A common variant in CCDC93 protects against myocardial infarction and cardiovascular mortality by regulating endosomal trafficking of low-density lipoprotein receptor

Antoine Rimbert 1†, Nawar Dalila 2†, Justina C. Wolters 1, Nicolette Huijkmann 1, Marieke Smit 1, Niels Kloosterhuis 1, Marijn Riemsma 1, Ydwine van der Veen 1, Amika Singla 3, Freerk van Dijk 5, Biobank-Based Integrative Omics Studies Consortium 6, Ruth Frikke-Schmidt 2,7, Ezra Burstein 3,4, Anne Tybjærg-Hansen 2,7,8,9‡, Bart van de Sluis 1‡, and Jan Albert Kuivenhoven 1*‡

1Section Molecular Genetics, Department of Pediatrics, University of Groningen, University Medical Center Groningen, Building 3226, Rm 04.14, Internal Zip Code EA12, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands; 2Section for Molecular Genetics, Department of Clinical Biochemistry, Righospitalet, Copenhagen University Hospital, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen, Denmark; 3Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA; 4Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, USA; 5Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; 6Biobank-Based Integrative Omics Studies Consortium; 7Department of Clinical Biochemistry, Righospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark; 8The Copenhagen General Population Study, Herlev and Gentofte Hospital, Herlev Ringvej 75, DK-2730 Herlev, Denmark and 9The Copenhagen City Heart Study, Bispebjerg and Frederiksberg Hospital, Nordre Fasanvej 57, DK-2000 Frederiksberg, Denmark

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Aims

Genome-wide association studies have previously identified INSIG2 as a candidate gene for plasma low-density lipoprotein cholesterol (LDL-c). However, we suspect a role for CCDC93 in the same locus because of its involvement in the recycling of the LDL-receptor (LDLR).

Methods and results

Characterization of the INSIG2 locus was followed by studies in over 107 000 individuals from the general population, the Copenhagen General Population Study and the Copenhagen City Heart Study, for associations of genetic variants with plasma lipid levels, with risk of myocardial infarction (MI) and with cardiovascular mortality. CCDC93 was furthermore studied in cells and mice. The lead variant of the INSIG2 locus (rs10490626) is not associated with changes in the expression of nearby genes but is a part of a genetic block, which excludes INSIG2. This block includes a coding variant in CCDC93 p.Pro228Leu, which is in strong linkage disequilibrium with rs10490626 (r² > 0.96). In the general population, separately and combined, CCDC93 p.Pro228Leu is dose-dependently associated with lower LDL-c (P-trend 2.5 × 10⁻⁶ to 8.0 × 10⁻⁶), with lower risk of MI (P-trend 0.04–0.002) and lower risk of cardiovascular mortality (P-trend 0.005–0.004). These results were validated for LDL-c, risk of both coronary artery disease and MI in meta-analyses including from 194 000 to >700 000 participants. The variant is shown to increase CCDC93 protein stability, while overexpression of human CCDC93 decreases plasma LDL-c in mice. Conversely, CCDC93 ablation reduces LDL uptake as a result of reduced LDLR levels at the cell membrane.

Conclusion

This study provides evidence that a common variant in CCDC93, encoding a protein involved in recycling of the LDLR, is associated with lower LDL-c levels, lower risk of MI and cardiovascular mortality.
Supplementary material online, ods is provided below. For more detailed descriptions refer to the endosomal recycling of the LDL-receptor (LDLR). Loss of the LDL-receptor (LDLR) is a well-established causal risk factor. Genome-wide association studies have identified numerous regions in the genome (loci) that are primarily associated with plasma LDL-c concentration. For approximately half of the LDL-c associated loci, it is not known which mechanisms underlie these associations, emphasizing that our knowledge of LDL metabolism is far from complete.

Here, we focus on the single nucleotide polymorphism (SNP) rs10490626, on the second chromosome at position 118835841 (GRCh37). The T allele is found in ~9% of non-Finnish Europeans (http://gnomad.broadinstitute.org) and has been reported to be associated with reduced plasma LDL-c levels. This variant is located in the locus 2q14 upstream of the INSulin Induced Gene 2 (INSIG2) which is implicated in the regulation of cholesterol synthesis. Loss of INSIG2 does, however, not affect plasma cholesterol levels in mice.

