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**Endothelial dysfunction and low-grade inflammation  
are associated with greater arterial stiffness over  
a 6-year period**

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**ABSTRACT**

Endothelial dysfunction and low-grade inflammation are associated with cardiovascular disease. Arterial stiffening plays an important role in cardiovascular disease and thus may be a mechanism through which endothelial dysfunction and/or low-grade inflammation lead to cardiovascular disease. We investigated the associations between, on the one hand, biomarkers of endothelial dysfunction (soluble endothelial selectin, thrombomodulin and both vascular- and intercellular adhesion molecules 1 and von Willebrand factor) and of low-grade inflammation (C-reactive protein, serum amyloid A, interleukin 6, interleukin 8, tumor necrosis factor  $\alpha$  and soluble intercellular adhesion molecule 1) and, on the other hand, arterial stiffness over a 6-year period, in 293 healthy adults (155 women). Biomarkers were combined into mean Z-scores. Carotid, femoral and brachial arterial stiffness and carotid-femoral pulse wave velocity were determined by ultrasonography. Measurements were obtained when individuals were 36 and 42 years of age. Associations were analyzed with generalized estimating equation and adjusted for sex, height and mean arterial pressure. The endothelial dysfunction Z-score was inversely associated with femoral distensibility [ $\beta$  (95%CI) -0.51 (-0.95;-0.06)] and compliance coefficients [-0.041 (-0.076;-0.006)], but not with carotid or brachial stiffness or carotid-femoral pulse wave velocity. The low-grade inflammation Z-score was inversely associated with femoral distensibility [-0.51 (-0.95;-0.07)] and compliance coefficients [-0.050 (-0.084;-0.016)], and with carotid distensibility coefficient [-0.91 (-1.81;-0.008)], but not with brachial stiffness or carotid-femoral pulse wave velocity. Biomarkers of endothelial dysfunction and low-grade inflammation are associated with greater arterial stiffness. This provides evidence that arterial stiffening may be a mechanism through which endothelial dysfunction and low-grade inflammation lead to cardiovascular disease.

## INTRODUCTION

From observational studies [1-5], it has become increasingly clear that biomarkers of endothelial dysfunction and low-grade inflammation are closely associated with (incident) cardiovascular disease (CVD). In part, these associations can be explained by the fact that endothelial dysfunction and low-grade inflammation play key roles in atherothrombosis [6]. However, other mechanisms may also be important. In this respect, arterial stiffening may constitute one such mechanism. First, greater arterial stiffness has been shown to be independently associated with cardiovascular morbidity and mortality [7,8]. Second, endothelial dysfunction and low-grade inflammation affect the composition of the subendothelial matrix, which is an important determinant of arterial stiffness [9].

Indeed, the association between biomarkers of endothelial dysfunction and low-grade inflammation, and greater arterial stiffness is receiving increasing attention [10,11]. However, previous studies on this topic were cross-sectional [10-26] and most focused on either biomarkers of endothelial dysfunction [24] or low-grade inflammation [12,16-23,25,26], and were done in middle-aged or elderly populations [10,11,13-15,18-23,26]. Importantly, endothelial dysfunction and low-grade inflammation are closely linked and difficult to separate conceptually [27], and any relationship with arterial stiffening may therefore be interdependent. In addition, the process of arterial stiffening is known to start at an early age [28].

In view of the above, we hypothesize that the development of biomarkers of endothelial dysfunction and low-grade inflammation is associated with arterial stiffening in early adulthood. Therefore, we have measured biomarkers of endothelial dysfunction, low-grade inflammation and arterial stiffness twice in a population-based cohort, i.e. for the first time at the age of 36 years and for the second time at the age of 42 years. We have investigated the 6-year longitudinal associations between biomarkers of endothelial dysfunction and low-grade inflammation, on the one hand, and stiffness estimates of the carotid, femoral and brachial arteries and of the carotid-femoral segment, on the other, in apparently healthy adults of the Amsterdam Growth and Health Longitudinal Study (AGAHLs).

## METHODS

### Study population

Data were derived from the AGAHLs, an observational, longitudinal study that started in 1976 with a group of 698 boys and girls (details described elsewhere) [29]. Briefly, its initial goal was to study the natural development of the growth, health and lifestyle of adolescents and to investigate longitudinal relationships between biological and lifestyle variables. The mean age of the individuals at the start of the study was  $13.1 \pm 0.8$  [mean  $\pm$  standard deviation (SD)] years. Since then, extensive follow-up measurements have been done and the cohort is still under investigation. At each follow-up measurement, anthropometric (body height, weight and skinfolds), biological (blood pressure, serum lipoprotein levels and physical fitness), lifestyle (nutritional habits, smoking behavior and daily physical activity) and psychological variables were assessed [29].

In addition, in blood samples of the individuals attending the 2000 and 2006 follow-up examinations (one batch), five biomarkers of endothelial dysfunction and six biomarkers of low-grade inflammation were measured and at both examinations arterial properties were assessed by ultrasonography. At the first ultrasound examination, 377 individuals participated and complete data on stiffness estimates of the carotid, femoral and brachial arteries were obtained in 373 individuals. From these, complete data on all three arteries were obtained in 293 individuals during the follow-up ultrasound examination, all of whom had full data on biomarkers of endothelial dysfunction and low-grade inflammation. The present study was therefore conducted with these 293 individuals.

The study was approved by the local ethics committee of the Vrije Universiteit University Medical Center and all participants gave their written informed consent.

