Remnant Cholesterol, Low-Density Lipoprotein Cholesterol, and Blood Pressure as Mediators from Obesity to Ischemic Heart Disease

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ABSTRACT

Rationale: Obesity leads to increased ischemic heart disease (IHD) risk, but the risk is thought to be mediated through intermediate variables and may not be caused by increased weight per se.

Objective: To test the hypothesis that the increased IHD risk due to obesity is mediated through lipoproteins, blood pressure, glucose, and/or C-reactive protein.

Methods and Results: ~90,000 participants from Copenhagen were included in a Mendelian randomization design with mediation analyses. Associations were examined using conventional measurements of body mass index (BMI) and intermediate variables, and using genetic variants associated with these. During up to 22 years of follow-up 13,945 participants developed IHD. The increased IHD risk due to obesity was partly mediated through elevated levels of nonfasting remnant cholesterol and low-density lipoprotein (LDL) cholesterol, through elevated blood pressure, and possibly also through elevated nonfasting glucose levels; however, reduced high-density lipoprotein cholesterol and elevated C-reactive protein levels were not mediators in genetic analyses. The three intermediate variables that explained the highest excess risk of IHD from genetically determined obesity were LDL cholesterol with 8%, systolic blood pressure with 7%, and remnant cholesterol with 7% excess risk of IHD. Corresponding observational excess risks using conventional BMI were 21%, 11%, and 20%, respectively.

Conclusions: The increased IHD risk due to obesity was partly mediated through elevated levels of nonfasting remnant and LDL cholesterol, and through elevated blood pressure. Our results suggest there may be benefit to gain by reducing levels of these risk factors in obese individuals not able to achieve sustained weight loss.

Keywords: Lipoprotein, lipid, cardiovascular diseases, genetics, risk factor, ischemic heart disease, obesity, Mendelian randomization, triglycerides.

Nonstandard Abbreviations and Acronyms:
BMI body mass index
CCH Copenhagen City Heart Study
CIHDS Copenhagen Ischemic Heart Disease Study
CGPS Copenhagen General Population Study
CRP C-reactive protein
HDL high-density lipoprotein
IHD ischemic heart disease
LDL low-density lipoprotein
INTRODUCTION

It has previously been shown that obesity is associated with increased risk of ischemic heart disease (IHD)\textsuperscript{1-3}, and that the association between obesity and IHD is likely to be causal\textsuperscript{4}; however, since it is probably not increased weight \textit{per se} that leads to the increased risk, it is likely that this risk is mediated through variables like lipoprotein levels, blood pressure, glucose levels, and/or inflammation. Obesity is an increasing problem for public health in many countries, and even though elevated body mass index (BMI) is a modifiable risk factor, it is difficult to encourage individuals to lose weight, and even more difficult to maintain weight loss. Therefore, it is important to delineate which of the intermediate variables from obesity to IHD are likely to be mediators.

In this study we use Mendelian randomization in the analysis of data from participants from the Copenhagen General Population Study (CGPS) (N=69,535), the Copenhagen City Heart Study (CCHS) (N=10,099), and the Copenhagen Ischemic Heart Disease Study (CIHDS) (N=5,050) to test the hypothesis that the increased risk of IHD due to obesity is mediated through 1) elevated nonfasting remnant cholesterol, 2) reduced high-density lipoprotein (HDL) cholesterol, 3) elevated low-density lipoprotein (LDL) cholesterol, 4) elevated systolic blood pressure, 5) elevated diastolic blood pressure, 6) elevated glucose levels, and/or 7) elevated C-reactive protein (CRP). In Mendelian randomization, the random assortment of alleles at conception is used to circumvent confounding and reverse causation that can bias observational associations. Assumptions for Mendelian randomization are first, that the genetic variants are associated with the phenotype, second, that the association between the genetic variants and the outcome is not confounded by the same factors that confound the observational association, third, that the genetic variants are not in linkage disequilibrium with other genetic variants associated with the outcome, and finally, that the genetic variants are not associated with the outcome through other ways than through the phenotype, that is, there is no pleiotropy\textsuperscript{5}.

To assess potential benefits if these intermediate variables are targeted in obese individuals unable to obtain sustained weight loss, we also performed mediation analyses to assess how much excess risk of IHD from obesity each intermediate variable mediates. Besides conventional mediation analyses using observational data, we used the novel approach of genetic mediation analyses to quantify how much of the risk of IHD, due to genetically determined obesity, is mediated through the different intermediate variables as this is presently not known. Each association was first examined in observational analyses, i.e. analyses prone to confounding and reverse causation, and second in genetic analyses not prone to the same confounding and reverse causation as the observational studies.

METHODS

Studies were approved by Herlev Hospital and Danish ethical committees (H-KF-01-144/01;KF-100.2039/91;KF-01-144/01;KA-93125;KA-99039), and were conducted according to the Declaration of Helsinki with informed consent from participants. All participants were white and of Danish descent, and none appeared in more than one study.

\textit{The Copenhagen General Population Study (CGPS).}

The CGPS is a prospective study of the general population initiated in 2003 with ongoing enrollment\textsuperscript{4}. Participants were randomly selected from the national Danish Civil Registration System to reflect the adult population aged 20-100+ years. Data collection included a questionnaire, a physical examination, and blood sampling for biochemical analyses and DNA extraction.
The Copenhagen City Heart Study (CCHS).

The Copenhagen Ischemic Heart Disease Study (CIHDS).
This study comprises 5,050 patients referred for coronary angiography to Rigshospitalet, Copenhagen University Hospital during the period 1991-2011. Beside a diagnosis of IHD as described below, patients also had stenosis/atherosclerosis on coronary angiography and/or a positive exercise electrocardiography test.

IHD.
Information on a diagnosis of IHD(ICD8:410-414, ICD10:I20-I25) was collected and verified from 1977 until April 2013 by reviewing all hospital admissions and diagnoses entered in the national Danish Patient Registry and all causes of death entered in the national Danish Causes of Death Registry, as described. These registers contain information on all participants and none were lost to follow-up. During up to 22 years of follow-up 13,945 participants developed IHD.

Laboratory analyses.
Standard hospital assays measured nonfasting total cholesterol, triglycerides, HDL cholesterol, high-sensitivity CRP, and glucose (Boehringer Mannheim, Dako, Dade Behring, and Konelab). LDL cholesterol was calculated using the Friedewald equation when plasma triglycerides were ≤4mmol/L, and otherwise measured directly (Konelab). Nonfasting remnant cholesterol was nonfasting total cholesterol minus HDL cholesterol minus LDL cholesterol6-8.