This, combined with recent insight, led us to suspect a role for coiled-coil domain-containing protein 93 (CCDC93), another gene in this locus. This because we have recently shown that CCDC93 participates in an evolutionary conserved multiprotein complex, annotated as the CCC complex (COMMD/CCDC22/CCDC93; see Supplementary material online for additional information), that orchestrates the endosomal recycling of the LDL-receptor (LDLR). Loss of the CCC complex studied thus far causes hypercholesterolaemia in mammals including man.

The current study contributes to the unravelling of a mechanism that offers an explanation of how rs10490626 is associated with reduced plasma levels of LDL-c and protection against myocardial infarction (MI) and death from cardiovascular disease. Several lines of evidence suggest that stabilization of the endosomal sorting machinery through a coding variant of CCDC93 increases LDLR recycling, thereby lowering levels of LDL-c in plasma.

Methods
A flowchart of epidemiological and genetic analyses is shown in Supplementary material online, Figure S1. A brief description of the methods is provided below. For more detailed descriptions refer to Supplementary material online, Appendix.

Characterization of the chromosomal region 2q14
Expression quantitative trait loci (eQTL) analyses aim to connect SNPs with the expression of genes. We first tested whether the T allele of rs10490626 was associated with expression levels of protein-coding genes in the locus 2q14 (within 1 Mb surrounding this SNP) using a publicly available mRNA dataset (GTEx v7 release). In addition, we used an eQTL dataset from Biobank-based Integrative Omics Studies (BIOS) Consortium of the BBMRI-NL (http://wiki.bbmri.nl/wiki/BIOS_bios) which is composed of whole blood samples from 2116 donors.

Pairwise linkage disequilibrium (LD) values were obtained from the Phase 3 release of the 1000 Genomes project. In addition, LD between rs10490626 and rs17512204 (CCDC93 p.Pro228Leu) was determined in individuals in the studied populations (see below).

Participants
We included individuals from two prospective studies of the Danish general population, The Copenhagen General Population Study (CGPS) and The Copenhagen City Heart Study (CCHS) that have been described in detail elsewhere. All individuals were white and of Danish descent.

Combining the participants in the CGPS and CCHS yielded a total of 107 063 participants at baseline. During a mean follow-up of 36 years (range: <1–40 years) which ended in March 2017, MI developed in 5291 individuals and 3635 died from ischaemic cardiovascular disease. In both studies, follow-up was 100% complete, i.e. we did not lose track of a single individual. DNA was available for all individuals, and lipid values were available for more than 98%.

The study was approved by institutional review boards and Danish ethics committees and was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all individuals. For additional information on participation rates, clinical end-points, laboratory analyses, and other covariates (Supplementary material online, Appendix).

Genotyping and statistical analyses
CCDC93 p.Pro228Leu (rs17512204; C>T) which is in complete LD with rs10490626 (see Results section), was genotyped using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) and TaqMan-based assays.

Data were analysed using Stata/SE 14.0. The χ² test evaluated Hardy–Weinberg equilibrium. To compare baseline characteristics by CCDC93 p.Pro228Leu (rs17512204) genotype, we used Cuzick’s test for trend. For trend tests, genotypes were coded 0, 1, and 2. The genotype associated with the highest LDL-c levels was used as the reference (coded 0). P-values were by tests for trend from linear regression models. Cox proportional hazards regression models, using age as time scale and delayed entry (left truncation) which implies that age is automatically adjusted for, were used to estimate hazard ratios for MI and cardiovascular mortality as a function of CCDC93 p.Pro228Leu (rs17512204) genotypes. Multifactorial adjustments were for well-known risk factors for MI: age, gender, body mass index, hypertension, diabetes mellitus, physical activity, smoking, alcohol consumption, hormone replacement therapy
(women only), and lipid-lowering therapy. Analyses were conducted from 1 January 1977 (start of the national Danish Patient Registry and the national Danish Causes of Death Registry) through March 2017.