### Assessment of endothelial dysfunction and low-grade inflammation

Serum biomarkers of endothelial dysfunction [soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble endothelial selectin (sE-selectin) and soluble thrombomodulin (sTM)], and of low-grade inflammation [C-reactive protein (CRP), serum amyloid A (SAA), interleukin 6

(IL-6), interleukin 8 (IL-8), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and sICAM-1] were assessed by an electro-chemiluminescence detection system using multi-array technology (SECTOR Imager 2400, Meso Scale Discovery, USA) as described elsewhere [30] (please see Supplement).

In addition, a plasma biomarker of endothelial dysfunction [von Willebrand factor (vWf)] was determined in citrated plasma by means of ELISA as described elsewhere [30] (please see Supplement).

### **Arterial stiffness**

We assessed local arterial stiffness of the carotid, femoral and brachial arteries and central arterial stiffness [the carotid-femoral pulse wave velocity (cfPWV)] [31,32] (please see Supplement).

### **Other measurements**

Weight, height, body mass index, heart rate and blood pressure were determined according to international standards and smoking behavior was assessed by questionnaire as previously described[29,31]. Total and high-density lipoprotein cholesterol, triglycerides and glycated haemoglobin were determined as previously described[29,31]. Hypertension was defined as a systolic blood pressure  $>140$  mmHg and/or a diastolic blood pressure  $>90$  mmHg, and/or treatment for hypertension.

### **Statistical analyses**

All analyses were performed with SPSS (Statistical Package for Social Sciences, version 15, USA). Variables with a skewed distribution (CRP, SAA, IL-6 and triglycerides) were transformed using a natural logarithm (ln). Overall Z-scores were calculated for endothelial dysfunction or low-grade inflammation (please see supplemental material). We used generalized estimating equations (GEE) to examine the associations between either the overall Z-score for endothelial dysfunction or the overall Z-score for low-grade inflammation and arterial stiffness over the 6-year study period. In these analyses an exchangeable correlation structure was used. GEE analysis is a method for longitudinal data analyses and takes into account the correlation of repeated measurements within individuals over time [33].

First, we investigated the associations between the overall Z-scores for either endothelial dysfunction or low-grade inflammation on the one hand and arterial stiffness estimates on the other with adjustments for sex, height, and mean arterial pressure.

Second, the analyses were repeated with mutual adjustments for the overall Z-scores for either low-grade inflammation or endothelial dysfunction. Third, to gain further insight into which of the individual elements of the stiffness formulas might have been primarily responsible for any relationships between endothelial dysfunction or low-grade inflammation and arterial stiffness estimates, the analyses were repeated with the individual elements of the stiffness formulas (diameter, distension, pulse pressure and intima media thickness) as dependent variables.

Data are presented as mean ( $\pm$  SD) or median (interquartile range) for skewed variables and regression coefficients ( $\beta$ ) with their 95% confidence intervals (95%CI). A two-sided p-value  $< 0.05$  was considered statistically significant.

## RESULTS

Table 1 shows the general characteristics, the biomarker concentrations and the stiffness estimates of the study population. Systolic blood pressure remained stable (116 mmHg), diastolic blood pressure increased (+5.5 mmHg), pulse pressure decreased (-5.6 mmHg) and heart rate decreased (-8.9 bpm) during the 6-year follow-up. Biomarkers of low-grade inflammation remained fairly stable over 6-year follow-up, except for a decrease in TNF- $\alpha$ . Biomarkers of endothelial dysfunction remained stable or increased over 6-year follow-up, except for a decrease in sE-selectin. Pulse pressure and femoral and brachial artery stiffness decreased during 6-year follow-up. Nevertheless, carotid artery stiffness increased [for distensibility coefficient (DC)  $-1.00 \cdot 10^{-3}/\text{kPa}$  and for Young's elastic modulus (YEM)  $+0.025 \cdot 10^3/\text{kPa}$ ] during the 6-year follow-up.