Genotypes.
Using information from our previous studies and from genome-wide association studies, we chose well-known genetic variants for each allele score so that the allele score was associated with BMI or the particular intermediate variable of interest, and ideally with nothing else: 1) For BMI we chose the five genetic variants FTO rs9939609, MC4R rs17782313, TMEM18 rs6548238, BDNF rs10767664, and GNPDA2 rs10938397 with the largest reported effects on BMI. 2) For nonfasting remnant cholesterol we chose TRIB1 rs2954029, GCKR rs1260326, and APOA5 rs6518217. 3) For HDL cholesterol we chose LIPC -480C/T, ABCA1 N1800H, and ABCA1 R2144X. 4) For LDL cholesterol we chose APOB rs5742904, LDLR W23X, LDLR W66G, LDLR W556S, and PCSK9 rs11591147. 5) For systolic and diastolic blood pressure we chose ATP2B1 rs2681472 and CYP17A1 rs11191548. 6) For glucose we chose GCK rs4607517, G6PC2 rs560887, ADCY5 rs11708067, DGKB rs2191349, and ADRA2A rs10885122. 7) For CRP we chose CRP rs1205, CRP rs1130864, CRP rs3091244, and CRP rs3093077.

Genotyping was by TaqMan (Applied Biosystems) or by restriction enzyme assays(details available in the Supplemental Material). Genotypes were verified by genotyping of randomly selected samples of each variant by two different methods (TaqMan plus sequencing or restriction enzyme assay). Call rates for genotypes were >99% for all assays. ATP2B1 rs2681472 and CYP17A1 rs11191548 were determined by LGC Genomics using a competitive allele-specific PCR system (KASPar).

Other covariates.
BMI was measured weight (kg) divided by measured height squared(m²). Systolic and diastolic blood pressures were measured at study inclusion. Smokers were current smokers. Lipid-lowering therapy was self-reported.
**Statistical analysis.**
We conducted analyses according to the order shown in Figure 1. We first analysed the observational association between BMI and the seven intermediate variables (Figure 1, #1). Second, we investigated how a BMI allele score associated causally with levels of the intermediate variables (Figure 1, #2). Third, we combined information from #1 and #2 in instrumental variable analyses, to examine whether observational associations between BMI and intermediate variables are causal, and how large a difference in levels of intermediate variables an observational and a causal 10kg/m² higher BMI was associated with, corresponding to the difference between being normal weight and obese (Figure 1, #3). Fourth, using the genetic variants only associated with BMI as well as other well-known genetic variants associated only with individual intermediate variables, we investigated how much the observational and causal change in the intermediate variable (caused by a 10kg/m² higher BMI) would translate into in observational and causal risk of IHD (Figure 1, #4a and b). Finally and fifth, we combined all information from #1 to #4 and previous knowledge from randomized clinical intervention trials, to conduct observational and causal mediation analyses to examine how much excess risk of IHD caused by obesity is mediated through the seven intermediate variables (Figure 1, #5a and b).

For a detailed description of the statistical analyses performed, please see the Supplemental Material.

**RESULTS**

Table 1 shows characteristics of 84,684 participants in the CCHS, CGPS, and CHIDS; however, number of participants varies between the different analyses according to availability of genotypes, intermediate variables, and covariates. Online Table II shows baseline characteristics of participants in the CGPS by genotypes and shows that the allele scores used as instruments for the different intermediate variables were not associated with other conventional risk factors for IHD, except for the expected associations of the remnant cholesterol allele score and the LDL cholesterol allele score with lipid-lowering therapy; that is, the allele scores were largely without pleiotropic effects on other cardiovascular risk factors. Genotype distributions for all studies were in Hardy-Weinberg equilibrium (P-values>0.1).

**Observational and genetic associations: BMI and IHD (Figure 1, Pa and Pb).**

As described previously using the same studies⁴, a 4kg/m² higher BMI was associated with increased risk of IHD with an observational odds ratio of 1.26(95%CI:1.19-1.34) and a genetically derived odds ratio of 1.52(1.12-2.05), corresponding to an observational odds ratio of 1.78(1.54-2.08) and a genetically derived odds ratio of 2.85(1.33-6.02) for a 10 kg/m² higher BMI. Also corresponding to the previous findings, BMI in quintiles was associated with a stepwise increased risk of IHD (Online Figure 1, left panel), and the BMI allele score was associated with stepwise increased risk of IHD for increasing number of alleles (Online Figure 1, right panel).

**Observational associations: BMI and intermediate variables (Figure 1, #1).**

Increasing BMI levels were observationally associated with higher levels of nonfasting remnant cholesterol, LDL cholesterol, glucose, and CRP, with higher systolic and diastolic blood pressure, and with lower levels of HDL cholesterol (Figure 2).
Genetic associations: BMI allele score and intermediate variables (Figure 1, #2).

The BMI allele score was associated with higher levels of nonfasting remnant cholesterol, and CRP, with higher systolic and diastolic blood pressure, and with lower HDL cholesterol levels for increasing number of BMI increasing alleles (Figure 3). The BMI allele score did not show strong evidence for an association with LDL cholesterol or nonfasting glucose levels; however, there was a tendency toward higher levels for increasing number of BMI alleles. Online Figure II show similar results as Figure 3 for the five genetic variants used in the BMI allele score separately.

Combined observational and genetic associations: BMI and intermediate variables (Figure 1, #3).

Figure 4 shows that both an observational and a genetically determined 10kg/m² higher BMI was associated with higher levels of remnant cholesterol, LDL cholesterol, glucose, and CRP, with higher systolic and diastolic blood pressure, and with lower HDL cholesterol levels. Even though P-values for comparison showed significant differences between observational and genetic estimates for remnant cholesterol, HDL cholesterol, and diastolic blood pressure, all genetic estimates were in the same direction as observational estimates indicating causality.

Online Figure III shows results for individuals with a BMI below 30kg/m² and Online Figure IV results for those with a BMI above 30kg/m².

Observational and genetically determined changes in intermediate variables, associated with a 10kg/m² higher BMI, translated into risk of IHD(Figure 1, #4).

Figure 5 shows observational and genetic risk estimates for IHD for levels of the intermediate variables corresponding to the magnitude of the observational and genetic associations of a 10kg/m² higher BMI with the intermediate variables shown in Figure 4. That is, Figure 5 shows what the difference in intermediate variable associated with an observational or a genetically determined 10kg/m² higher BMI translated into risk of IHD. For nonfasting remnant cholesterol and LDL cholesterol, both observational and genetic estimates for IHD risk were in the same direction indicating that the associations may be causal, with observational hazard ratios of 1.10(1.08-1.11) and 1.05(1.04-1.06) for remnant cholesterol and LDL cholesterol, respectively, and with corresponding genetically derived risk ratios of 1.20(1.07-1.34) and1.28(1.20-1.36), respectively. Genetic estimates were higher than observational estimates, which can probably be explained by genetic variants causing lifelong higher levels of remnant and LDL cholesterol, while observational estimates are based on a single measurement of remnant and LDL cholesterol.

For HDL cholesterol levels, systolic and diastolic blood pressure, and for glucose levels, the observational estimates indicate increased IHD risk; however, the genetically derived risk ratios were not statistically different from 1(Figure 5) indicating that the association may not be causal, and/or that the observational estimate may be confounded. For elevated CRP levels there was an increased observational risk of IHD, but the genetically derived risk ratio was in the opposite direction.

The allele scores for the intermediate variables were combined from genetic variants known to be associated with the intermediate variable of interest so that they had as large as possible an effect, and with the effect mainly on the intermediate variable of interest and not on the other intermediate variables(Online Figure V).

