Fat Distribution and Arterial Stiffness

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Higher leptin-to-adiponectin ratio is associated with carotid and femoral stiffening in young adults: a 6-yr longitudinal study

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ABSTRACT

Objective: To investigate, in healthy young adults, the longitudinal associations of leptin, adiponectin and the leptin-to-adiponectin ratio (LAR) with carotid, femoral and aortic stiffness, over a 6-yr follow-up period.

Methods: We investigated 286 subjects (151 women) in whom levels of adiponectin, leptin and measures of arterial stiffness [i.e., carotid and femoral distensibility (DC) and compliance (CC) coefficients, carotid Young's elastic modulus (YEM) and carotid-femoral pulse wave velocity (cfPWV)] were assessed when subjects were 36-years old and again at the age of 42.

Results: Throughout the 6-year period, and after adjustments for sex, height, mean arterial pressure and potential confounders, lower levels of adiponectin and higher levels of leptin as well as, and in particular, of the LAR were associated with greater carotid and femoral stiffness: e.g. in associations of the LAR with carotid DC [standardized longitudinal regression coefficient (b)=-0.18(95%CI: -0.26;-0.10), p<0.001]; CC [b=-0.13(-0.22;-0.04), p<0.01] and YEM [b=0.17(0.09;0.25), p<0.001] and femoral DC [b=-0.12(-0.21,-0.03), p<0.01] and CC [b=-0.16(-0.25;-0.06), p<0.01]. Adjustments for total-to-HDL cholesterol, tryglicerides, glycated haemoglobin and total body fat attenuated most of these associations, particularly those with femoral stiffness estimates, but most remained largely independent of these adjustments: with DC [b=-0.12(-0.24;-0.01), p<0.05], CC [b=-0.15(-0.29;-0.00), p<0.05], YEM [b=0.14(0.02;0.25), p<0.05], and with femoral CC [b=-0.13(-0.26;-0.00), p<0.01]. No associations were found between the adipocytokines or their ratio with cfPWV.

Conclusions: Adverse levels of leptin and adiponectin, as captured by their ratio, are independently associated with greater carotid and femoral stiffening throughout the course of young adult life, and may thus constitute a pathobiological mechanism involved in accelerated arterial ageing and related cardiovascular sequelae.

INTRODUCTION

Hyperleptinaemia [1] and/or hypoadiponectaemia [2] have been proposed as molecular mechanisms that link adiposity to arterial stiffness [3,4]. Arterial stiffness is an important determinant of cardiovascular disease (CVD) [5,6], and as such, deleterious associations of high levels of leptin and/or low levels of adiponectin with arterial stiffness, could explain, at least in part, the increased CVD risk associated with these adipocytokines [7-10].

Several observational studies have reported positive associations of leptin [1,11-13] and inverse associations of adiponectin [1,2,14-20] with arterial stiffness. However, the beneficial associations of adiponectin with arterial stiffness have not been confirmed in all studies [13,21,22]. 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 [23]. This finding was confined to obese individuals with Type 2 diabetes (T2D), and the extent to which they may apply to the general population remains unknown.

Apart from differences in studied populations, inferences are limited by their cross-sectional designs. In fact, evidence to support a causal role of adipocytokines in arterial stiffening in humans has been derived mainly from small intervention studies with short duration (3-6 months), and data have been confined to individuals with impaired glucose metabolism/metabolic syndrome [24] or T2D [25-27]. Furthermore, whether changes in leptin impact on changes in arterial stiffness is largely unknown. We are aware of only one small intervention study among severely obese subjects that reported significant decreases in both leptin and arterial stiffness after a 1-yr weight-loss program, but these beneficial changes were not associated with each other [28].

In view of these considerations, we have conducted the present longitudinal study, in order to better understand the etiological role, if any, of these adipocytokines in the process of arterial stiffening. Specifically, we investigated, in a cohort of apparently healthy young adults, over a 6-yr follow-up period, the associations between levels of leptin, adiponectin and the LAR, on the one hand, and arterial stiffness on the other.

METHODS

Subjects and study design

Data were derived from the *Amsterdam Growth and Health Longitudinal Study*, an observational longitudinal study that started in 1976 with a total inclusion of 698 boys and girls [29]. Briefly, 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 (±SD) age of the subjects at the start of the study was 13.1±0.8 years. Since then, subjects have been examined 3-9 times during a 30-y follow-up period. At each follow-up examination, anthropometric, biological and lifestyle variables were assessed, as detailed elsewhere [29].