**Table 1.** Characteristics of the study population

	2000	2006	p-value
Age, yrs	36.6 ± 0.6	42.6 ± 0.6	<0.001
Women, %	52.9	52.9	-
Smoker, %	64, 21.9	45, 15.4	<0.001
Systolic blood pressure, mmHg	116.3 ± 11.7	116.2 ± 14.4	0.858
Diastolic blood pressure, mmHg	64.9 ± 7.2	70.4 ± 8.1	<0.001
Pulse pressure, mmHg	51.4 ± 6.5	45.8 ± 9.0	<0.001
Mean arterial pressure, mmHg	82.1 ± 8.7	85.1 ± 9.9	<0.001
Prevalence of hypertension, %	5.1	9.6	0.015
Heart rate, bpm	70.5 ± 11.4	61.6 ± 9.0	<0.001
Body height, cm	177.0 ± 9.2	177.4 ± 9.0	<0.001
Body weight, kg	75.8 ± 12.7	77.9 ± 13.7	<0.001
Body mass index, kg/m <sup>2</sup>	24.1 ± 3.2	24.7 ± 3.6	<0.001
Total cholesterol, mmol/l	5.0 ± 0.9	5.0 ± 0.8	0.315
High density lipoprotein cholesterol, mmol/l	1.4 ± 0.4	1.7 ± 0.4	<0.001
Triglycerides, mmol/l	1.0 (0.8 – 1.5)	1.0 (0.7 – 1.4)	0.018
HbA1c, %	5.2 ± 0.4	5.4 ± 0.4	<0.001
<b>Biomarkers</b>			
C-reactive protein, mg/l	0.8 (0.3-2.0)	0.8 (0.3-1.8)	0.946
Serum amyloid A, mg/l	1.2 (0.7-2.2)	1.3 (0.7-2.3)	0.099
Interleukin 6, ng/l	2.3 (1.7-3.7)	2.4 (1.8-3.7)	0.664
Interleukin 8, ng/l	9.2 ± 3.4	9.5 ± 4.1	0.190
Tumor necrosis factor α, ng/l	9.3 ± 3.4	9.0 ± 3.3	0.006
Soluble intercellular adhesion molecule 1, µg/l	203.3 ± 52.4	200.3 ± 41.2	0.142
Soluble vascular cellular adhesion molecule 1, µg/l	332.8 ± 81.2	333.9 ± 69.2	0.767
Soluble endothelial selectin, µg/l	10.5 ± 4.7	10.1 ± 4.6	0.010
Soluble thrombomodulin, µg/l	2.47 ± 0.66	2.53 ± 0.62	0.023
von Willebrand factor, %	102.5 ± 40.0	112.8 ± 42.4	<0.001
<b>Carotid artery</b>			
Distensibility coefficient, 10 <sup>-3</sup> /kPa	26.7 ± 6.1	25.7 ± 7.2	0.014
Compliance coefficient, mm <sup>2</sup> /kPa	1.0 ± 0.3	1.0 ± 0.3	0.143
Young's elastic modulus, 10 <sup>3</sup> /kPa	0.44 ± 0.13	0.47 ± 0.16	0.007
Diameter, mm	6.9 ± 0.6	7.1 ± 0.7	<0.001
Distension, mm	0.6 ± 0.1	0.5 ± 0.1	<0.001
Pulse pressure, mmHg	51.3 ± 7.1	45.9 ± 9.4	<0.001
Intima-media thickness, mm	0.62 ± 0.10	0.66 ± 0.12	<0.001
<b>Femoral artery</b>			
Distensibility coefficient, 10 <sup>-3</sup> /kPa	7.1 ± 3.7	8.3 ± 4.5	<0.001
Compliance coefficient, mm <sup>2</sup> /kPa	0.51 ± 0.24	0.63 ± 0.33	<0.001
Diameter, mm	9.8 ± 1.3	10.0 ± 1.5	<0.001
Distension, mm	0.22 ± 0.10	0.23 ± 0.10	0.149
Pulse pressure, mmHg	51.4 ± 7.2	46.5 ± 9.8	<0.001
<b>Brachial artery</b>			
Distensibility coefficient, 10 <sup>-3</sup> /kPa	14.6 ± 9.2	17.6 ± 11.2	<0.001
Compliance coefficient, mm <sup>2</sup> /kPa	0.17 ± 0.09	0.21 ± 0.11	<0.001
Diameter, mm	4.0 ± 0.7	4.0 ± 0.7	0.187
Distension, mm	0.18 ± 0.09	0.19 ± 0.10	0.205
Pulse pressure, mmHg	51.7 ± 7.2	45.0 ± 9.5	<0.001
<b>Central arterial stiffness</b>			
Pulse wave velocity, m/s	7.7 ± 1.6 *	8.3 ± 1.6 †	<0.001

Data are reported as mean ± SD, median (interquartile range) or percentage, as appropriate; p-value, paired T-test or McNemar's test, as appropriate; n=293, \* n=241, † n= 292



**Endothelial dysfunction, low-grade inflammation and arterial stiffness (Table 2)**

The endothelial dysfunction overall Z-score was inversely associated with the femoral artery DC [ $\beta$ (95%CI), -0.51 (-0.95; -0.06),  $p=0.025$ ] and compliance coefficient (CC) [-0.041 (-0.076; -0.006),  $p=0.020$ ] after adjustment for sex, height and mean arterial pressure, whereas it was not associated with carotid or brachial artery stiffness (Table 2, models 1). Additional adjustment for the low-grade inflammation overall Z-score decreased the regression coefficient ( $\beta$ ) for the femoral artery DC by 32% and for the femoral artery CC by 46% (Table 2, models 2). In addition, the endothelial dysfunction overall Z-score was not associated with cFPWV after adjustment for sex, height and mean arterial pressure [0.001 (-0.22; 0.22),  $p=0.994$ ].

The low-grade inflammation overall Z-score was inversely associated with the carotid artery DC [-0.91 (-1.81; -0.008),  $p=0.048$ ] and femoral artery DC [-0.51 (-0.95; -0.07),  $p=0.024$ ] and CC [-0.050 (-0.084; -0.016),  $p=0.004$ ] after adjustment for sex, height and mean arterial pressure, whereas it was not associated with carotid artery CC or YEM or brachial artery stiffness (Table 2, models 1). Additional adjustment for the endothelial dysfunction overall Z-score decreased the regression coefficient ( $\beta$ ) for the femoral artery DC by 47% and for the femoral artery CC by 24%, whereas the result for the carotid artery DC did not materially change (Table 2, models 2). In addition, the low-grade inflammation overall Z-score was not associated with cFPWV after adjustment for sex, height and mean arterial pressure [-0.074 (-0.37; 0.24),  $p=0.626$ ].

**Endothelial dysfunction, low-grade inflammation and arterial diameter, distension, pulse pressure and intima-media thickness (Supplemental Table 1)**

The inverse associations between the endothelial dysfunction overall Z-score and the femoral artery DC and CC and the low-grade inflammation overall Z-score and the femoral artery DC and CC were primarily driven through inverse associations with femoral artery distension [for endothelial dysfunction -0.013 (-0.025; -0.001),  $p=0.029$ ; for low-grade inflammation -0.015 (-0.027; -0.004),  $p=0.010$ ; Supplemental Table 1]. In contrast, the inverse association between the low-grade inflammation overall Z-score and the carotid artery DC was not primarily driven through any of the elements of the stiffness formulas (Supplemental Table 1).