All intermediate variables were associated observationally with risk of IHD(Online Figure VI); however, only allele scores for remnant cholesterol, LDL cholesterol, and glucose were associated with risk of IHD(Online Figure VII).
Observational and genetic mediation analyses (Figure 1, #5).

For all intermediate variables, the observational excess risk (Figure 6, left panel) was higher than the genetically determined excess risk (Figure 6, right panel). P-values for comparison showed significant differences between observational and genetic estimates for remnant cholesterol, LDL cholesterol, and diastolic blood pressure; however, estimates were in the same direction, and the differences between observational and genetic estimates could be explained by the genetic variants explaining only part of the variation in the phenotypes, and/or the observational estimates being prone to confounding. Genetically determined excess risk is only shown for the intermediate variables with results suggesting genetically determined risk for IHD in Figure 5, except for blood pressure and glucose where previous randomized clinical intervention trials or Mendelian randomization studies suggest causality [16,18,19], and where the lack of genetic association in Figure 5 may be explained by lack of power because of weak instruments. The three intermediate variables that explained the highest excess risk of IHD from genetically determined obesity were LDL cholesterol with 8%, systolic blood pressure with 7%, and remnant cholesterol with 7% excess risk of IHD, with corresponding observational excess risks of 21%, 11%, and 20%, respectively. Results were similar when using the percent excess risk mediated (PERM) method [20], except that the excess risk for LDL cholesterol was only 1% (Online Figure VIII).

Online Table III shows P-values for interaction between the intermediate variables and BMI or BMI allele score on predicting risk of IHD. After correction for multiple comparisons, there was a significant interaction between systolic blood pressure and BMI in the observational association.

DISCUSSION

In this study, we show that the previously established increased risk of IHD due to obesity [4] is partly mediated through elevated levels of nonfasting remnant cholesterol and LDL cholesterol and through elevated blood pressure, and possibly also through elevated nonfasting glucose levels; however, HDL cholesterol and CRP levels did not seem to be causal mediators.

We and others have previously examined the association between BMI and IHD and the association between BMI and some of the intermediate variables [4,21-23]; however, we now use the novel approach of genetic mediation analyses to quantify how much of the risk of IHD, due to genetically determined obesity, is mediated through the different intermediate variables. Our data contribute important information because IHD is a growing problem worldwide, partly due to increased prevalence of obesity. BMI is a modifiable risk factor, but it can be difficult to obtain sustained weight loss, and thus an alternative approach could be risk reduction of IHD by modifying the intermediate risk factors. Results from this study, integrating results from randomized intervention trials of blood pressure lowering [18], indicate that there may be a potential for reducing IHD risk by reducing levels of remnant cholesterol, LDL cholesterol, and blood pressure in obese individuals unable to achieve sustained weight loss. Thereby, the findings from this study reinforce existing recommendations regarding lowering of LDL cholesterol and blood pressure, and stresses the need for large clinical intervention trials examining whether a lowering of remnant cholesterol in individuals with elevated levels will reduce IHD risk.

Genetically elevated levels of nonfasting remnant and LDL cholesterol and genetically elevated blood pressure explained only 27% of the excess IHD risk from obesity in our study, and most of the risk remained unexplained. This could be because both the lipoprotein metabolism and blood pressure control are complicated processes influenced by many different pathways as well as by the environment, and even though we carefully chose genetic variants associated with the phenotypes, they only explained a small fraction of the overall phenotypes. The observational estimates for levels of nonfasting remnant and
LDL cholesterol and elevated blood pressure explained a larger proportion of the mediated risk with 63% excess IHD risk; however, these estimates can be confounded by other cardiovascular risk factors correlated with the phenotypes and/or influenced by reverse causation.

Mechanistically, the explanation for elevated nonfasting remnant cholesterol and LDL cholesterol causing IHD most likely is that the lipoproteins enter and get trapped in the arterial wall, followed by cholesterol accumulation and development of atherosclerosis. Remnant cholesterol is the cholesterol content of the triglyceride-rich lipoproteins composed of very-low-density lipoproteins and some intermediate-density lipoproteins in the fasting state, and of these two lipoproteins together with chylomicron remnants in the nonfasting state. We have previously found elevated levels of remnant cholesterol to be associated with elevated levels of C-reactive protein in both observational and genetic analyses. This is probably because remnant cholesterol causes low-grade inflammation in the arterial wall after accumulation in the arterial intima and/or elsewhere in the body.

Triglyceride and remnant cholesterol levels are highly correlated. In this study we calculate remnant cholesterol as nonfasting total cholesterol minus LDL and HDL cholesterol. In participants with triglyceride levels below 4mmol/L, LDL cholesterol was calculated from the Friedewald equation and calculated remnant cholesterol is therefore a rescaling of triglycerides; however, in participants with triglyceride levels above 4mmol/L LDL cholesterol was directly measured and the correlation between triglycerides and remnant cholesterol for these participants cannot be explained by this, and is probably explained by the composition of remnant; that is, triglycerides and remnant cholesterol are part of the same molecules, i.e. remnants. However, since most cells can degrade triglycerides, and no cells can degrade cholesterol, it is plausible that it is the cholesterol content of remnants that causes atherosclerosis and IHD development. In the fasting state where there is very little cholesterol in chylomicrons, the Friedewald equation estimates LDL cholesterol as total cholesterol minus HDL and estimated very-low-density cholesterol from triglycerides; however, participants in our study were nonfasting, which means that our estimation of remnant cholesterol as total cholesterol minus LDL and HDL cholesterol also includes cholesterol in chylomicron remnants, and also the part of intermediate-density lipoprotein cholesterol that is not included in the LDL fraction.

We found no genetic association of elevated systolic and diastolic blood pressure with increased risk of IHD; however, since randomized clinical trials have consistently shown that lowering of blood pressure is associated with reduced risk of IHD, lack of genetic association in this study was probably explained by weak instruments. We chose two top-hits from genome-wide association studies, but they only explained a very small fraction of the variation in blood pressure.

A potential limitation to our study is that results were reported for an increase in BMI of 10kg/m² as this corresponds to going from being normal weight to being obese. Another increase in BMI could have been chosen instead; however, this would not have changed the overall interpretation of the results, but only the scaling of results. Another potential limitation in our study is the adjustment for lipid-lowering therapy and antihypertensive therapy as categorical covariates. A more detailed adjustment for different types/doses of lipid-lowering therapy and antihypertensive therapy might have made our estimates more accurate. Also, repeated measures of the phenotypes could have made our observational estimates more accurate, and this is why we also use genetic estimates that are presumably more accurate, since genetic variants are associated with lifelong altered levels of the phenotypes.

