Genome-wide Association Study of Genetic Determinants of LDL-c Response to Atorvastatin Therapy: Importance of Lp(a)

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Conflicts of Interest
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ABSTRACT

We carried out a genome wide association study of LDL-c response to statin using data from participants in the CARDS trial (n=1156), ASCOT trial (n=895) and the observational phase of ASCOT (n=651), all prescribed atorvastatin 10 mg. Following genome wide imputation, we combined data from the three studies in a meta-analysis. We found associations of LDL-c response to atorvastatin that reached genome-wide significance at rs10455872 (p=6.13 x 10⁻⁹) within the LPA gene and at two SNPs within the APOE region (rs445925; p=2.22 x 10⁻¹⁶ and rs4420638; p=1.01 x 10⁻¹¹) that are proxies for ε2 and ε4 variants in APOE respectively. The novel association with the LPA SNP replicated in the PROSPER trial (P=0.009). Using CARDS data we further showed that atorvastatin therapy did not alter Lp(a) and that Lp(a) levels, accounted for all of the association of SNPS in LPA gene and apparent LDL-c response levels. However statin therapy had a similar effect in reducing CVD in patients in the top quartile for serum Lp(a) levels (HR=0.60) compared with those in the lower three quartiles (HR=0.66) (p=0.8 for interaction. The data emphasise that high Lp(a) levels affect the measurement of LDL-c and the clinical estimation of LDL-c response. Therefore, an apparently lower LDL-c response to statin therapy may indicate a need for measurement of Lp(a). However statin therapy seems beneficial even in those with high Lp(a).

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INTRODUCTION

Statin therapy is now widely accepted for the primary and secondary prevention of cardiovascular disease (CVD) in certain patient groups. However, there is considerable variation in response to statin therapy that remains poorly understood. For example in the CARDS trial [1] among self-reported and pill count-validated compliant recipients of atorvastatin 10 mg daily, the absolute change in LDL-c at one month post-randomisation varied from -2 mmol/L to -0.6 mmol/L, (5th and 95th centiles of the range) and the percentage lowering from baseline varied from 67% to 22. Understanding the pathways and determinants involved in this variation in response to therapy could lead to improved treatments. Even without understanding pathways, identifying predictors of poorer response could identify those most in need of additional or alternative therapeutic strategies.

Two genome-wide studies of statin response and several candidate gene association studies have been reported [2,3,4,5]. From these the only consistent finding is that variants in the APOE gene region are associated with variation in LDL response. Here we report a genome wide analysis of LDL-c response from two randomised clinical trials of atorvastatin - the Collaborative atorvastatin Diabetes Study (CARDS) and the Anglo-Scandinavian Outcomes trial (ASCOT) [6] to investigate genetic effects on LDL-c response to atorvastatin. We chose to model genetic determinants of LDL-c response to atorvastatin among those assigned to atorvastatin in these trials. An alternative approach would be to model the interaction of genotype on the effect of atorvastatin on LDL-c using data from both placebo and active treatment groups. However we did not consider this latter approach as optimal since testing for interactions is much less powerful than direct tests of association and since in any case we did not consider genetic effects on change LDL-c in the placebo groups to be plausible.

RESULTS

Table 1 compares baseline characteristics of participants in the three studies. Figure 1 shows a QQ plot of the –log10 P-values for association of each SNP with LDL-c response to treatment, obtained by meta-analysing effect size estimates across the CARDS and ASCOT datasets. This shows that the cumulative distribution of test statistics approximates the null distribution over most of its range, but there is a tail of extreme results. Figure 2 shows a Manhattan plot of the -log10 p-values by map position. Table 2 shows all loci at which the summary test for association yielded a nominal p-value < 10^-6. The estimates of effect shown (beta) are for the transformed response variable (see methods). In CARDS, the response variable was transformed on-treatment LDL-c with transformed pre-treatment LDL-c entered as a covariate in the model. This is mathematically equivalent to modelling change in LDL-c with pre-treatment LDL-c as a covariate (i.e. the difference in transformed on–treatment and adjusted for pre-treatment LDL) as was done in ASCOT. A negative beta for an allele means that the modelled allele is associated with a bigger reduction in post treatment LDL-c and a better response to statins.

The strongest associations were with rs10455872 in the LPA gene on chromosome 6, and with SNPs in the BCAM/PVRL2/APOE/APOC1 gene region on chromosome 19, where genome-wide significant associations were found. The SNPs in the LPA and APOE region explained 4 % of the variance in LDL-C response in CARDS. The next most significant p-value was that for the ALG10 region on chromosome 12, but this did not reach genome-wide
significance. There was no evidence of gene-gender interaction for all the top SNPs reported in the study. The effect sizes for all top SNPs were similar in CARDS where all the participants had Type 2 Diabetes and ASCOT where 21% of the participants had Type 2 Diabetes (Table 2) suggesting that diabetes per se was not a strong determinant of the genetic effect of the top SNPs.

