Novel insights into blood markers and cardiovascular disease: Results of the Netherlands Epidemiology of Obesity study

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SECTION I

INFLAMMATION AND ADIPOKINES

CHAPTER 2

THE ROLE OF INFLAMMATION IN THE ASSOCIATION BETWEEN OVERALL AND VISCERAL ADIPOSITY AND SUBCLINICAL ATHEROSCLEROSIS

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Nutrition, Metabolism and Cardiovascular Disorders, 2019

Abstract

Background and aims

Inflammation may underlie the association between obesity, atherosclerosis and cardiovascular disease. We investigated to what extent markers of inflammation mediate associations between overall and visceral body fat and subclinical atherosclerosis.

Methods and Results

In this cross-sectional analysis of the Netherlands Epidemiology of Obesity study we estimated total body fat (TBF) by bio-impedance analysis, carotid artery intima media thickness (cIMT) by ultrasound, C-reactive protein (hs-CRP) and glycoprotein acetyls (GlycA) concentrations in fasting blood samples (n=5,627), and visceral adipose tissue (VAT) by magnetic resonance imaging (n=2,247). We examined associations between TBF and VAT, and cIMT using linear regression, adjusted for potential confounding factors, and for mediators: cardiometabolic risk factors (blood pressure, glucose and low-density lipoprotein cholesterol), and inflammation using CRP and GlycA as proxies.

Mean (SD) cIMT was 615 (90) µm. Per SD of TBF (8%), cIMT was 19 µm larger (95% confidence interval, CI: 10, 28). This association was 17 µm (95% CI: 8, 27) after adjustment for cardiometabolic risk factors, and did not change after adjustment for markers of inflammation. Per SD (56 cm^2) VAT, cIMT was 9 µm larger (95% CI: 2, 16) which changed to 5 µm (95% CI: -3, 12) after adjustment for cardiometabolic risk factors, and did not change after adjustment for inflammatory markers.

Conclusion

Our results suggest that associations between measures of overall and visceral body fat and subclinical atherosclerosis are not mediated by inflammation as measured by CRP and GlycA. Obesity may exert cardiovascular risk via other markers of systemic inflammation.

Introduction

Obesity, and in particular abdominal adiposity, is a well-established cause of coronary heart disease, stroke, and atherosclerosis [68, 69]. Between 40 and 50% of the association between obesity and cardiovascular diseases is mediated by three major cardiometabolic risk factors blood pressure, low-density lipoprotein cholesterol (LDL-c) and fasting glucose concentrations. [18] A low-grade systemic state of inflammation may partly mediate the relation between measures of body fat and cardiovascular disease (Figure 1).

Body fat, and in particular visceral fat, is infiltrated by pro-inflammatory (M1)-type macrophages, which contribute to a low-grade systemic inflammatory state by producing and secreting pro-inflammatory cytokines like IL-6, TGF-β, which is considered to have a detrimental effect on atherosclerosis [3, 70, 71]. Systemic inflammation is further marked by C-reactive protein (CRP) and glycoprotein acetyls (GlycA) [72]. CRP is produced by the liver upon stimulation by inflammatory cytokines, while GlycA is a composite marker of glycosylated acute phase protein concentrations [73]. Both markers are observationally associated with arterial stiffness and cardiovascular disease and can therefore be used as a proxy for low-grade systemic inflammation [74-78].

We aimed to investigate the role of low-grade systemic inflammation, in addition to the main known cardiometabolic risk factors, in the associations between overall and visceral body fat with subclinical atherosclerosis.

