Genetically elevated non-fasting triglycerides and calculated remnant cholesterol as causal risk factors for myocardial infarction

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Aims
Elevated non-fasting triglycerides mark elevated levels of remnant cholesterol. Using a Mendelian randomization approach, we tested whether genetically increased remnant cholesterol in hypertriglyceridaemia due to genetic variation in the apolipoprotein A5 gene (APOA5) associates with an increased risk of myocardial infarction (MI).

Methods and results
We resequenced the core promoter and coding regions of APOA5 in individuals with the lowest 1% (n = 95) and highest 2% (n = 190) triglyceride levels in the Copenhagen City Heart Study (CCHS, n = 10 391). Genetic variants which differed in frequency between the two extreme triglyceride groups (c.-1131T>C, S19W, and c.*31C>T; P-value: 0.06 to <0.001), thus suggesting an effect on triglyceride levels, were genotyped in the Copenhagen General Population Study (CGPS), the CCHS, and the Copenhagen Ischemic Heart Disease Study (CIHDS), comprising a total of 5705 MI cases and 54 408 controls. Genotype combinations of these common variants associated with increases in non-fasting triglycerides and calculated remnant cholesterol of, respectively, up to 68% (1.10 mmol/L) and 56% (0.40 mmol/L) (P, 0.001), and with a corresponding odds ratio for MI of 1.87 (95% confidence interval: 1.25–2.81). Using APOA5 genotypes in instrumental variable analysis, the observational hazard ratio for a doubling in non-fasting triglycerides was 1.57 (1.32–2.68) compared with a causal genetic odds ratio of 1.94 (1.40–1.85) (P for comparison = 0.28). For calculated remnant cholesterol, the corresponding values were 1.67 (1.38–2.02) observational and 2.23 (1.48–3.35) causal (P for comparison = 0.21).

Conclusion
These data are consistent with a causal association between elevated levels of remnant cholesterol in hypertriglyceridaemia and an increased risk of MI. Limitations include that remnants were not measured directly, and that APOA5 genetic variants may influence other lipoprotein parameters.

Keywords
Genetics • Lipoproteins • Triglycerides • Remnant cholesterol • Myocardial infarction

Introduction
Elevated non-fasting plasma triglyceride is a marker of elevated remnant cholesterol,1–3 and associated with an increased risk of ischaemic cardiovascular disease.1–7 Remnant cholesterol is the cholesterol content of triglyceride-rich remnant lipoproteins, which in the fasting state comprise very low-density lipoproteins (VLDLs) and intermediate-density lipoproteins (IDLs), and these two lipoproteins together with chylomicron remnants in the non-fasting state. Remnant lipoproteins share with low-density lipoproteins (LDLs) the potential to infiltrate the arterial intima, and thus to accumulate and cause atherosclerosis due to their cholesterol...
content, whereas triglycerides per se are unlikely to cause atherosclerosis.\(^4\)

Apolipoprotein A-V (apoA-V) is located on the surface of triglyceride-rich lipoproteins, where it modulates activation of lipoprotein lipase (LPL)\(^11,12\) and increases liver uptake of remnant particles.\(^13\) Studies of ApoA5 knockout mice show a 400% increase in triglyceride levels,\(^14\) and genetic deficiency of apoA-V in humans is associated with hypertriglyceridaemia.\(^14–18\) Furthermore, genome-wide association studies have consistently shown that polymorphisms in or near the apolipoprotein A5 gene (APOA5), located in the APOA1/APOC3/APOA4 gene cluster, are among the strongest genetic determinants of plasma triglyceride levels.\(^19,20\) Resequencing APOA5 to identify variants with an effect on triglyceride levels therefore provides an excellent instrument to explore causality between elevated non-fasting triglycerides, remnant cholesterol, and risk of myocardial infarction (MI), even if the exact role of APOA5 genetic variants in the regulation of plasma triglycerides and remnant cholesterol is not known.\(^21\)

Using a Mendelian randomization approach, we tested the following hypotheses: (i) genetic variants in APOA5 affect levels of non-fasting triglycerides and remnant cholesterol in the general population; (ii) genetic variants in APOA5 associate with risk of MI to the extent predicted by their effects on plasma levels of non-fasting triglyceride and remnant cholesterol; and (iii) remnant cholesterol is causally associated with risk of MI, using instrumental variable analysis. A Mendelian randomization approach circumvents most confounding and reverse causation,\(^21\) but may have limitations such as a need for huge statistical power, and for genetic instruments without major pleiotropic effects. This may pose a particular challenge when studying triglycerides and remnant cholesterol because of the inverse association with high-density lipoprotein (HDL) cholesterol.\(^22\)