Finally, we performed meta-analyses of CCDC93 rs17512204 (p.Pro228Leu) on LDL-c, risk of coronary artery disease (CAD) and MI combining data from the Danish general population with data from: (i) the Global Lipids Genetics Consortium (Ref.9 and http://lipidgenetics.org) for LDL-c; (ii) summary data from the UK Biobank (http://geneatlas.roslin.ed.ac.uk) and CARDIoGRAMplusC4D 1000 Genomes (Ref.17 and http://www.cardiogramplusc4d.org/data-downloads/), for risk of ischaemic heart disease and MI using the ‘metan’ command in Stata.

**In vitro characterization of CCDC93 Pro228Leu**

FLAG-tagged CCDC93 (pcDNA3.1-CCDC93-FLAG) was purchased from Genescript (O-Hu01865D), pcDNA3.1-CCDC93-p.Pro228Leu-FLAG was generated with the QuikChange II Site-Directed Mutagenesis Kit. Wildtype (CCDC93WT) and CCDC93 p.Pro228Leu were transiently overexpressed in HEK293T cells. The protein stability of both variants was assessed after blocking protein synthesis at an optimal concentration of cycloheximide which did not affect cell viability, i.e. 50 μg/mL (CHX – Sigma C4859). In short, 24 h after transfection, cells were cultured in medium supplemented with cycloheximide and harvested at sequential time points (from 4 to 36 h). Cell lysates were used for western blotting using anti-FLAG antibody. Statistical comparison of protein concentrations between CCDC93WT vs CCDC93p.Pro228Leu was calculated using the Student’s t-test.

**Overexpression of human CCDC93 in Ldlr^{−/−} mice**

Liver overexpression of human CCDC93 (hCCDC93) in Ldlr^{−/−} mice (n = 5) was achieved through intravenous administration of adenovirus 8 (AAV8) harbouring hCCDC93, or with a matched viral dose of empty AAV8 particles as controls (n = 5). Animals were single housed and fed a standard chow diet. Three weeks after injections, animals were fasted and sacrificed by cardiac puncture under anaesthesia. Plasma cholesterol concentrations in the main lipoprotein classes (very low-density lipoprotein, LDL, and high-density lipoprotein (HDL) were determined using fast protein liquid chromatography in plasma of each animal and compared using the Student’s t-test. This study was approved by the Institutional Animal Care and Use Committee, University of Groningen (the Netherlands).

**Generation and characterization of a CCDC93 deficient liver cell line**

Huh7 CCDC93 knockout cells (CCDC93^{−/−}) were generated using CRISPR/Cas9 editing technology. To quantify protein concentration, we used custom targeted liquid chromatography-mass spectrometry (LC/MS) based proteomic assays. Low-density lipoprotein uptake capacity of the cells [Huh7 CCDC93^{−/−} vs. wild-type (wt)] was evaluated using Dil-labelled LDL. Cells were deprived of serum for 16 h and subsequently incubated with Dil-LDL (5 mg/mL) containing medium for 2.5 h. After washing, fixation, and mounting, Dil-LDL uptake was quantified and normalized by fluorescent microscopy in a blinded fashion. More than 700 cells per condition were recorded.

To assess the level of the LDLR at the cell surface, we used a biodistribution assay which targets proteins at the plasma membrane. Briefly, cells were incubated with biotin and lysed. The biotinylated membrane fraction was isolated using neutravidin beads. Both, membrane and whole cell lysate fractions were analysed using western blotting. For both LDL uptake and biodistribution assays, the Student’s t-test was used to compare different conditions.

**Results**

**Genetic association and characterization of the 2q14 locus**

Among SNPs spanning the region of chromosome 2 (locus 2q14) (Figure 1A), rs10490626 shows the strongest association (r^2 > 0.8, data from the Phase 3 release of the 1000 Genomes project) that includes CCDC93, but not INSIG2 and DDX18 (Figure 1A). This LD block includes rs17512204, a SNP that leads to the substitution of a leucine for a proline residue at position 228 of CCDC93 (NM_019044.4: rs17512204_c.683C>T, p.Pro228Leu). Importantly, rs17512204 is in complete LD with rs10490626 (r^2 = 0.96, P-value: <0.0001; data from 1000 Genomes project) (Figure 1A) implying that these SNPs are co-inherited.