**LPA**

In the LPA gene SNP, rs10455872 showed a genome wide significant association with LDL-c response (Figure 3). The effect at rs10455872 was modest; the beta shown in table 2 is not easily directly interpretable given the transformation used but in CARDS for example the percentage change in LDL-c with statin therapy at one month was approximately -43% in those with at least one ‘G’ allele at rs10455872 (Minor allele Freq = 8%) compared with -46.5% in homozygotes for the ‘A’ allele. There was no significant effect of this SNP on change in LDL-c post randomisation in those in the placebo group (p=0.28).

To investigate the association with LPA genotype further, we first confirmed that LPA genotypes predicted serum Lp(a) levels, which had been measured in CARDS but not in ASCOT. Figure 4 shows the results of the GWAS for serum Lp(a) levels; all significantly associated loci were in the LPA region, consistent with other reports[7]. In a linear regression model that included age, sex and population structure covariates 12 SNPs in the LPA region had independent effects on serum Lp(a) (rs10455872 rs5014650 rs783147 rs6919346 rs3103349 rs2063347 rs6415084 rs10455782 rs394487 rs6926458 rs316174 rs3127569). Together these SNPs explained 40% variation in the serum Lp(a) levels in CARDS but most of this was attributable to the rs10455872 SNP (30%) with median levels being 7.6 mg/dl (Interquartile range 4.1-14), 50.5 mg/dl (IQR 37-68) and 55.2 mg/dl (IQR 51-113) in those with AA AG and GG genotype respectively. We then adjusted the association of genotype at SNPs in the LPA gene with LDL-c response to statin for measured serum Lp(a) levels in the CARDS data to test whether the genetic effects seen are likely to be mediated through the effect of LPA on serum Lp(a) levels (Table 3). The estimate of the standardized regression coefficient at the associated SNP (rs10455872) in LPA in CARDS was reduced from -0.35 (±0.08) to -0.09 (±0.08) consistent with the effect of genotype on apparent response to statin being mediated through Lp(a) levels. We noted that Lp(a) levels had an independent association with apparent LDL-c response to statin beyond genotype in these analyses (p=0.001). Further analysis in CARDS also confirmed that there is no effect of statin on Lp(a) levels; Beta was -0.23 mg/dl, (95% CI -2.25 to 1.80) for difference in Lp(a) levels with atorvastatin versus placebo at one year post randomisation, adjusted for baseline Lp(a), age and sex.

To assess whether serum Lp(a) levels might alter efficacy of statin therapy on CVD itself, we examined whether there was any evidence of interaction (deviation from a multiplicative model of joint effects on a hazard scale) between high serum Lp(a) levels and atorvastatin on CVD end points in CARDS. The hazard ratio for CVD events associated with statin use was 0.60 (95% CI: 0.32-1.13) among those in the top quartile for serum Lp(a) (> 22 mg/dl) compared with 0.66 (95% CI: 0.46 to .93) among those with serum Lp(a) below this level (likelihood ratio test for interaction p=0.8).

Nor was there any evidence of interaction between rs10455872 genotype at LPA and
atorvastatin for effects on CVD end points (p=0.27 for the interaction of genotype at rs10455872 locus. Here the HR associated with atorvastatin in those with homozygous for the A allele was 0.58 (95% CI: 0.41, 0.83) and the HR in those with at least one G allele was 1.03 (0.38-2.78). However, the power to detect such an interaction was limited as there were only 16 events among the 294 trial participants with at least one copy of the G allele.

**Replication of the LPA SNP**

We tested the effect of LPA SNP (rs10455872) in 2550 participants in the PROSPER trial randomized to 40mg/day of pravastatin. In this study ‘A’ allele of rs10455872 was also associated with lower response to statins; with a scaled beta of -0.18 (±0.04), P value=0.009). The combined p-value for the three studies was 1.2E-09 (Beta= -0.28 ±0.04).

**APOE**

Several SNPs in the BCAM/PVRL2/APOE/APOC1/APOE gene region reached genome wide significance for statin response (Figure 5). The effect on LDL-c response to statin therapy associated with these SNPs at in this region was modest; in CARDS for example the % change in LDL-c with statin therapy at one month was approximately -51% in those with at least one ‘A’ allele at rs445925 compared with -45% in common GG homozygotes and was approximately -37% in those with at least one ‘G’ allele at rs4420638 compared with -47% in common ‘AA’ homozygotes. These effects were independent of the effect of genotype at rs10455872 in LPA and of Lp(a) levels. In the CARDS dataset we confirmed that there was no significant effect of these SNPs on change in LDL-c post randomisation in those in the placebo group (p=0.47).

We examined whether the effects in this region could be accounted for by the known ε2/ε3/ε4 protein polymorphism of apolipoprotein E which corresponds to APOE SNP haplotypes T-T, T-C, and C-C respectively at rs429358 and rs7412. The presence of the ‘T’ allele at rs7412 contrasts ε2 protein variant with other protein variants while presence of ‘C’ allele at the rs429358 contrasts the ε4 protein variant with other protein variants. These two SNPS were not directly typed and could not be imputed as they are not in the HapMap II.

The ‘A’ allele at rs445925 which we found to be associated with a higher statin response (beta= -0.44) is in LD with ‘T’ allele at rs7412 with a reported $r^2$ of 0.76 and is thus a proxy for ε2 protein variant [8] while the ‘G’ allele at rs4420638 which was associated with lower response to statin is in LD with ‘C’ allele at rs429358 with reported r-squared values of 0.62 but with a low r-squared for rs7412 of 0.01[9] and thus is a proxy for ε4 protein variant.

