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The associations of biomarkers of inflammation and extracellular matrix degradation with the risk of abdominal aortic aneurysm: the ARIC Study

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Abstract

Animal and human laboratory studies suggest that the pathogenesis of abdominal aortic aneurysms (AAA) involves inflammation and the degradation and remodeling of the extracellular matrix. This study prospectively assessed the association between biomarkers for these mechanisms and the presence of AAA during 24 years of follow-up in the Atherosclerosis Risk in Communities

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Author contribution

All authors contributed to: (1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, and, (3) final approval of the version to be published.

Declaration of Conflicting Interests

The Authors declare that there is no conflict of interest.

(ARIC) study. ARIC prospectively identified clinically-diagnosed AAAs in 15,792 men and women from baseline in 1987-1989 to 2011 using hospital discharge codes and death records. Additional asymptomatic AAAs were detected by an abdominal ultrasound scan in 2011-2013. Matrix metalloproteinase (MMP)-3, MMP-9, interleukin-6 (IL-6), N-terminal propeptide of Type III procollagen (PIIINP), and osteopontin were measured in blood samples collected between 1987 and 1992 in participants with AAA (544 clinically-diagnosed AAAs and 72 ultrasound-detected AAAs) and a random sample of 723 participants selected from baseline and matched with AAAs by age, race and sex. Higher concentrations of MMP-9 and IL-6 were associated with future risk of clinically-diagnosed AAA [hazard ratios (95% confidence intervals): 1.55 (1.22, 1.97) and 1.87 (1.48, 2.35), respectively, comparing highest versus lowest tertiles] after multivariable adjustment (p for trend <0.001). MMP-9 was also associated with ultrasound-detected AAA. In conclusion, blood concentrations of MMP-9 and IL-6 measured in middle age predicted the risk of AAA during 24-years of follow-up.

Keywords

abdominal aortic aneurysm; biomarkers; inflammation; extracellular matrix degradation; longitudinal study

INTRODUCTION

Abdominal aortic aneurysm (AAA) is characterized by progressive and irreversible dilatation of the aortic wall, and is associated with high mortality rate when it ruptures.[1,2] The prevalence of AAA increases with age and was reported to be 1-2% in women and 4-8% in men aged ≥65 years based on screening programs.[2] The etiology of AAA is incompletely understood. The key pathological feature of AAA is progressive degradation and remodeling of the extracellular matrix (i.e. elastin and collagen) in the aortic wall, which is largely mediated by matrix metalloproteinases (MMPs).[3,4] Animal and human studies document that the destructive remodeling of extracellular matrix in AAA is initiated and exacerbated by a sequence of events at the aortic wall, including activation of chronic inflammation, abnormal response of the innate or adaptive immune systems, and up-regulation of MMPs and other proteinases, which are accompanied by impaired compensatory repair of extracellular matrix.[3–7]

Established cardiovascular risk factors, including age, male sex, smoking, and increased low-density lipoprotein (LDL) or total cholesterol, contribute to the etiology of AAA,[2,8] possibly through these etiopathogenic pathways.[3,4,9,10] Several biomarkers in these pathways have been consistently reported to be elevated in the circulation of AAA patients. These biomarkers, which are involved in extracellular matrix degradation and remodeling, include MMP-3, MMP-9, and N-terminal propeptide of Type III procollagen (PIIINP); [11,12] and inflammatory biomarkers including interleukin-6 (IL-6) and osteopontin. All of these biomarker associations with AAA have been reported in cross-sectional case-control studies, [11,12] but there is no evidence from population-based prospective studies. Cross-sectional studies are not able to provide information on time sequence between the synthesis of biomarkers and occurrence of disease outcome, which is important for inferring causality.

As part of an research project grant (R01) study funded by the National Institutes of Health, we measured biomarkers of inflammation and extracellular matrix degradation and remodeling (i.e. MMP-3, MMP-9, IL-6, osteopontin and PIIINP) in plasma samples from middle-aged participants of the Atherosclerosis Risk in Communities (ARIC) Study, a large community-based cohort, and prospectively related their levels to the incidence of AAA during 24 years of follow-up.

MATERIALS AND METHODS

Study Population

ARIC is a population-based study investigating the risk factors for atherosclerosis and cardiovascular diseases in a cohort of 15,792 adults aged 45-64 years recruited in 1987-1989 from Forsyth County, North Carolina; Jackson, Mississippi (African Americans only); the northwest suburbs of Minneapolis, Minnesota; and Washington County, Maryland.[13] ARIC examined participants at Visit 1 in 1987-1989 and followed them by annual or semi-annual telephone contact and 4 additional re-examinations in 1990-1992 (Visit 2), 1993-1995 (Visit 3), 1996-1998 (Visit 4) and 2011-2013 (Visit 5). The study was approved by the institutional review board of all participating institutions (leading institution: University of Minnesota, IRB # 8412M01053). All participants provided written informed consent.

Ascertainment of AAAs

The overall design of the study is illustrated in Supplementary Figure I. As previously reported,[8] we prospectively ascertained incident, clinically-diagnosed AAAs by searching hospitalization and death records that were obtained after reports of interim hospitalizations and deaths identified from regular telephone calls with participants (or proxies). ARIC also conducted surveillance of local hospitals to identify additional hospitalizations or deaths. Moreover, for participants over 65 years we linked participants' identifiers with Medicare data from the Centers for Medicare and Medicaid Services (CMS) for 1991-2011, to find additional hospital or outpatient AAAs. We defined clinically-diagnosed AAAs as those who had a hospital discharge diagnosis of AAA from any of the above sources, or 2 Medicare outpatient claims that occurred at least 1 week apart, with *ICD-9-CM* codes of 441.3 or 441.4, or procedure codes of 38.44 or 39.71, or the following cause of death codes: *ICD-9* 441.3 or 441.4 or *ICD-10* code I71.3 or I71.4. AAAs based on procedure codes were required to be verified by diagnosis codes. Some of these clinical diagnosis codes could include asymptomatic AAAs that were medically documented. We treated thoracic, thoracoabdominal, or unspecified aortic aneurysms as non-events.