Using the GTEx Consortium mRNA dataset (Release V7), we assessed whether rs10490626 is associated with the expression of DDX18, CCDC93, or INSIG2. In line with previous findings, the data show that rs10490626 is probably not an eQTL (plotted association data in adipose, liver, and intestine, Supplementary material online, Figure S2). Taken the small number of samples of these relevant tissues (n = 385, 313, 153, and 125 for subcutaneous adipose tissue, visceral adipose tissue, liver, and intestine, respectively) combined with the frequency of rs10490626, these eQTL analyses were, however, underpowered. Of note, GTEx suggests that rs10490626 is an eQTL in testis tissue for the pseudogene HTR5BP, which is exclusively expressed in testis (data not shown) and is very unlikely relevant in regard to the plasma-cholesterol metabolism.

In a next step, we took advantage of the fact that DDX18, CCDC93, and INSIG2 are all ubiquitously expressed (according to GTEx [https://www.gtexportal.org] and The Human Protein Atlas [https://www.proteinatlas.org]19) and ran an eQTL analysis using RNA-seq data set of whole human blood (n = 2116). Figure 1B shows that rs10490626 is not associated with changes in the expression of DDX18 (P = 0.31), CCDC93 (P = 0.17), or INSIG2 (P = 0.94) in blood, which suggests that rs10490626 is not an eQTL for the genes studied.

**CCDC93 genotype, plasma lipids, lipoproteins, and apolipoproteins in the Danish general population cohorts**

In agreement with publicly available data, LD between the minor alleles of the intergenic lead GWAS SNP, rs10490626 and rs17512204 (CCDC93 p.Pro228Leu) was near complete (r^2 = 0.98) in both the CGPS and the CCHS population cohorts, thereby validating the use of the latter variant in our study. Baseline characteristics of the 107 063 individuals from the CGPS and CCHS, combined and individually, as a function of CCDC93 p.Pro228Leu genotype (rs17512204; C>T) are shown in Supplementary material online.
Tables S1–S3. Characteristics as a function of genotype were similar. In agreement with the corresponding lower LDL-c levels (Figure 2A, second panel), the use of lipid-lowering therapy tended to be slightly less frequent as a function of increasing number of T-alleles ($P$ for trend < 0.05) (Supplementary material online, Table S1). Taken together, these data suggest that the genetic data are largely unconstrained by the main known measured risk factors for MI. Finally, genotype distribution did not differ from Hardy–Weinberg equilibrium.

$CCDC93$ p.Pro228Leu (rs17512204; C>T) was associated with lower plasma levels of total cholesterol up to 0.9% (-0.05 mmol/L, $P$ for trend = 4.8 x $10^{-8}$), LDL-c up to 2.2% (-0.07 mmol/L, $P$ for trend = 8.0 x $10^{-8}$), and apolipoprotein B up to 1.0% (-1.14 mg/dL, $P$ for trend = 3.4 x $10^{-6}$), in homozygotes for the minor T-allele vs. non-carriers (Figure 2A, Panels 1–3 from left) but was not associated with HDL cholesterol, apolipoprotein AI (ApoAI), triglycerides, calculated remnant cholesterol (which mirrors triglycerides), or lipoprotein(a) (Figure 2A, Panels 4–7; data not shown for calculated remnant cholesterol). Results were similar for the CGPS and the CCHS separately (Supplementary material online, Figure S3). In individuals who were not on lipid-lowering therapy ($N$ = 95 512), total cholesterol, LDL-c, and apolipoprotein B were even further reduced (compare Figure 2A and Supplementary material online, Figures S3 with S4).

### CCDC93 genotype and risk of myocardial infarction

The multifactorially adjusted hazard ratios for MI decreased stepwise as a function of rs17512204 genotype to 0.88 (95% confidence interval: 0.81–0.96) in CT heterozygotes and 0.67 (0.39–1.16) in TT homozygotes ($P$ for trend = 0.002) (Figure 2B). In the individual studies, the corresponding hazard ratios were 0.92 (0.83–1.01) and 0.67 (0.36–
1.24) in the CGPS (P for trend = 0.04), and 0.77 (0.63–0.93) and 0.71 (0.23–2.20) in the CCHS (P for trend = 0.007) (Supplementary material online, Figure S5A).