These two proxy SNPs are in the HapMap and could be imputed in this analysis with percent information content (i.e. imputation quality) of 77% and 56% respectively. Thus we tested for residual effects of SNP haplotypes conditioning either on rs445925 (as a proxy for rs7412) or on rs4420638 (as a proxy for rs429358). When conditioned on rs4420638 the ε4 proxy, the additional percentage variance explained by residual haplotype effects is 0.7% (F statistic with 8 and 854 df=3.21, p=0.001). When conditioned on rs445925, the proxy for ε2, the additional percentage variance explained by residual haplotype effects only 0.2 % (F statistic with 8 and 854 df =1.76, p=0.08) suggesting that ε2 accounts for most of the variance in response at this locus.
**ALG10**

Beyond these associations of LDL-c response with APOE and LPA, no other genome wide significant associations were found. The next most significant SNPS were those in the ALG10 gene region (Figure 6) on chromosome 12 where several SNPs had P<10^{-6}. ALG10 codes for asparagine-linked glycosylation protein 10 homolog A. Of these SNPs most map to inter-genic regions either side of the ALG10 gene itself with one imputed SNP within ALG10 having a p value for association with statin response of 6.79x10-6.

**Effect of pre-treatment LDL-C**

To demonstrate that these findings are unlikely to be confounded by baseline LDL-c Table 4 shows unadjusted, adjusted, and corrected estimates of the direct effect of genotype on post-treatment LDL at the strongest SNPs for the APOE region LPA and ALG10 in the CARDS dataset. At the APOE ε2 proxy SNP (rs445925), without adjusting for baseline LDL-c the apparent LDL-c response to statins would be more than double that observed in our baseline adjusted model (beta= -1.01 versus -0.44 per copy of ‘A’ allele) emphasising the effect of adjusting for baseline LDL-c. However adjusting our effect size estimate further by modelling measurement noise at baseline reduced the apparent effect just slightly to beta= -0.30, suggesting there is little residual effect of baseline due to measurement noise. At the APOE ε4 proxy SNP and at the LPA SNP the estimated effect of baseline LDL-c adjustment is much less, and thus the adjustment for measurement noise alters the association only slightly.

**Other genes of interest**

Previously reported variants associated with statin response in the PCSK9 (rs11591147), HMGCR (rs1047443, rs17671591, rs6453131), KIF-6 (rs20455), ABCB1 (1236/2677/3435 TTT haplotype), CLMN gene (rs80141914, associated with total cholesterol response to statin), and GCKR (rs1260326 associated with triglyceride level response to statin) were not significantly associated at with LDL-c response to statin in this study at an accepted genome-wide association threshold (P≤10^{-8}) or even at thresholds typically expected to declare replication (say P≤10^{-2}). However, PCSK9 (rs11591147), and GCKR (rs1260326) were significant at a threshold of 0.05 (see supplementary Table 1). We have refrained from comparing the directionality and magnitude of these effects in the present study because of the different phenotype characterization and transformations across the studies and in some studies, lack of information about the modelled alleles.
DISCUSSION

In this genome wide association study of LDL-c response to atorvastatin therapy we report that those with genotypes in the LPA gene that lead to higher Lp(a) levels have an apparently lower LDL-c response to statin and we replicate the previously reported association of a higher response to statin in those with the A allele at the APOE ε2 locus. The top 3 SNPs in the study, rs10455872 in LPA and the APOE ε2, APOE ε4 variants explained only 4% variance in the LDL-c response to statin treatment, however it is possible that that larger studies might detect more SNPS with smaller effect sizes or that there are larger effects at rarer variants not captured by our imputed genotypes.

LPA

Lipoprotein(a) is a plasma lipoprotein consisting of a cholesterol-rich LDL particle with one molecule of apolipoprotein B100 and an additional protein, apolipoprotein(a), attached via a disulfide bond. Serum levels of Lp(a) have a highly skewed distribution; for example in CARDS the median serum Lp(a) was 8.9 mg/dl with an interquartile range of 4.5-21.3 mg/dl and with values as high as 238 mg/dl. Approximately 30% of variance in Lp(a) levels has been reported as determined by the kringle IV type 2 (KIV-2) copy number variant in LPA which is known to encode variability the size of apo(a). Some variance in measured Lp(a) attributable to genes is also due to Apo(a) size heterogeneity affecting the results of the immunochemical methods used to quantify Lp(a) as is the case with the assay we have used [10]. That is genotype can induce some measurement error in Lp(a) though recent data from the Framingham study suggest the measurement error is likely to be of little practical importance [11]. The Lp(a) raising genotype associated with the kringle repeat and high Lp(a) levels themselves have also been reported to be associated with increased cardiovascular risk in several studies [12,13,14,15,16]. As such recent guidelines emphasise the importance of detection of high Lp(a) phenotype and possible intervention with niacin [14].