We conducted an abdominal ultrasound scan at the ARIC Visit 5 to identify additional asymptomatic AAAs in the survival ARIC cohort (Supplementary Figure I).[8] A radiologist with special vascular imaging expertise centrally trained cardiac ultrasonographers in the technique of abdominal aortic scanning. The abdominal aorta was defined for this study as the aorta from the level immediately below the superior mesenteric artery origin to the aortic bifurcation. After certification, the sonographers obtained transverse images of the abdominal aorta on which they made anterior-posterior and transverse diameter measurements. Imaging was carried out with a Philips iE33 high-resolution duplex scanner

and a Philips C5-1 transducer (Philips Healthcare, Bothell, WA) following a standardized protocol.[8] A fasting regimen (i.e. nothing by mouth) for 6 h was required before the ultrasound scan was performed. Rare exceptions were given if the participant had to eat before the ultrasound exam could be completed. Vascular imaging physicians over-read all images of abdominal aortas that had a ≥ 2.8 cm maximal infrarenal diameter or probable aortic pathology identified by sonographers, plus a 5% random sample of the remaining cohort. The correlation coefficient for the maximum infrarenal diameter was 0.92 between the physician readers and ultrasonographers. We defined asymptomatic AAA by a maximal infrarenal aortic diameter ≥3 cm.[1] Of 15,792 ARIC participants who were recruited at baseline, 10,036 participants were still alive through August 2013, 6,538 of them had a home or clinic ARIC examination and 5,911 of them had interpretable abdominal aortic ultrasonograms.

Case-cohort design

A case-cohort design was employed to prospectively investigate the role of the biomarkers (i.e. MMP-3, MMP-9, IL-6, osteopontin and PIIINP) measured in blood samples from Visit 1 (90% of samples) or Visit 2 (10% of samples) in predicting the subsequent risk of AAA through Visit 5 (Supplementary Figure I).

In the entire ARIC cohort, we ascertained 671 AAAs, including 596 incident, clinically-diagnosed AAAs identified between Visit 1 and the year 2011 during a median of 22.5 years of follow-up, and 75 additional asymptomatic AAAs detected by the ultrasound exam at Visit 5 in 2011-2013. We excluded 2 clinically-diagnosed AAAs who were diagnosed between Visits 1 and 2 and whose Visit 2 samples were used in the biomarker measurements; we further excluded 50 clinically-diagnosed and 3 ultrasound-detected AAAs, which occurred in individuals who were neither white nor African American or for which there was missing information on important covariates or blood samples. The final study included 616 AAAs, 544 of which were clinically-diagnosed AAAs and 72 were ultrasound-detected AAAs.

The subcohort comparison group was selected randomly from the entire ARIC cohort at baseline without regard to their AAA status during follow-up. Before the subcohort selection, we excluded participants with uncertain AAA status during follow-up (n = 29) or who were missing important covariates (n= 1,025). A total of 766 subcohort members were drawn from the eligible ARIC cohort within strata defined by race, gender, and baseline age (>55 vs. ≤ 55 years) so that the distribution of these variables was comparable between the AAA cases and those in the subcohort group. Of the 766 subcohort members, 43 did not have blood samples from Visits 1 and 2, leaving 723 participants in the subcohort group (including 43 with incident, clinically-diagnosed AAA and four with ultrasound-detected AAAs diagnosed during the follow-up). Of the 723 subcohort members, 227 had a Visit 5 ultrasound exam and thus were eligible to serve as reference for the 72 ultrasound-detected AAAs. Combining the final AAA cases and subcohort members resulted in a total of 1,292 non-overlapping participants in this study.

Measurement of traditional cardiovascular risk factors

At each visit ARIC measured cardiovascular risk factors and conditions including anthropometrics, behavior risk factors, history of physician-diagnosed cardiovascular conditions, and medication use. Details have been described elsewhere.[13] Briefly, ARIC staff measured weight and height with participants in scrub suits and took 3 blood pressure measurements with a random-zero sphygmomanometer. Pack-years of smoking were calculated for current and former smokers as the average number of cigarettes smoked per day multiplied by the years of smoking divided by 20. At each visit, blood samples were mostly drawn fasting from an antecubital vein. Based on standard protocols, the ARIC central laboratory measured plasma total cholesterol,[14] triglycerides,[15] and HDL cholesterol (HDL-C),[16] and calculated LDL cholesterol (LDL-C).[17]

Measurements of biomarkers

Using EDTA plasma samples that had been collected at Visit 1 (90% of samples) or Visit 2 (10% of samples) and stored unthawed at −70°C until analysis in 2014-2015, ARIC measured plasma concentrations of MMP-3, MMP-9, IL-6, osteopontin, and PIIINP of the AAA cases and subcohort members. Visit 2 samples were used for participants whose Visit 1 samples had been exhausted.

According to manufacturers' protocols, the ARIC laboratory measured MMP-3 and MMP-9 using a fluorokine multianalyte profiling assay (R&D Systems, Inc., Minneapolis, MN) on a Bio-plex 200 System (BioRad, Hercules, CA), IL-6 by Quantikine high-sensitivity sandwich ELISA (R&D Systems, Inc., Minneapolis, MN), osteopontin by Quantikine sandwich ELISA (R&D Systems, Inc., Minneapolis, MN), and PIIINP by sandwich ELISA (Cloud-Clone Corp., Houston, TX cat# SEA573Hu). The intra- and inter-assay coefficients of variations (CV) % for the ARIC samples are presented in Table 1. In addition, we analyzed repeatability of 58-59 blind duplicate pairs of ARIC Visit 1 samples split at the time of blood draw and stored until the lab assay of this study. The intraclass correlation coefficients for the duplicate pairs are reported in Table 1 as well.