These hypotheses were tested in white individuals of Danish descent from Copenhagen, including 5705 MI cases and 54 408 controls. Importantly, on purpose we did not measure chylomicron remnants or VLDL plus IDL directly, because we wanted a simple accessible estimate of remnant cholesterol, here defined as all cholesterol in non-fasting plasma not present in LDL or HDL. Thus, the presently used remnant cholesterol can be calculated, without any additional cost by any doctor, from a standard lipid profile, provided plasma is drawn in the non-fasting state.

Methods

Subjects

Studies were approved by institutional review boards and Danish Ethics Committees and conducted according to the Declaration of Helsinki. Informed consent was obtained from participants. All participants were white and of Danish descent. No participant was included in more than one study. In all studies, follow-up was 100% complete, that is, no individual was lost to follow-up.

The Copenhagen General Population Study

This is a prospective study of the general population initiated in 2003 with ongoing enrolment.\(^1\) Individuals were selected based on the National Danish Civil Registration System to reflect the adult Danish population aged 20–100 years. Data were obtained from a questionnaire, a physical examination, and from blood samples, including DNA extraction. At the time of analyses, 50 628 had been included. Of these, 11 216 were used as controls in the Copenhagen Ischemic Heart Disease Study (CIHDS) (see below) leaving 39 412 participants. An additional 2559 participants with ischaemic heart disease (IHD), but without MI were excluded from risk analyses, leaving 36 853 participants. Of these, 1803 had MI.

The Copenhagen City Heart Study

This is a prospective study of the general population initiated in 1976–78 with follow-up examinations in 1981–83, 1991–94, and 2001–03. Participants were recruited and examined exactly as in the Copenhagen General Population Study (CGPS). Blood samples for DNA extraction were drawn at the 1991–94 and 2001–03 examinations. A total of 10 391 participants were included. Of these, 1151 with IHD but without MI were excluded from risk analyses, leaving 9240 participants. Of these, 1098 had MI.

The Copenhagen Ischemic Heart Disease Study

This study comprises 2804 patients with an MI referred for coronary angiography in a stable phase unrelated to MI to Copenhagen University Hospital during the period 1991–2009, and 11 216 controls without IHD matched by age and gender from the CGPS. Besides a diagnosis of MI as described below, the cases also had stenosis/atherosclerosis on coronary angiography and/or a positive exercise electrocardiography test.

Myocardial infarction and ischaemic heart disease

In all three studies, diagnoses of MI (WHO International Classification of Diseases; ICD8:410, ICD10:121; I22) and IHD (ICD8:410-414; ICD10:120-125) were collected from 1976 to June 2011 and verified by reviewing all hospital admissions and diagnoses entered in the National Danish Patient Registry, all causes of death entered in the National Danish Causes of Death Registry, and medical records from hospitals and general practitioners.\(^1\) A diagnosis of MI followed the changing definitions over time.\(^23,24\)

Gene screening and genotyping

To increase the likelihood of identifying genetic variants with an effect on plasma triglyceride levels, we used an extreme phenotype approach. We screened the core promoter, coding region, and intron–exon boundaries (~50 base pairs upstream and downstream each exon) of APOA5 for genetic variants in individuals in the Copenhagen City Heart Study (CCHS) with the 1% lowest (\(n = 95; 190\) alleles) and 2% highest (\(n = 190; 380\) alleles) triglyceride levels for age and gender, as mutations in those with the highest levels may be the most important clinically. We used denaturing high-performance liquid chromatography followed by DNA sequencing.