**Table 2.** Longitudinal associations between, on the one hand, endothelial dysfunction and low-grade inflammation and low-grade inflammation Z-scores during 6-year follow-up and, on the other hand, arterial stiffness indices during 6-year follow-up (n=293)

Endothelial dysfunction	Carotid artery		Femoral artery		Brachial artery	
	b	95%CI	b	95%CI	b	95%CI
<b>Distensibility coefficient</b>						
1: + sex, height, MAP	-0.19	-1.02;0.64	-0.51	-0.95;-0.06	-0.94	-2.26;0.39
2: 1 + low-grade inflammation Z-score	0.38	-0.51;1.27	-0.34	-0.89;0.20	-1.46	-3.15;0.24
<b>Compliance coefficient</b>						
1: + sex, height, MAP	-0.012	-0.049;0.026	-0.041	-0.076;-0.006	-0.009	-0.023;0.004
2: 1 + low-grade inflammation Z-score	-0.001	-0.042;0.041	-0.022	-0.065;0.021	-0.017	-0.034;0.001
<b>Young 's elastic modulus</b>						
1: + sex, height, MAP	-0.001	-0.020;0.018	-	-	-	-
2: 1 + low-grade inflammation Z-score	-0.005	-0.028;0.017	-	-	-	-
Low-grade inflammation						
<b>Distensibility coefficient</b>						
1: + sex, height, MAP	-0.91	-1.81;-0.008	-0.51	-0.95;-0.07	0.19	-1.11;1.49
2: 1 + endothelial dysfunction Z-score	-1.12	-2.14;-0.11	-0.32	-0.86;0.22	0.99	-0.64;2.62
<b>Compliance coefficient</b>						
1: + sex, height, MAP	-0.022	-0.061;0.018	-0.050	-0.084;-0.016	0.005	-0.008;0.017
2: 1 + endothelial dysfunction Z-score	-0.021	-0.066;0.023	-0.038	-0.080;0.004	0.014	-0.002;0.030
<b>Young 's elastic modulus</b>						
1: + sex, height, MAP	0.006	-0.013;0.024	-	-	-	-
2: 1 + endothelial dysfunction Z-score	0.009	-0.013;0.030	-	-	-	-

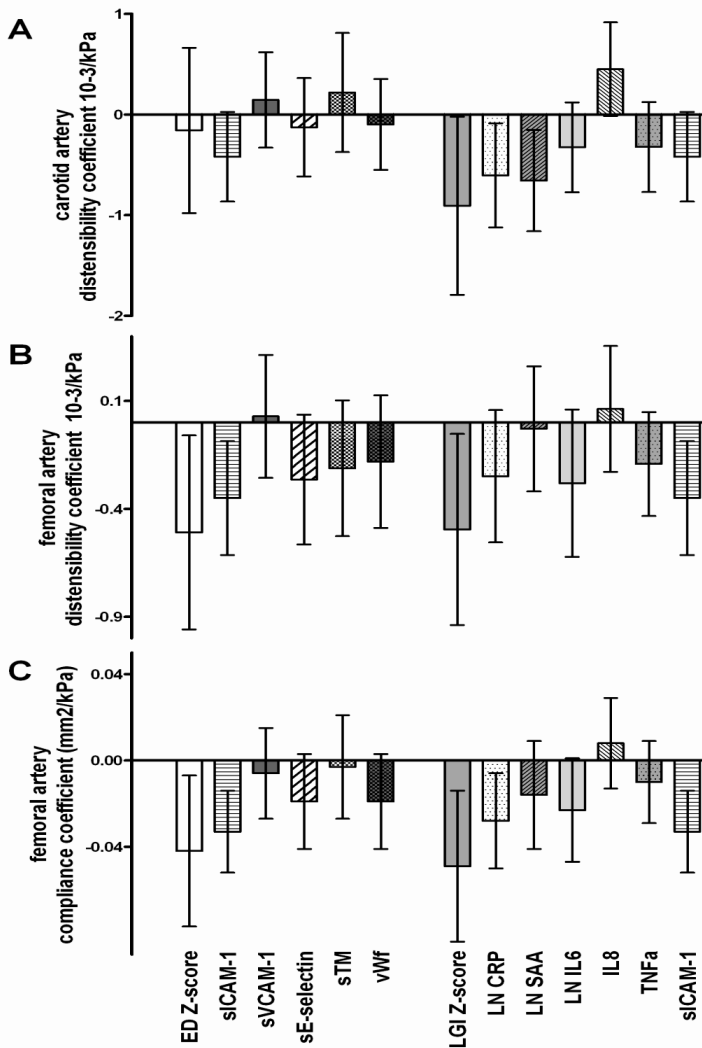
$\beta$ s, longitudinal regression coefficients that express the relationships between the longitudinal development of endothelial dysfunction and low-grade inflammation, on the one hand, and the longitudinal development of arterial stiffness on the other; 95%CI, indicate 95% confidence intervals and p-value; MAP = mean arterial pressure.

**Additional analyses**

Additional adjustment for age (the age range by design being extremely narrow), smoking behavior, total and HDL cholesterol, and hypertension did not materially change the associations, whereas adjustment for weight did attenuate the regression coefficients with ~50% (Supplemental Table 2). None of the individuals had diabetes mellitus.