In the year 2000 (8^{th} examination, baseline in the present study), when subjects' mean age was 36.6 (±0.6) years, complete measurements of large artery properties were performed for the first time, by means of non-invasive ultrasound, in 373 subjects [30-33]. In 2006, follow-up arterial measurements were obtained in 297 of these individuals, 286 of whom had also complete data on adiponectin and leptin at both examinations (the sample of the present study). Baseline characteristics from the 87 individuals without complete follow-up data did not differ from those included (data not shown).

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

Adiponectin and leptin

Serum leptin and plasma adiponectin were measured in blood samples collected in the 2000 and 2006 examinations and stored at -80°C till time of assay. 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 using 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) [34]. 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 [35]. 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.

Arterial stiffness

Arterial properties for the estimation of arterial stiffness were assessed according to guidelines for user procedures and with the use of reproducible and valid methods and devices [5,36,37]. Measures obtained in the year 2006 (by F.S.) followed the exact same protocol as conducted in 2000 (by I.F., described in detail before [30,32,33]), with good levels of inter-observer reproducibility [38].

Briefly, all subjects abstained from smoking and caffeine-containing beverages on the day the measurements were performed. At the time of measurements of arterial properties, subjects had been resting in a supine position for 15 minutes in a quiet, temperature-controlled room. Properties of the right common carotid (10 mm proximal to the beginning of the bulb) and the common femoral (20 mm proximal to the flow divider) were obtained by trained vascular sonographers with the use of an ultrasound scanner equipped with a 7.5-MHz linear array probe (Pie Medical, Maastricht, the Netherlands). The ultrasound scanner was connected to a

personal computer equipped with an acquisition system and a vessel wall movement detector software system (Wall Track System 2 (WTS_2), Pie Medical, Maastricht, the Netherlands). This integrated device enables measurements of arterial diameter (D), distension (ΔD), pulse wave transit time (TT), and intima-media thickness (IMT) as detailed elsewhere [32,36]. The carotid artery was measured approximately 10 mm proximal to the beginning of the bulb; the exact distance (ie, placement of the M-line) was video-recorded and a print-out was obtained in order to ensure that follow-up measures, as obtained in 2006, were obtained at the exact same arterial site [38].

Throughout the entire period of ultrasound imaging and while the subjects were in a supine position, systolic BP (SBP), diastolic BP (DBP), mean arterial pressures (MAP) and resting heart rate were assessed in the left arm at 5-min intervals with an oscillometric device (in 2000: Colin Press-Mate, model BP-8800, Komaki-City, Japan; in 2006: Dinamap ProCare, GE Healthcare, Tampa, Florida, USA). Brachial artery pulse pressure (PP) was defined as SBP minus DBP, and PP at the level of the common carotid and femoral arteries was calculated by calibration of the diameter-distension waveforms obtained at this and at the brachial arteries as described by Van Bortel *et al.* [39].

The mean IMT, D, ΔD and local PP of 3 consecutive measurements (each including 3 to 7 heart beats) were used to estimate the distensibility (DC) and compliance (CC) coefficients in the carotid and femoral artery, and the Young's elastic modulus (YEM) in the carotid artery as follows:

$$DC = (2\Delta D * D + \Delta D^2)/(PP * D^2) \quad \text{in } 10^{-3}/\text{kPa} \quad (1)$$

$$CC = \pi * (2D * \Delta D + \Delta D^2)/4PP \quad \text{in } mm^2/\text{kPa} \quad (2)$$

$$YEM = D/(IMT * DC) \quad \text{in kPa} \quad (3)$$

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 (calculated by subtracting the travel time of the pressure wave from the heart to the femoral artery by that from the heart to the carotid artery) [30,33,38]. For technical reasons (mainly faulty ECG triggering), cfPWV was obtained only in 235 subjects (123 women) of the 286 subjects included in the present study. Baseline

and follow-up levels of leptin and arterial stiffness estimates of these subjects did not differ from those of the 51 subjects with missing cfPWV data, but their levels of adiponectin at baseline were slightly lower (data not shown).

