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**Associations of total and central fatness with carotid
and femoral stiffness: analyses of the mediating role
by adipokines, endothelial dysfunction and low-grade
inflammation**

The Amsterdam Growth and Health Longitudinal Study

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ABSTRACT

Objective: To investigate to what extent levels of adipokines (adiponectin, leptin, the leptin-to-adiponectin ratio (LAR)), biomarkers of low-grade inflammation (LGI) and endothelial dysfunction (ED) explain the relationship between total and/or central fatness, on the one hand, and arterial stiffness, on the other.

Design: 6-year longitudinal study with 277 participants (145 women) from the Amsterdam Growth and Health Longitudinal Study.

Methods: Plasma adiponectin and serum leptin levels, biomarkers of LGI (ie, C-reactive protein, serum amyloid A, interleukin 6, interleukin 8, tumor necrosis factor α and intercellular adhesion molecule 1) and of ED (ie, endothelial selectin, thrombomodulin, vascular- and intercellular adhesion molecules 1 and von Willebrand factor), total and trunk fat (by dual-energy X-ray absorptiometry), and arterial properties (carotid and femoral stiffness; by ultrasound) were measured when individuals were 36 years old and again 6 years later. Data were analysed using generalized estimating equations.

Results: Greater levels of total and trunk fat were adversely associated with carotid and femoral stiffness. Leptin, adiponectin and in particular the LAR, but not LGI or ED, explained a substantial portion (55-90%) of the relationship between total fat and arterial stiffness, and 43-90% of the relationship between trunk fat and carotid stiffness. In contrast, the association between trunk fat and femoral stiffness was not markedly attenuated after adjustment for adipokines, LGI or ED.

Conclusions: This study suggests that, in middle-aged adults, adipokines, rather than low-grade inflammation and endothelial dysfunction, play an important mediating role in the relationship between overall fatness and arterial stiffness, and also between central fatness and carotid stiffness.

INTRODUCTION

Obesity, in particular central fatness, is associated with greater arterial stiffness, [1-3] which is an important determinant of cardiovascular disease (CVD) [4,5]. The pathobiological mechanisms explaining why central body fatness is associated with arterial stiffness remain largely unknown. An altered profile of adipokine secretion, such as hyperleptinaemia [6-9] and/or hypoadiponectinaemia [10-18] may be one such mechanism. The extent to which the adipokines mediate the adiposity-arterial stiffness relationship has only been investigated in a few cross-sectional studies, which have shown inconsistent results, however [7,10,17]. The leptin-to-adiponectin ratio (LAR), which captures the adverse effects of leptin relative to the beneficial effects of adiponectin in one measure, has been proposed as a stronger determinant of arterial stiffness than either leptin or adiponectin alone [19]. Whether the LAR explains the association between central fatness and arterial stiffness relationships better than each adipokine alone is not known. In addition, other adiposity-related factors may also explain, at least in part, the relationship between central body fatness and arterial stiffness. These include increased levels of low-grade systemic inflammation (LGI) [12,20,21] and endothelial dysfunction (ED) [21,22].

We have recently shown, among apparently healthy adults, that higher levels of total body fatness and a pattern of central fat accumulation (i.e., higher levels of trunk fat mass) were both associated with higher stiffness of the carotid and the femoral arteries [3]. In addition, we have also observed significant associations of adverse adipokine (i.e., leptin, adiponectin and LAR) [23], and LGI and ED profiles [24] with higher stiffness of these arteries. The extent to which these potential biological mechanisms link (central) fatness to arterial stiffness, and/or whether some have a predominant role here in, is not clear however [1]. Unravelling these questions may help a better understanding of the aetiology of (central) obesity-related arterial stiffening and cardiovascular sequelae.

In view of these considerations, we have conducted the present 6-year longitudinal study in order to investigate the mediating role, if any, of adipokines (i.e. leptin, adiponectin, LAR), and biomarkers of LGI [C-reactive protein (CRP), serum amyloid A (SAA), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor α (TNF- α)

and sICAM-1] and ED [soluble intercellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble endothelial selectin (sE-selectin), soluble thrombomodulin (sTM) and von Willebrand factor (vWf)], in the relationship between total body fatness, trunk fat mass and arterial stiffness.