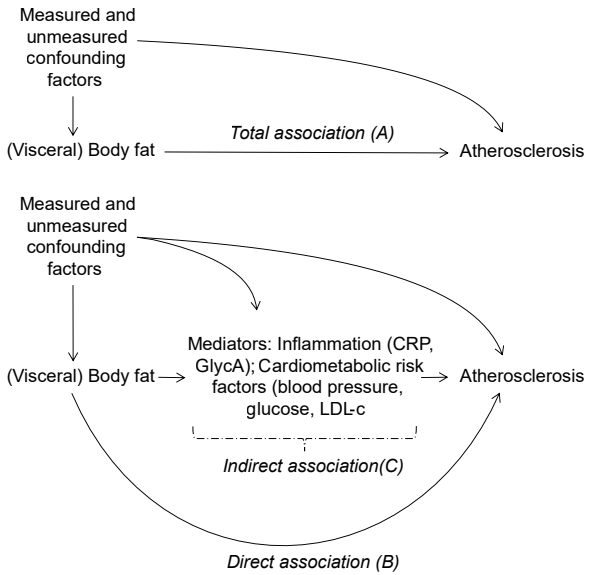

Figure 1 – Directed acyclic graph of the hypothesized relations between measures of body fat and (subclinical) atherosclerosis, the total association (A) may be separated in a direct association (B), and an indirect association (C) via inflammation

CRP, C-reactive protein; GlycA, glycoprotein acetyls; LDL-c, low density lipoprotein cholesterol

Methods

Study population

The present study is a cross-sectional analysis of baseline measurements of the Netherlands Epidemiology of Obesity (NEO) study, a population-based, prospective cohort study (n=6671). The study design and population are described in detail elsewhere [59].

Men and women aged 45-65 years with a BMI ≥27 kg/m^2 were eligible to participate in the NEO study. In addition, inhabitants of Leiderdorp were invited irrespective of their BMI to serve as a reference group with a BMI distribution of the general population. Prior to the study visit, participants completed questionnaires at home with respect to demographic, lifestyle, and clinical information. Research nurses recorded names and dosages of current medication.

The design of the NEO study was approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC). All participants gave their written informed consent.

Data collection

Self-reported ethnicity was regrouped into white and other. The highest completed level of education was reported in ten categories and regrouped into two categories: low education (no education, primary education or lower vocational education) and high education (other). Smoking was reported in three groups: never, former, and current smoker. The SQUASH questionnaire was used to self-report physical activity during leisure time in hours per week of metabolic equivalents of task [79]. Participants self-reported their medical history of diabetes and cardiovascular disease (defined as myocardial infarction, angina, congestive heart failure, stroke or peripheral vascular disease). Height was measured without shoes using a calibrated, vertically fixed tape measure. Blood pressure was measured seated on the right arm with a validated automatic oscillometric device (OMRON, Model M10-IT; Omron Health Care Inc, IL, USA). The mean of three measurements was used to calculate the mean systolic and diastolic blood pressure levels.

Measures of overall and visceral adiposity

Body weight and total body fat percentage (TBF) were assessed using a bio-impedance device (TBF-310, Tanita International Division, UK). BMI was calculated by dividing body weight in kilograms by body height in meters squared. Abdom-

inal visceral adipose tissue (VAT) was assessed by magnetic resonance imaging (MRI, 1.5 Tesla MR imaging, Philips Medical Systems, Best, the Netherlands) using a turbo spin echo imaging protocol in a random subgroup of 2580 individuals without contraindications for MRI (implanted metallic devices, a body circumference >1.70 m, or claustrophobia). At the level of the fifth lumbar vertebra, three 10 mm transverse images were obtained during a breath-hold. Visceral fat areas was quantified by converting the number of pixels to cm^2 for all three slices, of which the mean area was used in the analyses.

Carotid intima media thickness

To obtain cIMT, ultrasonography of the common carotid arteries (CCA) was performed. A 15 mm long section 10 mm proximal of the CCA bifurcation was measured while the subject was in supine position. IMT was measured using a 7.5–10 MHz linear-array probe and the Art.Lab system (ART.LAB version 2.1, Esaote, Maastricht, The Netherlands) in B-mode setting and using a wall-track system to detect boundaries between lumen and intima, and between media and adventitia. cIMT was reported as the mean of three measurements in angles of 180, 135 and 90 degrees (right CCA) and 180, 225 and 270 degrees (left CCA). In 183 randomly selected participants (n=183) we validated measurements of intima media thickness with an intra-observer coefficient of variation of 5.8%, and an inter-observer coefficient of variation of 9.0% [80].