Covariates

In both the baseline and follow-up examinations we measured subjects' levels of body fat and composition by anthropometry and dual x-ray absorptiometry (DXA) [38], total and high-density lipoprotein (HDL)-cholesterol, triglycerides, and glycated hemoglobin (HbA1c) according to standard methods [30] and obtained information on subjects' smoking and drinking status, and daily physical activity levels (expressed in metabolic equivalents - METs/week) by means of questionnaires and structured interviews [29]. Due to the lack of creatinine assessments at baseline, we estimated glomerular filtration rate (eGFR) using the short Modification of Diet in Renal Disease equation [40] at follow-up only.

Statistical Analysis

We used generalized estimating equations (GEE) to examine the associations of either leptin, adiponectin or the LAR with each arterial stiffness estimate throughout the 6-yr longitudinal period. GEE analyses take into account the correlation of repeated measurements within individuals over time. Results of these analyses were expressed by standardized regression coefficients, and respective 95% confidence intervals (CI) to enable comparison of the strength of the associations of each adipocytokine with each of the stiffness estimates. These regression coefficients are interpretable as longitudinal correlation coefficients that combine the between-subjects (cross-sectional) and the within-subjects (longitudinal) associations between variables over time [41].

Variables with a skewed distribution were $\log_e$ transformed prior to analyses. All analyses were adjusted, first, for sex, height, and MAP (model 1) and further for lifestyle factors (smoking status, alcohol intake, levels of physical activity and eGFR - model 2). The analyses between each adipocytokine with arterial stiffness estimates were also mutually adjusted for each other, to ascertain whether any of them exerted a predominant effect on arterial stiffness (model 3). Finally, we investigated

the extent to which adjustment for metabolic variables (i.e. total-to-HDL cholesterol, triglycerides and HbA1c - model 4) and total body fat (model 5) attenuated the associations between adipocytokines and arterial stiffness.
We also investigated whether any associations differed by sex by adding interaction terms between leptin, adiponectin or LAR and sex to the regression models described above. However, no consistent interactions were found between males and females, and accordingly, all results were presented for men and women combined.
All statistical analyses were performed using the Statistical Package for Social Sciences for Windows (SPSS version 17.0, SPSS Inc, Chicago, IL, USA).

RESULTS

Tables 1 and *2* show the main characteristics of the study population at baseline, and after 6-yr of follow-up. In both men and women, levels of both leptin and LAR increased whereas in men only, levels of adiponectin decreased during 6-year follow-up. Also in both sexes, the carotid IMT and the diameter of both the carotid and the femoral arteries increased whereas the distension of the carotid but not the femoral artery decreased with ageing. These changes in arterial properties, combined with decreases in (local) pulse pressure, resulted in lower DC and higher YEM of the carotid artery but greater DC and CC of the femoral artery at follow-up. As expected, cfPWV increased with ageing.

Table 1. Characteristics of the study population

	Men (n=135)		Women (n=151)	
	Baseline	6-yr follow-up	Baseline	6-yr follow-up
Age (years)	36.5 ± 0.6	42.6 ± 0.6	36.6 ± 0.6	42.6 ± 0.6
Leptin (μg/L)	3.34 [1.77-5.90]	3.56 [1.77-6.13]	10.68 [6.08-18.53]	10.90 [6.33-22.31]
Adiponectin (mg/L)	7.20 [5.42-8.74]	7.04 [5.67-9.38]	10.71 [8.73-12.61]	11.53 [8.86-13.86]‡
Leptin-adiponectin ratio	0.46 [0.21-1.04]	0.48 [0.23-0.99]	0.94 [0.57-1.98]	1.04 [0.53-2.14]
Body mass index (kg/m²)	24.6 ± 2.6	25.2 ± 2.9‡	23.7 ± 3.6	24.2 ± 4.1†
Total body fat[a] (%)	21.4 ± 6.3	23.7 ± 4.6‡	32.3 ± 7.2	32.0 ± 6.2
Systolic blood pressure (mm Hg)	121.8 ± 10.3	123.0 ± 13.6	111.9 ± 10.5	110.7 ± 12.5
Diastolic blood pressure (mm Hg)	67.2 ± 6.6	73.5 ± 7.4‡	63.1 ± 7.2	67.9 ± 7.9‡
Pulse pressure (mm Hg)	54.6 ± 6.0	49.5 ± 9.3‡	48.8 ± 5.6	42.8 ± 7.4‡
Mean arterial pressure (mm Hg)	85.8 ± 7.5	88.9 ± 9.1‡	79.0 ± 8.2	82.0 ± 9.6‡
Total/HDL-cholesterol (mmol/L)	4.5 ± 1.3	3.6 ± 1.0‡	3.2 ± 0.9	2.7 ± 0.7‡
Triglycerides (mmol/L)	1.3 [0.9-1.9]	1.1 [0.8-1.8]†	0.9 [0.6-1.2]	0.8 [0.7-1.1]
Glycated hemoglobin (%)	5.3 ± 0.4	5.5 ± 0.5‡	5.2 ± 0.4	5.3 ± 0.2†
eGFR (ml/min/1.73m²)	-	84.0 ± 10.7	-	78.7 ± 12.7
Smokers (%)	25.4	16.4*	19.2	14.1*
Alcohol drinkers (%)	94.7	95.4	84.0	89.9*
Physical activity (10³METS/wk)[b]	3.39 [2.18-5.81]	2.68 [2.19-3.13]‡	4.54 [3.22-6.73]	2.74 [2.28-3.35]‡