Data analysis

Since some of the cardiovascular risk factors that were associated with the survival of the patients who had an ultrasound are also risk factors for AAA, analyzing the clinically-diagnosed AAAs and ultrasound-detected AAAs together might cause bias in the estimates of associations between risk factors and AAA. Therefore, we analyzed the associations between AAA and each of the biomarkers separately for clinically-diagnosed and ultrasound-detected AAAs and adjusted for the potential selection bias caused by differential attrition in the analysis of the ultrasound-detected AAA (see details in the analysis of ultrasound-detected AAAs below). The entire subcohort group served as the comparison for clinically-diagnosed AAAs and the subcohort members who attended the ultrasound exam at Visit 5 served as the comparison for ultrasound-detected AAAs. The corresponding visit for biomarker samples was treated as baseline.

A total of 1,224 participants (544 AAA cases and 723 subcohort members with 43 subjects overlapping in the two groups) were included in the clinically-diagnosed AAA analysis and

295 (72 AAA cases and 227 subcohort members with 4 overlapping) in the ultrasound-detected AAA analysis. In the analysis of each specific biomarker, we further excluded samples with missing or extreme values for the biomarker (>6SD from the mean; no samples had values <6SD from the mean): MMP-3 (n=6), MMP-9 (n=3), IL-6 (n=26), PIIINP (n=14) and osteopontin (n=30). We also conducted a sensitivity analysis to winsorize these extreme values rather than excluding them.

Association analyses of the biomarkers with clinically-diagnosed AAAs: We analyzed the time-to-event data using the Cox proportional hazard models and weighted each stratum of the subcohort group by the reciprocals of the sampling rates. To account for some participants being in both the case and subcohort groups, we applied the weighting method and robust variance estimation proposed for case-cohort studies by Barlow et al.[18,19] Subjects were categorized into tertiles of each biomarker based on the distribution in the subcohort group. We calculated hazard ratios (HR) and 95% confidence intervals (CI) of AAA in relation to the tertiles and tested trends by modeling tertile as an ordinal variable. The analysis was adjusted for age, sex, and combination of race-field center in a basic model (model 1) and further adjusted for the following traditional risk factors in model 2 based on our prior knowledge of risk factors for clinically-diagnosed AAA: height, pack-years of smoking, hypertension, diabetes, total cholesterol and HDL-C.[8] All of the covariates were measured at the same visit as the sample collection for the biomarkers. As cigarette smoking is a very strong risk factor for AAA, we also stratified the analyses in model 2 by smoking status (ever, never) and tested for multiplicative interactions of biomarkers by smoking.

Association analysis of the biomarkers with ultrasound-detected AAAs: We employed generalized linear model (GLM) to estimate the odds ratios (OR) and 95% CI for the association of ultrasound-detected AAA with each biomarker in tertiles. The Schouten *et al.* sandwich estimator of the covariance matrix for log(OR) was used to adjust for the overlap of some AAA cases among the subcohort members in the variance estimate for the log(OR).[20] Since a number of participants were lost to follow-up or died prior to the Visit 5 ultrasound exam, we used inverse probability of attrition weighting (IPAW), as previously described,[21] to adjust for the potential selection bias caused by differential attrition due to loss to follow-up. We first calculated the ultrasound attendance probability as the product of the probability of being alive at Visit 5 and the probability of having an abdominal ultrasound conditional on being alive given relevant covariates measured at baseline and during follow-up.[8,22] A composite weight was then derived as the product of the ultrasound attendance probability and the sampling fractions of cases and subcohort members. The regression coefficients and 95% CIs were obtained from GLMs using a logit link function. The covariance matrix was estimated using the sandwich estimator, and each subject was inverse weighted by the composite weight as described above. Similar to the analysis of clinically-diagnosed AAAs, we adjusted for covariates in models 1 and 2 based our prior knowledge on risk factors for ultrasound-detected AAA in ARIC[8] and tested for trend across tertiles of the biomarkers.

Secondary analyses of the biomarker-AAA associations: To evaluate if significant biomarker associations with AAA might have been confounded by mix of samples from

different visits and if the associations were independent of other cardiovascular risk factors (i.e. body mass index (BMI), waist circumference, peripheral arterial disease (PAD), coronary heart disease (CHD), and use of antihypertensive or lipid-lowering medications), and the previously reported biomarkers in ARIC,[22] we further adjusted for sample visit, these cardiovascular risk factors, and biomarkers in the following potential pathways for AAA:[22] (1) inflammation (white blood cell count (WBC) and fibrinogen measured in Visit 1 samples and high sensitivity C-reactive protein (CRP) in Visit 4 samples), (2) thrombin generation (D-dimer in Visit 3 samples), (3) cardiac injury (troponin T (cTnT) in Visit 4 samples), and, (4) myocardial stretch and vascular stiffness (*N*-terminal pro-brain natriuretic peptide (NT-proBNP) in Visit 4 samples). Since there was a varying degree of missing data for these biomarkers (n of missingness=6 to 345), we focused on effect sizes, instead of p-values, in assessing the influence of the adjustment on the biomarker-AAA associations.

To infer a possible role of the biomarkers in early versus late stage development of AAA, we re-ran the analysis of clinically-diagnosed AAAs after excluding AAAs who were diagnosed within 10 years of the baseline.