For genotyping in the entire CCHS, CGPS, and CIHDS, we selected variants which differed in frequency between the extreme triglyceride phenotype groups (\(P < 0.06\), thus suggesting an effect on plasma levels of triglycerides in the general population. Genotyping of APOA5 c.-3A > G (rs651821; tagging the haplotype c.-1131T > C/ c.-3A > G/ c.162-43G > A/c.*158T > C), S19W (rs3135506; tagging the haplotype S19W/I44I), and c.*31C > T (rs619054) in the CCHS, CGPS, and CIHDS was by TaqMan (Applied Biosystems), and for LPL genotypes D9N (rs1801177), N291S (rs268), and S447X (rs328) in the CCHS by restriction enzyme assays.\(^25–27\) Genotypes were verified by sequencing of 50 randomly selected samples for each of the three APOA5 variants. There was 100% agreement between TaqMan and
sequencing results. Owing to two rounds of re-runs, call rates for genotypes where >99.9% for all assays.

**Laboratory analyses**

Colorimetric and turbidimetric assays were used to measure non-fasting plasma levels of triglycerides, HDL cholesterol, total cholesterol, and apoB (Boehringer Mannheim, Mannheim, Germany and Konelab, Helsinki, Finland); fasting plasma samples were not available. Low-density lipoprotein cholesterol was calculated using the Friedewald equation when plasma triglycerides were <4.0 mmol/L (<352 mg/dL), and otherwise measured directly using an assay validated for use in samples with triglycerides up to 11.4 mmol/L ( Diasys, Diagnostic Systems, Holzheim, Germany and Konelab, Helsinki, Finland). To validate the use of the Friedewald equation for non-fasting lipid values when triglycerides were <4.0 mmol/L, we also measured LDL cholesterol using a direct assay on 5631 individuals. In the LDL cholesterol range from 1 to 10 mmol/L, LDL cholesterol levels measured directly were similar to those calculated from non-fasting total cholesterol, triglycerides, and HDL cholesterol (R² = 0.85; P < 0.001; slope 0.95; intercept 0.29 mmol/L). Remnant cholesterol was calculated as non-fasting total cholesterol minus HDL cholesterol minus LDL cholesterol; thus, when triglycerides were <4.0 mmol/L, remnant cholesterol was triglycerides × 0.45, while for the 4% with triglycerides >4.0 mmol/L, remnant cholesterol was total cholesterol minus directly measured HDL and LDL cholesterol. In addition, in the 5631 individuals with directly measured LDL cholesterol, we also determined remnant cholesterol as total cholesterol minus directly measured LDL and HDL cholesterol.

**Other covariates**

Diabetes was self-reported disease, use of anti-diabetic medication, and/or a non-fasting plasma glucose >11.0 mmol/L. As HbA1c was not determined, and an oral glucose tolerance test was not carried out, diabetes mellitus was likely slightly underestimated. Smoking was current smokers. Hypertension was systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, and/or use of antihypertensive medication. The body mass index was measured weight (kg) divided by measured height squared (m²). Alcohol consumption was fraction of individuals consuming alcohol twice or more weekly. Lipid-lowering therapy was self-reported; in Denmark from 1995 to 2011, 96% of individuals on lipid-lowering therapy received statins (68% simvastatin; 14% atorvastatin; 9% pravastatin; 4% lovastatin; 3% fluvastatin).

**Statistical analysis**

Data were analysed by ABJ, RF-S, and AT-H using Stata/S.E. 12.0. χ² tests evaluated Hardy–Weinberg equilibrium. The Mann–Whitney U test and the Pearson χ² test were used in two-group comparisons. Tests for trend as a function of genotypes were by Cuzick’s extension of a Wilcoxon rank-sum test.

Association of genotypes with an observed risk of MI was by odds ratio (OR) from logistic regression analysis in the CGPS, CCHS, and CIHDS combined. Theoretically predicted risk of MI was estimated from the genotype-associated increases in non-fasting triglycerides and calculated remnant cholesterol levels and the observational associations of these parameters with MI in the CCHS, as determined by Cox proportional hazards regression models with age as time scale and delayed entry. Individuals diagnosed with IHD (angina pectoris and MI) before study entry were excluded, and those dying or emigrating during the follow-up were censored at their death or emigration date, respectively. Theoretically predicted hazard ratios (HRs) as a function of non-fasting triglycerides and calculated remnant cholesterol were corrected for regression dilution bias, using re-measurement of these variables 10 years apart in the CCHS. For both observed and predicted risks of MI, multifactorial adjustment was for age, gender, hypertension, diabetes, and smoking.