Blood measurements

Plasma glucose, serum triglyceride, serum high-density lipoprotein-cholesterol (HDL-c), and serum total cholesterol concentrations were determined in fasting blood samples at the central clinical chemistry laboratory of the LUMC using standard assays. LDL-c concentrations were calculated using the Friedewald equation.

Serum concentrations of CRP were determined in a fasting blood sample using a high sensitivity CRP assay (hs-CRP, TINA-Quant CRP HS system, Roche, Germany and Modular P800, Roche, Germany). GlycA concentrations were measured in plasma that had undergone one previous freeze-thaw cycle, using a high-throughput proton nuclear magnetic resonance (NMR) spectroscopy (Nightingale Health Ltd., Helsinki, Finland) [81]. We calculated intraclass coefficients for 94 participants with repeated CRP and GlycA measurements during a second visit within 3-5 months of the first visit. For CRP, the intraclass coefficient was 0.31, and for GlycA 0.64. To examine multicollinearity, variance inflation factors (VIF) were calculated, which were all under 10 and therefore considered acceptable.

Statistical analysis

For the present analysis, we excluded participants with a medical history of cardiovascular disease, participants with hs-CRP concentrations ≥10 mg/L, or using medication that targets systemic inflammation (corticosteroids, lumiracoxib or naproxen [82, 83]), and participants with missing data.

Participants with a BMI ≥27 kg/m^2 were oversampled in the NEO study. To correct for this oversampling, analyses were weighted towards the BMI distribution of the NEO participants from Leiderdorp, whose BMI distribution was similar to the general Dutch population. [16, 60] All results were based on weighted analyses. Consequently, the results apply to a population-based study without oversampling of individuals with a BMI ≥ 27 kg/m^2.

Baseline characteristics were calculated for the total population, and for men and women separately, and expressed as mean (standard deviation, SD), median (25th-75th percentile) or percentage. The measures of adiposity were standardized to a mean of zero and standard deviation of one. Hs-CRP and GlycA concentrations were skewed, and therefore log-transformed to the natural logarithm.

Mediation analyses

To examine the role of inflammation in the associations between visceral and total body fat and atherosclerosis, we performed mediation analysis according to Baron and Kenny and evaluated the assumptions [84].

First, we estimated the total association between measures of body fat and intima media thickness with linear regression analyses, and adjusted crude analyses for potential confounding factors age, sex, tobacco smoking, physical activity, statin use, ethnicity, education, and the other measure of body fat and calculated regression coefficients with 95% confidence intervals.

Second, we examined the assumptions for the Baron and Kenny method of mediation analyses. One of the assumptions is the absence of interaction between the exposure and the mediator. To examine this assumption, we included an interaction term of the measures of body fat with the markers of inflammation in the model of the total association. We assessed linearity by plotting residuals, kernel density, P-P, and Q-normal distributions.

Furthermore we estimated the associations between the potential mediators and the exposure and the outcome variables by linear regression analysis. We reported the regression coefficients and 95% confidence intervals, which can be

interpreted as the difference in cIMT per standard deviation of measure of body fat. The regression coefficients from the models with log-transformed markers of inflammation as outcome were back-transformed, and can be interpreted as a relative change in inflammatory marker per standard deviation of body fat.

Subsequently, we assessed mediation of the total association between measures of body fat and intima media thickness by known cardiometabolic risk factors for cardiovascular disease: systolic blood pressure, LDL-c, and fasting glucose concentrations, and by the markers of inflammation separately and simultaneously. Therefore we additionally adjusted the regression models for the cardiometabolic risk factors and markers of inflammation. We reported regression coefficients with 95% confidence intervals, which can be interpreted as the direct effect [84].

To assess the robustness of the models, we repeated all analyses including previously excluded participants with a history of CVD. Initial exclusion of participants with hs-CRP concentrations ≥10 mg/L, may introduce selection bias [85]. Therefore, we performed sensitivity analyses including these participants. Furthermore, mediation analysis with a mediator that is measured with error may lead to strongly biased results [85]. Therefore, we performed a sensitivity analysis with an assumed random measurement error of 35% using the *eivreg* command in STATA (Statacorp, College Station, Texas, USA), version 14.0.

