C. Routine laboratory markers were analysed immediately at the Department of Clinical Chemistry, Skåne University Hospital, Malmö (SWEDAC approved according to European norm 45001). For the non-routine biomarkers: p-ET-1, p-TNF-α, p-IL-6 and p-suPAR samples were frozen at -80°C and the analyses were performed successively at the Wallenberg Laboratory, Skåne University Hospital, Malmö. P-TNF-α and p-IL-6 levels were analysed with commercially available enzyme-linked immunosorbent assay (ELISA) test kits (R&D Systems, Inc, MIN, USA). The minimum detectable dose (MDD) was 0.106 pg/mL for p-TNF-α and 0.7 pg/mL for p-IL-6. The intra-assay coefficient of variability (CV) and inter-assay CV were 8.5% and 10.6% for p-TNF-α and 4.2% and 6.4% for p-IL-6 respectively. P-ET-1 was analysed with an ELISA test kit (R&D Systems, Inc, MIN, USA) with a MDD of 0.087 pg/mL and intra- and inter-assay CVs of 4.0% and 7.6%. P-suPAR levels were evaluated with the suPARnostic® ELISA kit (ViroGates, Birkeröd, Denmark). The detection limit was 0.1 ng/mL and the intra- and inter-assay CVs were 5.3% and 3.5%. For non-routine markers where blood levels deviated more than two standard deviations (SD), samples were re-analysed.

Statistical analyses

Statistical calculations were done in SPSS® (SPSS Inc, IBM, New York, USA) version 20. P-values <0.05 were considered significant. All variables were compared with univariate analysis between subjects with and without AAA. The Mann-Whitney U test was used for quantitative variables and the chi square test was used for nominal variables. Quantitative variables were expressed as the median, and the lower and upper quartiles. Nominal variables were stated as frequencies and percentages. Variables with a significant difference in univariate analysis were included in a multivariate analysis; **logistic regression, and stepwise excluded** until all remaining variables had a p-value of <0.1. Spearman's rank correlation coefficient test was used to evaluate correlations between aortic diameter and inflammatory markers. Sensitivity, specificity and the receiver-operating characteristic (ROC) curve were calculated to evaluate screening potential for some of the markers.

Ethical considerations

All subjects gave written consent to participate in this study, and it was approved by the Ethics Committee of Lund University (2010/239).

RESULTS

Study compliance

Of the 355 men included in the study (116 with AAA and 239 without), all 355 underwent physical examination, 316 completed the health questionnaire, and 343 underwent blood sample analysis.

Table 1
Clinical characteristics in 65-year-old men with and without abdominal aortic aneurysm (AAA) at ultrasound examination of the abdominal aorta. (Median [lower and upper quartiles]).

Variables	All subjects (n = 355)	Without AAA (n = 239)	With AAA (n = 116)	P-value
Systolic blood pressure (mmHg)	143 (134-157)	143 (134-158)	143 (133-151)	0.342
Diastolic blood pressure (mmHg)	85 (80-91)	85 (80-91)	85 (80-91)	0.553
Ankle brachial index- right	1.08 (1.01-1.15)	1.10 (1.03-1.17)	1.04 (0.95-1.11)	<0.001
Ankle brachial index-left	1.08 (1.00-1.14)	1.09 (1.01-1.15)	1.04 (0.95-1.11)	<0.001
p-creatinine (μmol/L)	85 (76-95)	83 (76-92)	88 (78-98)	0.049
p-cholesterol (mmol/L)	5.2 (4.3-5.9)	5.3 (4.5-5.9)	4.6 (3.9-5.8)	<0.001
p-TG (mmol/L)	1.6 (1.1-2.4)	1.5 (1.0-2.3)	1.6 (1.2-2.8)	0.065
p-HDL (mmol/L)	1.3 (1.1-1.6)	1.4 (1.2-1.6)	1.2 (0.9-1.4)	<0.001
p-LDL (mmol/L)	3.2 (2.5-4.0)	3.3 (2.7-4.1)	2.8 (2.2-3.9)	0.001
p-Hb (g/L)	148 (141-155)	148 (141-155)	147 (141-155)	0.801
p-TLC (10^9/L)	6.5 (5.6-7.5)	6.2 (5.2-7.1)	7.2 (6.2-8.7)	<0.001
p-PC (10^9/L)	216 (189-252)	215 (188-252)	220 (190-253)	0.518
p-glucose (mmol/L)	5.6 (5.2-6.4)	5.6 (5.1-6.4)	5.8 (5.3-6.6)	0.064
p-homocysteine (μmol/L)	14 (12-17)	14 (12-16)	16 (13-19)	<0.001
p-TNF-α (pg/mL)	0.98 (0.77-1.38)	0.96 (0.76-1.25)	1.11 (0.82-1.62)	0.023
p-IL-6 (pg/mL)	1.84 (1.09-3.44)	1.53 (0.89-2.51)	3.16 (1.66-5.98)	<0.001
p-ET-1 (pg/mL)	1.04 (0.82-1.36)	0.99 (0.80-1.32)	1.14 (0.93-1.48)	0.002
p-suPAR (pg/mL)	3.63 (2.94-4.49)	3.37 (2.82-4.12)	4.28 (3.49-5.13)	<0.001