A potential causal relationship between genetically elevated levels of non-fasting triglycerides and calculated remnant cholesterol and risk of MI was assessed using instrumental variable analysis and two-stage least squares regression. The combined genotypes associated with elevated non-fasting triglycerides and calculated remnant cholesterol were included as instruments in both the first- and the second-stage regression. The strength of the genetic instrument was evaluated by F-statistics from the first-stage regression, where an F-statistic >10 indicates sufficient strength to ensure validity of the instrumental variable analysis.

Lipid and lipoprotein levels were not available for all participants (1.7% missing) and were therefore imputed from age, sex, and genotypes, and from the known distributions in the CCHS and CGPS. Risk associated with a doubling in non-fasting triglycerides and calculated remnant cholesterol was calculated using logarithms of triglycerides and remnant cholesterol to base 2 in regression models and exponentiating them to give the HRs/ORs. Causal genetic estimates from instrumental variable analysis were compared with an observed risk of MI from observational epidemiology using the Altman and Bland method.

**Results**

Characteristics of subjects with and without MI in the CGPS, CCHS, and CIHDS are shown in Supplementary material online, Table S1.

**Genetic variation**

Resequencing the core promoter, coding regions, and exon—intron boundaries of APOA5 in individuals from the CCHS with the 1% lowest (n = 190 alleles; mean triglyceride = 0.55mmol/L) and 2% highest (n = 380 alleles; mean triglyceride = 8.2mmol/L) plasma triglyceride levels identified 23 genetic variants (Supplementary material online, Table S2). Six common variants representing two haplotypes (c.-1131T > C/c.-3A > G/c.162-43G > A/c.*158T > C, and S19W/I44I) and a common single nucleotide polymorphism, c.*31C > T (Supplementary material online, Figure S1 for linkage disequilibrium), were differentially distributed between the extreme triglyceride groups, suggesting an effect on triglyceride levels (Supplementary material online, Table S2; P-values 1% lowest vs. 2% highest triglycerides: 0.06 to <0.001). We chose to include the S19W variant with a P-value of 0.06, as this variant has been associated with triglyceride levels in the previous studies. We genotyped the CGPS, CCHS, and CIHDS for c.-3A > G and S19W [both minor allele frequencies (MAF): 0.06] tagging the two haplotypes mentioned above, and for c.*31C > T (MAF: 0.23) (Supplementary material online, Table S2). Genotype frequencies were in the Hardy–Weinberg equilibrium (P = 0.10–0.74).

**Lipids, lipoproteins, and apolipoproteins**

Non-fasting lipid, lipoprotein, and apolipoprotein levels as a function of APOA5 genotype are shown for the CGPS including the controls in the CIHDS (total n = 50 628) (Figure 1). The median time since last meal was 2–3 h (inter-quartile range 1–4 h).
For c.-3A > G, S19W and c.*31C > T (note that the reference genotype is TT in Figure 1) there were stepwise increases in triglycerides as a function of genotypes of up to, respectively, 51% (0.86 mmol/L), 37% (0.63 mmol/L), and 8% (0.13 mmol/L), in homozygotes vs. non-carriers (Figure 1; \( P \)-values for trend \( <0.001 \)). When combining these genotypes into 10 genotype combinations, there was a stepwise increase in levels of triglycerides of up to 56% (0.92 mmol/L) when carrying genotype combination 10 (GG CC CC) vs. genotype combination 1 (AA CC TT) (\( P < 0.001 \)). Associations were similar between individual and combined genotypes and calculated remnant cholesterol (Figure 1), and between individual and combined genotypes and remnant cholesterol determined as total cholesterol minus directly measured LDL and HDL cholesterol in 5631 participants in the CCHS (Supplementary material online, Figure S2). Apolipoprotein B and HDL cholesterol levels differed less by the genotype, while the effect on LDL cholesterol was minimal (Figure 1). Data from the CCHS were similar (Supplementary material online, Figure S2), while lipid levels in the CIHDS cases were not examined because of the possibility of reverse causation. When participants in the CGPS on lipid-lowering therapy were excluded, non-fasting lipid, lipoprotein, and apolipoprotein levels as a function of the \( APOA5 \) genotype were similar to those in Figure 1 (Supplementary material online, Figure S4). Also, when stratifying participants in the CGPS on time since last meal (\( \leq 2 \) h), lipid and lipoprotein levels as a function of the combined genotypes were similar to those in Figure 1 (Supplementary material online, Figure S5). Finally, we performed linear regression of logarithmically transformed levels of triglycerides and calculated remnant cholesterol as a function of \( APOA5 \) genotypes and genotype combinations adjusted for sex, age, and \( LPL \) genotypes D9N, N291S, and S447X in the CCHS (Supplementary material online, Table S3). Regression coefficients were similar in models adjusted and not adjusted for \( LPL \) genotypes.