An assumption for mediation analysis is lack of interaction between exposure and mediators on the endpoint. We found an interaction between systolic blood pressure and BMI in the observational association, and estimates from the observational mediation analysis for systolic blood pressure should therefore be interpreted with some caution.
Mendelian randomization studies are a way of circumventing confounding and reverse causation seen in observational epidemiology; however, some limitations apply such as canalization, population stratification, linkage disequilibrium, and pleiotrophy. The most important for this study is pleiotropy, which is when the genetic variant used as an instrument for a given risk factor is associated with yet other risk factors. To minimize influence from pleiotropy, we carefully selected genetic instruments and used several genetic variants combined in allele scores for BMI and for the intermediate variables. However, because of the lipoprotein metabolism with many complex interactions, it is difficult to find genetic variants associated with a specific lipoprotein only. By combining several genetic variants for each type of lipoprotein cholesterol, we have minimized pleiotropy on other types of lipoprotein cholesterol, but not eliminated it completely; however, these pleitropic effects on other types of lipoprotein cholesterol were only small, and can probably not explain our results. To avoid population stratification, we only studied white individuals of Danish descent which potentially could affect the generalizability of our results; however, we are not aware of data suggesting that our results should not apply to most races and countries where obesity is prevalent.

In conclusion, we found that the increased risk of IHD due to genetically determined obesity was partly mediated through elevated levels of nonfasting remnant cholesterol and LDL cholesterol, and through elevated blood pressure. This indicates that there may be benefit to gain by reducing levels of these risk factors in obese individuals not able to achieve sustained weight loss. Thereby, the findings from this study reinforce existing recommendations regarding lowering of LDL cholesterol and blood pressure, and stresses the need for large clinical intervention trials examining if lowering of remnant cholesterol in individuals with elevated levels will reduce IHD risk.

ACKNOWLEDGEMENTS
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DISCLOSURES
BGN has received lecture and/or consultancy honoraria from Omthera, Sanofi-Aventis/Regeneron, Aegerion, AstraZeneca, Merck, Pfizer, Fresenius, and ISIS Pharmaceuticals. GDS has received lecture/consultancy honoraria from Merck. AV, MB, NJT, and ATH have no conflicts of interest.
REFERENCES


FIGURE LEGENDS

Figure 1. Observational and genetic associations/mediations investigated in this study. Arrows indicate the associations/mediations studied with number corresponding to the order of analyses in the text, and with P indicating the previously studied associations. Double pointed arrows indicate observational analyses prone to confounding and reverse causation while single pointed arrows indicate genetic analyses not prone to confounding or reverse causation. Full lines indicate that participants from the Copenhagen General Population Study were included in the analyses, broken lines indicate that participants from the Copenhagen General Population Study and the Copenhagen City Heart Study were included in the analyses, and finally, dotted lines indicate that participants from the Copenhagen General Population Study, the Copenhagen City Heart Study, and the Copenhagen Ischemic Heart Disease Study were included in the analyses. BMI=body mass index, C=cholesterol, CRP=C-reactive protein, HDL=high-density lipoprotein, IHD=ischemic heart disease, LDL=low-density lipoprotein.

Figure 2. Observational associations between body mass index and intermediate variables. Levels of the intermediate variables for increasing body mass index categories for 84,888 participants from the Copenhagen General Population Study. Values are median and interquartile range. P-values for trend were estimated by Cuzick’s extension of a Wilcoxon rank-sum test. These analyses represent #1 in Figure 1. BMI=body mass index, HDL=high-density lipoprotein, LDL=low-density lipoprotein.

Figure 3. Genetic associations between body mass index allele score and intermediate variables. Levels of the intermediate variables as a function of body mass index allele score in 65,653 participants from the Copenhagen General Population Study with information on all genetic variants and intermediate variables. Bars indicate mean values with standard error whiskers for lipoproteins, blood pressure, and glucose, and geometric means and standard errors for C-reactive protein. These analyses represent #2 in Figure 1. BMI=body mass index, BP=blood pressure, C=cholesterol, CRP=C-reactive protein, HDL=high-density lipoprotein, LDL=low-density lipoprotein.

Figure 4. Combined observational and genetic associations of a 10kg/m² higher body mass index with levels of the intermediate variables. Observational and genetic associations were estimated in 65,653 participants from the Copenhagen General Population Study with information on all genetic variants and intermediate variables. F-statistics=59 and R²=0.5%. These analyses represent #3 in Figure 1. BMI=body mass index, BP=blood pressure, C=cholesterol, CRP=C-reactive protein, HDL=high-density lipoprotein, LDL=low-density lipoprotein.

Figure 5. Observational and genetically determined change in the intermediate variable, associated with a 10kg/m² higher body mass index, translated into risk of ischemic heart disease. Observational hazard ratios were estimated in 85,592 participants from the Copenhagen General Population Study and the Copenhagen City Heart Study combined. Genetically derived risk ratios were estimated in participants from the Copenhagen General Population Study, the Copenhagen City Heart Study, and the Copenhagen Ischemic Heart Disease Study combined with number of participants varying according to availability of genotypes. Hazard ratios and genetically derived risk ratios are for the corresponding increase/decrease of the intermediate variable associated with a 10kg/m² higher observational or genetically determined body mass index found in Figure 4. These analyses represent #4a and #4b in Figure 1. BMI=body mass index, C=cholesterol, CI=confidence interval, HDL=high-density lipoprotein, IHD=ischemic heart disease, LDL=low-density lipoprotein.

Figure 6. Observational and genetic mediation analyses. Percent excess risk of ischemic heart disease from observational and genetically determined obesity mediated by the intermediate variables was estimated in 70,743 participants from the Copenhagen General Population Study and the Copenhagen City Heart Study combined, using the product of coefficients method. Genetic estimates are shown for the
intermediate variables with evidence suggesting genetically derived risk of IHD in Figure 5 and/or evidence from randomized clinical intervention trials suggesting causality. P-values are for significance of the mediated effect (a*b) or for comparison of observational and genetic estimates. These analyses represent #5a and #5b in Figure 1. HDL=high-density lipoprotein, LDL=low-density lipoprotein.
Table 1. Characteristics of participants in the three studies.

<table>
<thead>
<tr>
<th></th>
<th>Copenhagen City Heart Study</th>
<th>Copenhagen General Population Study</th>
<th>Copenhagen Ischemic Heart Disease Study</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
<td>10,099</td>
<td>69,535</td>
<td>5,050</td>
</tr>
<tr>
<td>Women</td>
<td>5,601 (55%)</td>
<td>38,468 (55%)</td>
<td>1,486 (71%)</td>
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<tr>
<td>Age (years)</td>
<td>58 (43-69)</td>
<td>58 (48-67)</td>
<td>64 (66-71)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25 (22-28)</td>
<td>26 (23-28)</td>
<td>27 (24-29)</td>
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<tr>
<td>Remnant cholesterol (mmol/L)</td>
<td>0.7 (0.5-1.0)</td>
<td>0.6 (0.4-0.9)</td>
<td>N.A.</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.5 (1.2-1.8)</td>
<td>1.6 (1.2-1.9)</td>
<td>N.A.</td>
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<td>LDL cholesterol (mmol/L)</td>
<td>3.6 (2.9-4.4)</td>
<td>3.2 (2.6-3.9)</td>
<td>N.A.</td>
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<td>Systolic blood pressure (mmHg)</td>
<td>135 (121-151)</td>
<td>140 (125-154)</td>
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<td>Diastolic blood pressure (mmHg)</td>
<td>83 (75-91)</td>
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<td>Glucose (mmol/L)</td>
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<td>5.1 (4.7-5.7)</td>
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<td>C-reactive protein (mg/L)</td>
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<td>Diabetes mellitus</td>
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<td>39,695 (60%)</td>
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<td>4,716 (47%)</td>
<td>13,799 (20%)</td>
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<td>3,736 (67%)</td>
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<td>Lipid-lowering therapy</td>
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<td>1,136 (11%)</td>
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<td>Ischemic heart disease</td>
<td>2,503 (23%)</td>
<td>6,592 (9%)</td>
<td>5,050 (100%)</td>
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</tbody>
</table>

Data are from the 1991-94 or 2001-2003 examinations of the Copenhagen City Heart Study when DNA was collected, from study enrolment in 2003-2013 for the Copenhagen General Population Study and from study enrolment in 1991-2011 in the Copenhagen Ischemic Heart Disease Study. Values are median and interquartile range or number of participants and percentages. LDL= low-density lipoprotein, HDL= high-density lipoprotein, N.A.= Not available. Number of participants vary slightly according to availability of variables.
Novelty and Significance

What Is Known?