In the analyses for the individual elements of the low-grade inflammation Z-score, the results showed that lnSAA and lnCRP were the strongest determinants of the carotid artery DC (Figure 1A), whereas sICAM-1 and lnIL6 were the strongest determinants of the femoral artery DC (Figure 1B), and sICAM-1 and lnCRP were the strongest determinants for the femoral artery CC (Figure 1C). For the endothelial dysfunction Z-score, sICAM-1 and sE-selectin were the strongest determinants for the femoral artery DC (Figure 1B) and CC (Figure 1C).

To investigate whether the longitudinal associations (by GEE) were primarily determined by the between- or the within-subject associations over the 6 year study period, we calculated changes (i.e. within-subject) in the endothelial dysfunction overall Z-score, the low-grade inflammation overall Z-score and the arterial stiffness estimates. Then, we re-analyzed the data with the use of linear regression analyses. Changes in the endothelial dysfunction overall Z-score or in the low-grade inflammation overall Z-score were not associated with changes in arterial stiffness estimates (data not shown). This suggests that the reported associations were primarily determined by the between-subject associations over the 6-year study period.



**Figure 1.** Bars are regression coefficients that indicate the associations between either the endothelial dysfunction Z-score (ED), or the low-grade inflammation (LGI) Z-score or each of the individual biomarker Z-scores and the carotid distensibility coefficient (A), the femoral distensibility coefficient (B) and the femoral compliance coefficient (C) over the 6-year study period. Whiskers indicate the 95% confidence intervals. All results are adjusted for sex, height and mean arterial pressure.

sICAM-1 = soluble intercellular adhesion molecule 1; sVCAM-1 = soluble vascular cell adhesion molecule 1; sE-selectin = soluble endothelial selectin; sTM = soluble thrombomodulin; vWf = von Willebrand factor; CRP = C-reactive protein; SAA = serum amyloid A; IL-6 = interleukin 6; IL-8 = interleukin 8; TNF- $\alpha$  = tumour necrosis factor  $\alpha$ ; LN = log normalised.

## DISCUSSION

The present investigation is the first to prospectively evaluate, in apparently healthy adults, the relationship between the development of an extensive array of biomarkers of endothelial dysfunction and low-grade inflammation on the one hand and arterial stiffness on the other. The study had three main findings. First, biomarker scores for endothelial dysfunction and low-grade inflammation were associated with greater arterial stiffness over a 6-year period. The biomarker score for endothelial dysfunction was associated with greater femoral artery stiffness, whereas the biomarker score for low-grade inflammation was associated with both greater carotid and femoral artery stiffness. However, both the biomarker scores for endothelial dysfunction and low-grade inflammation were not associated with stiffness of the carotid-femoral segment. Endothelial dysfunction and/or low-grade inflammation may thus affect arterial stiffening in a way that depends upon the arterial territory under study [34]. Second, mutual adjustment for either the biomarker score for low-grade inflammation or endothelial dysfunction showed that the associations between either the biomarker score for endothelial dysfunction or low-grade inflammation with femoral artery stiffness were interdependent. Finally, the associations between each of the biomarker scores and greater femoral artery stiffness were primarily driven through decreased distension, whereas the association between low-grade inflammation and greater carotid stiffness was not primarily driven through any of the arterial parameters.

The endothelium has many functions and is itself heterogeneous [35,36]. The concept of endothelial dysfunction therefore has many dimensions [37]. vWf, sVCAM-1, sE-selectin, sTM and sICAM-1 are all synthesized by endothelial cells [6,35,36] and higher concentrations of these biomarkers are associated with (incident) cardiovascular disease [1-5]. Consequently, it is plausible to assume that higher circulating concentrations of these biomarkers reflect greater dysfunction.

A limitation of this study is that a measure of NO-mediated dilation, which represents an important function of the endothelium [35-37], was not available. Nevertheless, both biomarkers of the endothelium and NO-mediated dilation (such as flow-mediated dilation) have been associated with (incident) cardiovascular disease [1-5,38].

Furthermore, endothelial dysfunction is closely linked with low-grade inflammation and therefore these concepts are difficult to separate [27]. We, indeed, show that the associations with femoral artery stiffening are dependent on both endothelial dysfunction and low-grade inflammation, whereas this was not the case for carotid artery stiffening. This suggests that endothelial dysfunction and low-grade inflammation might affect arterial stiffening in concert or independently, dependent on the arterial territory under study.

The present investigation was comprehensive and had advantages over previous ones, which investigated either biomarkers of endothelial dysfunction [24] or low-grade inflammation [12,16-23,25,26], measured a less extensive array of biomarkers of endothelial dysfunction and low-grade inflammation [10-26], concerned middle-aged to elderly individuals [10,11,13-15,18-23,26], targeted one type of artery only [10-25] and were cross-sectional by design [10-26].

On aggregate, previous studies have shown higher CRP levels to be associated with greater arterial stiffness of elastic [10-14,16-23], muscular [25] or both types of arteries [26], and higher IL6 levels to be associated with greater elastic arterial stiffness [11,15]. Taken together, these studies [10-23,25,26] and the present results support the concept that low-grade inflammation may affect arterial stiffness. In particular, we show that this process is present even in young, apparently healthy adults.

With regard to biomarkers of endothelial dysfunction, previous studies have shown heterogeneous results for vWf and sICAM-1, some reporting positive associations with elastic arterial stiffness [15,24], whereas others did not [11,13,14]. We show that the overall endothelial dysfunction Z-score was significantly associated with femoral artery stiffness (Figure 1B and 1C), whereas none of the individual endothelial dysfunction biomarkers was, except for sICAM-1. This may be explained by the fact that in these young, apparently healthy individuals, endothelial dysfunction may not be very far advanced and therefore only the sum of endothelial dysfunction biomarkers reveals its association with greater arterial stiffness.