Results

Participants that used medication with effects on markers of inflammation (n=137) or had missing data (n=231), with a history of cardiovascular disease (n=437), or with hs-CRP concentrations ≥10 mg/L (n=267) were excluded. In total, 5627 participants with a mean age of 56 years (SD: 6), mean BMI was 26 kg/m^2 (SD: 4) and of whom 43% men were included in the analysis. Of these, 2247 underwent MRI and had assessment of VAT.

Total association

The analyses for the total association between body fat and intima media thickness showed that one standard deviation of total body fat (8%) was associated in the crude model with a 6 µm (95% CI: 2, 9) larger intima media thickness, which changed to 19 µm (95% CI: 10, 28) after adjustment for potential confounding factors. One SD of VAT (56 cm^2) was associated in the crude model with 24 µm (95% CI: 19, 29) larger cIMT, which changed to 9 µm (95% CI: 2, 16) per SD in the adjusted model.

Assessing the associations between exposure, mediators and outcome

We first examined associations between the measures of body fat and markers of inflammation. Adjusted linear regression analysis showed that per standard deviation of total body fat and visceral fat, hs-CRP concentrations were 71% (95% CI: 63, 79) and 33% (95% CI: 24, 42) higher. Per standard deviation of total body fat and visceral fat, GlycA concentrations were 9% (95% CI: 8, 9) and 6% (95% CI: 5, 7) higher. Measures of body fat were also associated with the cardiometabolic risk factors glucose, LDL-c, and blood pressure (data not shown). As for the association between the markers of inflammation and cIMT, the difference in cIMT was 1 µm (95% CI: -4, 7) per standard deviation of hs-CRP, and 2 µm (95% CI: -2, 5) per standard deviation of GlycA.

Table 1 – Baseline characteristics of participants of the NEO study aged between 45 and 65 years, without history of cardiovascular disease. (n = 5627).

	Total population	Men (43 %)	Women (57%)
Age (years)	56 (6)	56 (6)	55 (6)
BMI (kg/m^2)	26 (4)	27 (3)	25 (4)
Total body fat (%)	31 (8)	25 (5)	36 (7)
Visceral adipose tissue (cm^2) (n=2247)	90 (56)	115 (58)	67 (43)
Current smoking (%)	16	18	14
Alcohol intake (g/day)	10 (3-21)	16 (6-28)	8 (2-15)
Physical activity (MET h/week)	30 (16-50)	31 (15-51)	29 (16-49)
Education level (% high)	47	49	45
Diabetes (%)	5	7	3
Statin use (%)	8	11	6
Systolic blood pressure (mmHg)	131 (17)	135 (15)	127 (18)
Diastolic blood pressure (mmHg)	83 (10)	85 (10)	82 (10)
Triglycerides (mmol/L)	1.2 (0.8-1.7)	0.9 (0.7-1.3)	0.9 (0.7-1.3)
LDL cholesterol (mmol/L)	3.6 (0.9)	3.6 (1.0)	3.6 (1.0)
Glucose (mmol/L)	5.6 (1.2)	5.3 (0.8)	5.3 (0.7)
hs-CRP (mg/L)	1.1 (0.6-2.1)	1.2 (0.6-2.5)	1.1 (0.6-2.3)
Glycoprotein acetyls (mmol/L)	1.2 (1.1-1.3)	1.2 (1.1-1.3)	1.2 (1.1-1.3)
Carotid intima media thickness (µm)	615 (90)	625 (93)	605 (86)

Values are represented as mean (SD), median (25th-75th percentile) or percentage. Results were based on analyses weighted towards the BMI distribution of the general population (n = 5627).

hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein MET, metabolic equivalent; NEO, Netherlands Epidemiology of Obesity; SAT, abdominal adipose tissue; TBF, total body fat mass; SD, standard deviation; VAT, visceral adipose tissue

Table 2 – Linear regression analyses between measures of body fat and markers of inflammation, adjusted for potential confounding factors (total body fat: n = 5627; VAT: n = 2247).

