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The association between leptin and subclinical cardiovascular disease explained by body fat: Observational and Mendelian randomization analyses

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Abstract *Background and aims:* Leptin has been associated with adverse effects on cardiovascular disease, but the effect of confounding by body fat in these associations remains unclear. To investigate associations between leptin and heart function and subclinical cardiovascular disease adjusted for total body fat, and to investigate the causal relation between leptin and cardiovascular disease using Mendelian randomisation.

Methods and results: Leptin concentrations, total body fat and diverse measures of subclinical cardiovascular disease were determined in participants of the Netherlands Epidemiology of Obesity study. Linear regression between leptin concentration and measures of heart function, ECG measures, and carotid intima media thickness as a measure of subclinical atherosclerosis was adjusted for potential confounding factors, and additionally including total body fat. We analysed the combined effects of genetic variants from a GWAS on leptin concentrations in publicly-available summary statistics of coronary heart disease GWAS (CARDIoGRAMplusC4D, n = 184,305). As many as 6107 men and women, mean (SD) age 56 (6) years, BMI 26 (4) kg/m^2, and median leptin concentration 12.1 μg (IQR: 6.7–22.6) were included.

In observational analyses, leptin was weakly associated with heart function and subclinical cardiovascular disease, but these associations attenuated when adjusting for total body fat. A doubling of genetically-determined leptin concentration was associated with an odds ratio of cardiovascular disease of 0.69 (0.37, 1.27).

Conclusion: Observational associations between leptin and subclinical measures of cardiovascular disease were largely explained by differences in total body fat. Results of analyses of genetically-determined leptin and coronary heart disease risk were inconclusive due to a large confidence interval.

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1. Introduction

Leptin is a satiety hormone derived from adipose tissue, and is important in the feedback loop of central regulation of body weight [1]. There are indications that leptin has a regulatory function in immunity and vasoconstriction [2–4]. Furthermore, in a meta-analysis on the association between leptin and clinical cardiovascular disease, individuals in the top tertile of leptin concentrations had a 36–50% higher risk of developing cardiovascular disease than those in the lowest tertile, which persisted after adjustment for BMI [5]. While leptin seems detrimental for cardiovascular health in population studies, studies on subclinical markers of cardiovascular disease show conflicting results. Leptin has been associated with detrimental effects on some risk factors for cardiovascular disease, such as decreased muscle mass, hypertension, kidney damage, and increased arterial stiffness with consequent cardiac morphological changes, but also beneficial effects on atherosclerosis and cardiac repolarisation [4,6–11]. Small human studies have also suggested that an increased sympathetic nervous system activity is a mechanism in the relation between leptin and detrimental outcomes like hypertension, kidney damage, and increased arterial stiffness [9]. Increased activity of the sympathetic nervous system can be reflected by the electrocardiogram (ECG) [12]. We hypothesized that the observed relation between leptin and detrimental cardiovascular outcomes is, in fact, a consequence of inadequate adjustment for the effects of body fat on these outcomes. Since leptin is mainly produced by white adipose tissue, a problem in many previous studies is the lack of adequate adjustment for total body fat. Some studies instead adjusted for body mass index (BMI) which is subject to misclassification of body fat due to its relation to height and muscle mass [13]. Therefore, residual confounding may have distorted the results of several previous studies. For this reason, we aimed to study the association between leptin and heart function and subclinical cardiovascular disease (in this study defined as having two domains: ECG parameters and subclinical atherosclerosis) while taking the influence of total body fat into account. Furthermore, we aimed to elucidate the causal effect of leptin on clinical coronary artery disease by a Mendelian randomisation approach.

2. Methods

2.1. Study design and study population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based, prospective cohort study including 6671 individuals aged 45–65 years, in which individuals with overweight or obesity were oversampled. The study design and population have been described in detail elsewhere [14].

Men and women living in the greater area of Leiden (in the West of the Netherlands) were invited by letters and by local advertisements. They were invited to respond if they were aged between 45 and 65 years and had a self-reported body mass index (BMI) of 27 kg/m^2 or higher. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited to participate irrespective of their BMI, allowing for a reference BMI distribution. The present analysis is a cross-sectional analysis using baseline measurements. We excluded participants with a medical history of cardiovascular disease (defined as myocardial infarction, angina, congestive heart failure, stroke, or peripheral vascular disease).

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

2.2. Data collection

Participants were invited to a baseline visit at the NEO study centre of the LUMC after an overnight fast. Prior to this study visit, participants completed a general questionnaire at home to report demographic, lifestyle and clinical information. The participants were asked to bring all medication they were using to the study visit. A research nurse registered all medication and dosing. At the baseline visit an extensive physical examination was performed, including anthropometry and blood pressure, and blood samples were drawn from the antecubital vein. At the study centre, participants completed a screening form, asking about anything that might create a health risk or interfere with magnetic resonance imaging (MRI), most notably metallic devices, claustrophobia or a body circumference of more than 1.70 m. Of the eligible participants, 2580 were randomly selected to undergo MRI of the abdomen, of whom a random subset underwent additional cardiac imaging (n = 1207).