**CCDC93 genotype and cardiovascular mortality**

The multifactorially adjusted risk of cardiovascular mortality (N = 4126 events) decreased stepwise as a function of rs17512204 genotype to 0.87 (95% confidence interval: 0.78–0.97) in CT heterozygotes and 0.64 (0.33–1.22) in TT homozygotes (P for trend = 0.004) (Figure 2C). Results were similar in the CGPS and CCHS separately (Supplementary Figure S5B and D).

**Meta-analyses on low-density lipoprotein cholesterol, risk of ischaemic heart disease, and myocardial infarction**

In meta-analyses of CCDC93 rs17512204 (p.Pro228Leu) on LDL-c including data from the Global Lipids Genetics Consortium (Ref.9 and http://lipidgenetics.org) and the Danish general population (CGPS + CCHS), the per T-allele reduction in LDL-c was -0.05 standard deviation for the inversed normalized distribution of LDL-c (95% CI: -0.06 to -0.04) using both random and fixed effects models (Figure 3A), corresponding to an approximate reduction of 0.05 mmol/L (2 mg/dL) in our study. There was no evidence of heterogeneity between studies (I^2 = 0%, P = 0.84).

In meta-analysis of risk of CAD, the per T-allele odds ratio was 0.97 (0.94–0.99) in both random and fixed effects models (Figure 3B). CAD was self-reported heart attack or MI in the UK Biobank. In the Danish general population, this diagnosis corresponded best to ischaemic heart disease and included angina pectoris and MI based on International Classification of Diseases codes (ICD8: 410–414 and ICD10: I20–25); MI was independently validated—for a detailed description see Supplementary material online, Methods. In CARDiogramPlusC4D 1000 Genomes (Ref.17 and http://www.cardiogramplus4d.org/data-downloads/), CAD was based on diagnostic criteria in 48 different studies which were harmonized across studies (for a detailed description of diagnoses in the individual studies see Appendix F).

1.24) in the CGPS (P for trend = 0.04), and 0.77 (0.63–0.93) and 0.71 (0.23–2.20) in the CCHS (P for trend = 0.007) (Supplementary material online, Figure S5A and C).
Figure 3 Meta-analyses of low-density lipoprotein cholesterol, risk of coronary artery disease, and myocardial infarction per rs17512204 C>T (CCDC93 p.P228L) T-allele. (A) Meta-analysis summarizing effect size on low-density lipoprotein cholesterol per T-allele. Global Lipids Genetics consor
tium (Ref.9 and http://lipidgenetics.org/). (B) Meta-analysis summarizing risk of coronary heart disease per T-allele. CARDIoGRAMplusC4D 1000 Genomes (Ref.17 and http://www.cardiogramplusc4d.org/data-downloads/); UK Biobank summary data from GeneAtlas (http://geneatlas.roslin.ed.ac.uk). The diagnosis coronary artery disease was self-reported heart attack/myocardial infarction in the UK Biobank (http://biobank.ctsu.ox.ac.uk/crystal/field.cgi?id=20002), coronary artery disease from CARDIoGRAMplusC4D 1000 Genomes (data as published in Ref.17) and ischaemic heart disease (including angina pectoris based on ICD code, and myocardial infarction based on ICD code and independently validated to a validity >99%). (C) Meta-analysis summarizing risk of myocardial infarction per T-allele. Myocardial infarction was myocardial infarction in the UK Biobank (ICD10 code I21 acute myocardial infarction, http://biobank.ctsu.ox.ac.uk/crystal/field.cgi?id=41202; summary data from http://geneatlas.roslin.ed.ac.uk) myocardial infarction from sub-phenotype analysis in CARDIoGRAMplusC4D, and myocardial infarction (ICD8: 410 and ICD10 I21 and I22; validated to a validity of >99% by cardiologists). In all three meta-analyses, horizontal lines correspond to 95% confidence intervals by forest plots. Diamonds and red broken vertical lines represent the summary estimates. The width of the diamonds represents the confidence intervals for the summary estimates. Grey shaded areas correspond to the weight of the study; weights on the graphs are from random effects models. Overall effect sizes are Cohen’s d with 95% confidence intervals. aCopenhagen General Population Study and Copenhagen City Heart Study combined.
Ref. Supplementary material online, Notes). There was no evidence of heterogeneity between studies \((I^2=0\%, \ P = 0.96)\) (Figure 3B).