		Relative change (%) in markers of inflammation per SD of measures of body fat	
		hs-CRP (%)	GlycA (%)
Total body fat (SD: 9%)	Crude	40 (35, 44)	3 (3,4)
	Adjusted	71 (63, 79)	9 (8, 9)
VAT (SD: 56 cm^2)	Crude	42 (35, 50)	9 (8, 10)
	Adjusted	33 (24, 42)	6 (5, 7)

Values represent relative change (%) in markers of inflammation per standard deviation of measures of body fat. Results were based on analyses weighted towards the BMI distribution of the general population (n= 5627; 2247 for VAT), and adjusted for potential confounding factors: age, sex, tobacco smoking, ethnicity, education and physical activity. Analyses with VAT were additionally adjusted for total body fat. GlycA, glycoprotein acetyls; hs-CRP, high-sensitivity C-reactive protein; SD, standard deviation; VAT, visceral adipose tissue

Table 3 – Linear regression analyses of associations between markers of inflammation and intima media thickness, adjusted for potential confounding factors (n = 5627).

	Model	
Difference in cIMT (μm) per SD of	Crude	Multivariate
hs-CRP	11 (5, 17)	1 (-4, 7)
GlycA	15 (9, 20)	2 (-2, 5)

Values represent difference in cIMT per standard deviation of markers of inflammation. Results were based on analyses weighted towards the BMI distribution of the general population (n= 5627), and adjusted for potential confounding factors: age, sex, tobacco smoking, ethnicity, education and physical activity, blood pressure, LDL-cholesterol, fasting glucose, and total body fat.

BMI, body mass index; cIMT, carotid intima media thickness; GlycA, glycoprotein acetyls; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; SD, standard deviation

No interaction was observed for associations between body fat and measures of inflammation in linear regression analyses between measures of body fat and intima media thickness.

Mediation analyses

The association between one SD of total body fat and intima media thickness decreased from 19 to 17 µm (95% CI: 8, 27) after adjustment for systolic blood pressure, LDL-c, and fasting glucose concentrations and to 16 µm (95% CI: 6, 25) after additional adjustment for hs-CRP and GlycA. The association between visceral fat and intima media thickness decreased from 9 to 5 µm (95% CI: -3, 12), and to 3 µm (95% CI: -5, 11) after additional adjustment for hs-CRP and GlycA.

Table 4 – Linear regression analyses of associations between measures of body fat and intima media thickness, adjusted for potential confounding factors (total association), hs-CRP and GlycA (direct association), and known cardiometabolic risk factors (systolic blood pressure, LDL-c, and glucose) (TBF: n = 5627; VAT: n = 2247).

	Difference in IMT (µm) per SD of:	
	Total body fat (SD: 9%)	VAT (SD: 55 cm^2)
Model 1 (crude)	6 (2, 9)	24 (19, 29)
Model 1 + age and sex	26 (22, 30)	19 (13, 24)
Model 2 (adjusted)	19 (10, 28)	9 (2, 16)
Model 3A (Model 2+ cardiometabolic risk factors)	17 (8, 27)	5 (-3, 12)
Model 3B (Model 2 + hs-CRP)	17 (8, 27)	7 (-0, 15)
Model 3C (Model 2 + GlycA)	17 (8, 26)	6 (-1, 14)
Model 3D (Model 2 + hs-CRP + GlycA)	16 (7, 26)	6 (-2, 13)
Model 4 (Model 2 + cardiometabolic risk factors + hs-CRP + GlycA)	16 (6, 25)	3 (-5, 11)

Results were based on analyses of the associations between measures of body fat and intima media thickness in participants of the Netherlands Epidemiology of Obesity study, weighted towards the BMI distribution of the general population (n=5627; 2247 for VAT). The adjusted analyses in Model 2 and further were adjusted for potential confounding factors: age, sex, tobacco smoking, ethnicity, education and physical activity. Model 3B-3D were additionally adjusted for hs-CRP, GlycA, and both. Model 4 was additionally adjusted for cardiometabolic mediating risk factors: systolic blood pressure, LDL-c and fasting glucose. Abbreviations: GlycA, glycoprotein acetyls; hs-CRP, high-sensitivity C-reactive protein; LDL-c, low-density lipoprotein cholesterol; SD, standard deviation; VAT, visceral adipose tissue

Sensitivity analyses

The observed associations did not change substantially when including participants with a history or with hs-CRP concentrations in excess of 10 mg/L (data not shown). Furthermore, the observed associations did not change after adjustment for measurement error in the mediators (Supplementary Table 1).