SUBJECTS AND METHODS

Subjects and study design

Data were derived from the AGAHLs, an observational longitudinal study which started in 1976 with a total inclusion of 698 boys and girls [25]. Its initial goal was to describe the natural development of growth, health and lifestyle of adolescents, and to investigate longitudinal relationships between biological and lifestyle variables. The mean age of the subjects at the start of the study was 13.1 ± 0.8 (mean \pm SD) years. Since then, subjects have been examined 3 to 9 times during a 30-year follow-up period. At each examination, anthropometrical, biological and lifestyle variables have been assessed, as described in detail elsewhere [25].

In 2000 (8th examination, baseline in the present study), when the mean age of the subjects was $36.6 (\pm 0.6)$ years, the following measurements were added to the study for the first time: body composition by means of dual-energy X-ray absorptiometry (DXA, $n=355$), and large artery properties by non-invasive ultrasound imaging ($n=373$ subjects); both measures were obtained in 336 subjects (175 women) [26]. In 2006, a follow-up examination was performed, including similar measurements of body composition ($n=293$) and large artery properties ($n=311$). The current study consists of 277 subjects (145 women) in whom complete data on body composition, arterial stiffness, adipokines and circulating biomarkers of LGI and ED were obtained in both 2000 and 2006.

The medical ethical committee of the VU University Medical Centre in Amsterdam approved the study protocol and all participants gave their written informed consent.

Adipokines

Serum leptin and plasma adiponectin were measured in blood samples collected in the 2000 and 2006 examinations and stored at -80°C. Leptin was determined in serum samples with a 2-plex multi-array (MesoScale Discover -MSD, Gaithersburg, MD, USA) as measured in a 96-well MULTI-SPOT plate. All reagents were provided with the MSD kit. Each 96-well plate has 2 spots per well with each spot pre-coated with anti-leptin antibodies. Samples, standards and controls were added at 25 µl per well and the plate was incubated for 2h at room temperature. At the end of the incubation, the wells were washed and the electrochemiluminiscent-labeled detection antibody was added at 25 µl per well and incubated for 1h at room temperature. For the detection, 150 µl of the MSD Read Buffer was added to each well and the MSD plates were measured on the MSD Sector Imager 2004 plate reader as electro-chemiluminescence signal (light) detected by photodetectors and analyzed by the Discovery Workbench 3.0 software (MSD). A logistic fit curve was generated for each marker using the standards and the concentration of each sample was calculated. Plasma total adiponectin was determined, as previously described, by a in-house time-resolved immunofluorometric assay based on two monoclonal antibodies and recombinant human adiponectin (R&D Systems, Abingdon, UK).[27] All standards and unknown samples were analyzed in duplicate, with the exception of non-specific binding, which were analyzed in quadruplicate. The intra- and inter-assay coefficients of variation for both leptin and adiponectin assessments were <5% and <10%, respectively.

Endothelial dysfunction and low-grade inflammation

Serum biomarkers of endothelial dysfunction (sICAM-1, sVCAM-1, sE-selectin and sTM), and of low-grade inflammation (CRP, SAA, IL-6, IL-8, TNF- α and sICAM-1) were assessed by an electro-chemiluminescence detection system using multi-array technology (SECTOR Imager 2400, Meso Scale Discovery, Gaithersburg, MD, USA) as described elsewhere [28] (please see also Supplement).

In addition, a plasma biomarker of endothelial dysfunction (vWf) was determined in citrated plasma by ELISA as described elsewhere [28] (please see also Supplement).

Body composition

In both the baseline and follow-up examinations we measured total and regional (i.e. arms, legs, trunk and head) body fat and lean masses with a whole-body dual X-ray absorptiometry (DXA) scanner, as detailed previously.[3,26]

Arterial stiffness

Properties of the carotid and femoral arteries were obtained by trained vascular sonographers with the use of an ultrasound imaging device connected to a computer equipped with vessel wall movement detector software (Wall Track System 2, Pie Medical, Maastricht, the Netherlands). In both the baseline and follow-up examinations arterial measurement followed the exact same protocol, which has been described in detail [3,29] (and was designed according to international guidelines.[4] Briefly, we obtained measures of carotid and femoral diameter (D) and distension (ΔD) and carotid (IMT); during these assessments we measured blood pressure in the brachial artery with an oscillometric device. We estimated the levels of pulse pressure (PP) at both the carotid and femoral arteries by calibration of the distension waveforms [30]. On the basis of these data, the following estimates of arterial stiffness were calculated:

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

DC reflects the elastic properties, whereas the CC reflects the buffering capacity of the artery at given operating local pressures. The YEM (estimated for the carotid artery only) represents the intrinsic elastic properties of the vessel wall. In contrast to carotid DC and CC, higher values of carotid YEM indicate greater arterial stiffness.