Risk prediction analysis for clinically-diagnosed AAAs: We calculated Harrel's C-index in Stata 15.1 to assess the predictive ability for clinically-diagnosed AAA in 3 risk factor models using the approach proposed by Sanderson et al. for case-cohort studies.[23] Prentice weights[24] were used to incorporate the contribution of non-subcohort cases. A robust jackknife estimator for standard error was implemented. The 3 risk factor models are: model 1—traditional risk factors: age, sex, race-field center, height, smoking pack-years, hypertension, diabetes, total cholesterol and HDL-C; model 2—variables in model 1 plus the significant biomarkers identified in this study (MMP-9, IL-6 and osteopontin); model 3—variables in model 2 plus the biomarkers previously reported in ARIC and measured at the same visit as the 3 significant biomarkers of this study: WBC, fibrinogen, antithrombin III and lipoprotein(a).[22,25]

RESULTS

As shown in Table 2, the participants who developed clinically-diagnosed AAA and those with ultrasound-detected AAA at Visit 5 had poorer cardiovascular risk factor profiles at baseline compared with their subcohort comparison groups. Furthermore, participants who developed clinically-diagnosed AAA tended to have poorer risk factor levels than participants with ultrasound-detected AAA. The median and mean (SD) times to event for clinically-diagnosed AAAs were 15.8 and 14.5 (5.6) years, respectively.

The associations between the biomarkers and incident, clinically-diagnosed AAA are shown in Table 3. After adjustment for age, sex, and race-field center in model 1, baseline MMP-9, IL-6, PIIINP, and osteopontin were significantly and positively associated with clinically-diagnosed AAA, with respective HRs (95% CI) of 1.82 (1.46, 2.27), 2.31 (1.84, 2.90), 1.27 (1.02, 1.60), and 1.36 (1.08, 1.70) for the highest versus lowest tertiles. Further adjustment for other AAA risk factors in model 2 moderately attenuated the associations, with those for MMP-9, IL-6, and osteopontin remaining statistically significant with a dose-response relationship. The smoking-stratified analyses included 475 and 69 AAAs in ever smokers

and never smokers, respectively. As shown in Supplementary Figure II, the associations of MMP-9, IL-6, and osteopontin with AAA were positive and significant for ever smokers, with the strength of the associations being similar to that in the whole sample. The associations for these biomarkers were weaker and not significant in never smokers (p for interaction with smoking status <0.0001 for MMP-9, IL-6 and osteopontin).

Of the biomarkers that were significantly associated with clinically-diagnosed AAA, MMP-9 was significantly and strongly associated with Visit 5 ultrasound-detected AAA, independent of age, sex, race, height, total cholesterol, triglycerides, and pack-years of smoking (Table 4). The associations of PIIINP and osteopontin with ultrasound-detected AAAs were similar to those with clinically-diagnosed AAA but were not statistically significant (Table 4), likely due to limited statistical power. IL-6 was clearly not associated with ultrasound-detected AAA.

Secondary analyses of the biomarker-AAA associations:

1) Winsorization of outliers (>6SD from the mean) rather than excluding them did not result in appreciable changes in the associations between the biomarkers and clinically-diagnosed AAA (Supplementary Table I). The winsorization transformation was not applicable to the ultrasound-detected AAA analysis because the outlier samples did not participate in the abdominal ultrasound exam. 2) Exclusion of 116 clinically-diagnosed AAAs who were diagnosed within 10 years of baseline did not materially change the associations for MMP-9 or IL-6, while the association for osteopontin was strongly attenuated (Supplementary Table II). 3) Additional adjustment for sample visit, BMI, waist circumference, PAD, CHD, or use of antihypertensive medication did not appreciably change the associations of most of the biomarkers with either clinically-diagnosed AAA or ultrasound-detected AAA (data not shown). The only exception was that adjustment for PAD or CHD modestly attenuated the association between osteopontin and clinically-diagnosed AAA so that it was no longer significant: HR associated with the highest tertile of osteopontin (p for trend) =1.22 (0.10) and 1.21 (0.11) after adjusting for PAD and CHD, respectively (Supplementary Table III). Adjustment for the use of lipid-lowering medication did not appreciably change any of the associations for clinically-diagnosed AAA. This variable was not adjusted for as an additional covariate in the ultrasound-detected AAA analysis because too few participants (n=6) were on lipid-lowering medications. 4) In the subsamples with data on WBC, fibrinogen, CRP, D-dimer, cTnT, or NT-proBNP, further adjustment for each of these biomarkers did not result in appreciable changes in the associations, with the exception of WBC adjustment on MMP-9 analyses (Supplementary Tables IV and V). The adjustment for WBC moderately attenuated the associations of MMP-9 with both clinically-diagnosed AAA and ultrasound-detected AAA: clinically-diagnosed AAA HR (95% CI) associated with the highest tertile of MMP-9 =1.53 (1.20-1.94) and 1.26 (0.98-1.62) before and after the adjustment, respectively (Supplementary Table IV); ultrasound-detected AAA OR (95% CI) = 6.53 (2.38-17.89) and 4.49 (1.53-13.21) before and after the adjustment, respectively (Supplementary Table V).

Risk prediction analysis for clinically-diagnosed AAAs:

Table 5 presents C-index for the prediction of clinically-diagnosed AAA in 3 risk factor sets. The 3 biomarkers identified in our study did not significantly increase the predictive ability beyond the traditional risk factors, nor did the previously reported biomarkers beyond the 3 biomarker set sequentially. However, combining the previously reported biomarkers with the 3 biomarker set resulted in statistically significant increase in the predictive ability beyond the traditional risk factors (C-index increment = 0.011, p = 0.04) (Table 5).