The rs10455872 SNP that we found associated with LDL-c response is in strong LD with the KIV-2 CNV in Lp(a) [17]. Consistent with this, variation at rs10455872 accounted for 30% of variance in Lp(a) in the CARDS data. However, the explanation for the apparently lower LDL-c response in those with genotypes associated with high Lp(a) lies in understanding what LDL-c estimation actually captures. The standard Friedewald formula calculates LDL-c levels from total cholesterol, HDL-cholesterol, and plasma triglyceride and actually includes the cholesterol that resides in Lp(a). For most patients this is of little importance since usually only about 5% of what is measured as LDL-cholesterol is estimated to reside in Lp(a). However it is estimated that about 8% of apparent LDL-c resides in Lp(a) if Lp(a) levels are in the range 30-60 mg/dl and as much as 20% if Lp(a) is > 60 mg/dl [18]. As we show definitively here in the CARDS trial, statin therapy does not lower Lp(a) levels. Thus, individuals who have an appreciable fraction of their total plasma cholesterol carried on Lp(a) particles have some cholesterol in statin responsive LDL particles and some in statin unresponsive Lp(a) particles. For such patients true LDL-c response will be underestimated since apparent on treatment LDL-c will comprise truly falling LDL-c but static Lp(a) levels. This phenomenon has been noted in the context of nephrotic syndrome previously [19] and has been emphasised by Scanu and others[20]. Our estimate that those with at least one copy of the Lp(a)-raising G allele at rs10455872 have about a 5 percentage
points lower apparent statin response (45% in ‘GG’ and ‘AG’ genotype vs. 40% in ‘AA’ genotype), but that this association disappears when adjusted for Lp(a) levels, is consistent with this.

Although the effect of the G allele on statin response is modest, this allele only accounts for about 30% of variance in Lp(a) levels. The data highlight a more general clinical point that individuals with raised Lp(a) levels for any reason have a somewhat lower apparent response to statin therapy and therefore that an apparently lower LDL-c response to statin may be an indication for checking Lp(a) levels. However, we also show here that a similar relative protection from CVD with atorvastatin therapy was found in those with and without elevated Lp(a). It is increasingly accepted that elevated Lp(a) increases CVD risk [12,13]. Therefore it is important that although Lp(a) levels themselves are not changed and statin effects on LDL-c appear erroneously low that statin therapy be continued in individuals with high Lp(a).

We confirmed the association of LPA SNP rs10455872 – association of ‘A’ allele with a lower response to statins in an independent cohort of 2550 subjects randomized to 40 mg of pravastatin. This is the first report of a successful replication of genetic response to statin treatment beyond the APOe region in a genome-wide association study.

**APOE**

We replicated the previous finding that genotype at the APOE ε2 locus is associated with variation in statin response. Those with at least one ‘A’ allele at rs445925, which is in strong LD with the locus determining the ε2 protein variant, was associated both with higher baseline LDL-c and with greater response to statin whereas the proxy for ε4 protein variant and was associated with lower LDL-c response to statin. The conditional haplotype analysis suggests most of the variation is attributable to the number of ε-2 copies rather than to the number of ε-4 copies, but this is not definitive given that there is uncertainty in the haplotypes and that the HapMap SNPs are imperfect proxies. As noted previously individuals in whom a higher proportion of cholesterol is synthesized rather than taken up via diet such as ε2 carriers are more susceptible to inhibition of cholesterol synthesis [2] and in addition there may be more remnant and IDL-like particles contributing to apparent LDL-c in those patients with an APO ε2 allele. Statins are very good at removing these larger LDL particles through LDL receptor up-regulation.

**ALG10**

We found some suggestion of an association between statin response and variants in the ALG10 gene region but this did not reach the usual genome wide significance threshold of $P<10^{-8}$ so requires confirmation in other studies before considering it other than a spurious association. ALG10 codes for asparagine-linked glycosylation protein 10 homolog A that adds the third glucose residue to the lipid-linked oligosaccharide precursor for N-linked glycosylation. Its relevance to statin response remains to be established though of course protein modification by N-linked glycosylation is relevant to diverse aspects of human biology including functional modification of many enzymes [21].
Previous Studies

Two genome-wide association studies of LDL-c response to statin therapy have been reported previously [2,3]. In the TNT trial dataset with 1984 treated individuals typed with a genome-wide panel, there were no loci at which p-values for association were less than $10^{-6}$, but in a superset of 5745 individuals studied for candidate gene associations only, three SNPs in APOE and one SNP in PCSK9 reached genome-wide significance [2]. In a meta-analysis of three trials including 3932 treated subjects [3] a SNP in the CLMN gene was significant at $p < 10^{-7}$ for association with total cholesterol response and there was a weak association with SNP in APOE. In the same study polymorphism in GCKR gene, was shown to be associated with statin-induced change in triglyceride. Candidate gene analyses have shown that a common LDLR 3-UTR haplotype is associated with attenuated lipid-lowering response to simvastatin treatment [22]. In the same study, HMGCR gene polymorphisms were also associated with reduced plasma LDL-c and with reduced LDL-c response to simvastatin. The association of HMGCR gene with statin response was also reported in a population based cohort of patients with diabetes [23]. These effects were more evident in African-Americans than in European-Americans. In a separate study, carried out in acute coronary syndrome patients, carriers of a polymorphism in kinesin-like protein 6 (KIF-6) have been reported to have greater benefit from pravastatin versus placebo with respect to CVD outcome but not with respect to lipid or C-reactive peptide response [24]. Additionally, association with the ABCB1 gene with statin response have been reported [4,25]. Apart from the APOE association, none of these other associations were replicated here.