Statistical Analysis

Variables with a skewed distribution were log-transformed prior to analyses. For reasons of statistical efficiency and to reduce the influences of the biological variability of each measure, overall scores were determined for both ED and LGI in 2000 and 2006, according to predefined clusters of conceptually related biomarkers [22,31]. These overall 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. These individual Z-scores were then averaged into an overall score for either ED or LGI. The overall ED score consisted of the biomarkers sICAM-1, sVCAM-1, sE-selectin, sTM and vWf, and the overall LGI score consisted of the biomarkers CRP, SAA, IL-6, IL-8, TNF- α 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 [32].

All longitudinal associations were examined with the use of generalized estimating equations (GEE) with an exchangeable correlation structure, to properly take into account the correlation of repeated measurements within individuals over time [33]. First, we studied the associations between total body fat %, trunk fat mass (determinants) and the potential mediators, i.e., adiponectin, leptin, LAR, LGI and ED scores, adjusted for sex and also for trunk lean, peripheral fat and lean mass in analyses with trunk fat as main determinant. Second, we studied the associations between the potential mediators and carotid and femoral stiffness estimates (study main outcome variables), adjusted for sex, height and mean arterial pressure. Finally, we investigated to what extent the associations between total body fat %, trunk fat mass and arterial stiffness estimates, adjusted for sex, height and MAP (model 1) were attenuated after further adjustments for each of the potential mediators considered, by adding adiponectin, leptin, LAR, LGI and ED separately to the regression models (models 2a to 2e). Extent of mediation was quantified by expressing the change in the magnitude of the regression coefficients (in %) in models 2 vs. 1.

In all analyses, effect modification by sex was analyzed by adding interaction terms between the determinants and sex to the regression models. Because the associations did not differ consistently between men and women, results will be presented for men and women combined. All analyses were performed with Statistical Package for Social Sciences (SPSS, version 17, Chicago, IL, USA).

RESULTS

Tables 1 and 2 show the main characteristics of the study population at baseline (age 36), and after 6-yrs of follow-up. Subjects' mean levels of BMI, trunk fat and peripheral lean masses increased, whereas trunk lean mass remained fairly stable in both men and women. Peripheral fat mass increased in men but decreased in women. Adiponectin and leptin levels at baseline were higher in women than in men, and in both sexes leptin increased with age, whereas adiponectin only increased in women. Overall, biomarkers of LGI and ED remained fairly stable during the 6-year follow-up. Carotid stiffness increased, as depicted by decreases in DC and increases in YEM, whereas femoral stiffness decreased slightly with ageing.

Associations of total and central fatness with adipokines, inflammation and endothelial dysfunction

Throughout the 6-yr longitudinal period, higher levels of total fat and trunk fat were inversely associated with levels of adiponectin and positively with levels of leptin, LAR and LGI; only total fat % was associated with ED (Table 3).

Associations of adipokines, inflammation and endothelial dysfunction with arterial stiffness

Table 4 shows that lower levels of adiponectin, and higher levels of leptin, were associated with higher stiffness estimates of both the carotid and the femoral arteries. Consequently, higher values of LAR were adversely associated with all estimates of carotid and femoral stiffness.

Higher levels of LGI and ED scores were associated with increased stiffness of predominantly the femoral artery.