- Obesity is an increasing problem in many countries, and it is difficult to encourage individuals to lose weight, and even more difficult to maintain weight loss.

- Obesity leads to increased risk of ischemic heart disease (IHD).

- However, it may not be obesity per se that causes IHD, but rather changes in mediating factors like levels of lipoproteins, blood pressure, glucose levels, and low-grade inflammation caused by obesity.

What New Information Does This Article Contribute?

- In this study we combine observational and genetic evidence from ~90,000 individuals from the Danish general population to delineate which mediating factors contribute causally to the increased risk of IHD from obesity.

Obesity is a growing problem and even though elevated body mass index is a modifiable risk factor, it is very challenging to achieve persistent weight loss in obese individuals. It is therefore important to delineate which intermediate factors from obesity to IHD that are causal, to be treated with benefit. We used the novel approach of combining observational analyses, Mendelian randomization, and observational and genetic mediation analyses to circumvent confounding and reverse causation, both of which are sources of bias in conventional observational epidemiology. The top three intermediate factors in this study that mediated the increased risk of IHD from obesity were elevated levels of low-density lipoprotein cholesterol, elevated levels of remnant cholesterol, and elevated blood pressure. Current guidelines on IHD prevention includes recommendations for treatment of elevated levels of low-density lipoprotein cholesterol and elevated blood pressure; however, there is no consensus on whether elevated levels of remnant cholesterol should be treated, mainly because of a lack of evidence from randomized clinical intervention trials. Results from this study, combined with previous studies, highlights the need for randomized clinical intervention trials aimed at reducing levels of remnant cholesterol in individuals with elevated levels.
Increased BMI Mediators
Remnant-C, HDL-C, LDL-C, systolic and diastolic blood pressure, glucose, and CRP

Mediator genetic variants

BMI genetic variants

IHD

Pa

Pb

Figure 1
Figure 3

BMI (kg/m²)

- Remnant-C (mmol/L)
  - P=0.001

- HDL-C (mmol/L)
  - P=0.001

- LDL-C (mmol/L)
  - P=0.2

- Systolic BP (mmHg)
  - P=0.004

- Diastolic BP (mmHg)
  - P=0.1

- Glucose (mmol/L)
  - P=0.4

- CRP (mg/L)
  - P<0.001

BMI increasing allele score (FTO, MC4R, TMEM18, BDNF, GNPDA2)
**Figure 4**

**Lipoprotein change for a BMI increase of 10kg/m²**

**Remnant-C**
- Observational: 0.35(0.34-0.36)
- Genetic: 0.19(0.08-0.31)

**HDL-C**
- Observational: -0.40(-0.41- -0.39)
- Genetic: -0.26(-0.39- -0.12)

**LDL-C**
- Observational: 0.28(0.26-0.30)
- Genetic: 0.27(0.02-0.53)

**Blood pressure change for a BMI increase of 10kg/m²**

**Systolic BP**
- Observational: 12.23(11.86-12.61)
- Genetic: 10.43(4.82-16.04)

**Diastolic BP**
- Observational: 6.84(6.63-7.04)
- Genetic: 2.97(-0.08-6.03)

**Glucose change for a BMI increase of 10kg/m²**

- Observational: 0.34(0.32-0.36)
- Genetic: 0.39(0.06-0.72)

**CRP change for a BMI increase of 10kg/m²**

- Observational: 103.97(100.79-107.20)
- Genetic: 109.99(66.09-165.49)
Figure 5

Risk of IHD by intermediary parameter for a BMI increase of 10 kg/m²

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N total</th>
<th>N cases</th>
<th>R²</th>
<th>F</th>
<th>Risk estimate (95% CI)</th>
<th>P for comparison</th>
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<td><strong>Remnant-C</strong></td>
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<tr>
<td>Observational</td>
<td>85,592</td>
<td>4,599</td>
<td></td>
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<tr>
<td>Genetic (TRIB, GCKR, APOA5)</td>
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<tr>
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<tr>
<td><strong>HDL-C</strong></td>
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<tr>
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<tr>
<td>Observational</td>
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<td>4,599</td>
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<tr>
<td>Genetic (APOB, LDLR, PCSK9)</td>
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<td>12,668</td>
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<td><strong>Systolic blood pressure</strong></td>
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<tr>
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<td>4,599</td>
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<tr>
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<td>4,599</td>
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<td><strong>Glucose</strong></td>
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<tr>
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<td>Genetic (GCK, G6PC2, ADCY5, DGKB, ADRA2A)</td>
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<td><strong>C-reactive protein</strong></td>
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<tr>
<td>Observational</td>
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<td>4,599</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Genetic (CRP)</td>
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<td>12,455</td>
<td>1.1</td>
<td>84</td>
<td>0.84(0.76-0.94)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Hazard ratio / genetically derived risk ratio for IHD
Figure 6

**Observational**

- **Remnant cholesterol**: 20% (P-value <0.001)
- **HDL cholesterol**: 22% (P-value <0.001)
- **LDL cholesterol**: 21% (P-value <0.001)
- **Systolic blood pressure**: 11% (P-value <0.001)
- **Diastolic blood pressure**: 11% (P-value <0.001)
- **Glucose**: 6% (P-value <0.001)
- **C-reactive protein**: 7% (P-value <0.001)

**Genetic**

- **Remnant cholesterol**: Not causal (P-value <0.001)
- **HDL cholesterol**: 8% (P-value 0.03, P-value for comparison 0.009)
- **LDL cholesterol**: Not causal (P-value <0.001)
- **Systolic blood pressure**: 7% (P-value <0.001, P-value for comparison 0.2)
- **Diastolic blood pressure**: 5% (P-value 0.005, P-value for comparison 0.04)
- **Glucose**: 4% (P-value 0.04, P-value for comparison 0.2)
- **C-reactive protein**: Not causal (P-value <0.001)
Remnant Cholesterol, Low-Density Lipoprotein Cholesterol, and Blood Pressure as Mediators from Obesity to Ischemic Heart Disease

Anette Varbo, Marianne Benn, George Davey Smith, Nicholas J Timpson, Anne Tybjaerg-Hansen and Børge G Nordestgaard

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Supplemental material

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Anette Varbo, Marianne Benn, George Davey Smith, Nicholas J. Timpson, Anne Tybjærg-Hansen, and Børge G. Nordestgaard.