In meta-analysis of MI, the overall odds ratio for MI per T-allele was 0.95 (0.90–0.99) using a random-effects model, and 0.95 (0.92–0.98) using a fixed-effects model (Figure 3C). There was modest, non-significant heterogeneity between studies \((I^2 = 53\%, \ P = 0.12)\) with overlapping confidence intervals. The estimates became significant for both CARDioGRAMplusC4D 1000 Genomes \([0.96 \ (0.92–0.99)]\) and our study \([0.88 \ (0.81–0.96)]\), but not different from unity for the UK Biobank, on the hard endpoint MI.

**Sensitivity analyses**

To determine whether CCDC93 p.Pro228Leu affected other phenotypes than LDL-c which might additionally explain the effect on risk of CAD, MI, and cardiovascular mortality, or affect other endpoints, we first determined the per T-allele effect on 34 different phenotypes and an additional five endpoints in our own study (Supplementary material online, Figure S6). Only total cholesterol, LDL-c, apolipoprotein B, and risk of MI were significant. Second, we collected all available data from GWAS studies and chip data from the Cardiovascular Disease Knowledge Portal (29 March 2019; http://broadcvdi.org/variantInfo/variantInfo/rs17512204) (for a description of the studies included, see legend to Supplementary material online, Figure S7). In these meta-analyses including 30 phenotypes or endpoints, the per T-allele effect reached genome-wide significance for total cholesterol and LDL-c only (Supplementary material online, Figure S7). Third, we included summary ‘pshews’ (phenomewide association study) data from the UK Biobank for rs17512204 (http://geenatlas.roslin.ed.ac.uk). In that study, ‘high cholesterol’ was the most significant hit (note that the reference allele in the UK Biobank was the wild-type G-allele, implying that the results are opposite using the minor T-allele as the reference in our study, i.e. low cholesterol), while there were indications that the variant might affect anthropometric parameters (for T-allele: increased leg fat mass, increased weight and hip circumference, but also increased basic metabolic rate, and lower physical activity) (Supplementary material online, Table S4). Combined, these latter findings are unlikely to reduce risk of CAD.

**Proportional risk of coronary artery disease and myocardial infarction as a function of per allele genetically determined increase in low-density lipoprotein cholesterol in the general population (CCHS and CGPS)**

The proportional risk of CAD and MI as a function of the per allele genetically determined increase in LDL-c for variants in well-known LDL-c genes (NPC1L1, PCSK9, ABCG8, APOB, and the LDLR) in the CCHS and CGPS is shown in Supplementary material online, Figure S8 and compared to the effect for CCDC93 p.Pro228Leu (G-allele). First, the proportional risk of CAD and MI for CCDC93 p.Pro228Leu (G-allele) was comparable to that of other LDL-c genes. Second, per allele risk of CAD for genetic variants in these genes was similar to risks reported in the EAS consensus paper. These data together with data from the meta-analyses suggest that CCDC93 p.Pro228Leu is likely associated with risk of CAD and MI through the LDL-c effect per se. Finally, as expected, risk of MI, a more valid endpoint, was higher than risk of CAD for most variants in our study, and therefore, these two endpoints are not directly comparable, as also shown for CARDioGRAMplusC4D 1000 Genomes in the meta-analyses (Figure 3).

**CCDC93 p.Pro228Leu increases protein stability**

In a next step, we investigated whether the substitution of a Leucine for a Proline residue at position 228 in the CCDC93 protein could explain our genetic and epidemiologic observations. To this purpose, we transiently overexpressed wildtype (WT) and the p.Pro228Leu variant in HEK293T cells and investigated their half-life (\(t_{1/2}\)) following incubation with a protein synthesis inhibitor (cycloheximide) (Figure 4A and B). We found that the protein stability of CCDC93 p.Pro228Leu was significantly increased compared to CCDC93 WT after 24 and 36 h \((P < 0.05)\) (Figure 4A and B). This finding suggests that CCDC93 p.Pro228Leu increases the stability of CCDC93. Analysis of the whole CCC complex with LC-MS based targeted proteomics furthermore shows that CCDC93 p.Pro228Leu does not affect the stoichiometry of CCC complex (Supplementary material online, Figure S9).