Table 1. General characteristics of the study population

	Men (n=132)		Women (n=145)	
	Baseline	6-yr follow-up	Baseline	6-yr follow-up
Body composition				
BMI (kg/m ²)	24.6 ± 2.9	25.3 ± 2.9	23.7 ± 3.5	24.2 ± 4.1
Body fat (%)	21.3 ± 6.3	23.6 ± 4.5	32.3 ± 7.1	31.8 ± 6.1
Trunk fat mass (kg)	8.1 ± 4.4	10.2 ± 3.8	8.4 ± 4.0	9.5 ± 4.4
Trunk lean mass (kg)	30.3 ± 3.3	29.9 ± 3.2	22.6 ± 2.8	22.7 ± 2.6
Peripheral fat mass (kg)	8.7 ± 2.8	9.2 ± 2.5	13.1 ± 4.3	12.7 ± 4.5
Peripheral lean mass (kg)	26.7 ± 3.2	28.8 ± 3.3	17.1 ± 2.9	19.8 ± 2.9
Blood pressure				
Systolic blood pressure (mmHg) ^a	121.5 ± 10.3	122.4 ± 13.5	111.8 ± 10.1	110.5 ± 12.0
Diastolic blood pressure (mmHg) ^a	67.0 ± 6.7	73.0 ± 7.5	63.1 ± 6.9	67.9 ± 7.5
Pulse pressure (mmHg) ^a	54.5 ± 5.9	49.4 ± 7.3	48.7 ± 5.5	42.6 ± 7.3
Mean arterial pressure (mmHg) ^a	85.6 ± 7.5	88.5 ± 9.1	78.9 ± 8.0	82.0 ± 9.2
Adipokines				
Adiponectin (mg/l)	7.2 (5.5-8.8)	7.0 (5.7-9.4)	10.7 (8.7-12.5)	11.5 (8.8-13.7)
Leptin (µg/l)	3.3 (1.8-6.0)	3.6 (1.8-5.9)	10.6 (6.2-18.2)	11.1 (6.6-22.8)
Leptin-adiponectin ratio	0.46 (0.21-1.04)	0.48 (0.23-0.99)	0.95 (0.61-1.98)	1.04 (0.54-2.25)
Biomarkers				
<i>Low-grade inflammation</i>				
C-reactive protein (mg/l)	0.6 (0.3-1.3)	0.7 (0.3-1.4)	0.9 (0.3-2.9)	0.9 (0.3-2.3)
Serum amyloid A (mg/l)	1.0 (0.6-1.9)	1.1 (0.6-2.1)	1.4 (0.9-2.3)	1.4 (0.9-2.6)
Interleukin 6 (ng/l)	2.2 (1.6-3.6)	2.4 (1.7-3.5)	2.5 (1.7-3.8)	2.5 (1.8-4.1)
Interleukin 8 (ng/l)	8.8 ± 3.2	9.4 ± 3.4	9.6 ± 3.6	9.5 ± 4.7
Tumor necrosis factor α (ng/l)	9.7 ± 3.7	9.5 ± 3.9	9.0 ± 3.0	8.6 ± 2.7
Soluble intercellular adhesion molecule 1 (µg/l) ^b	207.6 ± 47.4	203.7 ± 40.6	200.0 ± 59.0	198.1 ± 42.6
<i>Endothelial dysfunction</i>				
Soluble vascular cellular adhesion molecule 1 (µg/l)	337.7 ± 83.4	337.8 ± 63.6	331.4 ± 79.5	335.6 ± 75.0
Soluble endothelial selectin (µg/l)	12.2 ± 4.8	10.8 ± 5.1	9.2 ± 4.0	9.4 ± 4.1
Soluble thrombomodulin (µg/l)	2.76 ± 0.64	2.68 ± 0.66	2.21 ± 0.56	2.39 ± 0.56
von Willebrand factor (%)	102.7 ± 37.2	108.6 ± 37.2	100.6 ± 40.3	117.4 ± 46.2

Data are means ± SD, percentages, or median (inter-quartile range);

^aMeasured in the supine position during arterial properties assessment;

^bIncluded also in the endothelial dysfunction score.

Table 2. Arterial stiffness estimates of the study population

	Men (n=132)		Women (n=145)	
	Baseline	6-yr follow-up	Baseline	6-yr follow-up
Carotid artery				
Distensibility coefficient ($10^{-3}/\text{kPa}$)	26.1 \pm 5.5	25.3 \pm 7.3	27.2 \pm 6.5	26.2 \pm 7.1
Compliance coefficient (mm^2/kPa)	1.06 \pm 0.28	1.09 \pm 0.35	0.94 \pm 0.26	0.96 \pm 0.29
Young's Elastic Modulus ($10^3 \bullet \text{kPa}$)	0.47 \pm 0.13	0.49 \pm 0.16	0.42 \pm 0.12	0.44 \pm 0.16
Femoral artery				
Distensibility coefficient ($10^{-3}/\text{kPa}$)	5.7 \pm 2.8	6.6 \pm 3.5	8.1 \pm 4.0	9.6 \pm 4.8
Compliance coefficient (mm^2/kPa)	0.50 \pm 0.24	0.61 \pm 0.32	0.51 \pm 0.24	0.63 \pm 0.33

Data are means \pm SD.