Endothelial dysfunction may influence arterial stiffening by affecting vascular smooth muscle cell tone due to the reduced availability of nitric oxide and the increased activity of vasoconstrictor substances such as endothelin-1 [9], and by affecting the composition of the extracellular matrix[9]. In addition, inflammation may induce both

functional and structural changes in the arterial wall via the increased production of reactive oxygen species [39], which in turn triggers an inflammatory process leading to the proliferation of smooth muscle cells, the influx of leucocytes and the production of pro-inflammatory substances and chemo-attractants [6]. Our data support the notion that both endothelial dysfunction and low-grade inflammation may cause increased muscular artery stiffness via similar pathophysiological pathways as in the femoral artery both endothelial dysfunction and low-grade inflammation primarily appeared to affect arterial distension (Supplemental table 1). In contrast, only low-grade inflammation affected (elastic) carotid artery stiffness, most likely through a pathophysiological mechanism independent of endothelial dysfunction, and not specifically driven through diameter, distension, pulse pressure or intima-media thickness. This suggests that low-grade inflammation may cause increased arterial stiffness of elastic arteries by affecting multiple elements of the vascular wall. These observations particularly align with the notion that the athero- and arteriosclerotic processes are, at least in part, inflammatory in origin and that inflammatory changes may lead to an altered matrix homeostasis (affecting vascular smooth muscle cells and matrix proteins), which in turn leads to a decrease in (femoral) distension. Taken together, the above suggests that endothelial dysfunction and low-grade inflammation may affect arterial stiffness of either muscular or elastic arteries differently. This might explain why endothelial dysfunction and low-grade inflammation were not associated with cfPWV, as the carotid-femoral segment includes both elastic and muscular arterial components. Alternatively, the adults investigated were young and did not have central arterial stiffening, which typically occurs after the age of 60 years [7].

Additional adjustment for potential confounders did not materially change the results, whereas adjustment for body weight attenuated the associations between biomarkers of endothelial dysfunction or low-grade inflammation and arterial stiffness. However, it is questionable whether body weight should be seen as a true confounder in this relationship. It is biologically more plausible that body weight is part of the causal pathway, i.e. body weight influences biomarkers of endothelial dysfunction and low-grade inflammation [40] and these influence arterial stiffening [9].

Finally, the timeframe in which endothelial dysfunction or low-grade inflammation have their possible impacts on arterial stiffness is unknown and might differ from the six-year period of the present investigation. (This might explain why changes in biomarkers of endothelial dysfunction or low-grade inflammation did not parallel changes in arterial stiffness.) The question remains for what period a person should have endothelial dysfunction or low-grade inflammation before it affects the vasculature. In any case, our results may suggest that any inflammatory changes that have led to arterial stiffening have already occurred before the age of 36 years.

The present investigation had some limitations. First, intrinsic to an overall Z-score is the assumption that each biomarker in the Z-score carries similar weight. This might have caused us to underestimate the reported associations, due to error because of a mathematical approach that might not optimally reflect pathophysiology. Second, the increase in diastolic blood pressure of 6 mmHg and the practically unchanged levels of systolic blood pressure between the ages of 36 and 42 in this study population led to a decrease in pulse pressure. Although this may seem 'unexpected', increases in pulse pressure with ageing are often observed after the 5<sup>th</sup> or 6<sup>th</sup> decades of life as a consequence of arterial stiffening [7]. In addition, these changes were in line with previous life course trends in changes of the two blood pressure components in this cohort, showing steeper increases in (sitting) diastolic (0.6 mmHg/year) than systolic blood pressure (0.2 mmHg/year) between late adolescence (age 15) and age 36 (data not shown). Still, as different blood pressure devices were used during the two measurement periods, it is possible that 2000's diastolic blood pressure data may have been underestimated as compared to the 2006 data. As a consequence, at the population level, pulse pressure and all arterial stiffness estimates are most likely underestimated in 2006 as compared to 2000. Nevertheless, a systematic underestimation of stiffness does not materially change the reported associations. Third, on the population level, heart rate was higher in 2000 as compared to 2006. This might be explained by the fact that the ultrasound examination was introduced for the first time in 2000, and, at the second ultrasound examination, individuals might have been more relaxed as they knew what was going to happen. As a (patho)physiological explanation is not readily at hand, such an effect might explain the difference in heart rate. Again, however, this would not be expected to affect the associations observed. Fourth, renal function was only determined in 2006.



Although additional adjustment did not materially change our results, the present study, therefore, can only partly exclude a role for renal function in the reported associations. Finally, our data were obtained in a young, Caucasian population and therefore inferences to older individuals and (or) other ethnicities should be made with caution.

### **Perspectives**

Both scores of biomarkers for endothelial dysfunction and low-grade inflammation are associated with greater arterial stiffness in apparently healthy adults, over a 6-year period. This suggests that arterial stiffness may be a mechanism through which endothelial dysfunction and low-grade inflammation lead to cardiovascular disease. In addition, the results showed that early in the development of arterial stiffening, endothelial dysfunction and low-grade inflammation act differentially along the arterial tree. These data may help to understand the pathophysiology of early arterial stiffening and may provide potential targets for intervention.