**Risk of myocardial infarction**

Assuming that non-fasting triglycerides and calculated remnant cholesterol are causally associated with risk of MI, we would theoretically expect genetically elevated levels to associate with risk of MI in the same direction and to at least the same extent as in observational studies, as previously shown for triglycerides.\(^1,3,7\) In the CGPS, CCHS, and CIHDS combined (MI cases \( n = 5705/ \) controls \( n = 54408 \)), for both non-fasting triglycerides and calculated remnant cholesterol, the observed associations of the 10 genotype combinations with risk of MI were in the same direction and more pronounced than the corresponding theoretically predicted risk (Figures 2 and 3). Importantly, potential confounders were equally distributed among the 10 genotype combinations, while
Figure 2  Non-fasting triglyceride levels, theoretically predicted hazard ratios, and observed odds ratios for myocardial infarction, as a function of the combined c.-3A > G, S19W, and c.*31C > T genotypes in the Copenhagen General Population Study, the Copenhagen City Heart Study and the Copenhagen Ischemic Heart Disease studies combined (n = 60 113). Adjustment was for age, sex, smoking, hypertension, and diabetes mellitus. Triglyceride values are mean ± standard error. P-values are tests for trend.

Figure 3  Calculated remnant cholesterol levels, theoretically predicted hazard ratios, and observed odds ratios for myocardial infarction, as a function of the combined c.-3A > G, S19W, and c.*31C > T genotypes in the Copenhagen General Population Study, the Copenhagen City Heart Study and the Copenhagen Ischemic Heart Disease studies combined (n = 60 113). Adjustment was for age, sex, smoking, hypertension, and diabetes mellitus. Calculated remnant cholesterol values are mean ± standard error. P-values are tests for trend.
lipid-lowering therapy increased slightly from genotype combinations 1–10 (Supplementary material online, Table S4). The wider confidence intervals around observed vs. predicted risk estimates is because of less statistical power in the former vs. the latter model. For genotype combination 10 vs. 1, the increase in triglyceride levels was 68% (1.10 mmol/L), and the theoretically predicted risk of MI was 1.40 (95% confidence interval: 1.23–1.59) (Figure 2, middle). For calculated remnant cholesterol, the corresponding increase was 56% (0.40 mmol/L), and the theoretically predicted risk of MI was 1.39 (1.23–1.57) (Figure 3, middle). The observed increase in risk of MI for genotype combination 10 vs. 1 was 1.87 (1.25–2.81) (Figures 2 and 3, right). When further adjusting the risk estimate for apoB in the CCHS and CGPS combined (Supplementary material online, Figure S6), and for LPL genotypes in the CCHS (Supplementary material online, Figure S7), results were similar. The three separate haplotypes tagged by c.-3A>G, c.56C>G (S19W), and c.*31C>T all associated with an increased risk (borderline for c.*31C>T) of MI in the combined study, when compared with the haplotype associated with the lowest triglyceride levels (=ACT) (Supplementary material online, Figure S8).

Risk of myocardial infarction: causal estimates

We also examined a potential causal association of non-fasting triglycerides and calculated remnant cholesterol with risk of MI in a Mendelian randomization approach, using the combined genotypes as the genetic instrument in instrumental variable analysis. This analysis incorporates both the genotype effect on non-fasting triglycerides and calculated remnant cholesterol and the effect of genotype on MI risk, to derive a causal estimate of non-fasting triglycerides and calculated remnant cholesterol directly on MI risk. The F-statistic for this instrument was 82, indicating substantial strength to ensure the validity of the genetic instrument. For non-fasting triglycerides and calculated remnant cholesterol causal risk, estimates for genetically elevated levels were in the same direction and slightly higher than the corresponding risk estimates for a similar increase in plasma levels from the observational study (Figure 4). The causal OR for MI for a doubling in genetically elevated levels of non-fasting triglycerides was 1.94 (1.40–1.85), with a corresponding observational HR of 1.57 (1.32–2.68) (Figure 4, top; P for comparison = 0.28). For calculated remnant cholesterol, the corresponding values were 2.23 (1.48–3.35) causal and 1.67 (1.38–2.02) observational (Figure 4, bottom; P for comparison = .21).