Table 3. Associations of total body fat % and trunk fat mass with adiponectin, leptin, LAR, and LGI and ED scores throughout the 6-yr follow-up period

Dependent variables	Total body fat (per 10%)		Trunk fat mass (per 10 kg)	
	b	95% CI	b	95% CI
Ln Adiponectin	-0.08	-0.13; -0.04‡	-0.26	-0.37; -0.14‡
Ln Leptin	1.02	0.93; 1.11‡	1.08	0.88; 1.28‡
Leptin-to-Adiponectin ratio	1.07	0.97; 1.16‡	1.34	1.10; 1.59‡
Low-grade inflammation score	0.27	0.19; 0.36‡	0.40	0.22; 0.58‡
Endothelial dysfunction score	0.12	0.04; 0.20†	0.08	-0.11; 0.27

β , regression coefficient as obtained from GEE analyses – indicates the difference in dependent variable per 10% or per 10-kg increase in total or trunk fat; CI, confidence intervals; Data are adjusted for sex and, for the associations with trunk fat as independent variable, also for trunk lean, and peripheral (i.e. arms and legs) fat and lean masses; † $P < 0.01$, ‡ $P < 0.001$.

Table 4. Associations of adiponectin, leptin, LAR, LGI and ED levels with arterial stiffness throughout the 6-yr follow-up period

Dependent variables	Ln Adiponectin		Ln Leptin		LAR		LGI		ED	
	b	95% CI	b	95% CI	b	95% CI	b	95% CI	b	95% CI
<i>Carotid artery</i>										
Distensibility coefficient (10^{-3} /kPa)	2.12	0.58; 3.66†	-1.04	-1.61; -0.47‡	-1.06	-1.57; -0.54‡	-0.76	-1.68; 0.17	-0.24	-1.10; 0.61
Compliance coefficient (mm^2/kPa , *100)	9.94	1.93; 17.95**	-3.09	-5.84; -0.34**	-3.48	-6.00; -0.96†	-1.59	-5.70; 2.52	-1.40	-5.29; 2.49
Young's elastic modulus ($10^3 \cdot \text{kPa}$, *100)	-4.20	-7.79; -0.61**	2.28	1.01; 3.55‡	2.32	1.23; 3.42‡	0.48	-1.47; 2.42	-0.19	-2.21; 1.82
<i>Femoral artery</i>										
Distensibility coefficient (10^{-3} /kPa)	1.57	0.66; 2.48†	-0.31	-0.71; 0.09	-0.40	-0.75; -0.06**	-0.52	-1.00; -0.06**	-0.41	-0.87; 0.04*
Compliance coefficient (mm^2/kPa , *100)	13.21	6.42; 20.00‡	-3.45	-6.34; -0.55**	-4.03	-6.51; -1.55†	-5.38	-8.91; -1.85†	-3.30	-6.82; 0.23*

LAR, leptin-to-adiponectin ratio ; LGI, low-grade inflammation; ED, endothelial dysfunction;

β , regression coefficient as obtained from GEE analyses – indicates the difference in dependent variable per unit increase in adipokines or LGI or ED scores; CI, confidence interval; Data are adjusted for sex, height and mean arterial pressure; * $P < 0.10$; ** $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

Table 5. Six-year longitudinal associations of trunk fat with arterial stiffness and the mediating role of adiponectin, leptin, LGI and ED herein