Venous blood samples from the antecubital vein were obtained after an overnight fast of >10 h. Fasting serum total cholesterol, HDL-cholesterol, triglycerides, C-reactive protein, and plasma glucose and insulin were determined in the fasting blood samples at the central clinical chemistry laboratory of the LUMC using standard assays. LDL cholesterol concentrations were calculated using the Friedewald equation [15]. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as the product of plasma glucose and insulin, divided by 22.5 [16]. Leptin concentrations were determined in fasting plasma samples using enzymatic colorimetric reactions with a human leptin competitive RadioImmunoAssay (RIA) [17] (Cat Nr HL-81HK, Merck Millipore, Darmstadt, Germany) and the Roche P800 automated analyser (Roche Diagnostics, Almere, The Netherlands), according to standard laboratory protocols. Analytical total CV's as determined with internal control materials were calculated based on 22 runs over 105 days and was 12–14% at levels between 19 and 55 μg/L. Samples with a concentration >100 μg/L were diluted 1:5 with buffer from the kit.

Furthermore, aliquots of plasma and serum were stored after centrifugation at −80 °C. DNA was extracted and genotyping was performed by the Centre National de Génotypage (Evry Cedex, France), using the Illumina HumanCoreExome24 BeadChip (Illumina Inc., San Diego,

California, United States of America). Subsequently, genotypes were imputed to the 1000 Genome Project reference panel (v3 2011) using IMPUTE (v2.2) software [18,19]. Five known genetic variants for leptin were extracted, that were discovered in a BMI-adjusted genome-wide association study for leptin concentrations [20]. As these variants are located in independent genome regions and were associated with the expression of different genes, we assumed all genetic effects to be additive.

2.3. Subclinical cardiovascular outcome measures

2.3.1. Electrocardiographic measurements

A 12-lead electrocardiogram (ECG) was obtained in all participants using a Mortara Eli-350 electrocardiograph (Mortara Instrument Inc., Best, the Netherlands) after a resting period of at least 10 min. Standard 10-s ECGs were stored in an 8-lead (I, II, II, V1−V6), 5000 sample comma-separated-value file. The Kors matrix was used to calculate a vector cardiograms (VCG) from the eight independent ECG leads [14]. ECGs and VCGs were analysed using the automatic MATLAB-based (The MathWorks, Natick, MA) program BEATS and the semiautomatic program LEADS [21,22]. BEATS was used to detect the timings of all QRS complexes and calculated R-R intervals (ms) and coefficient of variation. All ECGs were checked for falsely identified QRS complexes or nonsinus beats, and the timings were manually adjusted. Also, complexes surrounding the nonsinus beat were removed from the mean R-R interval calculations. Mean HR in beats/min was calculated as 60 divided by the mean R-R interval in seconds. LEADS was used to calculate QT time (ms), QTc (corrected according to the Bazett formula), and Tpeak-end duration (ms). The QRS and T integral vectors were approximated by calculating the numerical sum of x-y-z deflections (amplitudes of positive deflections are added and those of negative deflections subtracted). The spatial QRS-T angle was defined as the angle (°) between the integral QRS vector and the integral T vector. The spatial ventricular gradient (mV*ms) was calculated as the vectorial sum of these vectors.

2.3.2. Intima media thickness

Carotid intima media thickness (cIMT) was used as a measure of subclinical atherosclerosis. cIMT was assessed by ultrasonography of the common carotid arteries in all participants. A 15 mm long section 10 mm proximal of the carotid artery bifurcation was measured while the subject was in supine position. cIMT was measured using a 7.5−10 MHz linear-array probe and the Art.Lab system in B-mode setting and using a wall-track system (ART.LAB version 2.1, Esaote, Maastricht, The Netherlands) to detect boundaries between lumen and intima, and adventitia. cIMT was measured during six heart beats in angles of 180, 135 and 90° (right CCA) and 180, 225 and 270° (left CCA).

2.3.3. Cardiac imaging

In a random subset of the MRI population (n = 1207), through-plane flow measurements of the ascending, proximal descending, mid-descending, and distal descending aorta were acquired in participants with MRI imaging. Aortic peak wave velocity (PWV) was calculated by dividing the aortic path length between the measurement sites by the transit time between the arrival of the systolic wave front at these sites, and it is expressed in meters per second. The heart was imaged in the short-axis orientation by using electrocardiographically gated breath-hold balanced steady-state free precession imaging to assess ventricular dimensions and mass. An electrocardiographically gated gradient echo sequence was performed with velocity encoding to measure blood flow across the mitral valve to determine diastolic function. Systolic parameters included ejection fraction (EF). Diastolic parameters included peak filling rates of the early filling phase (E) and atrial contraction (A) and their ratio (E/A ratio). Image postprocessing was performed with in-house−developed software packages (MASS and FLOW; Leiden University Medical Center, Leiden, the Netherlands).

