MATERIALS AND METHODS

Subjects and study design
The present study consists of cross-sectional analyses of data from participants in the baseline examination of the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) Study\(^1-3\) and in the last follow-up examination of the Hoorn Study\(^4-8\), which were designed to allow future pooling\(^9\).

The CODAM study is an ongoing prospective cohort study in the Netherlands that was originally designed to study the effects of obesity, glucose and lipid metabolism, lifestyle and genetics on cardiovascular disease (CVD), as described in detail elsewhere\(^1-3\). Briefly, between 1999-2001 subjects were selected from a large population-based cohort and included if they had Caucasian ethnicity, were older than 40 years and had one or more of the following: BMI>25 kg/m\(^2\), positive family history of type 2 diabetes mellitus (T2DM), history of gestational diabetes, postprandial blood glucose>6.0 mmol/L, glucosuria, or use of antihypertensive medication. In total, 574 subjects were included and extensively characterized with regard to their metabolic, cardiovascular and lifestyle risk profiles during two visits to the research unit\(^1-3\).

The Hoorn study started in 1989 as a population-based cohort study investigating glucose metabolism status and cardiovascular risk factors and CVD among a sample of the general population of Hoorn, the Netherlands (n=2484)\(^7\). In 2000-2001, individuals still alive and who gave permission to be contacted were invited for a follow-up examination; specifically, these consisted of a random sample of those with normal and with impaired glucose metabolism and all those with T2DM in the previous examination (1996-98)\(^7\). Of the 1074 invited 648 subjects participated\(^4-6\); this number was further enriched with individuals with T2DM from the Hoorn Screening study\(^8\) such that the 2000-2001 follow-up examination comprised a total of 822 individuals\(^4, 6\).

After excluding individuals with missing data on plasma CML levels, markers of LGI and/or important covariates (n=126) a total of 1270 individuals (532 from CODAM and 738 from Hoorn) were included in the present study. We have also analyzed the subsample from the Hoorn study who underwent DXA measures of body composition for the first time during the 2000-2001 follow-up examination (n=623 of the 648 individuals mentioned above)\(^5, 6\); these analyses were confined to 576 individuals with complete data on the variables of interest for the present study.

For purposes of illustration only, we have also investigated the levels of CML and LGI in a clinical sample of 37 patients with severe/morbid obesity (i.e. at the highest levels of BMI) who were consecutively admitted to the Surgical Department of the Maastricht University Medical Centre (MUMC) between April 2006 and November 2007 to undergo bariatric surgery.

A flow-chart describing the selection of the population examined in the present study from the cohorts described above is presented in Figure I. All participants gave written informed consent for participation in the present study, which was undertaken with approval from the local Medical Ethics Committees.

Measurement of plasma CML concentrations
Circulating protein-bound CML concentrations were measured by ultra performance liquid chromatography (UPLC)-tandem mass spectrometry in EDTA-plasma samples for the CODAM and in serum plasma for the HOORN study, both stored at 80\(^\circ\)C until analyses\(^9\). Briefly, total plasma proteins were reduced with 100 mM sodium borohydride in 0.2 M sodium borate buffer at pH 9.2 for 2h at room temperature. The proteins were precipitated with trichloroacetic acid and hydrolysed with 6 M HCl overnight at 110\(^\circ\)C. After drying the hydrolysates under nitrogen, the residues were resolved in 0.5 mM tridecafluoroheptanoic acid. Analysis was performed using a C18 reverse phase column with a linear gradient of acetonitrile. CML levels were
measured using a positive ionization mode with D4-CML as internal standard. Intra- and inter-assay coefficients of variation (CV) were 5.3% and 15.6%, respectively.

**Measurement of markers of low-grade inflammation**

Six biomarkers of LGI (i.e. CRP, SAA, IL-6, IL-8, TNF-α and sICAM-1) were measured by a multi-array detection system based on electro-chemiluminescence technology (MesoScaleDiscovery MSD, Gaithersburg, MD, USA), as described in detail elsewhere\textsuperscript{10, 11}. All reagents were provided with the MSD kit and measurements were performed according to the manufacturer’s instructions. The intra- and inter-assay CVs for all markers were <11%.

Because some of these biomarkers had already been determined in the CODAM study population by conventional single-biomarker laboratory techniques (i.e. CRP by immunoturbidimetry and SAA, IL-6 and sICAM-1 by ELISA)\textsuperscript{2, 3}, we realigned individuals’ levels as obtained by these conventional techniques to those as obtained by the multi-array technique\textsuperscript{10} and used the average values of both methods, after realignment, in the analyses.

**Low-grade inflammation score.** To obtain a more robust characterization of the overall level of LGI than that can be obtained on the basis of each biomarker separately, we computed a LGI score by averaging the z-scores [i.e. (individual’s observed value – study mean)/SD] of each inflammatory marker (after log\textsubscript{e} transformation)\textsuperscript{1-3, 5, 10, 11}. Biomarkers’ z-scores were study-specific to properly accommodate differences in methodolgy (i.e. use of EDTA-plasma samples in the CODAM study and use of serum samples in the Hoorn study), and thus possibly differ in absolute mean and distribution concentrations, between cohorts. In addition, this score has the advantage of reducing the influence of measurement error/biological variability and avoids the problem of multiple testing in analyses performed with each biomarker separately\textsuperscript{1-3, 5, 10, 11}.