Dependent variable Model: Adjustments	Total body fat (per 10%)				Trunk fat (per 10 kg)			
	β	95% CI	P-value	$\Delta\beta$	β	95% CI	P-value	$\Delta\beta$
Carotid Distensibility Coefficient (10^{-3}/kPa)								
1.	-1.57	-2.42; -0.72	<0.001	-	-2.35	-4.32; -0.38	0.019	-
2a: + Ln adiponectin	-1.43	-2.30; -0.55	0.001	-9%	-1.71	-3.78; 0.36	0.105	-27%
2b: + Ln leptin	-1.01	-2.17; 0.14	0.086	-36%	-1.83	-3.97; 0.32	0.095	-22%
2c: + LAR	-0.71	-1.83; 0.42	0.218	-55%	-1.34	-3.58; 0.90	0.239	-43%
2d: + LGI	-1.49	-2.38; -0.60	0.001	-5%	-2.20	-4.20; -0.21	0.031	-6%
2e: + ED	-1.57	-2.42; -0.71	<0.001	0%	-2.26	-4.24; -0.27	0.026	-4%
Carotid Compliance Coefficient (mm^2/kPa, *100)								
1.	-5.09	-8.97; -1.21	0.010	-	-5.57	-15.59; 4.44	0.276	-
2a: + Ln adiponectin	-4.36	-8.47; -0.25	0.038	-14%	-2.69	-12.97; 7.58	0.607	-52%
2b: + Ln leptin	-3.80	-9.80; 2.19	0.214	-25%	-2.92	-14.07; 8.23	0.607	-48%
2c: + LAR	-2.26	-8.44; 3.93	0.475	-56%	-0.58	-12.13; 10.98	0.922	-90%
2d: + LGI	-5.07	-8.91; -1.22	0.010	-0%	-5.37	-15.26; 4.53	0.288	-4%
2e: + ED	-5.05	-8.96; -1.13	0.012	-1%	-5.36	-15.52; 4.79	0.300	-4%
Carotid Young's Elastic modulus ($10^3 \cdot \text{kPa}$, *100)								
1.	3.19	1.29; 5.09	0.001	-	4.55	-0.73; 9.83	0.092	-
2a: + Ln adiponectin	2.90	0.89; 4.90	0.005	-9%	3.35	-2.46; 9.15	0.259	-26%
2b: + Ln leptin	1.63	-0.88; 4.13	0.202	-49%	3.37	-2.19; 8.92	0.235	-26%
2c: + LAR	1.00	-1.57; 3.57	0.444	-69%	2.12	-3.82; 8.06	0.483	-53%
2d: + LGI	3.32	1.38; 5.26	0.001	4%	4.90	-0.42; 10.21	0.071	8%
2e: + ED	3.25	1.35; 5.14	0.001	2%	4.60	-0.71; 9.91	0.089	1%
Femoral Distensibility Coefficient (10^{-3}/kPa)								
1.	-0.38	-0.92; 0.16	0.167	-	-1.19	-2.42; 0.04	0.058	-
2a: + Ln adiponectin	-0.27	-0.81; 0.28	0.342	-29%	-0.86	-2.11; 0.40	0.180	-28%
2b: + Ln leptin	-0.22	-1.00; 0.56	0.575	-42%	-1.41	-2.75; -0.08	0.038	19%
2c: + LAR	0.04	-0.71; 0.78	0.927	-90%	-1.22	-2.57; 0.13	0.076	10%
2d: + LGI	-0.27	-0.85; 0.31	0.357	-29%	-1.14	-2.40; 0.12	0.076	-6%
2e: + ED	-0.38	-0.94; 0.18	0.183	0%	-1.23	-2.48; 0.02	0.053	3%

Femoral Compliance Coefficient (mm ² /kPa, *100)									
1.	-4.76	-8.62; -0.90	0.016	-	-11.63	-20.27; -2.99	0.008	-	-
2a: + Ln adiponectin	-3.84	-7.69; 0.02	0.051	-19%	-9.08	-17.76; -0.39	0.041	-22%	-
2b: + Ln leptin	-3.09	-8.58; 2.40	0.269	-35%	-12.11	-21.67; -2.55	0.013	4%	-
2c: + LAR	-1.05	-6.26; 4.16	0.693	-78%	-10.61	-20.09; -1.12	0.028	-9%	-
2d: + LGI	-3.72	-7.83; 0.39	0.076	-22%	-10.91	-19.77; -2.05	0.016	-6%	-
2e: + ED	-4.84	-8.85; -0.84	0.018	2%	-12.20	-20.96; -3.44	0.006	5%	-

β , regression coefficient as estimated by GEE analyses – indicates change in dependent variables (i.e. carotid or femoral stiffness estimates) per 10% or 10-kg increase in total body fatness or central fat; $\Delta\beta$, indicates change (in %) in the magnitude of the association displayed in model 1 after further adjustment for variables in models 2a to 2e, and was calculated as $(\beta \text{ in model 2} - \beta \text{ in model 1})/\beta \text{ in model 1} * 100$; MAP, mean arterial pressure; LAR, leptin-to adiponectin ratio; LGI, low-grade inflammation; ED, endothelial dysfunction; CI, confidence interval; Model 1: adjusted for sex, height, mean arterial pressure, trunk lean mass, peripheral fat and lean mass.