2.3.4. UK Biobank

In the UK Biobank, a population-based cohort study of approximately 500,000 individuals from the United Kingdom, who were genotyped, and phenotypic data was collected in subgroups of the study population. Pulse wave velocity was measured in 117,867 genotyped participants using an infra-red sensor at the tip of a finger of the warm hand while the participant was sitting. The sensor detected the blood flow velocity, and recorded the velocity over time. The time between the two peaks of flow velocity was divided by the participant's height to obtain the pulse wave velocity index (PWVi).

2.3.5. Other variables

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). Three measurements were performed with 5-min rests in between measurements, and the mean systolic and diastolic blood pressure levels were calculated.

Height was measured without shoes using a calibrated, vertically fixed tape measure. Body weight and percent total body fat (TBF) were estimated by the Tanita bioimpedance balance (TBF-310, Tanita International Division, UK) without shoes and 1 kg was subtracted to correct for the weight of clothing. Body mass index (BMI) was calculated by dividing body mass in kilograms by body height in meters squared.

2.4. Statistical analyses

Baseline characteristics were presented as means (standard deviation), medians (25th-75th percentile) or percentages. As the NEO study population included an oversampling of participants with BMI of 27 kg/m^2 or higher, we weighted all analyses towards the BMI distribution of participants from the Leiderdorp municipality, who had a similar BMI distribution to the general Dutch population [23]. Consequently, the results apply to a population-based study without oversampling of

individuals with a BMI≥27 kg/m^2. For comparison of the results in the figures, all measures of subclinical cardiovascular disease were standardized to a mean of zero and standard deviation of one.

We performed linear regression analyses between leptin and EF and E/A ratio, heart rate and heart rate variability, QRS and P duration, PR and QT interval, P, QRS, and T axis, and carotid intima-media thickness as outcomes. These analyses were adjusted for the potential confounding factors age, sex, smoking status, HOMA-IR, triglyceride, glucose, HDL-, and LDL-cholesterol concentrations, blood pressure, use of antihypertensive and glucose and lipid lowering medication. We additionally adjusted the association between leptin and cardiovascular risk factors for total body fat. The regression coefficients can be interpreted as SD difference in the outcome measure per 10 μg/ml difference in leptin concentration. We repeated all analyses stratified by sex.

For the Mendelian randomisation analysis with genetically-determined leptin concentrations, we calculated a weighted genetic risk score including five known genetic variants from a recent genome-wide association study (GWAS) on leptin: rs10487505 (LEP), rs780093 (GCKR), rs900400 (CCNL1), rs6071166 (SLC32A1), and rs6738627 (COBLL1) [20]. The genetic risk score was calculated by multiplying the number of leptin concentration-increasing alleles per variant with the BMI-adjusted per-allele effect of the variant from the genome-wide association study. We performed several analyses to (1) assess the effects of leptin of markers of subclinical cardiovascular disease, defined as heart function and subclinical atherosclerosis; (2) assess the effect of leptin on arterial stiffness, defined as pulse wave velocity; and (3) assess the effect of leptin on clinical cardiovascular disease. Details on these analyses are presented below.

1. We estimated the effect of the genetically-determined leptin on the measures of heart function and subclinical cardiovascular disease measured in the NEO study using two-stage least squares regression analysis. The regression coefficients can be interpreted as SD difference in the outcome measure per 10 μg/ml difference in genetically-determined leptin concentration.
2. We performed additional analyses on publicly-available data on the pulse wave velocity index, as recorded in the UK Biobank [24]. We used the MR-base application to estimate the inverse-variance weighted effect of our set of BMI-adjusted genetic variants for leptin [20,25]. To perform this estimation, we performed a weighted regression of the association (beta) between the gene and leptin, and the betas between the gene and the pulse wave velocity.
3. We estimated the effect of genetically-determined leptin on clinically overt coronary artery disease by combining data on our genetic instruments for leptin with a publicly available database of summary statistics of genome-wide associations for coronary artery disease: CARDIoGRAMplusC4D. Data on coronary artery disease (CAD) in 60,801 cases and 123,504 controls of European descent have been contributed by CARDIoGRAMplusC4D investigators and have been downloaded from www.CARDIoGRAMplusC4D.org [26]. Of these individuals, 6688 had also been included in the GWAS of leptin concentrations [20,26,27]. We used the MR-base application to estimate the inverse-variance weighted effect of the BMI-adjusted genetic variants for leptin on clinical CAD [20,25]. A weighted regression of the beta of the association between the gene and leptin and the betas between the gene and CAD was performed. The regression was weighted towards the inverse of the standard error of the betas, and the regression line was constrained to intersect the origin. The resulting regression coefficient can be interpreted as the change in the outcome per doubling of genetically-determined leptin concentration. One genetic variant (rs10487505, LEP) was not indexed in the CARDIoGRAMplusC4D dataset, and therefore we used a proxy in perfect linkage (rs6979832; LD 1.0) [28].