Data are presented as mean ± SD, percentage, or median (inter-quartile range) as appropriate;
[a] As estimated by DXA; [b] Absolute measurements at baseline and follow-up are not comparable due to different instruments/equipment used at the two time points; *p<0.05; †p<0.01; ‡p<0.001.

Table 2. Large artery properties of the study population

	Men (n=135)		Women (n=151)	
	Baseline	6-yr follow-up	Baseline	6-yr follow-up
Carotid artery				
Diameter (mm)	7.18 ± 0.51	7.41 ± 0.63‡	6.62 ± 0.51	6.85 ± 0.62‡
Distension (μm)	623 ± 141	515 ± 123‡	522 ± 120	450 ± 115‡
Local pulse pressure (mmHg)	52.6 ± 8.1	45.1 ± 11.1‡	46.0 ± 7.6	40.2 ± 9.5‡
Intima-media thickness (mm)	0.62 ± 0.10	0.66 ± 0.12‡	0.63 ± 0.10	0.66 ± 0.11†
Distensibility coefficient (10^{-3}/kPa)	26.1 ± 5.5	25.1 ± 7.1	27.2 ± 6.4	26.2 ± 7.2
Compliance coefficient (mm^2/kPa)	1.06 ± 0.28	1.08 ± 0.35	0.94 ± 0.25	0.96 ± 0.28
Young's Elastic Modulus (10^3•kPa)	0.47 ± 0.13	0.50 ± 0.16*	0.42 ± 0.12	0.44 ± 0.16
Femoral artery				
Diameter (mm)	10.63 ± 1.05	11.0 ± 1.09‡	9.00 ± 1.05	9.16 ± 1.22†
Distension (μm)	213 ± 97	220 ± 96	229 ± 99	238 ± 98
Local pulse pressure (mmHg)	54.0 ± 9.2	50.3 ± 13.4†	50.0 ± 9.95	43.9 ± 10.8‡
Distensibility coefficient (10^{-3}/kPa)	5.77 ± 2.81	6.51 ± 3.36*	8.07 ± 3.85	9.66 ± 4.77‡
Compliance coefficient (mm^2/kPa)	0.51 ± 0.24	0.61 ± 0.31‡	0.51 ± 0.23	0.63 ± 0.33‡
Carotid-femoral pulse wave velocity (m/s)[a]	8.06 ± 1.55	8.65 ± 1.47†	7.48 ± 1.54	8.05 ± 1.39‡

Data are presented as mean ± SD; [a]Data available on 235 subjects only (112M/123F);
*p<0.05; †p<0.01; ‡p<0.001.

Longitudinal associations between adipocytokines and arterial stiffness

Throughout the 6-year longitudinal period, and after adjustments for sex, height and MAP, higher levels of leptin and LAR were adversely whereas higher levels of adiponectin were favourably associated with carotid and femoral stiffness (*Table 3*, model 1). These associations did not materially change after adjustments for potential confounders (model 2). Mutual adjustment for either adiponectin or leptin (model 3) showed that the associations between each adipocytokine with carotid and femoral stiffness were only partially interdependent.

Owing to the opposite associations between leptin and adiponectin, higher levels of the LAR were also adversely associated with carotid and femoral stiffness. None of the adipocytokines or their ratio was associated with cfPWV, however.

Additional adjustment for levels of metabolic variables (total- to-HDL cholesterol, triglycerides and HbA1c - model 4) only slightly attenuated the associations between both adipocytokines and carotid stiffness estimates, but more strongly so with regard to femoral stiffness. Importantly, adjustment for total body fat (model 5) attenuated the associations between leptin and arterial stiffness estimates, whereas associations between adiponectin and the LAR with the stiffness estimates were to a greater extent independent of fatness.