Association between total and central fatness with arterial stiffness and the mediating role of adipokines, inflammation and endothelial dysfunction herein

Table 5 shows the associations of total body fat % (adjusted for sex, height and MAP) and trunk fat (also adjusted for trunk lean, peripheral fat and lean mass) with carotid and femoral stiffness (model 1), after additional adjustments for each adipokine or their ratio, or LGI or ED scores (model 2a to 2e). Overall, and as judged from the attenuation in the magnitude of the regression coefficients in models 2a to 2e vs. model 1, these analyses can be summarized as follows: 1) the LAR was the strongest mediating factor, explaining a large portion (~55-90%) of the adverse association between total body fat % and both carotid and femoral stiffness; 2) noteworthy, this portion explained by the LAR exceeded the one that could be expected by summation of the portions explained by each adipokine alone, of which leptin contributed the most; 3) a similar extent of mediation by the LAR was also observed in the associations of central fatness with carotid stiffness (~43-90%), though these did not exceed the contribution by the summation of adiponectin and leptin, each explaining a similar portion of the association with carotid stiffness. 4) adiponectin was the single mediator explaining a relevant, albeit modest, portion of the associations between central fatness and femoral stiffness; 5) LGI only seemed to play a mediating role in the associations between total body fatness and femoral stiffness, though of considerably less magnitude than the LAR; and 6) ED did not appreciably explain the associations of either total or trunk fat with carotid or femoral stiffness.

Additional analyses

Additional adjustment for metabolic variables (ie, total-to-HDL-cholesterol ratio, triglycerides, glycated haemoglobin and heart rate) did not materially change the associations between trunk fat, carotid stiffness and the adipokines, nor the degree by which adipokines, LGI or ED explained associations between total body fat, trunk fat and arterial stiffness (data not shown).

DISCUSSION

To the best of our knowledge, this is the first population-based study that has examined the mediating role of adipokines, LGI and ED in the association of total and trunk fat with arterial stiffness of both the (elastic) carotid and the (muscular) femoral arteries. The key findings of our study are that adipokines, in particular the LAR, explained to a large extent the relationship between total fat and both carotid and femoral stiffness, and also between central fatness and carotid stiffness. The association between central fatness and femoral stiffness appeared to be largely independent of leptin and the LGI and ED profiles, and was only affected to a modest extent by adiponectin. LGI, but not ED, also played a modest mediating role in the associations investigated but this was only found for the associations between total fatness and femoral stiffness.

The mediating role of adipokines in the associations between central fatness and arterial stiffness has been examined before in two cross-sectional studies among older individuals [7,10]. In the study where both adipokines were investigated, leptin seemed to be of greater influence than adiponectin in the central fatness-arterial stiffness associations [10], whereas in our study the contribution of adiponectin was of similar strength in the carotid and even more powerful in the femoral stiffness associations. Our study thus provides additional information by extending earlier observations also to: apparently healthy and younger adults; the associations between *total* body fat and arterial stiffness; the context of a longitudinal and thus more robust study design. Furthermore, we have now examined, for the first time, the mediating role of the LAR in the associations between (central) fatness and arterial stiffness.

The LAR has been proposed as a comprehensive measure of adipokine secretion that combines the joint effects of leptin and adiponectin on metabolic [34,35] and cardiovascular health outcomes [19,36,37]. Indeed, in both individuals with type 2 diabetes (T2D) as well as in the present cohort consisting of an apparently healthy adults, the LAR was more strongly associated with arterial stiffness than either leptin or adiponectin alone [19,23]. We now show that the LAR may also explain the associations between total fatness and arterial stiffness beyond the summation of

the mediation 'effects' conferred by each adipokine. This suggests that the LAR may constitute a relevant tool for the cardiovascular risk prediction among the obese, but this needs to be further investigated.