DISCUSSION

To the best of our knowledge, this is the first population-based, prospective study of long follow-up to investigate the association of these biomarkers of inflammation and extracellular matrix degradation and remodeling with the risk of AAA. Based on a median of 22.5 years of follow-up, we found that higher MMP-9 and IL-6 measured at baseline were associated with greater risk of clinically-diagnosed AAA, independent of the influence of established risk factors for AAA. MMP-9 was also significantly and positively associated with the risk of asymptomatic AAA detected by the ARIC ultrasound exam at the end of the follow-up.

The associations of MMP-9 and IL-6 were also present for clinically-diagnosed AAAs detected in the more distant future, i.e. after 10 years of follow-up. As the pathogenesis of AAA may have started years before an aortic diameter meets the diagnostic criteria or a clinical event occurs, the robust findings in this sensitivity analysis indicate that these two biomarkers are not likely the by-product during the complex process of AAA development, thus proving support for the inference of a causal relationship between these biomarkers and AAA.

MMP-9, also named gelatinase B, is produced by many types of cells including leukocytes.[26,27] It is capable of degrading intact elastin fibers,[4] and is the most abundant MMP produced by AAA tissues in vitro.[28,29] The mRNA expression of MMP-9 in AAA tissues correlates with aneurysm diameter.[30] MMP-9 knockout mice are resistant to experimentally induced AAA[31] and wild-type bone marrow transplantation removed the resistance.[4] Of all MMPs that have been investigated cross-sectionally in the circulation of AAA patients and normal controls, MMP-9 showed the most consistent association with AAA.[11,12] In a recent genome-wide association study of AAA, Jones et al.[32] identified direct interaction of MMP-9 gene with several genome-wide significant genes at gene expression level, including *ERG, IL6R* and *LDLR*. These and our findings together support the hypothesis that elevated MMP-9 plays an important role in the etiology of AAA.

In our study, adjustment for WBC moderately attenuated the associations of MMP-9 with both clinically-diagnosed AAAs and ultrasound-detected AAAs, but the association with ultrasound-detected AAAs remained strong and significant. It is debatable whether WBC should be adjusted for as a confounder because MMP-9 is not only an effector but also a regulator of leukocyte biology. Leukocytes may be one of the sources for circulating MMP-9.[33] Circulating levels of MMP-9 correlated with WBC, with a stronger correlation in current smokers (r^2=0.21) than never smokers (r^2=0.07).[33] In humans, chemokines such as

IL-8 stimulate the release of MMP-9 by neutrophils, which in turn further stimulates the activation and chemotaxis of neutrophils via its effect on IL-8.[34] In transgenic mouse models, MMP-9 was found to play a role in the process of hematopoiesis, i.e. recruitment of blood cells from the bone marrow.[35] In the context of the mutual regulation between leukocytes and MMP-9, adjustment for WBC in the analysis of MMP-9 with AAA may be "over-adjustment".

IL-6 is a pleiotropic cytokine that participates in the regulation of homeostasis via pro- and anti-inflammatory properties.[36] IL-6 exerts a protective role in the body's acute response to many infections but seems harmful when it is chronically elevated, as elevated plasma IL-6 is associated with increased risk of chronic conditions such as coronary heart disease[37] and rheumatoid arthritis.[36,38] IL-6 has been identified as an important pro-inflammatory cytokine in the pathogenesis of AAA. It promotes MMP expression by directly stimulating MMP production by inflammatory cells.[4,39] IL-6 levels were significantly higher in AAA biopsies than controls.[40] Experimentally-induced AAA was suppressed in mice with targeted deletion of IL-6 or in wild-type mice treated with anti-IL6 antibody.[3] In addition to previously reported cross-sectional associations with AAA,[11,12,41] circulating IL-6 was positively correlated with aortic diameters in individuals without AAA,[42] suggesting that IL-6 may be involved in the early stage of AAA formation. Moreover, a Mendelian randomization study linked an IL6R genetic variant with AAA risk.[41] In our study, the prospective association of IL-6 with AAA remained significant after we controlled for the other inflammatory biomarkers, supporting an independent contribution of the IL-6 pathway to the etiology of AAA.

IL-6 was not associated with ultrasound-detected AAA in our study. Compared with the entire subcohort group, participants who survived and attended the Visit 5 ultrasound exam had a healthier risk factor profile and lower degree of inflammation, as reflected by lower levels of CRP, fibrinogen, and IL-6 (Table 2). It is possible that the IL-6 pathway may not have been activated sufficiently to contribute to the risk of AAA in the relatively healthy survival cohort. Alternatively, and quite likely, the absence of association between IL-6 and ultrasound-detected AAA may be due to poor statistical power resulting from a limited number of cases.

Osteopontin is an acidic glycoprotein and exerts pleiotropic functions in tissue repair, remodeling, inflammation and immune response.[43–45] It has been demonstrated to up-regulate pro-MMP-9 activation[46] and was increased in human AAA tissue.[47] Osteopontin-deficient mice had reduced AAA formation induced by angiotensin II infusion and exhibited decreased activity of MMPs including MMP-9.[48] In our study, the association of osteopontin with AAA was modestly attenuated after additional adjustment for PAD or CHD, and largely attenuated after excluding clinically-diagnosed AAAs who were diagnosed within the first 10 years of baseline. This suggests that osteopontin may be involved in a later stage of AAA development and its association with AAA is not independent of the influence of PAD and CHD.