3. Results

3.1. Effects of (genetically determined) leptin on heart function and subclinical cardiovascular disease: cardiac MRI, ECG and cIMT

3.1.1. Baseline characteristics

A total of 6671 participants were included in the NEO study. After exclusion of participants with missing blood samples (n = 44), missing values for leptin (n = 35), and a history of cardiovascular disease (n = 485), data of 6107 participants of whom 2844 men and 3263 women were used for the observational analyses. Further exclusion of related participants, or participants with genetic data of insufficient quality yielded 5639 participants for genetic analyses. Cardiac MRI analyses were performed in 1270 participants, of whom 1060 participants had genetic data of sufficient quality.

Table 1 presents baseline characteristics of the NEO study, stratified by leptin concentration above or below the median (12.1 μg/L). Mean (standard deviation, SD) age was 55 (6) years, and 43% were men. Median (25th −75th percentile) leptin concentrations in men were 6.9 μg/L (4.5−10.7), and in women 19.0 μg/L (11.4−29.7).

The results of the observational and Mendelian randomisation analyses of the association between leptin and MRI measures of heart function are presented in Fig. 1 (n = 1060), between leptin and ECG parameters in Fig. 2 (n = 5639), and between leptin and measures of subclinical atherosclerosis presented in Fig. 3 (n = 5639). In adjusted observational models, 10 μg/L increased leptin concentration was associated with 0.18 SD (95% confidence interval: 0.13, 0.23) increased right ventricular ejection fraction, and 0.04 SD (95% CI: 0.00, 0.08) increased left ventricular ejection fraction, and weak associations with ECG measures of cardiac conduction with confidence intervals including the null. Additional adjustment for total body fat reduced these and other associations towards the null: 10 μg/L increased leptin concentration became associated with 0.05 SD (95% CI: -0.01, 0.12) of RVEF, and 0.04 SD (95% CI: -0.02, 0.1) of LVEF. All associations were similar

Table 1 Characteristics of participants in the Netherlands Epidemiology of Obesity (NEO) study (n = 6107), stratified by leptin concentration below or above median (12.1 μg/L).

	Total population	Leptin <12.1 μg/L	Leptin ≥12.1 μg/L
Age (y)	55 (6)	56 (6)	55 (6)
Sex (men, %)	43	68	17
BMI (kg/m^2)	26 (4)	25 (3)	28 (4)
Total body fat (%)	31 (8)	25 (6)	37 (6)
Smoking (current, %)	16	18	14
Diabetes (%)	5	4	6
Antihypertensive use (%)	20	16	25
Glucose lowering medication use (%)	2	2	3
Lipid lowering medication use (%)	8	7	10
Glucose (mmol/l)	5.4 (1.0)	5.4 (1.1)	5.5 (0.8)
HOMA-IR	1.8 (1.2, 2.9)	1.5 (1.0, 2.3)	2.1 (1.4, 3.4)
HDL cholesterol (mmol/l)	1.6 (0.5)	1.6 (0.5)	1.6 (0.4)
LDL cholesterol (mmol/l)	3.6 (1.0)	3.6 (0.9)	3.6 (1.0)
Systolic blood pressure (mmHg)	130 (17)	131 (17)	130 (17)
Diastolic blood pressure (mmHg)	83 (10)	83 (11)	84 (10)
Carotid intima media thickness (μm)	615 (90)	611 (91)	619 (89)
Genetic risk score leptin	4.51 (1.43)	4.43 (1.44)	4.62 (1.41)
Leptin (μg/l)	12.1 (6.7−22.6)	6.7 (4.5, 9.0)	22.6 (16.0, 32.7)

Values are represented as mean (SD), median (25th −75th percentile) or percentage. Results were based on analyses weighted towards a normal BMI distribution (n = 6107).
Abbreviations: BMI, Body mass index; HDL, high density lipoprotein HOMA-IR, homeostatic model of insulin resistance; LDL, low density lipoprotein; SD, standard deviation.