Discussion

In this study, we used genetic variation in APOA5 to explore a possible causal association between elevated non-fasting triglycerides and calculated remnant cholesterol and risk of MI. The principal findings are that three APOA5 genotypes defined 10 common genotype combinations which associated with substantial stepwise increases in non-fasting triglycerides and calculated remnant cholesterol, and with corresponding increases in risk of MI. A doubling of non-fasting triglycerides and calculated remnant cholesterol associated with, respectively, 1.9-fold and 2.2-fold causal risks of MI, supporting a causal role of remnant cholesterol in hypertriglyceridaemia for risk of MI.

Compared with a recent meta-analysis studying the association between the APOA5 c.-1131T>C promoter variant, triglycerides, and risk of coronary disease, novel aspects of the current study include the use of a stronger genetic instrument (three genetic variants located in the APOA5 gene in a combined genotype), up to
68% higher genetically elevated triglycerides vs. 16% higher,\textsuperscript{30} non-fasting rather than mainly fasting triglycerides, which provides better assessment of the triglyceride exposure as most individuals are in the non-fasting state the majority of their life, calculated remnant cholesterol, uniform assessment of the harder endpoint MI rather than the less well-standardized coronary heart disease, individual participant data allowing for multifactorial adjustment of risk estimates, and use of formal instrumental variable analysis in a homogenous white population. Additional strengths of our study include that we corrected the theoretically predicted HRs for regression dilution bias, and that the F-statistic for the combined genotype was 82, documenting substantial strength to ensure the validity of our genetic approach.\textsuperscript{23}

Remnant lipoproteins carry large amounts of cholesterol and share with LDL the potential to enter and get trapped in the intima of the arterial wall.\textsuperscript{9,10,31} This would lead to intimal accumulation of cholesterol and atherosclerosis, ultimately leading to IHD and MI.\textsuperscript{3,4,12} The present data are also consistent with the occurrence of premature atherosclerosis in conditions with elevated levels of remnant lipoproteins and triglycerides, such as type III hyperlipidaemia, familial combined hyperlipidaemia, chronic renal failure, and non-insulin-dependent diabetes mellitus.\textsuperscript{33–36}

Because genetic determinants of lipids and lipoproteins are randomly distributed at conception, they are unconfounded by other cardiovascular risk factors, and therefore ideal instruments to explore causality between a given lipid trait and risk of IHD. Nevertheless, the impact of genetic variation in APOA5 on risk of cardiovascular disease has until now not been conclusive. Some studies have suggested that the promoter variant c.-1131T > C, which is tagged by the c.-3A > G variant in our study, associates with IHD.\textsuperscript{15,37–40} While others have failed to find such an association.\textsuperscript{41,42} The same is true for S19W.\textsuperscript{15,39–43} Most persuasive so far, a recent meta-analysis showed that c.-1131T > C associated with triglyceride levels in a dose-dependent manner and with a corresponding increase in cardiovascular risk.\textsuperscript{30} Combining three genetic variants into 10 genotype combinations in the largest study to date, we show that the combined genotypes are associated with stepwise increases in non-fasting triglycerides, calculated remnant cholesterol and risk of MI. These risk estimates are robust to confounding factors due to the use of genetic instruments. That the causal risks of MI, as estimated by instrumental variable analysis, were slightly higher for both non-fasting triglycerides and calculated remnant cholesterol than the observational risks, suggests that a life-long exposure to genetically elevated levels of triglyceride-rich remnants may have a larger effect on risk than suggested from observational data alone. This is in accordance with previous results for genetic variants affecting LDL cholesterol.\textsuperscript{14,45}