Table 3. Associations between leptin, adiponectin and leptin-to-adiponectin ratio with arterial stiffness

		Leptin		Adiponectin		Leptin-to-adiponectin ratio	
Dependent variables	Model	b	95% CI	b	95% CI	b	95% CI
Carotid DC	1	-0.19	-0.28; -0.10‡	0.14	0.05; 0.23†	-0.19	-0.27; -0.11‡
	2	-0.18	-0.27; -0.08‡	0.14	0.05; 0.23†	-0.18	-0.26; -0.10‡
	3	-0.15	-0.25; -0.06†	0.11	0.02; 0.20*	-	-
	4	-0.16	-0.25; -0.06†	0.11	0.02; 0.21*	-0.17	-0.25; -0.08‡
	5	-0.07	-0.20; 0.07	0.11	0.01; 0.20*	-0.12	-0.24; -0.01*
Carotid CC	1	-0.12	-0.22; -0.03*	0.13	0.03; 0.23*	-0.14	-0.23; -0.05†
	2	-0.12	-0.22; -0.02*	0.13	0.02; 0.23*	-0.13	-0.22; -0.04†
	3	-0.10	-0.20; 0.01	0.11	0.00; 0.22*	-	-
	4	-0.09	-0.20; 0.01	0.10	-0.01; 0.20	-0.11	-0.21; -0.01*
	5	-0.10	-0.26; 0.06	0.12	0.00; 0.23*	-0.15	-0.29; -0.00*
Carotid YEM	1	0.20	0.10; 0.29‡	-0.13	-0.22; -0.03*	0.19	0.11; 0.28‡
	2	0.17	0.08; 0.26‡	-0.12	-0.22; -0.02*	0.17	0.09; 0.25‡
	3	0.15	0.06; 0.25†	-0.09	-0.19; 0.01	-	-
	4	0.17	0.07; 0.26‡	-0.12	-0.23; -0.01*	0.18	0.09; 0.26‡
	5	0.10	-0.02; 0.23	-0.09	-0.19; 0.02	0.14	0.02; 0.25*
Femoral DC	1	-0.08	-0.18; 0.02	0.17	0.09; 0.26‡	-0.12	-0.20; -0.03†
	2	-0.08	-0.19; 0.02	0.16	0.07; 0.24‡	-0.12	-0.21; -0.03†
	3	-0.05	-0.16; 0.05	0.15	0.06; 0.24†	-	-
	4	-0.02	-0.13; 0.09	0.10	0.01; 0.19*	-0.05	-0.15; 0.05
	5	0.03	-0.13; 0.18	0.12	0.03; 0.21†	-0.06	-0.19; 0.07
Femoral CC	1	-0.13	-0.24; -0.03*	0.19	0.10; 0.28‡	-0.16	-0.25; -0.07†
	2	-0.13	-0.24; -0.02*	0.18	0.08; 0.27‡	-0.16	-0.25; -0.06†
	3	-0.10	-0.21; 0.01	0.16	0.06; 0.25†	-	-
	4	-0.05	-0.16; 0.07	0.09	-0.01; 0.19	-0.07	-0.17; 0.04
	5	-0.05	-0.21; 0.10	0.14	0.05; 0.24†	-0.13	-0.26; -0.00*
*Carotid-femoral PWV*ª	1	0.03	-0.10; 0.16	0.05	-0.08; 0.19	0.01	-0.11; 0.12
	2	0.03	-0.10; 0.17	0.03	-0.11; 0.16	0.01	-0.10; 0.13
	3	0.04	-0.10; 0.18	0.04	-0.11; 0.18	-	-
	4	0.05	-0.09; 0.20	0.01	-0.14; 0.15	0.04	-0.09; 0.17
	5	0.01	-0.19; 0.20	0.05	-0.10; 0.19	-0.03	-0.19; 0.13

βs are standardized regression coefficients as estimated by GEE; a β of 0.1 indicates that when Log_e leptin or Log_e adiponectin or Log_e leptin-to-adiponectin ratio increases by 1 SD unit, the estimate of arterial stiffness increases by 0.1 SD unit; CI, confidence interval; ªData available on 235 subjects only (123 women); Model 1: adjusted for sex, height and mean arterial pressure (MAP); Model 2: model 1 further adjusted for smoking status, alcohol consumption, physical activity and eGFR; Model 3: model 2 with mutual adjustment between adipocytokines; Model 4: model 2, additionally adjusted for total to HDL cholesterol, triglycerides and HbA1c; Model 5: model 2, additionally adjusted for total body fat; *p<0.05; †p<0.01; ‡p<0.001.