n, number; *BP,* blood pressure; *ABI,* ankle brachial index; *TG,* triglycerides; *HDL,* high-density lipoprotein; *LDL,* low-density lipoprotein; *Hb,* haemoglobin; *TLC,* total leukocyte count; *PC,* platelet count; *TNF*-α, tumour necrosis factor-α; *IL-6,* interleukin-6; *ET-1*, endothelin-1; *suPAR,* soluble urokinase-type plasminogen activator receptor; p, plasma

Univariate analysis

When comparing subjects with and without AAA, significant differences were found for the following variables: ABI in both right (p<0.001) and left (p<0.001) legs, p-creatinine (p = 0.049), p-total cholesterol (p<0.001), p-HDL (p<0.001) and LDL (p = 0.001) cholesterol, p-TLC (p<0.001), p-homocysteine (p<0.001), p-TNF-α (p = 0.023), p-IL-6 (p<0.001), p-ET-1 (p = 0.002), p-suPAR (p<0.001), smoking habits (p<0.001), and use of antihypertensive (p<0.001) and lipid-lowering (p = 0.001) drugs (*tables 1-2*).

Logistic regression analysis

When the 15 variables that differed significantly between men with and without AAA were entered into a **logistic regression model and stepwise excluded**, the following variables were found to differ between groups in a multivariate analysis: ABI in the right (p = 0.005) and left (p = 0.094) leg, p-HDL cholesterol (p = 0.004), p-IL-6 (p = 0.002), p-homocysteine (p = 0.015), smoking habits (p = 0.003), and antihypertensive drug use (p = 0.045).

Correlations

Among all study subjects, significant correlations with aortic diameter were found for p-TNF-α (r_s = 0.126; p = 0.028), p-IL-6 (r_s = 0.364; p<0.001), p-ET-1 (r_s = 0.168); p = 0.002) and p-suPAR (r_s = 0.297; p<0.001). In men with AAA, p-TNF-α (r_s = 0.228; p = 0.023), p-ET-1 (r_s = 0.248; p = 0.009) and p-suPAR (r_s = 0.322; p = 0.001) correlated significantly with aortic diameter (*table 3*).

IL-6 as a screening marker for AAA

A receiver-operating characteristic (ROC) curve for p-IL-6 and AAA showed an area under the curve (AUC) of 0.730 (standard error [SE] 0.029, 95% confidence interval [CI] 0.674-0.786, p<0.001) (*figure 1*).

Table 2
Smoking habits and drug use in 65-year-old men with and without abdominal aortic aneurysm (AAA) at ultrasound examination of the abdominal aorta. (N[%]).

Variables	All subjects (n = 355)	Without AAA (n = 239)	With AAA (n = 116)	P-value
Smoking habits				
Current/Previous/Never	70 (20%)/169 (48%)/89 (25%)	31 (13%)/112 (47%)/83 (35%)	39 (34%)/48 (41%)/6 (5%)	<0.001
Antihypertensive drug use				
Yes/No	160 (45%)/121 (34%)	96 (40%)/98 (41%)	64 (55%)/23 (20%)	<0.001
Lipid-lowering drug use				
Yes/No	116 (33%)/164 (46%)	67 (28%)/126 (53%)	49 (42%)/38 (33%)	0.001

n, number

Table 3
Spearman's rank correlation coefficient () of different variables with different aortic diameters in 65-year-old men undergoing ultrasound examination of the abdominal aorta.