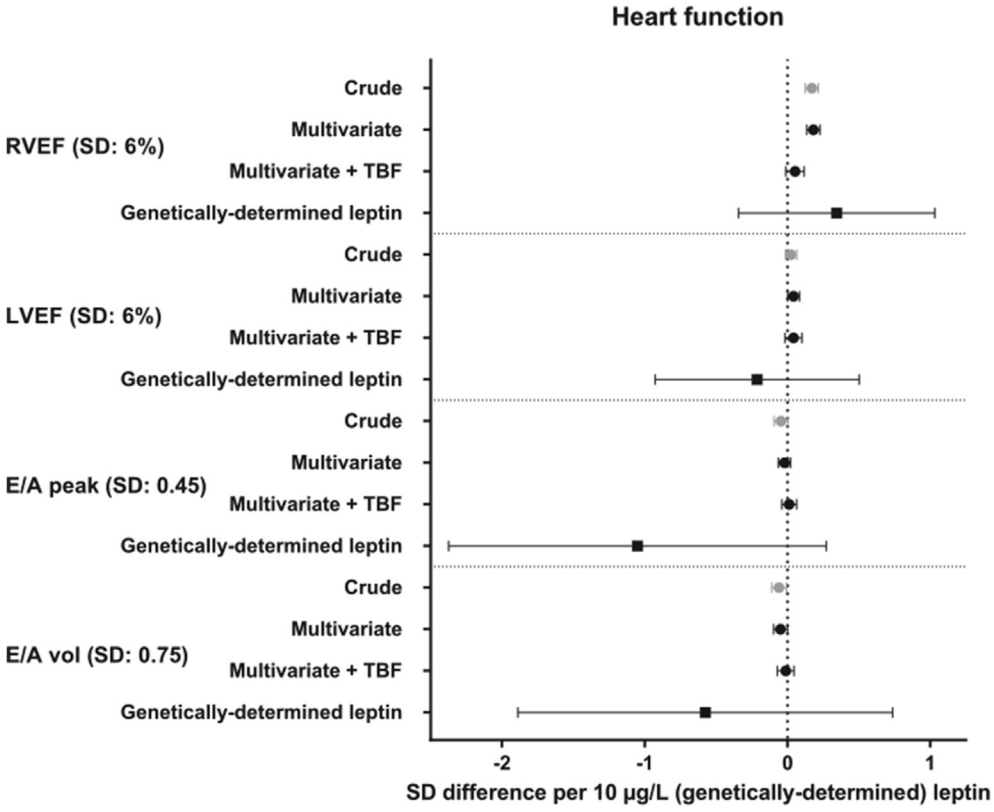

Figure 1 Standardized differences in MRI measures of heart function with 95% confidence intervals as associated with observational (circles) and genetically-determined (squares) leptin concentration. Multivariate analyses were adjusted for age, sex, smoking status, HOMA-IR, triglyceride, glucose, HDL- and LDL-cholesterol concentrations, blood pressure, use of antihypertensive and glucose and lipid lowering medication. Multivariate + TBF analyses were additionally adjusted for total body fat. Results were based on weighted analyses (n = 1060).Abbreviations: CI, confidence interval; E/A, early/atrial (filling phase) HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin response; LDL, low-density lipoprotein; LVEF, left ventricle ejection fraction; RVEF, right ventricle ejection fraction; SD, standard deviation; TBF, total body fat.