Given the biological relationship between IL-6, MMP-9, and osteopontin, we further included the 3 biomarkers simultaneously in model 2 for clinically-diagnosed AAA analysis

to investigate the independence of the biomarkers. After simultaneous adjustment for each other, the association of MMP-9 and IL-6 with AAA remained significant (MMP-9: HR=1.15 and 1.32 for the 2nd and 3rd tertiles versus the 1st tertile, p for trend=0.03; IL-6: HR=1.38 and 1.78, p<0.001), while that for osteopontin was no longer significant (HR=0.99 and 1.14, p=0.30). This observation further corroborates our postulation that osteopontin may be a downstream reactor to MMP-9 and IL-6 and its elevation probably occurs at a later stage of AAA development.

Smoking is the strongest risk factor for AAA, increasing AAA lifetime risk by 5-fold compared with never smokers.[8] The association of MMP-9, IL-6, and osteopontin were mainly present in ever smokers, but not in never smokers in our study. While the role of these biomarkers in never smokers needs further investigation in larger samples, our findings in ever smokers is in line with current understanding of the pathophysiology of AAA. In the etiopathologic pathways for AAA, cigarette extracts activate many downstream events, including the expression and activity of MMPs, systemic and local inflammation, and apoptosis of smooth muscle cells,[49,50] all of which contribute to the pathogenesis of AAA. While smoking prevention and cessation are the most effective strategies in AAA prevention, the effect of smoking on AAA lasts for at least 10 years after smoking cessation.[2,8] Therefore, our study provides insights into the relative etiologic importance of these biomarkers for AAA in people with a smoking history, which may improve risk stratification and prevention of AAA as well as aid in the identification of drug targets for AAA treatment in this high risk population. Future research is warranted to investigate longitudinal changes in these biomarkers in relation to smoking cessation to better understand the role of these biomarkers in AAA development due to smoking.

The strengths and limitations of this study warrant discussion. This study reports data from a large population-based prospective cohort that has been followed for >20 years. The longitudinal design provides supportive evidence for causality. We included both clinically-diagnosed AAAs ascertained from event follow-up and additional asymptomatic AAAs detected at ultrasound screening. The biomarkers were selected based on accumulating evidence from animal and human studies. However, we also recognize the following limitations. Firstly, due to the lack of an ultrasound screening at baseline, we might have still included some prevalent AAAs in the cohort at baseline even after exclusion of subjects with AAA repair prior to the baseline evaluation. However, the prevalence of AAA should have been low at the baseline age of 45-64 years, and the associations for MMP-9 and IL-6 remained consistent and significant after exclusion of clinically-diagnosed AAAs ascertained within 10 years of baseline. Secondly, the biomarkers in this study were measured in blood samples after long-term freezer storage. If there is any change in the biomarkers due to sample degradation, the change should be non-differential with regard to AAA status. Consequently, the influence would have been minimal if the relative ranking of individuals in the population based on the biomarkers was not changed or would most likely have diluted the strength of associations if the relative ranking of the biomarkers was changed. We noticed that the reliability coefficients of the blind duplicate pairs were poorer (<0.5) for MMP-3 and MMP-9 than the other assays. These blind pairs were drawn in separate vacuatiners in one draw, but were processed and analyzed separately and thus reflect processing, storage and laboratory variability. One possibility is that the MMP

analytes were more sensitive to long-term storage than the others. The influence of the measurement errors for these analytes should be non-differential with regard to case status and thus would have unlikely resulted in spurious positive associations between the biomarkers and AAA. Thirdly, in our study we used an ELISA-type method to measure circulating levels of total MMPs (both pro-MMPs (inactive form) and cleaved MMPs (active form)). Since we did not specifically measure plasma MMP activities we cannot comment on possible associations of MMP activities with future risk of developing AAA in this ARIC cohort. Fourthly, residual confounding by other risk factors (e.g. smoking) could have occurred although we have adjusted for those risk factors in model 2. In the analysis adjusting for the previously reported biomarkers, there might still have been residual influences of some of these biomarkers because they were measured in samples obtained at later visits (i.e. CRP, D-dimer, cTnT and NT-proBNP). Fifthly, other biomarkers that were reported to be different in AAA patients vs. controls (e.g. tissue inhibitor of matrix metallo-proteinase 1 (TIMP-1) and α1-antitrypsin)[12] are not available in ARIC and warrant future investigation in prospective studies. Notably, TIMP-1 is a physiological inhibitor of MMP-9 and a1-antitrypsin is a protease inhibitor that protects tissues from the action of various proteases including elastase.[12] Therefore, these two proteins might play a role in the pathogenesis of AAA. Finally, we used case-cohort design with slightly more controls than a 1:1 ratio. The estimates of biomarker-AAA associations and risk prediction analysis would be more accurate and associated with better statistical power if more controls were included, ideally by inclusion of the whole ARIC cohort. Future studies in different populations with similar or larger sample size are needed to confirm the findings from our study.

In summary, in this community-based prospective study with a long follow-up, higher concentrations MMP-9 and IL-6 were associated with future risk of AAA, independent of the established AAA risk factors. Our findings corroborate the evidence from animal and human laboratory studies and highlight the role of inflammation and extracellular matrix degradation in the development of AAA. Future studies with longitudinal measurements of both the biomarkers and ultrasound exam are needed to assess if the longitudinal trend of these biomarkers predicts AAA progression and rupture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Indices of laboratory quality for the biomarker assays in the Atherosclerosis Risk in Communities (ARIC) study.

	ARIC Samples		Blind Duplicate Pairs in ARIC	
Biomarker	**Intra-assay CV**	**Inter-assay CV**	**n Pairs**	**Repeatability***
MMP-3	3.1%	5.3%	59	0.24
MMP-9	2.9%	7.1%	59	0.42
IL-6	9.7%	20.1%	58	0.87
Osteopontin	8.6%	19.2%	59	0.70
PIIINP	7.2%	14.9%	59	0.56

CV=coefficient of variation; MMP=matrix metalloproteinase; IL-6=interleukin-6; PIIINP=N-terminal propeptide of Type III procollagen;

*the intraclass correlation coefficient

Table 2.