**Increased expression of human CCDC93 in the liver reduces low-density lipoprotein cholesterol plasma levels in mice**

The increased protein stability of CCDC93 p.Pro228Leu and its association with reduced plasma LDL-c led us to evaluate whether increasing the levels of wild-type CCDC93 in the liver of mice could reduce plasma LDL-c. To this end, we injected heterozygous Ldlr knockout mice (C57Bl6; Jackson Labs) with AAV8 harbouring wild-type human (h) CCDC93. Three weeks after virus administration, we observed a 1.8-fold increase of CCDC93 protein in the liver compared to controls (Figure 5A). This was associated with a small but significant reduction of LDL-c \((P = 0.049)\) in mice overexpressing hCCDC93 compared to controls (Figure 5B; Supplementary material online, Table S5). Using LC-MS based targeted proteomics, we confirmed that CCDC93 protein concentration was increased \((1.6 \text{ fold}) \ (P < 0.001)\) in liver homogenates, which was accompanied by an increase of the CCC complex component CCDC22 \((P < 0.001)\) in mice that were treated with AAV8 hCCDC93 compared to controls (Supplementary material online, Figure S10). Although the overexpression of the WT form of CCDC93 does not mimic a gain of function mutation, these results support the idea that increasing the amount of hepatic CCDC93 can lower plasma LDL-c.

**CCDC93 is required for low-density lipoprotein receptor recycling and low-density lipoprotein uptake in liver cells**

To validate the role of CCDC93 on endosomal LDLR recycling, we ablated CCDC93 in human hepatocyte carcinoma cells (Huh7) (Figure 6A). While this cell line cannot recapitulate all aspects of human lipid metabolism, the involvement of the CCC complex in facilitating endosomal trafficking of LDLR has also been shown in HEK293T, HepG2 as well as primary murine hepatocytes and several animal models.12,13 Loss of CCDC93 decreased the protein levels of...
the CCC core components CCDC22 and C16orf62 which is in line with previous observations\textsuperscript{12,13} (Figure 6B). This destabilization of the CCC complex was accompanied by a \textasciitilde 50% reduction of LDLR at the plasma membrane (P < 0.001) (Figure 6C) and a \textasciitilde 40% reduction in the uptake of fluorescently labelled LDL (Dil-LDL) compared to control cells (P < 0.001) (Figure 6D). Combined, these results suggest that CCDC93 is involved in the endosomal trafficking of LDLR back to the plasma membrane for the uptake of LDL (Figure 6E).

Discussion
This study suggests that a common variant in CCDC93, p.Pro228Leu (rs17512204), is associated with increased functioning of the CCC complex, an endosomal sorting protein complex that orchestrates LDLR recycling. In agreement with these findings, CCDC93 p.Pro228Leu is associated with per allele lower levels of plasma LDL-c and a corresponding lower risk of MI and cardiovascular mortality in over 107,000 individuals from the general population (Take home figure). These results were validated for LDL-c, risk of both CAD and MI in meta-analyses including from 194,000 to >700,000 participants.

The LDLR has, since its discovery by Goldstein and Brown in the 70s,\textsuperscript{21} been recognized as the principal regulator of LDL clearance from the circulation. Its central role in this process is illustrated by the notion that changes in its transcription,\textsuperscript{22} internalization,\textsuperscript{23,24} stability,\textsuperscript{25} subcellular localization, and degradation\textsuperscript{26,27} all affect the concentration of LDL-c in blood.

Central to the current study is that Goldstein and Brown also reported that the LDLR can be re-used up to 100 times after its internalization into early endosomes.\textsuperscript{28} On the one hand, the re-use of LDLR can be prevented by the binding of pro-protein convertase subtilisin-kexin type 9 (PCSK9) to LDLR. This protein directs the LDLR to the lysosomal compartment for degradation,\textsuperscript{19} and it has been established that genetic variants in PCSK9 strongly affect the capacity of the liver to clear LDL from the circulation. On the other
hand, little is known about the mechanisms that transport the LDLR back to the cell membrane for re-use, and how this may affect plasma LDL-c levels.30 Figure 6E illustrates our current working model.