3 Online Tables and 9 Online Figures
Detailed methods

Genotyping by restriction enzyme assays

Participants from the 1991-94 examination of the Copenhagen City Heart Study (CCHS) were genotyped for genetic variants in \( LIPC \) and \( APOB \) by restriction enzyme assays. Please see Online Table I for information on assays.

Statistical analysis

We used Stata/S.E.12.0. Non-normally distributed variables were log-transformed to approach normal distribution. Chi-square tests evaluated Hardy-Weinberg equilibrium. Participants with a body mass index (BMI) <18.5 or >50 kg/m\(^2\) were excluded to approach linearity (Online Figure IX) from all analyses (except for the observational association between BMI and mediators shown in Figure 2).

Observational associations: BMI and intermediate variables (Figure 1, #1).

In Figure 2, levels of intermediate variables are median and interquartile range for increasing BMI categories for 84,888 participants from the Copenhagen General Population Study (CGPS) with information on BMI and all intermediate variables. P-values for trend were by Cuzick’s extension of a Wilcoxon rank-sum test.

Genetic associations: BMI allele score and intermediate variables (Figure 1, #2).

In Figure 3, trend of level of the intermediate variables across BMI allele score were estimated by Cuzick’s extension of a Wilcoxon rank-sum test in 65,653 participants from the CGPS with information on BMI, intermediate variables, and BMI allele score. C-reactive protein (CRP) levels were log-transformed to approach a normal distribution, and effect sizes in Figure 3 therefore
indicate mean and standard errors for lipoproteins, blood pressure, and glucose, and geometric means and standard errors for CRP.

**Combined observational and genetic associations: BMI and intermediate variables (Figure 1, #3).** In Figure 4, observational associations of a 10kg/m\(^2\) higher BMI with level of the intermediate variables were estimated using linear regression in 65,653 participants from the CGPS. Genetic associations were estimated in the same participants by two-stage least squares regression in an additive model\(^3\) using the BMI allele score. Strength of the instrument was evaluated by F-statistics from the first-stage regression where an F-statistic >10 has been used to indicate sufficient instrument strength\(^4\). R\(^2\) indicates the variation in BMI contributed by the BMI allele score. P for comparison were estimated using the method of Bland and Altman\(^5\).

**Observational and genetically determined changes in intermediate variables, associated with a 10kg/m\(^2\) higher BMI, translated into risk of ischemic heart disease (IHD) (Figure 1, #4a and #4b).** Hazard ratios and genetically derived risk ratios in Figure 5 are for the corresponding increase/decrease of the intermediate variable associated with a 10kg/m\(^2\) higher observational or genetically determined BMI found in Figure 4. Levels of triglycerides, remnant cholesterol, and low-density lipoprotein (LDL) cholesterol in participants from the CGPS and CCHS using lipid-lowering therapy were multiplied by 1.33, 1.33, and 1.43 respectively, corresponding to average reductions of 25%, 25%, and 30% using common statin treatment regiments, as done previously\(^6\). 10mmHg was added to systolic blood pressure and 5mmHg to diastolic blood pressure in participants from the CGPS and CCHS using antihypertensive medication, as done previously\(^7\).

Observational hazard ratios for IHD were estimated using Cox proportional hazards regression models with age as time scale and with the use of left truncation and delayed entry in
85,592 participants with information on all intermediate variables and IHD from the CGPS and CCHS combined. Participants diagnosed with an endpoint before study entry were excluded from Cox regression analyses, and those dying during follow-up were censored at their death date. Multivariable adjustment was for age, sex, and smoking. Median follow-up time was 6 years (range: 0-22 years).

Genetically derived risk ratios for IHD were estimated by the multiplicative generalised methods of moments estimator in participants from the CGPS, CCHS, and the Copenhagen Ischemic Heart Disease Study (CIHDS) combined, and with genotypes included as allele scores or genotype combinations. F-statistics and $R^2$ were estimated from first-stage of a two-stage least squares regression. For participants from the CIHDS where existing disease could influence the risk factors (reverse causation), levels of intermediate variables entered into instrumental variable analyses were derived from the known association from a first stage regression between the different intermediate variable allele scores/genotype combinations with the intermediate variables in the CGPS. The number of participants in instrumental variable analyses varies corresponding to availability of the different genotypes.

$P$ for comparison were estimated using the method of Bland and Altman.

*Observational and genetic mediation analyses (Figure 1, #5a and #5b).*

In Figure 6, genetic mediation analyses were carried out using the product of coefficients method. The excess risk mediated is the proportion of the indirect effect, also called the mediated effect, to the total effect of the BMI allele score on IHD risk:

$$\text{The excess risk mediated} = \frac{(a*b)}{(c´ + (a*b))}$$

with regression coefficients $a$, $b$, and $c´$ derived from a) the association between the BMI allele score and the intermediate variable estimated by linear regression (Figure 1, #2), b) the association
between the intermediate variable and risk of IHD, adjusted for the BMI allele score, estimated by logistic regression (Figure 1, #4a), and c’) the association between the BMI allele score and IHD, adjusted for the intermediate variable, estimated by logistic regression (Figure 1, Pb). All regressions were adjusted for age, sex, and smoking.

The observational mediation analyses were done in exact the same way except for using conventional BMI instead of the BMI allele score with regression coefficients a, b, and c’ derived from a) the association between conventional BMI and the intermediate variable estimated by linear regression (Figure 1, #1), b) the association between the intermediate variable and risk of IHD, adjusted for conventional BMI, estimated by logistic regression (Figure 1, #4b), and c’) the association between conventional BMI and IHD, adjusted for the intermediate variable, estimated by logistic regression(Figure 1, Pa). All regressions were adjusted for age, sex, and smoking.

Observational and genetic mediation analyses were done in 70,743 participants from the CGPS and CCHS combined, with information on BMI, BMI allele score, intermediate variables, and IHD status. P-values are for significance of the mediated effect a*b. P for comparison were estimated using the method of Bland and Altman.

In sensitivity analyses, observational and genetic mediation analyses were also conducted using the percent excess risk mediated (PERM) method (Online Figure VIII).
Supplemental references


Online Table I. Restriction enzyme assay information.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genetic variant</th>
<th>Primers</th>
<th>Restriction enzyme</th>
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</thead>
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<td><em>LIPC</em></td>
<td><em>LIPC</em> -480C/T</td>
<td>5′TCCTGGCCAGAAATCTCTCTCT′3</td>
<td>NlaIII</td>
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<td>5′GACTTGTGTCCATTTCTCCGT′3</td>
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<td><em>APOB</em>-3500 rs5742904</td>
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<td>MspI</td>
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<td>5′GGGTGGCTTTGCTTGATG′3</td>
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<td></td>
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<td>5′TGCAGCTTCACCTGACACT′3</td>
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Online Table II. Characteristics of participants in the Copenhagen General Population Study by genotypes.