Additional analyses

To investigate whether the associations were primarily determined by the between- or the within-subject associations over the 6 year study period, we also examined associations between *changes* (i.e. within-subject) in adipocytokines or their ratio and changes in arterial stiffness estimates with the use of linear regression analyses. Changes in adipocytokines or their ratio 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.

Because earlier evidence for a predominant role of the LAR as a determinant of arterial stiffness was confined to overweight/obese individuals we also investigated whether overweight status (defined on the basis of a BMI < or >25 kg/m^2) modified the associations reported herein. However, we found no such effect-modification by BMI.

DISCUSSION

To the best of our knowledge, this is the first study that has examined, in apparently healthy adults, the relationships of leptin, adiponectin, and the LAR with carotid, femoral and aortic stiffness over a 6-yr period. Main findings were that lower levels of adiponectin and higher levels of leptin, and more consistently, of the LAR, were adversely associated with carotid and femoral stiffness throughout the longitudinal period. However, neither the adipocytokines nor the LAR were associated with stiffness of the carotid-femoral segment. Thus, the adipocytokines may affect arterial stiffening in a way that depends upon the arterial territory under study. Furthermore, mutual adjustment for either adiponectin or leptin showed that the associations between either leptin or adiponectin with carotid and femoral stiffness were partially interdependent. This suggests that adverse arterial conditions may be more easily identified when the deleterious effects of high leptin and low adiponectin are examined in combination, rather than in relation to leptin or adiponectin alone.

Our findings are in agreement with a previous study, showing inverse associations between adiponectin and carotid but not aortic stiffness [2]. The remaining studies have examined associations of either leptin or adiponectin with estimates of one artery/arterial segment only, such as the carotid [17,25,42], brachial [11,19], carotid-femoral or aortic PWV [1,13-16,22,28], or a non-specific arterial segment, such as the ankle-brachial PWV [18,20,21,23,24,27]. These studies do not enable the appreciation of whether associations between adipocytokines and arterial stiffness differ across the arterial tree. Still, some but not all [22,28] studies did report associations between leptin [1,13] or adiponectin [1,14-16] with carotid-femoral PWV. The older age [1,13], hypertensive status [14,16] and black ethnicity [15] of the study populations in which such associations were found may explain the discrepancy with our findings. Noteworthy, in the only experimental study with carotid-femoral PWV as study outcome, decreases in both leptin and this stiffness estimate were observed after a 1-yr weight-loss program, but these beneficial decreases were not associated with each other [28].

Leptin and adiponectin differ from other adipocytokines in being almost exclusively secreted by adipocytes, and their systemic levels reflect the effects of fat accumulation in a reciprocal fashion [43]. Therefore, the LAR has been proposed as a more comprehensive measure of adipocytokine secretion that enables the investigation of their joint effects on metabolic [44,45] and cardiovascular health outcome [23,46,47]. In accordance, Bundy *et al* showed that adiponectin attenuated the relationship between adiposity and leptin in adolescents [48]. In addition, experimental evidence points to pathophysiological interaction/crosstalk between leptin and adiponectin in (cancer) cell cycle regulation and cell proliferation [49-51], but also in the domain of hepatic cells [52] and of metabolic actions [53]. In the only study so far that has investigated the LAR in relation to arterial stiffness, the LAR was more strongly associated with arterial stiffness than either leptin or adiponectin alone [23]. These findings were obtained in a cross-sectional study among individuals with T2D and were confined to those with overweight/obesity. Our current findings, based on a longitudinal data collection, suggest that the LAR is indeed more consistently associated with arterial stiffening than each of the adipocytokines alone, and extend that study by showing this among an apparently healthy population and across the whole range of levels of body fatness.