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## SUPPLEMENT

### **Assessment of endothelial dysfunction and low-grade inflammation**

The electro-chemiluminescence detection system uses multi-array plates fitted with multi-electrodes per well with each electrode being coated with a different catching antibody. The assay procedure then follows that of a classic sandwich enzyme linked immunosorbent assay (ELISA) with any of the analytes of interest captured on the relevant electrode. These captured analytes were then in turn detected by a secondary analyte-specific ruthenium-conjugated antibody, which is capable of emitting light after electrochemical stimulation. A particular advantage of this system is the ability to measure different biomarkers of endothelial dysfunction and (or) low-grade inflammation simultaneously in relatively small (25 or 50  $\mu$ l) serum samples. In our laboratory intra- and inter-assay coefficients of variation were for sVCAM-1, 3.8 and 6.9%; for sE-selectin, 4.1 and 8.6%; for sTM, 2.5 and 8.1%; for sICAM-1, 2.6 and 6.0%; for CRP, 2.3 and 4.3%; for SAA, 4.5 and 9.0%; for IL-6, 6.3 and 17.5%; for IL-8, 6.9 and 7.3%; and for TNF $\alpha$ , 5.9 and 12.6%, respectively. We also measured IL-1 $\beta$ , but levels were below the detection limit in 107 participants (resulting in intra- and inter-assay variation coefficients of 20.0 and 32.2%, respectively), and therefore we excluded IL-1 $\beta$  from the present investigation.

For the von Willebrand factor (vWf) ELISA, vWf rabbit anti-body was used as a catching antibody and a peroxidase-conjugate rabbit antibody as detecting antibody<sup>1</sup>. Levels were expressed as a percentage of vWf detected in pooled citrated plasma of healthy volunteers. Intra- and inter-assay coefficients of variation for vWf were 3.8 and 7.4%, respectively.

### **Assessment of arterial stiffness**

Arterial stiffness of the carotid, femoral and brachial artery was determined according to international guidelines<sup>2</sup> and the exact procedures for the AGAHL cohort, as executed in the 2000 follow-up, have been described elsewhere<sup>3</sup>. Briefly, arterial diameter (D), intima-media thickness (IMT) and distension ( $\Delta$ D) were quantified by ultrasonography<sup>3</sup> and local pulse pressure (PP) was determined according to the Van Bortel calibration method of distension waveforms<sup>4</sup>. Arterial stiffness was then calculated according to the following formulas:

- Distensibility Coefficient (DC)  

$$DC = (2\Delta D \cdot D + \Delta D^2) / (PP \cdot D^2) \quad (10^{-3} \cdot \text{kPa}^{-1})$$
- Compliance Coefficient (CC)  

$$CC = \pi \cdot (2D \cdot \Delta D + \Delta D^2) / 4PP \quad (\text{mm}^2 \cdot \text{kPa}^{-1})$$
- Young's elastic modulus (YEM)  

$$E_{inc} = D / (\text{IMT} \cdot DC) \quad (10^3 \cdot \text{kPa})$$

DC represents local arterial stiffness; CC represents the arterial buffering capacity; and YEM represents the intrinsic stiffness of the arterial wall.

In addition, central arterial stiffness [the carotid-femoral pulse wave velocity (cfPWV, in m/s)] was measured by dividing the length between the carotid and the femoral arterial sites by the carotid-to-femoral transit time. The carotid-to-femoral transit time was calculated by subtracting the travel time of the pressure wave from the heart (as triggered) to the femoral artery by that from the heart to the carotid artery. For technical reasons, cfPWV was obtained only in 241 individuals in 2000 and in 292 individuals in 2006.

### **Calculation of overall Z-scores for endothelial dysfunction and low-grade inflammation**

For reasons of statistical efficiency and to reduce the influences of the biological variability of each measure<sup>5</sup>, overall Z-scores were determined for both endothelial dysfunction and low-grade inflammation in 2000 and 2006, according to predefined clusters of conceptually related biomarkers<sup>5,6</sup>. These overall Z-scores were calculated as follows: first, for each individual biomarker a Z-score was calculated according to the formula: (individual value – population mean) / population standard deviation. The resulting individual Z-scores were then averaged into the overall Z-score for either endothelial dysfunction or low-grade inflammation. The endothelial dysfunction overall Z-score consisted of the biomarkers sICAM-1, sVCAM-1, sE-selectin, sTM and vWf. The low-grade inflammation overall Z-score consisted of the biomarkers CRP, SAA, IL-6, IL-8, TNF- $\alpha$  and sICAM-1. sICAM-1 levels were included in both overall Z-scores as it is expressed by both monocytes and the endothelium and is strongly affected by inflammatory stimuli<sup>7</sup>.

**Supplemental Table 1.** Longitudinal associations between, on the one hand, endothelial dysfunction and low-grade inflammation Z-scores during 6-year follow-up and, on the other hand, elements of the arterial stiffness indices during 6-year follow-up (n=293)

Endothelial dysfunction	Carotid artery		Femoral artery	
	b	95%CI	b	95%CI
<b>Diameter</b>				
1: + sex, height, MAP	-0.008	-0.09;0.08	0.02	-0.12;0.15
<b>Distension</b>				
1: + sex, height, MAP	0.007	-0.010;0.023	-0.013	-0.025;-0.001
<b>Pulse pressure</b>				
1: + sex, height, MAP	0.79	-0.53;2.13	0.53	-0.70;1.75
<b>Intima-media thickness</b>				
1: + sex, height, MAP	0.006	-0.009;0.022	-	-
<b>Low-grade inflammation</b>				
<b>Diameter</b>				
1: + sex, height, MAP	0.02	-0.08;0.11	-0.02	-0.15;0.10
<b>Distension</b>				
1: + sex, height, MAP	-0.007	-0.024;0.010	-0.015	-0.027;-0.004
<b>Pulse pressure</b>				
1: + sex, height, MAP	0.58	-0.86;2.00	-0.147	-1.53;1.23
<b>Intima-media thickness</b>				
1: + sex, height, MAP	0.012	-0.005;0.029	-	-

$\beta$ s, longitudinal regression coefficients that express the relationships between the longitudinal development of endothelial dysfunction and low-grade inflammation, on the one hand, and the longitudinal development of the elements of the arterial stiffness formulas on the other; 95%CI, indicate 95% confidence intervals and p-value; MAP = mean arterial pressure.