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<tr>
<th>Genes/Alleles</th>
<th>No.</th>
<th>Age years</th>
<th>Women %</th>
<th>Diabetes mellitus %</th>
<th>Menopause, women only %</th>
<th>Hypertension %</th>
<th>Anti-hypertensive medication %</th>
<th>Smoking %</th>
<th>Lipid-lowering therapy %</th>
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<tr>
<td><strong>BMI allelescore</strong></td>
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</table>

Remnant-C allelescore: P-trend <0.001
HDL-C allelescore: P-trend <0.001
LDL-C allelescore: P-trend <0.001
### Blood pressure allelescore

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>N</th>
<th>BMI</th>
<th>C</th>
<th>HDL</th>
<th>LDL</th>
<th>P-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP2B1</td>
<td>0-1</td>
<td>3,277</td>
<td>58(47-67)</td>
<td>55</td>
<td>4</td>
<td>66</td>
<td>58</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>2</td>
<td>14,869</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>65</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>27,274</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>66</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>P-trend</td>
<td></td>
<td></td>
<td>0.8</td>
<td>0.5</td>
<td>1.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

### Glucose allelescore

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>N</th>
<th>BMI</th>
<th>C</th>
<th>HDL</th>
<th>LDL</th>
<th>P-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCK</td>
<td>0-3</td>
<td>1,366</td>
<td>57(47-66)</td>
<td>57</td>
<td>3</td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td>G6PC2</td>
<td>4</td>
<td>4,355</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td>ADCY5</td>
<td>5</td>
<td>9,853</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td>DGKB</td>
<td>6</td>
<td>13,438</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td>ADRA2A</td>
<td>7</td>
<td>10,729</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td>8-10</td>
<td>5,679</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>66</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>P-trend</td>
<td></td>
<td>0.5</td>
<td>0.6</td>
<td>0.07</td>
<td>0.3</td>
<td>1.0</td>
</tr>
</tbody>
</table>

### C-reactive protein allele combination

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>N</th>
<th>BMI</th>
<th>C</th>
<th>HDL</th>
<th>LDL</th>
<th>P-trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td>1</td>
<td>5,160</td>
<td>57(47-67)</td>
<td>56</td>
<td>4</td>
<td>64</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9,253</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>66</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4,096</td>
<td>57(47-67)</td>
<td>55</td>
<td>3</td>
<td>64</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9,442</td>
<td>58(47-67)</td>
<td>55</td>
<td>4</td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8,657</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1,502</td>
<td>57(47-67)</td>
<td>55</td>
<td>4</td>
<td>65</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4,595</td>
<td>58(47-67)</td>
<td>55</td>
<td>4</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1,338</td>
<td>57(47-67)</td>
<td>54</td>
<td>3</td>
<td>63</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1,377</td>
<td>58(47-68)</td>
<td>55</td>
<td>3</td>
<td>66</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>P-trend</td>
<td></td>
<td>0.3</td>
<td>0.2</td>
<td>0.03</td>
<td>0.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Continuous values are median and interquartile range and categorical values are summarized in percent. P-values for trend are by Cuzick’s extension of a Wilcoxon rank-sum test. BMI=body mass index, C=cholesterol, HDL=high-density lipoprotein, LDL=low-density lipoprotein
Online Table III. P-values for interaction between the intermediate variables and body mass index or body mass index allele score on predicting risk of ischemic heart disease.

<table>
<thead>
<tr>
<th>Intermediate variable</th>
<th>Observational body mass index</th>
<th>Body mass index allele score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-value for interaction</td>
<td>P-value for interaction</td>
</tr>
<tr>
<td>Remnant cholesterol</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.03</td>
<td>0.4</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>$4 \times 10^{-8}$</td>
<td>$6 \times 10^{-7}$</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>0.005</td>
<td>0.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>0.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

BP= blood pressure, HDL=high-density lipoprotein, LDL= low-density lipoprotein.
Online Figure I. Observational and genetic associations of body mass index with risk of ischemic heart disease. Observational hazard ratios (left panel) for ischemic heart disease by body mass index quintiles were estimated using Cox proportional hazards regression models with age as timescale adjusted for age, sex, and smoking in 90,175 participants from the Copenhagen General Population Study and the Copenhagen City Heart Study combined with information on body mass index, covariates, and ischemic heart disease status. Participants with an event before study entry were excluded. Mean follow-up time was 6 years (range 0-22 years). Genetic odds ratios (right panel) for ischemic heart disease by increasing number of body mass index increasing alleles were estimated in 85,176 participants from the Copenhagen General Population Study, the Copenhagen City Heart Study, and the Copenhagen Ischemic Heart Disease Study combined using logistic regression. P-values for trend were estimated by Cuzick’s extension of a Wilcoxon rank-sum test. BMI=body mass index, CI=confidence interval.
Remnant-C (mmol/L) 0.74 0.76 0.78
Glucose (mmol/L) 5.28 5.32 5.36
CRP (mg/L) 1.4 1.5 1.6 1.7
HDL-C (mmol/L) 1.58 1.62
LDL-C (mmol/L) 3.2 3.3 3.4
Systolic BP (mmHg) 140 141 142
Diastolic BP (mmHg) 83.5 84.0 84.5
BMI (kg/m²) 26 27
LDL-C (mmol/L) 3.2 3.3 3.4
Systolic BP (mmHg) 140 141 142
Diastolic BP (mmHg) 83.5 84.0 84.5
Glucose (mmol/L) 5.28 5.32 5.36
CRP (mg/L) 1.4 1.6 1.7

Alleles | N   |
---------|-----|
0        | 23,585 |
1        | 51,202 |
2        | 10,566 |
0        | 37,125 |
1        | 24,475 |
2        | 4,053  |
0        | 1,832  |
1        | 18,563 |
2        | 23,067 |
0        | 31,568 |
1        | 39,100 |
2        | 10,802 |

FTO rs9939609   MC4R rs17782313   TMEM18 rs6548238   BDNF rs10767664   GNPDA2 rs10938397
Online Figure II. Levels of the intermediate variables as a function of FTO, MC4R, TMEM18, BDNF, and GNPDA2 genotypes. Bars indicate mean values with standard error whiskers for lipoproteins, blood pressure, and glucose, and geometric means and standard errors for C-reactive protein in 65,653 participants from the Copenhagen General Population Study with information on all genotypic variants and intermediate variables. P-values for trend were estimated by Cuzick’s extension of a Wilcoxon rank-sum test. BMI=body mass index, BP=blood pressure, C=cholesterol, CRP=C-reactive protein, HDL=high-density lipoprotein, LDL=low-density lipoprotein.
Participants with BMI < 30 kg/m²

Lipoprotein change for a BMI increase of 10kg/m²

- **Remnant-C**
  - Observational: 0.48(0.47-0.49)
  - Genetic (FTO, MC4R, TMEM18, BDNF, GNPDA2): 0.19(-0.08-0.46)

- **HDL-C**
  - Observational: -0.56(-0.57- -0.54)
  - Genetic (FTO, MC4R, TMEM18, BDNF, GNPDA2): -0.26(-0.59-0.07)