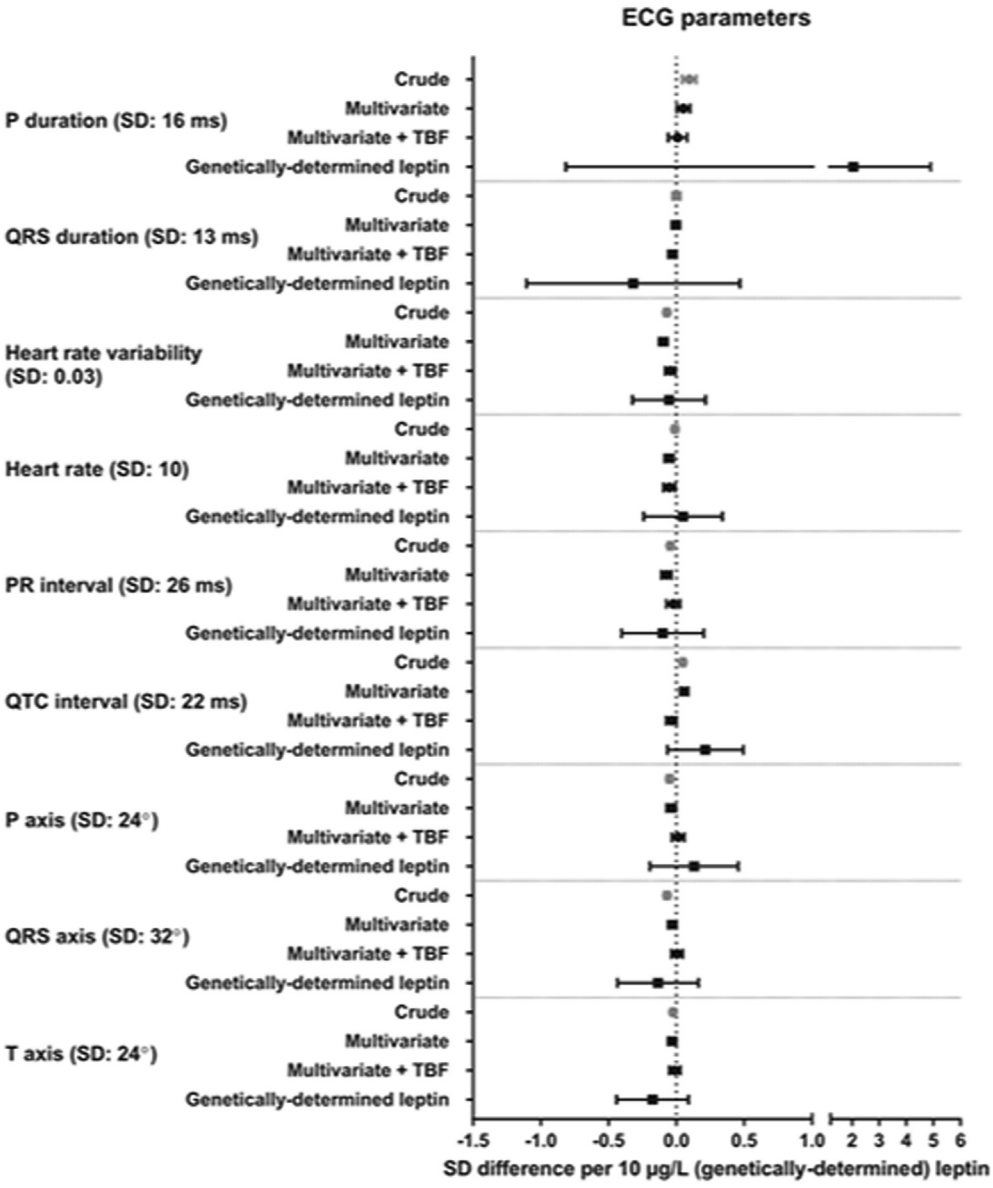

Figure 2 Standardized differences in ECG parameters with 95% confidence intervals as associated with observational (circles) and genetically-determined (squares) leptin concentration. Multivariate analyses were adjusted for age, sex, smoking status, HOMA-IR, triglyceride, glucose, HDL- and LDL-cholesterol concentrations, blood pressure, use of antihypertensive and glucose and lipid lowering medication. Multivariate + TBF analyses were additionally adjusted for total body fat. Results were based on weighted analyses (n = 6107).Abbreviations: CI, confidence interval; ECG, electrocardiogram; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin response; LDL, low-density lipoprotein; SD, standard deviation; TBF, total body fat.

in women and men (data not shown). Mendelian randomisation analyses showed no associations between genetically raised leptin and the subclinical markers of cardiovascular disease, and wide confidence intervals.

3.2. Effects of genetically determined leptin on subclinical cardiovascular disease: pulse wave velocity

3.2.1. Baseline characteristics

Measurement of pulse wave velocity were performed in 214,847 participants of UK Biobank. A detailed description of the population included in the UK Biobank cohort is reported elsewhere [24].

3.2.2. Leptin and subclinical cardiovascular disease

Analyses using the UK Biobank data indicated that leptin did not causally affect pulse wave velocity: per doubling of genetically-determined leptin concentration, the pulse wave velocity index was 0.02 lower (95% CI: −0.23, 0.18).

3.3. Effects of genetically determined leptin on coronary artery disease

3.3.1. Baseline characteristics

Analyses of the CARDIoGRAMplusC4D dataset were performed in 60,801 cases and 123,504 controls. Detailed characteristics of this population are described elsewhere [19].