Because LAR combines the two adipocytokines, we also investigated the associations of each adipocytokine with arterial stiffness following mutually adjustment for each other, as this may enable a better understanding of the driving forces confined within the LAR ratio. These analyses unveiled a partial interdependence between adiponectin and leptin. In apparent contrast to what we found, Windham *et al* has recently shown that leptin, rather than adiponectin (or resistin), explained the cross-sectional associations between adiposity and arterial stiffness [1]. In another study adiponectin but not leptin was associated with adverse changes in carotid distensibility over the course of 1-yr follow-up [42]. This study was confined to post-menopausal women with increased levels of carotid atherosclerosis (i.e. intima-media thickness) at baseline, who were enrolled on a randomized controlled trial to ascertain the effects of hormone replacement therapy on the progression of atherosclerosis. The differences in study population, research setting and/or duration of follow-up may explain the discrepancies between study results.

Adjustment for total body fat attenuated the associations between leptin and arterial stiffness more strongly than the associations with adiponectin and the LAR. This could be explained by the known biological effects of both leptin and adiponectin. Leptin provides signals about nutritional status and fat mass to neural centres that regulate appetite and energy metabolism and the observation of elevated levels of leptin with increasing fat mass are thus thought to reflect hypothalamic leptin resistance [10,54]. Adiponectin on the other hand plays a key role in metabolic disturbances, such as improvements in insulin sensitivity and tissue fat oxidation resulting in reduced circulating fatty acid levels and reduced liver and intramyocellular triglyceride content [9,55].

Further, it is questionable whether body fat should be seen as a true potential confounder in the associations between adipocytokines and arterial stiffness. It is biologically plausible that adipocytokines are part of the causal pathway linking (central) fatness to arterial disease, i.e. body fat influences adiponectin and leptin levels and these influence arterial stiffening. Noteworthy, adjustments for other biological risk factors did not explain the associations between adipocytokines and carotid stiffness, but partly did so for femoral stiffness. This suggests that elastic and muscular arteries may differ in their determinants of local vascular properties [56],

and that other mechanisms may link adipocytokines to (carotid) arterial stiffness. Several experimental studies have shown that both leptin and adiponectin may, respectively, promote or prevent endothelium dysfunction, oxidative stress, platelet aggregation, and migration, hypertrophy and proliferation of smooth muscle cells [9,10,54,55], all of which may lead to accelerated arterial stiffening [57].

The timeframe and thresholds that link changes in adiponectin and leptin levels to changes in arterial stiffness is unknown and might differ from the six-year period of the present investigation. This might explain why we did not find an association between changes in adiponectin and leptin levels and changes in arterial stiffness. The question remains for what period a person should have unfavourable adiponectin and leptin levels before it affects the vasculature. We observed that the majority of the study population had changes of close to zero in adiponectin and leptin levels. This 'consistency' phenomenon was confirmed in two other prospective studies on adiponectin, leptin and coronary disease [7,8]. In any case, our results suggest that any change in adipocytokines that have led to arterial stiffening may have already occurred before the age of 36 years. Still, we observed increases in mean levels of adiponectin over the 6-yr period, though these were confined to women. Although this could also be a chance finding, paradoxical increases in adiponectin with aging, which is often characterized by increases in (central) body fatness, have been shown previously [58,59]. In agreement with our study, such increases may, however, be modulated by sex-related hormonal changes, since steeper increases in adiponectin were observed already from the age of 30 onwards in women, whereas in men such increases were observed later in life, from the age of 50 onwards [59]. Alternatively, reduced clearance of adiponectin due to a decline in renal function could explain increases in adiponectin with ageing. Although we cannot fully discard this hypothesis since we were only able to estimate (and adjust for) GFR at follow-up, we do not think it explained our observations. First, such inverse associations between eGFR and adiponectin have been observed mainly among the elderly [59]; second, it is not clear why impaired renal glomerular filtration would lead to increases in adiponectin in women only; and finally, in the present cohort of young adults eGFR was not associated with adiponectin in women [b per 10 ml/min/1.73m^2 = -0.016 mg/L (95%CI=-0.055; 0.022), p=0.406 – cross-sectional analyses at follow-up].

There are some limitations to our study. First, we have not measured adiponectin fractions. 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, the use of different BP devices at both time points may have caused seemingly unexpected phenomena, such as a decrease in PP. Although this may seem unexpected, increases in PP with ageing are usually not observed until after the 5th or 6th decade [57]. 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 therefore, extrapolations to other populations should be interpreted with caution.

In conclusion, throughout the 6-year period, lower levels of adiponectin and higher levels of leptin and LAR were, in middle-aged adults, associated with greater carotid and femoral stiffness. This association was, at least partially, independent of body fatness.

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