Mechanistically, the potential interaction between adipokines and the vessel wall is complex. Although elevated leptin levels have been associated with poor vascular compliance in adolescents [6] and impaired coronary vasoreactivity in young obese individuals [38], evidence from experimental work suggests that leptin may have both vasoconstrictor and vasodilator effects through endothelium-dependent mechanisms [39]. Leptin induces vasoconstriction via the endothelin-1, but can also promote vasodilation via nitric oxide [40]. It is conceivable that leptin-induced vasodilation is blunted in obese individuals, enabling its vasoconstrictor actions to dominate [41]. Several experimental studies have further shown that both leptin and adiponectin may, respectively, promote or prevent ED, oxidative stress, platelet aggregation, and migration, hypertrophy and proliferation of smooth muscle cells [42-45], all of which may influence arterial stiffening [21].

In large agreement with the current literature, biomarkers of LGI were strongly associated with both total and trunk fat. Owing to the associations of LGI with predominantly femoral stiffness, LGI also explained a small portion of the association between total but not trunk fat and femoral stiffness. This is in contrast with one study among patients with type 2 diabetes showing that the association between visceral fat and carotid stiffness disappeared after adjustment for inflammatory markers (i.e., IL-6 and CRP) [20]. Differences in study populations may explain the discrepancies in findings. Still, in both older [46] and younger individuals [47], biomarkers of LGI and/or ED did not seem to explain the association between the metabolic syndrome, which is closely associated with central obesity, and femoral stiffness.

Our study also showed that biomarkers of ED did not explain a relevant portion of the association between trunk fat and arterial stiffness, given that there was no association between trunk fat mass and ED. However, biomarkers of ED were associated with total body fatness and with femoral stiffness, although less strongly so as compared with the other potential mediators such as adipokines, which may have 'overruled' any role for ED. Alternatively, it is possible that the circulating biomarkers as investigated herein, albeit extensive, may have insufficiently reflected

(e.g. in comparison with functional markers such as flow-mediated dilation) the process of vascular endothelial dysfunction likely to result in arterial stiffening.

Why the association between central fatness and femoral but not carotid stiffness remained largely independent of all the potential mediators examined, and if anything only mediated by adiponectin, is unclear. It is known, however, that carotid and femoral arteries are structurally different and their stiffness is differentially influenced by common determinants of arterial stiffening.[48] Other obesity and stiffness-related factors not examined herein may need to be further considered.

Main strengths of the present study are its longitudinal design, the robust assessment of both body composition and stiffness of both elastic (carotid) and muscular (femoral) arteries, and the extensive characterization of biomarkers reflecting underlying pathobiological mechanisms of interest. There are some limitations to our study, however. First, the adiponectin molecule is known to form a wide range of polymers, of which the predominant polymers include trimers, hexamers and highly congregated multimers. Previous experiments have demonstrated that both monoclonal antibodies used are able to detect several adiponectin polymers in serum, including the three major molecular forms. However, the high-molecular weight (HMW) sub-form has been suggested to be the biologically most active form of adiponectin. It is thus possible that associations between HMW adiponectin and arterial stiffness are stronger than the ones reported herein. Second, blood pressure was measured with different devices at baseline and at follow-up [3,24]. Such changes in measurement equipment may have caused seemingly unexpected phenomena, such as a decrease in pulse pressure. Although this may seem unexpected, increases in pulse pressure with ageing are usually not observed until after the 5th or 6th decade [21]. Our data may thus reflect real changes among young adults. Nevertheless, should any systematic under- or overestimation of blood pressure have occurred due to different devices, this does not impair inferences from the associations reported here. Further, our findings were obtained in a Caucasian, relatively young and apparently healthy adult population, and thus should be interpreted with caution with regard to older individuals, other ethnicities, and high-risk populations.

In conclusion, this study shows that an adverse adipokine profile, rather than low-grade inflammation and endothelial dysfunction, exert a predominant mediating role

in the association between total and central fat accumulation and arterial stiffness in healthy middle-aged adults. These data may help to understand the mechanisms of arterial stiffening linked to adiposity.

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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 μ 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 TNFa, 5.9 and 12.6%, respectively. We also measured IL-1 β , 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 β 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 antibody¹. 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.