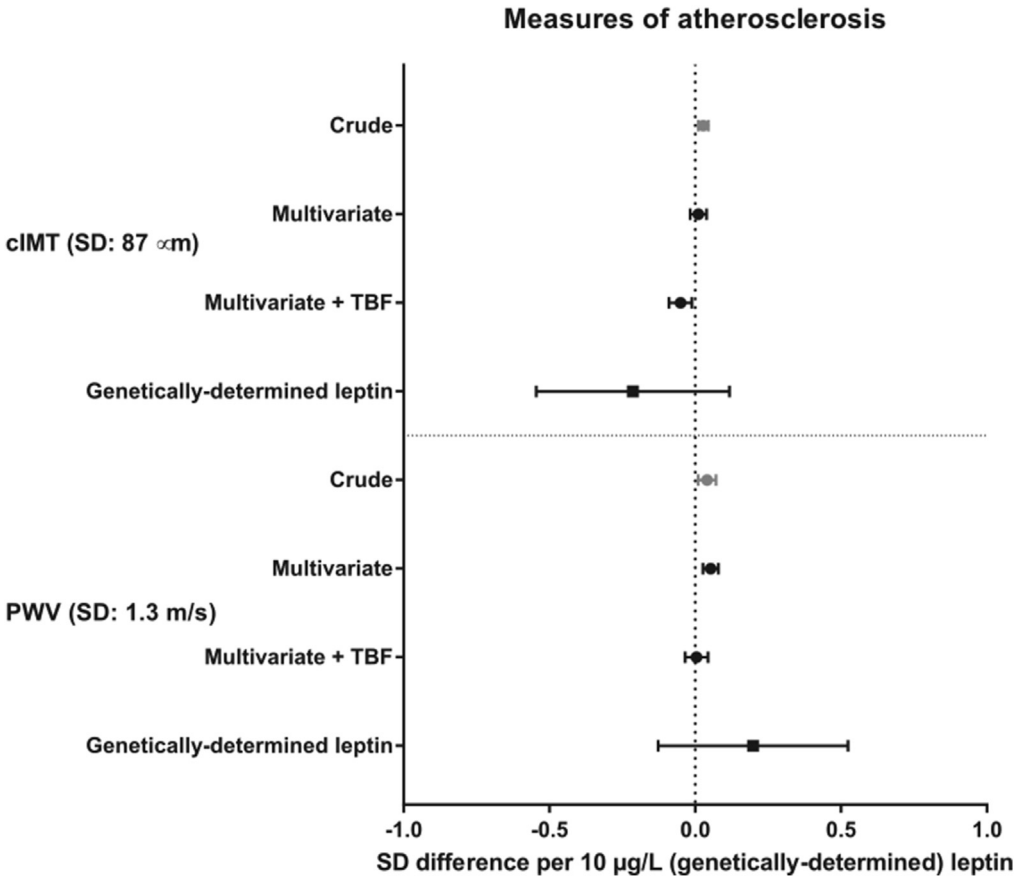

Figure 3 Standardized differences in measures of atherosclerosis with 95% confidence intervals as associated with observational (circles) and genetically-determined (squares) leptin concentration. Multivariate analyses were adjusted for age, sex, smoking status, HOMA-IR, triglyceride, glucose, HDL- and LDL-cholesterol concentrations, blood pressure, use of antihypertensive and glucose and lipid lowering medication. Multivariate + TBF analyses were additionally adjusted for total body fat. Results were based on weighted analyses (n = 6107 for cIMT, n = 2320 for PWV).Abbreviations: CI, confidence interval; cIMT, carotid intima media thickness; ECG, electrocardiogram; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin response; LDL, low-density lipoprotein; PWV, peak wave velocity; SD, standard deviation; TBF, total body fat.

Inverse variance weighted regression in publicly-available summary statistics on coronary artery disease (CARDIoGRAMplusC4D) showed that a doubling of genetically determined leptin concentration was associated with an odds ratio of 0.69 (95% CI: 0.37, 1.27) for coronary artery disease.

4. Discussion

Our results show that in a well-phenotyped population-based cohort, observational and genetically-determined leptin concentration was not associated with various measures of heart function and subclinical cardiovascular disease. Furthermore, Mendelian randomisation analysis in a large publicly-available database of coronary heart disease indicated no effect in either direction, with large uncertainty, but a strong detrimental effect seems unlikely.

The results of this study extend the current knowledge on the relation between leptin and cardiovascular disease, since we investigated these associations with adjustment for total body fat, which has been done in only a few studies [5]. We have shown that multivariable observational associations between leptin and subclinical measures of cardiovascular disease disappeared after adjustment for the main producer of leptin, total body fat. In the light of literature on leptin and cardiovascular disease, there are two potential explanations for the discrepancy between observational studies that consistently show an association between leptin concentration and several cardiovascular parameters [6–10].

First, adipose tissue may act as a confounding factor in the study of the association between leptin and subclinical cardiovascular disease, and was previously not adequately adjusted for. In this case, leptin is produced by adipose tissue, which is a cause of cardiovascular risk via other mechanisms [29]. Based on previous research, adipose tissue predominantly affects cardiovascular parameters through mechanisms that do not involve leptin, such as insulin resistance and diabetes [30] Also, previous studies often adjusted for body mass index instead of total body fat [31]. Body mass index is partly determined by lean mass, which is regarded as a cardioprotective factor, in part because of insulin sensitizing properties of muscle mass. Adjusting for body mass index therefore does not

completely solve the confounding by total body fat, and leaves residual confounding. The role of high leptin concentrations that are due to leptin resistance is unclear, but by adjustment for total body fat we attempted to correct for this as well. Also, the possibility exists that leptin is a mediator of the effect of body fat on cardiovascular disease. However, if this were true, any remaining causal effect of leptin on cardiovascular disease parameters would not disappear by adjustment for total body fat. Therefore our analyses would still have been able to identify such effects.