A limitation of our study is that we did not measure remnant lipoprotein cholesterol directly. However, previous studies have documented that elevated non-fasting triglycerides is a marker of elevated non-fasting remnant cholesterol measured using direct assays,\textsuperscript{46} and that elevated remnant cholesterol associates with an increased risk of coronary heart disease.\textsuperscript{47} Furthermore, the c.-1131T > C and the S19W variants have previously been associated with elevated levels of remnant lipoprotein cholesterol and VLDL particle concentration.\textsuperscript{15,30} Finally, as the results of Sarwar \textit{et al.}\textsuperscript{30} using mainly fasting triglycerides were similar to the results of the present study using non-fasting triglycerides, we cannot deduce whether IDL, VLDL, and chylomicron remnants combined or IDL and VLDL remnants combined are the main mediators of the increased risk of MI. An additional limitation is that we only examined whites and therefore our findings may not translate to populations of other ethnicities. However, both the c.-1131T > C and S19W variants have similar effects on triglycerides in Asian populations,\textsuperscript{16,38,40} suggesting that these effects are not specific to a particular ethnic group. Another potential limitation is that our genetic instrument had minor effects on HDL cholesterol. However, as the increase in non-fasting triglycerides and calculated remnant cholesterol was five- to six-fold larger than the corresponding decrease in HDL cholesterol, since genetic variants causing life-long reduced HDL cholesterol have not been associated with risk of IHD or MI\textsuperscript{22,48–52} and since HDL cholesterol raising has failed to reduce cardiovascular disease,\textsuperscript{53,54} elevated remnant cholesterol may be a more likely causal factor than reduced HDL cholesterol. The strongest genetic evidence for the absence of any increased cardiovascular risk at low HDL cholesterol levels was obtained in studies of ABCA1.\textsuperscript{48} In these studies, including 109 heterozygotes and a total of 41 961 participants of which 6666 had IHD, genetic variants in ABCA1 associated with a 17 mg/dL reduction in HDL cholesterol, with a corresponding 49% reduction in the cholesterol efflux, but not with risk of IHD. One could argue that heterozygosity for ABCA1 mutations in vivo may be compensated to some degree by other mechanisms such as non-specific desorption of cellular membrane cholesterol to apoAI/HDL. Furthermore, recent studies have suggested that the cholesterol efflux capacity from macrophages, a possible metric of HDL function, is a more reliable marker of cardiovascular risk than plasma levels of HDL cholesterol.\textsuperscript{52} In line with this, there is until now no basis on which to link the cholesterol content of HDL with its potential anti-atherogenic activities. Finally, deletion of apoAI has been associated with marked HDL deficiency, dramatically accelerated coronary atherosclerosis and peripheral accumulation of cholesterol in family studies.\textsuperscript{55,56}

In addition, as the metabolism of triglyceride-rich lipoproteins, HDL and LDL are closely linked, the genetically elevated levels of triglycerides might influence the quality and functionality of these lipoproteins. This could result in formation of small dense LDL particles\textsuperscript{38,57} and dysfunctional HDL particles,\textsuperscript{58} which could confound the causal estimate of remnant cholesterol in hypertriglyceridaemia on risk of MI in our study. Therefore, future studies of yet other genetic variants affecting triglycerides and remnant cholesterol in LPL, APOC2, and APOC3, with or without effects on HDL cholesterol, small dense LDL, HDL functionality, and other lipoprotein parameters, are needed. Such studies together with the present data may contribute to the development of a clinically valuable ‘genetic score’ approach to the question of cardiovascular risk associated with elevated levels of remnant cholesterol in hypertriglyceridaemia. Also, the slightly higher use of lipid-lowering therapy among the genotype combinations with the highest non-fasting triglycerides and calculated remnant cholesterol may have attenuated the risk between these genotypes and risk of MI, suggesting that the causal estimates on MI risk for non-fasting triglycerides and calculated remnant cholesterol is slightly
underestimated in the present study. Finally, it may also be a limitation that non-fasting plasma was not drawn at a specific time-point after a standard meal, as triglycerides and thus remnant cholesterol vary slightly depending on time since the last meal. However, such variation would only tend to bias our results towards the null hypothesis, and, therefore, cannot explain the results of the present study.

In conclusion, genetic variation in APOA5 associates with stepwise increases in levels of non-fasting triglycerides and calculated remnant cholesterol, and with corresponding increases in risk of MI. These data are consistent with a causal association between elevated levels of remnant cholesterol in hypertriglyceridaemia and increased risk of MI. However, limitations include that remnants were not measured directly, and that genetic variants in APOA5 may influence other lipoprotein parameters. Future studies should examine the potential benefit of reducing non-fasting triglycerides and remnant cholesterol in individuals with elevated levels.

Supplementary material
Supplementary material is available at *European Heart Journal* online.

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