**Supplemental Table 2.** GEE Longitudinal associations between, on the one hand, endothelial dysfunction and low-grade inflammation z-scores during 6-year follow-up and, on the other hand, arterial stiffness indices during 6-year follow-up (N=293)

	Carotid artery			Brachial artery			Femoral artery		
	b	95%CI	P-value	b	95%CI	P-value	b	95%CI	P-value
<b>Endothelial dysfunction</b>									
Distensibility coefficient									
1: + sex, height, MAP	-0.19	-1.02;0.64	0.656	-0.94	-2.26;0.39	0.165	-0.51	-0.95;-0.06	0.025
2: 1 + smoking, total & HDL cholesterol, age, hypertension*	-0.03	-0.81;0.756	0.947	-1.00	-2.29;0.30	0.131	-0.42	-0.87;0.02	0.060
3: 2 + weight	0.16	-0.62;0.94	0.681	-1.15	-2.45;0.14	0.081	-0.27	-0.72;0.19	0.249
4: 2 + eGFR in 2006 only**	-0.004	-0.88;0.77	0.992	-1.01	-2.30;0.29	0.128	-0.42	-0.86;0.02	0.063
Compliance coefficient									
1: + sex, height, MAP	-0.012	-0.049;0.026	0.539	-0.009	-0.023;0.004	0.163	-0.041	-0.076;-0.006	0.020
2: 1 + smoking, total & HDL cholesterol, age, hypertension*	-0.008	-0.044;0.029	0.687	-0.011	-0.024;0.003	0.117	-0.036	-0.071;0.000	0.048
3: 2 + weight	-0.008	-0.044;0.029	0.683	-0.013	-0.026;0.000	0.050	-0.027	-0.064;0.010	0.155
4: 2 + eGFR in 2006 only**	-0.008	-0.045;0.029	0.683	-0.011	-0.024;0.002	0.111	-0.037	-0.071;-0.002	0.038
Young 's elastic modulus									
1: + sex, height, MAP	-0.001	-0.020;0.018	0.919	-	-	-	-	-	-
2: 1 + smoking, total & HDL cholesterol, age, hypertension*	-0.005	-0.024;0.014	0.587	-	-	-	-	-	-
3: 2 + weight	-0.009	-0.028;0.009	0.315	-	-	-	-	-	-
4: 2 + eGFR in 2006 only**	-0.005	-0.024;0.013	0.572	-	-	-	-	-	-
<b>Low-grade inflammation</b>									
Distensibility coefficient									
1: + sex, height, MAP	-0.91	-1.81;-0.01	0.048	0.19	-1.11;1.49	0.777	-0.51	-0.95;-0.07	0.024
2: 1 + smoking, total & HDL cholesterol, age, hypertension*	-0.65	-1.54;0.24	0.153	0.30	-0.97;1.57	0.644	-0.30	-0.74;0.14	0.185
3: 2 + weight	-0.35	-1.25;0.54	0.438	0.10	-1.12;1.32	0.868	-0.03	-0.49;0.44	0.913
4: 2 + eGFR in 2006 only**	-0.64	-1.53;0.24	0.154	0.24	-1.01;1.50	0.705	-0.32	-0.80;0.12	0.157
Compliance coefficient									
1: + sex, height, MAP	-0.022	-0.061;0.018	0.288	0.005	-0.008;0.017	0.480	-0.050	-0.084;-0.016	0.004
2: 1 + smoking, total & HDL cholesterol, age, hypertension*	-0.016	-0.054;0.022	0.414	0.006	-0.006;0.018	0.350	-0.031	-0.065;0.003	0.076
3: 2 + weight	-0.017	-0.053;0.020	0.374	0.003	-0.009;0.015	0.651	-0.016	-0.052;0.020	0.379
4: 2 + eGFR in 2006 only**	-0.015	-0.053;0.024	0.448	0.006	-0.007;0.018	0.377	-0.034	-0.068;0.000	0.051
Young 's elastic modulus									
1: + sex, height, MAP	0.006	-0.013;0.024	0.556	-	-	-	-	-	-
2: 1 + smoking, total & HDL cholesterol, age, hypertension*	0.001	-0.018;0.020	0.925	-	-	-	-	-	-
3: 2 + weight	-0.006	-0.025;0.013	0.531	-	-	-	-	-	-
4: 2 + eGFR in 2006 only**	0.002	-0.016;0.021	0.808	-	-	-	-	-	-

βs, longitudinal regression coefficients that express the relationships between the longitudinal development of endothelial dysfunction and low-grade inflammation on the one hand and the longitudinal development of arterial stiffness on the other; 95%CI, indicate 95% confidence intervals; MAP, mean arterial pressure; eGFR, estimated glomerular filtration rate; MDRD, Modification of Diet in Renal Disease short formula as follows:  $175 \cdot (\text{serum creatinine})^{-1.154} \cdot (\text{age})^{-0.203} \cdot 1.212$  (if black)  $\cdot 0.742$  (if female); \* hypertension defined as systolic blood pressure >140, diastolic blood pressure <90 mmHg and/or treatment for hypertension; \*\* only determined in 2006.



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