Discussion

In this population-based study, we confirmed that in particular total body fat and to a lesser extent visceral fat were strongly associated with markers of inflammation, and modestly associated with cIMT. However, we observed no association between markers of inflammation and cIMT, which violated one of the main assumptions of a mediation analysis: the presence of an association between mediator and outcome. Consequently, we did not observe mediation of the association between measures of body fat and cIMT by markers of inflammation.

We observed a stronger association with markers of inflammation for total body fat than visceral fat, which was discordant with previous literature that found stronger associations for visceral fat. [86] A potential explanation is that we used an accurate measures of visceral fat using MRI, and adjusted the associations for total body fat. Although visceral fat secretes cytokines at a higher rate than subcutaneous fat, visceral fat has a limited volume, and therefore contributes less to total inflammatory marker concentrations [25].

The role of inflammation in the development of atherosclerosis and cardiovascular disease has been debated for several years. While atherosclerosis is an inflammatory disease [22], CRP has been shown to be non-causal in the development of atherosclerosis and cardiovascular disease [87]. GlycA is a biological proxy of inflammation, as it is a measure of *N*-linked acute-phase proteins, which are secreted by the liver in response to stimulation by IL-6 [88]. Higher GlycA concentrations also predict an increased risk of CVD, independent of CRP [74]. Previous studies suggested that up to eight percent of the association between obesity and cardiovascular disease is mediated by inflammation [18, 89]. However, we did not observe an association between markers of inflammation and subclinical atherosclerosis, which precluded mediation.

This inconsistency between our and previous studies may be attributed to a difference in populations and markers of (subclinical) cardiovascular disease that were studied. Previous studies were performed in individuals at high cardiovascular risk, while the present study was performed in individuals from the general population. The main clinical trial that investigated the effects of inhibition of inflammation with the strong antiinflammatory drug canakinumab on cardiovascular disease incidence was also performed in patients at very high cardiovascular risk, and found only a small cardiovascular risk reduction but no net effect on all-cause mortality [39]. Second, atherosclerosis is considered an inflammatory disease [3]. Therefore it is possible that the inflammation of interest originates from atherosclerotic lesions, which may not be reflected by serum concentrations of hs-CRP and GlycA given the short half-lives of most cytokines.

Strengths of this study are that the analyses were performed in a large and well phenotyped population in which an objective measure of subclinical atherosclerosis and precise measures of overall and visceral adiposity were available. Two separate inflammation markers have been analysed with similar results. The possibility of measurement error was taken into account and our results showed that this error had very limited potential implications for our conclusions.

This study also has some limitations. First, cIMT measures subclinical atherosclerosis with a degree of uncertainty. However, our measurement of cIMT had a fair replicability [80]. Second, hs-CRP and GlycA are merely non-specific markers of low-grade systemic inflammation. Therefore, these concentrations may measure the true degree of systemic inflammation with error. This measurement error may severely bias the results of a mediation analysis [85]. However, as the markers of inflammation were robustly associated with total body fat and visceral fat, it is likely that concentrations of these markers truly reflected low-grade chronic inflammation associated with adipose tissue. Future studies could investigate more clinical measures of inflammation, such as leukocyte or monocyte counts. Also, the results did not essentially change upon correction for measurement error in all mediators. Furthermore, exclusion of participants with a history of cardiovascular disease to prevent reverse causation may have restricted our study population to individuals without clinically relevant atherosclerosis. However, our results did not change upon including these participants in sensitivity analyses. Finally, due to the observational nature of this study, unmeasured confounding or reverse causation may be present, that could specifically influence mediation analysis [90].

In conclusion, in this population-based study we observed no association between two markers of subclinical inflammation and cIMT, and no mediation by these markers of inflammation of the association between measures of body fat and intima media thickness. Further research may reveal other mediators of the relation between overweight and obesity and atherosclerosis.