Finally, we note that the effects identified in this study are of modest size: the importance of further studying the genetics of response to statin therapy may be not in predicting who will benefit from statins, but in identifying other therapeutic targets.

MATERIALS AND METHODS

Study populations and phenotype definition

Both trials were conducted with Ethics Committee/ IRB approval, under good clinical practice guidelines and in accordance with the Declaration of Helsinki principles. Patients gave consent for genetic studies.

CARDS

Methods in CARDS have been described previously. In brief, 2838 patients with Type 2 diabetes and no previous CVD were randomized to receive either placebo or atorvastatin 10mg once daily and followed for a median of 3.7 years. Allocation was double blinded. Mean serum LDL-c concentration during baseline visits prior to randomisation had to be $\leq 4.14$mmol/L (160 mg/dl) and serum triglycerides $\leq 6.78$mmol/L (600mg/dl). After randomisation, total cholesterol, HDL-C, and triglycerides were measured at one, two, and three months and 6 monthly thereafter. Patients attended after an overnight fast. LDL-c was calculated with the Friedewald formula[26] or, if serum triglycerides exceeded 4·0 mmol/L, by removing VLDL by ultracentrifugation then measuring the change in infranatant cholesterol content when LDL was removed by precipitation of apolipoprotein B-containing lipoproteins. For this genome-wide study the analyses were restricted to those randomised to
atorvastatin and the mean of two pre-treatment LDL-c measurements was used as the baseline LDL-c and a weighted average of five post-randomisation values within the first year post-randomisation was the outcome measure or “on treatment LDL-c”, with weights (0.6 for month 1 and then 0.1 for measurements at 2, 3, 6 and 12 months). Lipoprotein(a) concentrations were determined by an immunoturbidimetric assay with Immuno LEIA® reagents from Technoclonle Ltd., Dorking, UK (now www.PathwayDiagnostics.com) which is calibrated against the IFCC Standard preparation PRM02.

ASCOT

Of 19 342 hypertensive patients, aged 40—79 years with at least three other cardiovascular risk factors, randomised to one of two antihypertensive regimens in the Anglo-Scandinavian Cardiac Outcomes Trial, 10 305 with non-fasting total cholesterol concentrations 6·5 mmol/L or less (measured at non-fasting screening visit) had been randomly assigned additional atorvastatin 10 mg or placebo. These patients formed the lipid-lowering arm of the study. For this genome-wide study two subpopulations from ASCOT were included; i) Those individuals randomised to 10mg atorvastatin in whom pre-treatment LDL-c was measured at the (fasting) randomisation visit and on-treatment LDL-c was calculated as the simple average of measures at the (fasting) visits 6 months and 12 months post randomisation. LDL-c was estimated using the Friedewald equation as in CARDS ii) Following the end of the randomisation phase there was an observational period. We included from all individuals not originally randomised to 10mg atorvastatin (i.e. those randomised to placebo and those not eligible for the LLA) those who were subsequently prescribed atorvastatin 10mg. For these individuals, pre-treatment LDL-c was defined as the measurement on the last visit before or equal to date of starting atorvastatin, and on-treatment LDL-c was defined as the measurement taken from the first visit after date of starting atorvastatin.

PROSPER (Replication Cohort)

All data come from the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER)

Phenotype Transformation

To maximise power to detect associations, and to improve test statistic behaviour under the null for low MAF SNPs, we transformed measured LDL-c levels to conform with the
distributional assumptions made by our association analysis model, using the same transformation for off- and on-treatment measures to preserve the relationship between the two. We maximised the fit of the residuals in a regression of on–treatment on the pre-treatment value to a Gaussian distribution we used a 2-parameter Box-Cox transform of the form $x - \beta^\alpha / \alpha$ applied to baseline and on-treatment LDL-c values. The parameter values $\alpha$ and $\beta$ were chosen by maximizing the likelihood of a model with linear regressions of the transformed pre-treatment and response (transformed pre-treatment minus transformed pre-treatment) values on the covariates (age and sex), with the joint distribution of the residuals from the two regression models being bivariate Gaussian.

The parameter values obtained were $\alpha = 0.156$, $\beta = -0.505$ mmol/L in CARDS. In ASCOT the parameters were $\alpha = 0.6807$, $\beta = 0.8850$ mmol/L in the randomised dataset and $\alpha = 0.4805$, $\beta = 0.5813$ mmol/L in the observational dataset. This transformation has the same motivation as the inverse normal transform used in some GWAS applications [28] [29] but the use of a parametric transform preserves the relationship between pre- and on-treatment measures thereby allowing the difference between the two, adjusted for pre-treatment value to be used as a response variable as was done in ASCOT or simply the on-treatment adjusted for pre-treatment value as in CARDS (these are equivalent). The effect sizes in discovery cohorts (CARDS and ASCOT) and the replication cohort (PROSPER) were scaled so that the residuals had unit variance thereby allowing studies using different transforms to be combined.