Variables	All subjects (n = 352)	Subjects with an aortic diameter of <25 mm (n = 228)	Subjects with an aortic diameter of 25-29 mm (n = 9)	Subjects with AAA (n = 115)
p-TNF-α (pg/mL)	r_s = 0.126 (p = 0.028)	r_s = 0.005 (p = 0.945)	r_s = -0.536 (p = 0.215)	r_s = 0.228 (p = 0.023)
p-IL-6 (pg/mL)	r_s = 0.364 (p<0.001)	r_s = -0.094 (p = 0.163)	r_s = -0.186 (p = 0.631)	r_s = 0.179 (p = 0.063)
p-ET-1 (pg/mL)	r_s = 0.168 (p = 0.002)	r_s = -0.061 (p = 0.365)	r_s = 0.203 (p = 0.600)	r_s = 0.248 (p = 0.009)
p-suPAR (pg/mL)	r_s = 0.297 (p<0.001)	r_s = -0.073, (p = 0.281)	r_s = 0.017 (p = 0.965)	r_s = 0.322 (p = 0.001)

n, number; *AAA,* abdominal aortic aneurysm; *TNF-α,* tumour necrosis factor-α; *IL-6,* interleukin-6; *ET-1*, endothelin-1; *suPAR,* soluble urokinase-type plasminogen activator receptor; p, plasma.

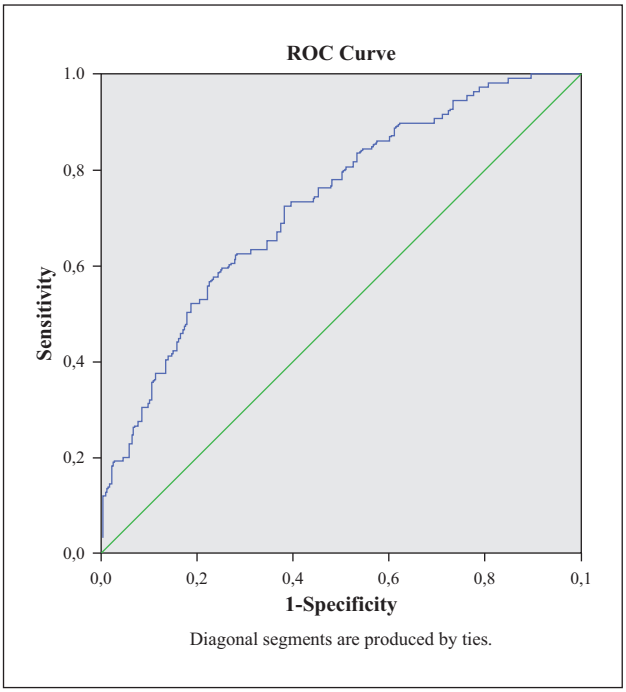

Figure 1
Receiving-operating curve (ROC) for interleukin-6 as a screening marker for abdominal aortic aneurysm.

DISCUSSION

Several variables differed between men with and without AAA. P-creatinine, p-TLC, p-homocysteine, p-TNF-α, p-IL-6, p-ET-1, and p-suPAR were all higher, whereas ABI, p-cholesterol, p-HDL and p-LDL were lower among men with AAA. Use of antihypertensive and lipid-lowering drugs, as well as smoking history, were also more common among subjects with AAA. These results are confirmatory in relation to previous data [1-5, 11], however, it should be noted that the logistic regression analysis revealed that significant differences remained only for ABI, p-HDL cholesterol, p-homocysteine, p-IL-6, use of antihypertensive drugs, and smoking habits. It is not surprising that significant differences for several variables in univariate analysis were lost in logistic regression analysis, as several variables are strongly influenced by each other.