**Anthropometrics**

Participants’ height (in cm) was measured with subjects standing upright against a stadiometer. Body weight was measured to the nearest 100g on electronic weight scales. BMI was calculated by dividing weight by height squared, and was used to categorize the participants as with normal weight (BMI\textsuperscript{•} 18.5-24.9 kg/m\textsuperscript{2}), overweight (BMI\textsuperscript{•} 25-29.9 kg/m\textsuperscript{2}), moderate obesity (BMI\textsuperscript{•} 30-34.9 kg/m\textsuperscript{2}), and severe obesity (BMI\textsuperscript{•} 35.0), according to the WHO criteria\textsuperscript{12}. Patients from the clinical cohort were all severely or morbid obese, and were kept as a separate category given their indication for bariatric surgery treatment. Waist circumference was measured with a flexible tape at the level midway between the lowest rib and the iliac crest.

**Dual x-ray absorptiometry (DXA)**

A sub-sample of the individuals attending the Hoorn Study follow-up examination underwent a whole body DXA scan using the fan beam technology (QDR-2000, software version 7.20D, Hologic, Brussels, Belgium) to obtain measures of fat mass, lean tissue mass and bone mineral content (BMC) for the total body and for the following standard body regions: head, trunk, arms and legs, which were distinguished with the use of specific anatomic landmarks\textsuperscript{5, 6}. In the present analyses we used fat and lean soft tissue (excluding BMC) masses from the trunk, arms and legs; central fat mass was that estimated as the fat mass in the trunk region whereas peripheral fat mass was calculated by adding the fat mass of the legs to that of the arms, and peripheral lean mass was calculated by adding the lean mass of the legs to that of the arms\textsuperscript{5, 13}.

**Covariates**

The following variables were determined as described in detail elsewhere: fasting plasma glucose, HbA1C, total- and HDL-cholesterol, triglycerides, serum creatinine,
systolic and diastolic blood pressure (and the difference between the two pressures calculated as pulse pressure and used as a marker of arterial stiffness), smoking habits (never, former and current), use of lipid-, glucose- and/or blood-pressure-lowering medication, T2DM status and prior CVD.

Statistical analyses
All analyses were performed with the Statistical Package for Social Sciences (SPSS) version 18.0 for Windows (SPSS Inc., Chicago, IL). Dependent variables with a skewed distribution were log-transformed prior to analysis. Tests for linear-trends of the subjects’ levels of CML across established categories of BMI or quartiles of waist circumference were conducted by using the categories’ median value of BMI or waist circumference as a continuous independent variable in linear regression models.

Multiple linear regression analyses, all adjusted for potential confounders (i.e. cohort, age, sex, serum creatinine, T2DM, smoking status and, when appropriate, height) were used to investigate, the associations between: 1) BMI or waist circumference and plasma CML (path a); 2) plasma CML and LGI, also independently of BMI or waist circumference (path b); and 3) BMI or waist circumference and LGI (path c) (see Figure II). A total of 1270 patients entered these analyses. We estimated that this sample size would be sufficient to detect a standardized regression coefficient of 0.079 or larger (at a two-sided 0.05 significance level and probability of 80 percent). In other words, a significant association would be detectable if one standard deviation (SD) higher level of the independent variable was associated with at least 0.079 SD higher levels of the dependent variables.

To investigate, quantitatively, whether and the extent to which concentrations of plasma CML explained (a significant portion of) the association between BMI or waist circumference and LGI, we used the statistical computations commonly used in multiple mediation analyses. At this stage, we explicitly clarify that plasma CML was not hypothesized to be a ‘mediator’ (M) in its strict definition (i.e. in the causal pathway between BMI or waist circumference and LGI) but as a ‘marker’ (Z) of such a mediator (i.e. CML accumulation in adipose tissue). The dashed (marker) and continuous (causal) lines in Figure II illustrate this conceptual distinction. The portion of the BMI or waist circumference-LGI associations independently explained by plasma CML and other variables considered as potential mediators (specifically, HbA1c, total-to-HDL cholesterol ratio, triglycerides and pulse pressure – M1-4), was quantified by calculating the magnitude of attenuation in the regression coefficients (β) reflecting these association before (path c) and after adjustment for CML and these other mediators (path c’), which is simply calculated as c-c’. To ascertain the statistical significance of this attenuation, and the portion of it attributable to CML and each of mediators considered, we used SPSS macros provided by Preacher and Hays to calculate, after 1000 bootstrap samples were drawn, bias-corrected 95% confidence intervals (CIs) around the estimated attenuation (deemed as statistical significant whenever the CI did not comprise 0). Multiple mediation analyses as described above were also used to quantify the portion of the trunk fat mass-LGI association explained by CML (vs. other risk factors) in analyses confined to the Hoorn sub-sample with DXA measures of body composition.
REFERENCES