**DNA extraction and genotyping**

**CARDS**

For CARDS DNA was extracted from whole blood EDTA samples. DNA was isolated from 10 ml of frozen blood using the Gentra Puregene DNA Isolation Kit from Qiagen (Cat no. 158389), USA. Briefly, RBC was lysed with an anionic detergent in the presence of a DNA stabilizer which limits the activity of intracellular DNases. WBC was collected by centrifugation at 2000g for 2 min. RNA was removed by treatment with RNase A. Protein was removed by salt precipitation (centrifugation at 2000g for 5 min). Genomic DNA was recovered by precipitation with isopropanol and centrifugation at 2000g for 5 min. Genomic DNA was removed by salt precipitation (centrifugation at 2000g for 5 min). Genomic DNA was recovered by precipitation with isopropanol and centrifugation at 2000g for 5 min. The DNA pellet was washed in 70% ethanol, air dried, and dissolved in hydration solution (1 mM EDTA, 10 mM Tris·Cl pH 7.5). Purified DNA was stored at -20C. DNA aliquots were genotyped at Perlegen Sciences using a proprietary SNP set comprising 599164 SNPs. 243 SNPs that had discrepant map positions between HapMap and Perlegen were dropped. We set a minimum SNP call rate threshold for including SNPs in the analysis of 80% and required that the p-value for a test of deviation from Hardy-Weinberg equilibrium (HWE) was not < $10^{-5}$. This gave 517746 SNPs for analysis. The average call rate was 98% with 86.25% SNPs with a call-rate of greater than 90%. SNP annotation was based on build 36 of the Human Genome Sequence. All SNPs were used in the analysis regardless of allele frequency but the allele frequency was considered when evaluating putative associations. Allele frequency was below 1% at 6% of SNPs. We selected samples from those people who had been allocated atorvastatin 10 mg daily, had given consent for genotyping and had a sample SNP call rate >80%. After applying the exclusions of HWE, we estimated relatedness with PLINK and those individuals with $Pi_HAT > 0.25$ (excluding first and second-degree relatives) were removed (n=0). Only LDL-c values from time points at which the person was
compliant with atorvastatin (based on pill count > 80/%) were used.

**ASCOT genotyping**

Genotyping was carried out on HumanCNV370 (Illumina) array on 3868 individuals at Centre National de Génotypage (CNG) in two batches. Samples were excluded if they had >= 5% missing data (2 samples). SNPs were excluded if they had been mapped to different chromosomes or positions in the different releases (2 SNPs) or if were polymorphic A/T or C/G in either release or in the combined dataset or had call rate ≤ 97% in either release or in the combined dataset (47744 SNPs) or had HW p-value ≤ 10^-7 in either release or in the combined dataset (8502 SNPs). After applying the above exclusions, samples were excluded if they had estimated relatedness > 0.1875 (halfway cut point between second and third degree relatives), estimated using a using a subset of 101954 SNPs obtained by LD based pruning (87 duplicates, 15 first degree relatives and 4 presumed second degree relatives removed. Then, SNPs were excluded if they showed significant differences in allele frequency between the different batches at p<10^-7 (20 SNPs) or were monomorphic in the combined dataset (3838 SNPs) or were not in HapMap r22 (12817 SNPs) or had different alleles to HapMap r22 (6 SNPs) or showed significant differences (p<10^-7 using Fisher's exact test) in allele frequency between the combined dataset and HapMap r22 (308 SNPs). After applying all the above exclusions, ancestry outliers were excluded (n=143) by using Ancestry Principal Component analysis[30] on a subset of 100905 SNPs selected by LD based pruning, and ancestry PCs were calculated for the remaining 3804 individuals.

**PROSPER genotyping**

A whole genome wide screening has been performed in the sequential PHASE project with the use of the Illumina 660K beadchip. Of 5,763 subjects, DNA was available for genotyping. Genotyping was performed with the Illumina 660K beadchip, after QC (call rate <95%) 5,244 subjects and 557,192 SNPs were left for analysis[31].

**Statistical analysis**

**Imputation of genotypes**

The CARDS genotype data were combined with phased haplotypes from HapMap phase II CEU r22 to compute posterior probability distribution of genotype at all HapMap loci using the IMPUTE program [32]. For ASCOT and PROSPER, genotypes at unmeasured SNPs were imputed using MACH [33] and phased haplotypes from HapMap CEU r22. For ASCOT, a randomly chosen subset of 400 individuals was used to estimate transition and emission probabilities (i.e. to estimate recombination rates between SNPs, and per-SNP genotyping error rates), using MACH options "--greedy -r 100" for each (entire) chromosome in turn. Using these estimated rates (the .rec and .erate files), genotypes were imputed for the whole sample of 3804 individuals, using MACH options "--greedy --mle --mldetails" for each (entire) chromosome in turn.