The fact that levels of all inflammatory biomarkers analysed were significantly higher in men with AAA in univariate analysis corroborates that the notion that inflammation plays an important role for development and growth of AAA [2, 3, 12]. However, p-IL-6 was the only independent biomarker for AAA. P-IL-6 has previously been shown to be elevated in AAA patients compared to controls [3, 13, 14]. Dowson *et al*. showed that when blood samples were collected from the entire length of the aorta in subjects with and without AAA, higher plasma levels of IL-6 were found closer to the aneurysm [13]. These findings support the hypothesis that IL-6 is produced and secreted into the circulation from aneurysm tissue [13]. Furthermore, the IL-6 signalling pathway might be involved in AAA development and expansion, and the IL-6 receptor (IL-6R) might be a possible target for pharmacological intervention in AAA subjects [15]. Our findings are somewhat inconclusive with regard to the correlation between aortic diameter and IL-6, as we saw a positive correlation in the entire study population, but no correlation in subgroups with and without AAA. IL-6 has previously been reported to increase with aneurysm size in some studies, whereas other studies reported no correlation between p-IL-6 and aneurysm diameter or growth rate [2, 14, 16]. Plasma levels of TNF-α, ET-1 and suPAR correlated with aortic diameter both in the entire population and among men with AAA. Higher levels of TNF-α have been found in AAA tissue compared to normal aortic tissue [17], and decreased levels of TNF-α in patients with large AAAs, suggesting the possibility that TNF-α might be able to be used as a marker for AAA development [18]. We found a positive correlation with aortic diameter among men with AAA, however, and other studies report that no evidence for a correlation of TNF-α to different growth rates of AAAs [6]. ET-1 has previously been dismissed as a marker for AAA growth or rupture, and there is a paucity of research with regard to suPAR [6]. Whereas Lindqvist *et al*. reported no correlation between aortic diameter and suPAR [19], but our findings suggest otherwise, highlighting the need for further evaluation. Of all the inflammatory biomarkers we set out to investigate, IL-6 seems to be the most important one as regard to AAAs. It is still not specific enough to be a contender to ultrasound screening, as determined from the ROC-curve however: further research is warranted to clarify its importance in AAA pathogenesis before it can be used in clinical practice.

HDL-cholesterol was the only independent lipid marker. This might be attributed to the fact that statin treatment, which mainly lowers LDL [20], was more common among men with (42%) compared to men without (28%) AAA, or might perhaps indicate a true association between HDL-cholesterol and AAA. Low levels of HDL-cholesterol have previously been found among subjects with AAA, and high levels have been proposed to decrease the risk of

AAA development [21]. Differences in levels of homocysteine, a non-protein amino acid, also remained significant in the logistic regression model. Homocysteine has previously been associated with with vascular incidents such as stroke and myocardial infarction [22], whereas reports on its relation to AAA are somewhat inconclusive [22].
Since smoking is the most important risk factor for the development of AAA [23, 24], and as men with atherosclerotic peripheral vascular disease have a greater incidence of AAA [25, 26], it is not surprising that significant differences between groups existed concerning these variables. Whereas there was no association between SBP or DBP and AAA in the univariate analysis, we found a significant association between use of antihypertensive drugs and AAA in the multivariate model. Similar associations suggesting that patients using antihypertensive drugs run an increased risk of developing AAA have been previously reported [27]. Subjects with high BP are more likely use antihypertensive drugs, and successful antihypertensive treatment might mask any potential relationship between AAA and BP, making an apparent association appear between AAA and antihypertensive drug use instead [27].

Limitations

This study has several limitations. It is a cross-sectional study and all measurements were performed only once, without prospective follow-up. Therefore, we have no possibility of drawing conclusions about aneurysm growth. Furthermore, the conclusions are only valid for 65-year-old men since we have no measurements from other age groups or from females. In addition, we only have self-reported data on medication and smoking habits from the health questionnaire. There is always a risk of reporting false significances when performing multiple comparisons. However, we tried to minimize this risk by using multivariate analysis in addition to the univariate tests.

CONCLUSION

Several inflammatory biomarkers were significantly elevated and correlated with aortic diameter among 65-year-old men undergoing ultrasound screening for AAA. In a logistic regression model, men with AAA showed higher levels of IL-6 and homocysteine, and more often used antihypertensive medication in addition to a higher prevalence of established risk markers for AAA such as smoking and atherosclerosis.

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