Endosomal trafficking of different proteins (cargo’s) is a long-standing topic in cell biology.31,32 We have recently taken a next step to study this process in vivo.12,13,30,33 These experimental studies in mice have shown that a loss of proteins of the endosomal sorting machinery causes hypercholesterolaemia and increased atherosclerosis.12,13 In the current study, we provide evidence that it is also possible to decrease plasma LDL-c in mice through overexpressing...
wild-type CCDC93 albeit it that the effect is small. Others have shown that over-expression of SNX17, another protein involved in the endosomal sorting machinery, also enhances LDL uptake via its positive role on LDLR sorting in cultured cells.34

Evidence that the endosomal LDLR recycling may also be relevant in humans was so far restricted to rare loss-of-function mutations in CCDC22 and WASHC5, which cause hypercholesterolaemia but also developmental defects and intellectual disability.13,35,36 This combined with the notion that a complete loss of components of the endosomal recycling machinery is lethal in mice12,13,37 indicated that impairment of this pathway is very harmful. In contrast, the current study shows that a common genetic variant, presumably associated with a ‘gain-of-function’, is associated with cardiovascular risk reduction including a reduction in cardiovascular mortality in the general population.

In meta-analyses, we found similar CCDC93 p.Pro228Leu per T-allele reductions in LDL-c and risk of CAD in all studies considered, comparable to data reported in a recent consensus statement from the European Atherosclerosis Society Consensus Panel.1 Taken together, the data suggest that the per T-allele reduction in risk of CAD and MI can be explained by the corresponding observed reduction in LDL-c.

The per T-allele reduction in risk of MI which is a harder endpoint than CHD/CAD was 5% overall, similar between studies (overlapping confidence intervals for all studies), and significant in both CARDioGRAMplusC4D and in our own study, but not different from unity in the UK Biobank. The most likely explanation for the latter finding is that the participation rate in that study was 5.5% (9 million invited, ~500 000 participants), limiting the representativeness of the study for the UK general population, and possibly leading to a healthy participant bias (the participation rates were 61% and 45%, respectively, in the CCHS and CGPS). The latter is supported by the observation that the proportion of smokers and the proportion with no qualifications—reflecting social position—were considerably lower than in the background population, and that the 5-year mortality rate was several-fold lower than in the UK population as a whole.38,39 Furthermore, the frequency of MI in the UK Biobank was <2% compared to >5% in the Danish general population, suggesting
that the UK Biobank represents a low-risk population. Therefore, a common genetic variant, as studied here, which typically associates with a modest change in risk, is at this point in time unlikely to be associated with a measurable risk reduction in the UK Biobank with the current limited follow-up and the low risk at the start of the study.

It is noteworthy that genome-wide association studies of human plasma lipid traits have thus far not pointed at a role for the genes encoding proteins involved in the endosomal recycling process, with the only exception being for \textit{CCDC93} as unveiled in this study. This can be explained by the fact that this process is primarily regulated at the post-translational (protein) level\textsuperscript{12,13,40}, which, in turn, supports our hypothesis that the \textit{CCDC93} variant (rs17512204) encodes a protein with improved function.

The current study provides evidence for a role of \textit{CCDC93} in human LDL metabolism, cardiovascular disease, and cardiovascular mortality with molecular clues from in vitro and in vivo experiments that is related to LDLR-mediated LDL-c clearance (Take home figure). Proof that loss of function mutations in \textit{CCDC93} may cause familial hypercholesterolaemia in man would strengthen our findings, but mutations in \textit{CCDC22}, another gene encoding for a protein of the CCC complex, have been shown to cause hypercholesterolaemia.\textsuperscript{13}

In conclusion, this study shows that large-scale genetic studies can help us find other angles to study LDL metabolism. It has implicated yet another pathway that was already known to control the activity and/or functionality of LDLR, i.e. it is endosomal sorting and recycling in humans. It remains to be seen whether this insight may lead to new tools for pharmaceutical intervention, but it has been shown that it is possible to pharmaceutically target the endosomal-sorting pathway with small molecules.\textsuperscript{41}

**Supplementary material**

\textit{Supplementary material} is available at \textit{European Heart Journal} online.

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