Baseline characteristics obtained at 1987-1992 in the Atherosclerosis Risk in Communities (ARIC) case-cohort samples for clinically-diagnosed abdominal aortic aneurysms (AAA) and ultrasound-detected AAA analyses: mean ± SD or percentage.

	Clinically-diagnosed AAA Analysis			**Ultrasound-detected AAA Analysis**		
Baseline characteristics	**Clinical AAA (n=544)**	**Sub-cohort Group[*] (n=723)**	**p[†]**	**Ultrasound AAA (n=72)**	**Sub-cohort Group[‡] (n=227)**	**p[†]**
Age, years	56.7 ± 5.1	56.1 ± 5.5	0.03	52.9 ± 5.0	53.8 ± 5.3	0.17
Male, %	70.2	71.8	0.54	79.1	73.6	0.34
White, %	86.8	86.6	0.93	87.5	93.0	0.15
Height, cm	172.5 ± 8.7	171.8 ± 9.1	0.15	175.8 ± 8.9	172.4 ± 8.5	0.004
Smoking status			<0.001			<0.001
Current smoker, %	50.6	25.9		43.1	15.9	
Former smoker, %	36.8	41.6		38.9	42.3	
Never smoker, %	12.6	32.5		18.0	41.8	
Pack-years smoking[§]	33.3 ± 24.9	20.6 ± 24.2	<0.001	24.9 ± 20.5	14.0 ± 19.7	<0.001
Hypertension, %	40.5	31.8	0.0015	23.6	16.7	0.19
PAD, %	4.4	2.0	0.01	1.5	0.5	0.40
Diabetes, %	7.2	11.8	0.006	4.2	4.0	0.94
Total cholesterol, mg/dL	223 ± 40	213 ± 39	<0.001	227 ± 33	208 ± 37	<0.001
LDL cholesterol, mg/dL	149 ± 37	138 ± 37	<0.001	155 ± 31	135 ± 36	<0.001
HDL cholesterol, mg/dL	44 ± 14	48 ± 16	<0.001	44 ± 11	49 ± 15	0.003
Triglycerides, mg/dL	151 ± 89	132 ± 69	<0.001	144 ± 73	123 ± 67	0.03
SBP, mmHg	123.9 ± 19.8	120.7 ± 18.0	0.003	116.9 ± 17.2	115.2 ± 14.3	0.45
DBP, mmHg	74.4 ± 11.8	72.9 ± 11.0	0.02	72.3 ± 12.4	71.6 ± 9.4	0.67
Ln[//](CRP), mg/L	1.07 ± 1.10	0.74 ± 1.05	<0.001	0.92 ± 1.09	0.53 ± 0.92	0.009
Fibrinogen, mg/dL	322 ± 68	304 ± 66	<0.001	299 ± 48	286 ± 56	0.05
MMP-3, pg/mL	11060 ± 6153	11073 ± 5722	0.97	11575 ± 5865	11063 ± 4797	0.50
MMP-9, pg/mL	17924 ± 9984	16003 ± 8844	<0.001	20844 ± 13535	15394 ± 8426	0.001
IL-6, pg/mL	3.23 ± 2.17	2.81 ± 2.08	<0.001	2.37 ± 1.25	2.30 ± 1.59	0.71
PIIINP, pg/mL	12518 ± 4844	12342 ± 5144	0.53	12040 ± 3947	11296 ± 4311	0.16
Ln[//](osteopontin), ng/mL	2.22 ± 0.90	2.15 ± 0.87	0.14	2.38 ± 0.96	2.12 ± 0.81	0.03

[*] Forty-three participants were identified as clinically-diagnosed AAA during follow-up;

[†] p was obtained in generalized linear model;

[‡] Four participants were diagnosed as asymptomatic AAA in the ultrasound exam at Visit 5; §including non-smokers; ‖natural log-transformed;

[§] including non-smokers;

[‖] natural log-transformed;

SD=standard deviation; DBP=diastolic blood pressure; SBP=systolic blood pressure; PAD=peripheral arterial disease; LDL=low-density lipoprotein; HDL=high-density lipoprotein; CRP=C-reactive protein; MMP=matrix metalloproteinase; IL-6=interleukin-6; PIIINP=N-terminal propeptide of Type III procollagen

Table 3.

Hazard ratio (HR) (95% CI) for the associations of biomarkers with clinically-diagnosed abdominal aortic aneurysms (AAA) in the Atherosclerosis Risk in Communities (ARIC) study, 1987 to 2011.

Biomarkers	Tertile 1	Tertile 2	Tertile 3	p for trend
MMP-3				
Median (range), pg/mL	5932 (644, 8088)	9988 (8096, 12147)	15789 (12188, 48933)	
n	420	421	420	
n AAA	184	178	178	
Hazard ratio (95% CI)				
Model 1	1	0.93 (0.74, 1.18)	1.00 (0.78, 1.27)	0.99
Model 2	1	0.91 (0.70, 1.17)	1.07 (0.82, 1.40)	0.61
MMP-9				
Median (range), pg/mL	9833 (538, 12160)	14432 (12164, 17697)	22543 (17713, 79054)	
n	421	422	421	
n AAA	155	180	207	
Hazard ratio (95% CI)				
Model 1	1	1.38 (1.10, 1.73)	1.82 (1.46, 2.27)	<0.001
Model 2	1	1.27 (1.00, 1.62)	1.55 (1.22, 1.97)	<0.001
IL-6				
Median (range), pg/mL	1.38 (0.15, 1.87)	2.37 (1.87, 3.07)	4.40 (3.07, 19.19)	
n	413	414	414	
n AAA	134	187	211	
Hazard ratio (95% CI)				
Model 1	1	1.61 (1.28, 2.02)	2.31 (1.84, 2.90)	<0.001
Model 2	1	1.36 (1.07, 1.71)	1.87 (1.48, 2.35)	<0.001
PIIINP				
Median (range), pg/mL	8173 (2552, 9721)	11471 (9725, 13345)	16427 (13352, 40105)	
n	417	418	418	
n AAA	162	197	179	
Hazard ratio (95% CI)				
Model 1	1	1.38 (1.11, 1.71)	1.27 (1.02, 1.60)	0.03
Model 2	1	1.33 (1.06, 1.67)	1.14 (0.90, 1.46)	0.27
Osteopontin				
Median (range), ng/mL	4.40 (0.70, 5.80)	7.40 (5.90, 9.50)	20.70 (9.60, 124.80)	
n	409	414	414	
n AAA	159	187	184	
Hazard ratio (95% CI)				
Model 1	1	1.22 (0.98, 1.52)	1.36 (1.08, 1.70)	0.02
Model 2	1	1.06 (0.84, 1.34)	1.29 (1.02, 1.63)	0.03