- **LDL-C**
  - Observational: 0.56(0.53-0.58)
  - Genetic (FTO, MC4R, TMEM18, BDNF, GNPDA2): 0.41(-0.21-1.03)

Blood pressure change for a BMI increase of 10kg/m²

- **Systolic BP**
  - Observational: 16.55(15.91-17.19)
  - Genetic (FTO, MC4R, TMEM18, BDNF, GNPDA2): 10.97(-2.67-24.60)

- **Diastolic BP**
  - Observational: 9.25(8.91-9.59)
  - Genetic (FTO, MC4R, TMEM18, BDNF, GNPDA2): -2.01(-9.52-5.51)

Glucose change for a BMI increase of 10kg/m²

- Observational: 0.28(0.25-0.32)
- Genetic (FTO, MC4R, TMEM18, BDNF, GNPDA2): 0.50(-0.24-1.23)

CRP change for a BMI increase of 10kg/m²

- Observational: 103.50(98.05-109.10)
- Genetic (FTO, MC4R, TMEM18, BDNF, GNPDA2): 153.98(42.24-353.49)
Online Figure III. Combined observational and genetic associations of a 10kg/m² higher body mass index with levels of the intermediate variables in participants with BMI<30kg/m². Observational and genetic associations were estimated in 54,885 participants from the Copenhagen General Population Study with a BMI<30kg/m². F-statistics=24 and R²=0.2%. BMI=body mass index, BP=blood pressure, C=cholesterol, CRP=C-reactive protein, HDL=high-density lipoprotein, LDL=low-density lipoprotein.
Participants with BMI > / = 30 kg/m²

Lipoprotein change for a BMI increase of 10kg/m²

**Remnant-C**
- Observational
- Genetic
  - (FTO, MC4R, TMEM18, BDNF, GNPDA2)
  - Change: 0.08 (0.05-0.11)
  - P for comparison: 0.1

**HDL-C**
- Observational
- Genetic
  - (FTO, MC4R, TMEM18, BDNF, GNPDA2)
  - Change: -0.34 (-1.06-0.39)
  - P for comparison: 0.1

**LDL-C**
- Observational
- Genetic
  - (FTO, MC4R, TMEM18, BDNF, GNPDA2)
  - Change: -0.07 (-0.13- -0.02)
  - P for comparison: 0.3

Blood pressure change for a BMI increase of 10kg/m²

**Systolic BP**
- Observational
- Genetic
  - (FTO, MC4R, TMEM18, BDNF, GNPDA2)
  - Change: 5.66 (4.46-6.85)
  - P for comparison: 0.1

**Diastolic BP**
- Observational
- Genetic
  - (FTO, MC4R, TMEM18, BDNF, GNPDA2)
  - Change: 2.37 (1.69-3.05)
  - P for comparison: 0.1

Glucose change for a BMI increase of 10kg/m²

- Observational
- Genetic
  - (FTO, MC4R, TMEM18, BDNF, GNPDA2)
  - Change: 0.35 (0.25-0.44)
  - P for comparison: 0.3

CRP change for a BMI increase of 10kg/m²

- Observational
- Genetic
  - (FTO, MC4R, TMEM18, BDNF, GNPDA2)
  - Change: 92.21 (83.48-101.35)
  - P for comparison: 0.3
Online Figure IV. Combined observational and genetic associations of a 10kg/m$^2$ higher body mass index with levels of the intermediate variables in participants with BMI≥30kg/m$^2$. Observational and genetic associations were estimated in 10,770 participants from the Copenhagen General Population Study with a BMI≥30kg/m$^2$. F-statistics=4 and R$^2=0.2\%$. BMI=body mass index, BP=blood pressure, C=cholesterol, CRP=C-reactive protein, HDL=high-density lipoprotein, LDL=low-density lipoprotein.
Online Figure V part 1
Online Figure V
part 2
Online Figure V. Levels of the intermediate variables as a function of allele scores and genotype combinations for the intermediate variables. Bars indicate mean values with standard error whiskers for lipoproteins, blood pressure, and glucose, and geometric means and standard errors for C-reactive protein in 45,420 participants from the Copenhagen General Population Study with information on body mass index, all intermediate variables, and all genotypic variants. Red color indicates the intermediate variable that the allele score is used as an instrument for, and grey color indicates the other intermediate variables that the allele score ideally should not be associated with. P-values for trend were estimated by Cuzick’s extension of a Wilcoxon rank-sum test. BMI=body mass index, BP=blood pressure, C=cholesterol, CRP=C-reactive protein, HDL=high-density lipoprotein, LDL=low-density lipoprotein.
Online Figure VI. Observational associations of the intermediate variables with risk of ischemic heart disease. Intermediate variables are in quintiles in 86,748 participants from the Copenhagen General Population Study and the Copenhagen City Heart Study combined. Hazard ratios were estimated using Cox proportional hazards regression models with age as timescale adjusted for age, sex, and smoking. Participants with an event before study entry were excluded. Mean follow-up time was 6 years (range 0-22 years). P-values for trend were estimated by Cuzick’s extension of a Wilcoxon rank-sum test. BP=blood pressure, CI=confidence interval, CRP=C-reactive protein, IHD=ischemic heart disease.
Online Figure VII. Associations of the allele scores or genotype combination for the intermediate variables with risk of ischemic heart disease. Odds ratios were estimated in 61,601 participants from the Copenhagen General Population Study, the Copenhagen City Heart Study, and the Copenhagen Ischemic Heart Disease Study combined, with information on all genotypic variants and ischemic heart disease status using logistic regression. P-values for trend were estimated by Cuzick’s extension of a Wilcoxon rank-sum test. C=cholesterol, CRP=C-reactive protein, HDL=high-density lipoprotein, LDL=low-density lipoprotein.
Online Figure VIII. Observational and genetic mediation analyses. Percent excess risk of ischemic heart disease was estimated in 70,743 participants from the Copenhagen General Population Study and the Copenhagen City Heart Study combined using the percent excess risk mediated (PERM) method. Body mass index, body mass index allele score, and intermediate variables were standardized as Z-scores before logistic regression. Observational percent excess risk of ischemic heart disease was estimated as: (the odds ratio of ischemic heart disease for a 1 Z-score higher body mass index adjusted multivariably) minus (the odds ratio of ischemic heart disease for a 1 Z-score higher body mass index adjusted multivariably with additional adjustment for the intermediate variable), divided with (the odds ratio of ischemic heart disease for a 1 Z-score higher body mass index adjusted multivariably) minus 1. Genetic excess risk was estimated in the same way except for using the odds ratio for ischemic heart disease for a 1 Z-score higher genetic body mass index (using the body mass index allele score) instead of observational body mass index. Participants with an event before study entry were excluded to avoid reverse causation. HDL=high-density lipoprotein, LDL=low-density lipoprotein.
Online Figure IX. Observational associations between body mass index and the intermediate variables. Dots are mean of intermediate variable as a function of body mass index in steps of 1 kg/m² estimated in 84,888 participants from the Copenhagen General Population Study with information on body mass index and all intermediate variables. BP=blood pressure, C=cholesterol, HDL=high-density lipoprotein, LDL=low-density lipoprotein.