Second, the previously observed associations between leptin and subclinical cardiovascular disease might be due to reverse causation, i.e., worsening of these cardiovascular parameters may affect leptin concentrations when subclinical disease leads to weight change. Therefore, in addition to observational analyses adjusted for total body fat, we used a Mendelian randomisation approach specifically to mitigate problems due to unmeasured confounding or reverse causation. In our Mendelian randomisation analyses we observed estimates of the effect of leptin on measures of subclinical cardiovascular disease close to the null, some of which with broad confidence intervals. This leaves the possibility of mostly small effects that may be observed in larger populations, but for most outcome measures this indicates the absence of large clinically relevant effects. The results of the CARDIoGRAMplusC4D GWAS suggested that if there is an effect of leptin on clinical cardiovascular disease, it is likely to be beneficial. However, this study showed large uncertainty despite a large population and reasonably strong genetic instruments. Therefore, this study should be repeated when stronger genetic instruments become available. It is unlikely that this study could be repeated in a larger cardiovascular disease cohort in the near future.

Potential beneficial effects of leptin may arise downstream of its receptor, which further signals via the JAK-STAT3 pathway [32]. This pathway is pivotal in regulation of gene expression and apoptosis, and small alterations in activation of this pathway may lead to diverse effects on homeostasis [33]. The main effect of leptin remains on the regulation of energy expenditure and body weight, but leptin may also have off-target effects that could be beneficial with regard to cardiovascular disease [34]. Previous studies have related leptin to inhibition of the angiotensin receptor in vascular smooth muscle cells [4], myocardial de- and repolarisation [11], and decreased atherosclerosis [35] in tissue culture and rodents, and to slightly improved heart function in a population-based study [36]. This is partly reflected in our results, indicating small effects on subclinical measures of cardiovascular disease in both adverse and beneficial directions. However, these effects were small and therefore may have a negligible impact on clinical cardiovascular risk. In addition, animal experiments indicate novel fat tissue remodeling pathways of leptin, contributing to the understanding of the way a potential causal relation between leptin and cardiovascular disease is constructed [37–40]. Further downstream effects of the leptin-adipose tissue-cardiovascular disease pathway may be further elucidated by research on fibroblast growth factor and its contributions to the development of fatty liver [41].

The major strength of this study is the large number of well-phenotyped participants from the general population. Furthermore, the use of genotyping allowed us to study causal associations as well. However, this study also has some limitations. First, the cross-sectional observational analyses may be subject to residual confounding and reverse causation. Since our population was genotyped, we were able to perform additional genetic analyses to validate our initial findings without the risk of unmeasured confounding. However, in genetic studies the risk of pleiotropy remains, i.e. the genetic variants may affect the outcome via other mechanisms than the exposure. A second potential limitation of genetic studies on leptin may be the possible introduction of collider stratification bias, that might have been caused by the adjustment for BMI of the genome-wide association study on leptin [20]. By correcting for BMI, the remaining significant instruments may have become associated with BMI. Therefore these instruments may affect measures of subclinical cardiovascular disease through BMI instead of through leptin. However, the loss of genome-wide significance of the variant in the *FTO* gene after adjustment for BMI indicates that at least a part of the BMI-mediated effects may be adjusted away. In addition, we presented mostly null findings. An effect of BMI would potentially be away from the null. Third, a disadvantage of genetic studies is the large number of participants needed in the presence of a weak genetic instrument, such as in the present study. Even the analyses using publicly-available summary data may be inadequately powered, as the broad confidence intervals suggest. Also, these data do not provide sex-stratified statistics, therefore analyses separate for women and men are not yet possible. However, a larger dataset on cardiovascular disease is not yet publicly available, therefore the current analysis is the most precise analysis we could have performed. A potential limitation of the analyses of publicly-available data is that the populations in which the GWAS on leptin was performed and CARDIoGRAMplusC4D partly overlap. Overlap is mainly a problem when weak instruments are used in strongly overlapping studies. However, the present study used strong genome-wide significant genetic instruments with a small overlap of 3.6%, therefore this sort of bias is unlikely to affect our results [27].

In summary, our results show that observationally, leptin concentration was associated with various measures of heart function and subclinical cardiovascular disease, but appropriate adjustment for total body fat reduced these associations towards the null. These results are supported by Mendelian randomisation analyses in the same study. However, Mendelian randomisation analysis of the causal relation between leptin and coronary heart disease in a large publicly-available dataset suggested large uncertainty in the effect of leptin on coronary heart disease.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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