Model 1: Adjusted for age, sex and race-field center;

Model 2: Model 1 further adjusted for height, smoking pack-years, hypertension, diabetes, total cholesterol and HDL cholesterol;

For each biomarker, 15-17 individuals were excluded from the analysis in model 2 due to missing data for the covariates, and the results from model 1 after exclusion of these individuals were highly consistent to those shown in the table;

p for trend from the analysis modeling tertile as an ordinal variable;

MMP=matrix metalloproteinase; CI=confidence interval; IL-6=interleukin-6; PIIINP=N-terminal propeptide of Type III procollagen

Table 4.

Odds ratio (OR) (95% CI) for the associations of biomarkers with ultrasound-detected abdominal aortic aneurysms (AAA) in the Atherosclerosis Risk in Communities (ARIC) study, 1987 to 2013.

Biomarkers	Tertile 1	Tertile 2	Tertile 3	p for trend
MMP-3				
Median (range), pg/mL	6413 (2364, 8392)	10572 (8399, 12526)	15808 (12702, 38099)	
n	99	100	100	
n AAA	23	23	26	
Odds Ratio (95% CI)				
Model 1	1	0.83 (0.38, 1.82)	1.25 (0.55, 2.87)	0.59
Model 2	1	0.97 (0.39, 2.39)	1.50 (0.56, 4.06)	0.42
MMP-9				
Median (range), pg/mL	9730 (3653, 11838)	14257 (11885, 16952)	21908 (16960, 79902)	
n	99	100	100	
n AAA	12	24	36	
Odds Ratio (95% CI)				
Model 1	1	2.24 (0.95, 5.27)	6.50 (2.73, 15.50)	<0.001
Model 2	1	2.93 (1.06, 8.14)	7.44 (2.62, 21.15)	<0.001
IL-6				
Median (range), pg/mL	1.15 (0.15, 1.54)	1.94 (1.55, 2.46)	3.28 (2.49, 10.50)	
n	97	98	98	
n AAA	20	20	31	
Odds Ratio (95% CI)				
Model 1	1	0.67 (0.28, 1.64)	1.15 (0.50, 2.62)	0.75
Model 2	1	0.63 (0.21, 1.90)	0.87 (0.32, 2.35)	0.79
PIIINP				
Median (range), pg/mL	7463 (3838, 8988)	10717 (9015, 12679)	15277 (12698, 29416)	
n	99	100	99	
n AAA	16	29	27	
Odds Ratio (95% CI)				
Model 1	1	2.23 (0.97, 5.13)	1.90 (0.86, 4.20)	0.11
Model 2	1	3.17 (1.26, 7.94)	1.93 (0.72, 5.15)	0.19
Osteopontin				
Median (range), ng/mL	4.55 (0.70, 5.50)	6.70 (5.60, 9.30)	19.65 (9.50, 79.80)	
n	94	98	98	
n AAA	23	18	27	
Odds Ratio (95% CI)				
Model 1	1	0.80 (0.32, 1.98)	1.49 (0.72, 3.12)	0.29
Model 2	1	0.57 (0.17, 1.85)	1.21 (0.55, 2.68)	0.64

Model 1: Adjusted for age, race and sex;

Model 2: Model 1 further adjusted for height, total cholesterol, triglycerides and smoking pack-years; p for trend from the analysis modeling tertile as an ordinal variable;

MMP=matrix metalloproteinase; CI=confidence interval; IL-6=interleukin-6; PIIINP=N-terminal propeptide of Type III procollagen

Table 5.

C-index for risk prediction of clinically-diagnosed abdominal aortic aneurysms (AAA) based on different risk factor sets in the Atherosclerosis Risk in Communities (ARIC) case-cohort samples, 1987 to 2011.

Model	C-index (SE)	Comparison Model	C-index Increment (p)*
Model 1	0.810 (0.013)	NA	NA
Model 2	0.816 (0.013)	Model 1	0.006 (0.14)
Model 3	0.821 (0.012)	Model 2	0.005 (0.20)
Model 3	0.821 (0.012)	Model 1	0.011 (0.04)

* Compared with the model listed under the Comparison Model column;

Model 1 included age, sex, race-field center, height, smoking pack-years, hypertension, diabetes, total cholesterol and HDL cholesterol;

Model 2 included risk factors in Model 1 plus MMP-9, IL-6 and osteopontin;

Model 3 included risk factors in Model 2 plus WBC, fibrinogen, antithrombin III and lipoprotein(a);

SE=standard error; NA=not applicable; MMP=matrix metalloproteinase; IL-6=interleukin-6; WBC=white blood cell count