**CARDS Data Analysis**
The EIGENSTRAT program [34] was used to adjust for population structure. Using PLINK[35], we generated a pruned subset of 152587 SNPs that are in approximate linkage equilibrium with each other in the CARDS dataset. Principal components analysis was undertaken using this subset of SNPs. Thirty-seven individuals identified as outliers in the initial principal components analysis were excluded from the subsequent computation of principal components leaving 1174 persons evaluable for statin response. The first three principal components were retained and included as covariates in all tests of association.

On-treatment values for LDL-c for each individual at 1, 2, 3, 6, and 12 months post-randomisation were available. We initially used the first available post-randomisation LDL-c and established that the previously reported APOE genotype at rs445925 was the strongest association in a genome-wide analysis of response at p= 1.1x 10^-13. To maximise the power to detect any further new associations we then trained the weighting of post-randomisation LDL-c time points to maximize the strength of the association of LDL response with APOE genotype at rs445925. Based on this the non-missing values for each individual were combined in a weighted average, with the 1-month value allocated a weight of 0.6 and the four subsequent values weights of 0.1 each (p-value for rs445925 with these weights=2.2x10^-16) SNPTST [32] was used to test for association of LDL response with genotype in a linear regression with weighted average post-randomisation LDL value as dependent variable and with covariates including transformed pre-treatment LDL-c, age, sex and scores on the first three principal components of population stratification. The missing-data likelihood option was used to allow for uncertainty of genotypes at each imputed locus. In practice, the use of several-weighted post-randomisation LDL-c values rather than a single first value made very little difference to the results (see supplementary table 2).

We used the conditional analysis test in PLINK [35] to test for independence of SNP associations over short regions within the same gene; a null model based on equating the effects of haplotypes that differed only at the SNP under test was compared with a more general model in which the effects of these haplotypes were unconstrained. The null hypothesis is that the SNP under test accounts for all associations of haplotypes with response. Other analyses included those carried out to explore initial associations included a test of whether LPA genotype modifies the effect of atorvastatin on CVD. This was carried out by estimating the hazard ratio associated with allocation to atorvastatin in a Cox regression model of time to first CVD event and using a likelihood ratio test comparing a model with this main treatment effect and one including also a term for interaction of genotype x treatment effect.

**ASCOT Data Analysis**

We regressed the response variable (transformed on-treatment minus transformed pre-treatment LDL-c) onto imputed expected genotype dosage as implemented in ProbABEL [33,36]. This is asymptotically equivalent to score test for taking into account uncertainty in imputed genotypes (as in SNPTST) but with improved finite sample size operating characteristics [37]. Age, sex, age*sex and transformed pre-treatment LDL were used as covariates, plus 10 ancestry principal components.

**PROSPER Data Analysis**

The response variable was regressed (natural log of transformed on-treatment minus natural
log of pre-treatment LDL-c) onto imputed expected genotype dosage as implemented in SNPTEST. Age, sex, transformed pre-treatment LDL and top three principal components were used as covariates.

**Meta-analysis**

The score and observed information for the effect parameter were summed over studies to obtain a summary score test. This is algebraically equivalent (based on the quadratic approximation of the log-likelihood) to obtaining a weighted average of the maximum likelihood estimates with weights inversely proportional to the squared standard errors, with the useful feature that the ratio of observed to complete information (calculated by summing numerators and denominators over the three studies) is obtained as a summary measure of the efficiency of genotype imputation. For concise presentation, we focus here on showing the results of the meta-analysis rather than each study separately and provide study-specific estimates of effect only at the most extreme significance levels. In the data presentation, those loci at which the overall proportion of information extracted was less than 30% across the studies have been excluded. We have used a p-value threshold of $<5 \times 10^{-8}$ as the threshold for declaring a genome wide significant association.

**Distinguishing indirect and direct effects of genotype on on-treatment LDL**

Effects of genetic variation on treatment response as measured by on-treatment LDL-c could be mediated through effects on the pre-treatment LDL-c. To evaluate whether genetic on on-treatment LDL-c likely reflects residual effect on pre-treatment LDL-c it is necessary to adjust for the pre-treatment LDL-c levels but also to correct the maximum likelihood estimate of the adjusted effect of genotype on on-treatment value for the noise in pre-treatment values (the noise is both random measurement error and intra-individual variation in usual LDL-c). From the rules of path analysis, we can calculate the direct effect $\gamma$ of genotype on on-treatment trait value as $\beta - \alpha \delta (1-\rho)/\rho$ where $\beta$ is the coefficient of regression for on-treatment value on genotype adjusted for measured pre-treatment value, rho is the intra-class correlation between replicate measurements of pre-treatment values, and $\delta$ is the coefficient of regression for on-treatment value on observed pre-treatment value. For these calculations we have used $\rho=0.8$ as a plausible value for the intra-class correlation based on the within person correlation in LDL-c values taken over two pre-treatment visits in CARDS.

**List of Figures**

Figure 1: Quantile-quantile plot of the Meta-analysis P-values for statin response

Figure 1 legend: A plot of the quantiles of observed and expected distribution of P-values against each other

Figure 2: Manhattan plot of P-values from meta-analysis of all SNPs that passed stringent quality control
Figure 2 legend: The Manhattan plots (also known as $-\log_{10}(P)$ association plots) show the chromosomal position of SNPs exceeding the genome-wide significance threshold ($P < 5 \times 10^{-8}$) as indicated by the solid red line.

Figure 3: Regional association Plot of LPA locus with statin response
Figure 3 legend: Correlations between the target SNP (the SNP with the lowest $P$ value, depicted in purple) and nearby SNPs within a 500 kb region. The $r^2$ values were based on the HapMap CEU population.

Figure 4: Regional association Plot of LPA locus with Lp (a) levels in the CARDS dataset
Figure 4 legend: Correlations between the target SNP (the SNP with the lowest $P$ value, depicted in purple) and nearby SNPs within a 500 kb region. The $r^2$ values were based on the HapMap CEU population.

Figure 5: Regional association Plot of APOE locus with statin response
Figure 5 legend: Correlations between the target SNP (the SNP with the lowest $P$ value, depicted in purple) and nearby SNPs within a 500 kb region. The $r^2$ values were based on the HapMap CEU population.

Figure 6: Regional association Plot of ALG10 locus with statin response before Lp(a) adjustments
Figure 6 legend: Correlations between the target SNP (the SNP with the lowest $P$ value, depicted in purple) and nearby SNPs within a 500 kb region. The $r^2$ values were based on the HapMap CEU population.

List of Tables

Table 1: Patient and Study Characteristics of Studies included in the meta-analysis
Table 2: SNPs associated with Statin response with a meta-analysis P-value <10E-6 and Rsq >0.30 before adjustment for LPA levels
Table 3: SNPs associated with Statin response with a meta-analysis P-value <10E-6 and Rsq >0.30 after adjustments for LPA levels in (CARDS only)
Table 4: Effect of genotype on post-treatment LDL-c with and without correction for measurement noise in baseline LDL (CARDS only)

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The authors thank the other investigators, the staff, and the participants of the CARDS study and the ASCOT study for their important contributions. A full list of CARDS investigators can be found in original CARDS (Lancet. 2004 Aug 21-27; 364(9435):685-96) and ASCOT papers (Lancet. 2003 Apr 5; 361(9364):1149-58).

AUTHOR CONTRIBUTIONS
Generated data for the study: Helen M Colhoun, D John Betteridge, John H Fuller, Shona
Livingstone, Valentine Charlton-Menys, Andrew Neil, Neil Poulter, Peter Sever, Denis Shields, Alice Stanton, Alu Chatterjee, Craig Hyde, Roberto A. Calle, David A DeMicco, Graham A Hitman, Mark Caulfield, Stella Trompet, Iris Postmus, Ian Ford, J. Wouter Jukema Analyzed the data: Paul M McKeigue, Toby Johnson, Harshal Deshmukh, Helen M Colhoun, Graham Hitman, Mark Caulfield, Stella Trompet, J. Wouter Jukema Drafted initial manuscript: Helen M Colhoun, Paul M McKeigue, Harshal Deshmukh, Toby Johnson, Graham Hitman, Mark Caulfield. Editing manuscript: all authors.

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REFERENCES


Table 1: Patient and Study Characteristics of Studies included in the meta-analysis

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<th>ASCOT-Obs f (n=691)</th>
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<td>% with Diabetes %</td>
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<td>Hypertension%</td>
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</table>

a) Inter-quartile range
b) P-value threshold for exclusion of SNPs not in Hardy-Weinberg equilibrium
c) Fasting status for LDL-c at baseline (2 cells above) and for response to statin measure
d) In N=656 with non-missing LDL-c at baseline; the missingness is non-random because these are individuals with baseline TG too high for Friedewald formula
e) Randomized arm of the ASCOT trial
f) Observational arm of the ASCOT trial
Table 2: SNPs associated with LDL-c response to Statins with a meta-analysis P-value <10^{-6} and Rsq >0.30

<table>
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<tr>
<th>Chr</th>
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<th>SNP</th>
<th>Modeled Allele</th>
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a) Rsq=an estimate of squared correlation between imputed and true genotypes.
b) A positive beta for an allele means that the modelled allele is associated with a bigger post treatment LDL-c and therefore a lower response to statins. A negative beta for an allele means that the modelled allele is associated with lower post-treatment LDL-c and therefore a better response to statins.

c) For SNPs that lie in the intergenic regions location of the nearby genes are shown.
Table 3: Effect of adjustment for serum Lp(a) levels in CARDS on the SNPs associated with LDL-c response to Statins with a meta-analysis P-value < $10^{-6}$

<table>
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<tr>
<th>CHR</th>
<th>POS(cM)</th>
<th>SNP</th>
<th>Modelled Allele</th>
<th>Minor Allele (Freq)</th>
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a) For SNPs that lie in the intergenic regions location of the nearby genes are shown
Table 4: Effect of genotype on post-treatment LDL-c with and without correction for measurement noise in baseline LDL (CARDS only)

<table>
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<tr>
<th>rs445925</th>
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<th>Beta adjusted for observed baseline LDL but uncorrected for measurement noise</th>
<th>Beta adjusted for baseline LDL and corrected for measurement noise</th>
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</table>
Figure 5

![Figure 5](image)

37 genes omitted