Rare sequence variants that disrupt *ASGR1* function lower non-HDL cholesterol and protect against coronary artery disease

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ABSTRACT

BACKGROUND

The level of non-high density lipoprotein (non-HDL) cholesterol is strongly correlated with the risk of coronary artery disease. Whole-genome sequencing may enable discovery of rare sequence variants with large effects on serum lipid levels and the risk of coronary artery disease.

METHODS

We sequenced the whole-genomes of 2,636 Icelanders and found variants that were imputed into ~398,000 additional Icelanders and tested for association with non-HDL cholesterol levels (n=119,146) followed by validation in other European populations. We then assessed the effects of associated variants on the risk of coronary artery disease in 41,648 cases and 247,374 controls from five European ancestry populations.

RESULTS

We found a rare non-coding 12 base pair (bp) deletion (minor allele frequency (MAF)=0.41%), NM_001671.4:c.284-36_283+33delCTGGGGCTGGGG (del12), in intron 4 of the gene encoding the asialoglycoprotein receptor 1 (*ASGR1*) that activates a cryptic splice site leading to a frameshift and consequently introduces a premature stop codon that disrupts the function of ASGR1. Del12 associates with lower levels of non-HDL cholesterol (-15.3 mg/dL; 95% confidence interval (CI) -18.9 to -11.7; $P=1.0\times10^{-16}$) and less risk of coronary artery disease (odds ratio 0.66; 95% CI 0.55 to 0.79; $P=4.0\times10^{-6}$). We found an additional rarer loss of function variant (p.W158X, MAF=0.027%) in a larger set of sequenced Icelanders that also reduced non-HDL cholesterol (-24.9mg/dL; 95% CI -40.6 to -9.3; $P=1.8\times10^{-3}$)

CONCLUSIONS

These results show that *ASGR1* haploinsufficiency lowers non-HDL cholesterol and protects against coronary artery disease and suggests that ASGR1 inhibition could be used as an approach to prevent coronary artery disease.

INTRODUCTION

Epidemiological and genetic studies have demonstrated a causal link between non-high density lipoprotein (non-HDL) and low density lipoprotein (LDL) cholesterol levels and the development of coronary artery disease and myocardial infarction[1-3]. Recent studies show that non-HDL cholesterol is a better predictor for cardiovascular risk than LDL cholesterol as it encompasses, in addition to LDL cholesterol all, cholesterol containing pro-atherogenic lipoproteins such as very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), Lp(a) and chylomicron (CR)[4]. Non-HDL cholesterol levels are calculated by subtracting HDL cholesterol levels from those of total cholesterol.

Through the discovery of sequence variants that affect both cholesterol and the risk of coronary artery disease, genetic studies have yielded targets for drugs for treating dyslipidemia and preventing coronary artery disease[5-10]. Recent advances in DNA sequencing technology provide the means to sequence the genomes of large numbers of individuals allowing for discovery of rare variants. We have previously reported methods to interrogate whole-genomes of large numbers of Icelanders and search for associations with various traits[11]. Here we apply this methodology to search for novel variants that affect non-HDL cholesterol levels and investigate the way in which they affect risk of atherosclerotic diseases like coronary artery disease.

METHODS

Study Participants

Details of the population sample sets from Iceland, Denmark and The Netherlands, used to measure the various lipids traits (non-HDL cholesterol, HDL cholesterol, LDL cholesterol and triglycerides), alkaline phosphatase (ALP) and vitamin B12, are outlined in Table S1 and in the Supplementary Methods. The ten coronary artery disease case-control sample sets that were a part of the study are outlined in Table S2 and Supplementary Methods.

Data Generation and Analysis

Whole-genome sequencing, SNP calling, and imputation

The Icelandic samples were genotyped using Illumina microarrays as previously described[11]. The whole-genomes of 2,636 Icelanders were sequenced using the standard TruSeq methodology (Illumina) to a mean depth of at least 10X (median 20X) as previously described[11] and outlined in Supplementary Methods.

For improved sequencing coverage of the GC rich intron 4 in *ASGR1* gene we analyzed whole-genome sequence data generated for 738 Icelanders using TruSeq PCR-free method from Illumina (mean depth of 30X). In this dataset the del12 variant in intron 4 of *ASGR1* was detected.

Single-Track assay SNP and microsatellite genotyping

We performed single SNP genotyping of rs186021206, using the Centaurus (Nanogen) platform[12]. The del12 variant was genotyped using a PCR based method with the following primers: forward primer (NED labelled) 5´-TTCATCTTTCTTCCCACATTGC-3´, reverse primer 5'-GGGCCTGAGAGAGACGTTCA-3'. An internal size standard was added to the resulting PCR products and the fragments were separated and detected on an Applied Biosystems model 3730 sequencer, using in-house Allele Caller software.

Statistical analyses

Associations between imputed genotypes and serum lipids (non-HDL cholesterol, HDL cholesterol, LDL cholesterol and triglycerides), ALP and vitamin B12 levels in the Icelandic dataset were tested using a generalized linear regression, assuming an additive genetic model as previously described[13,11] and as outlined in Supplementary Methods.

For the Icelandic dataset, logistic regression was used to test for association between the del12 variant and coronary artery disease and myocardial infarction, treating the disease status as the response and the number of copies of del12 in individual carrier as the explanatory variable as outlined in Supplementary Methods. Coronary artery disease case-control association analysis for the non-Icelandic sample sets was done using the NEMO software[14] assuming a multiplicative risk model. Results for the Icelandic and the non-Icelandic sample sets were combined using a Mantel-Haenszel fixed effects model.

To estimate the effect of the del12 variant on myocardial infarction free survival, we estimated the Kaplan-Meier curves for survival to first myocardial infarction in heterozygous carriers and non-carriers as outlined in Supplementary Methods.

Correction for familial relatedness in the Icelandic datasets was carried out using the method of genomic control[15] by dividing the corresponding chi-square statistic by 1.36 for non-HDL cholesterol, 1.57 for HDL cholesterol, 1.40 for triglycerides, 1.53 for ALP, 1.30 for vitamin B12, 1.71 for coronary artery disease and 1.48 for myocardial infarction.

To obtain a reliable imputation of the del12 variant, not easily called from the whole-genome sequence data, we genotyped 3,799 Icelandic individuals for the variant and used those genotypes as a training set for imputation of del12 into the rest of the Icelandic population. The imputation information for del12 was 0.99.

Functional Characterization of the del12 Variant in *ASGR1*

cDNA preparation, amplification, Sanger sequencing and next generation sequencing

RNA was isolated from blood samples from carriers and non-carriers of del12 as outlined in Supplementary Methods. Following cDNA generation, the region between exon 3 and 5 in *ASGR1* was PCR amplified and the identified PCR products (two for del12 carriers and one for non-carriers) Sanger sequenced using standard methodology to determine the sequence difference between the identified cDNA products. To quantify the ratio between the two amplified cDNA PCR products they were sequenced using Illumina MiSeq instrument coupled with the MiSeq v2 reagent kit.

Western blot analysis

The wild type *ASGR1* cDNA and *ASGR1* cDNA with the 22bp deletion were transiently overexpressed in HeLa cells to determine if *ASGR1* transcripts with the 22bp deletion generated stable truncated ASGR1 protein as evaluated by western blot analysis. Details on these experiments are outlined in Supplementary Appendix.

RESULTS

Association of sequence variants with non-HDL cholesterol

We identified 25.3 million sequence variants through whole-genome sequencing of 2,636 Icelanders, with standard TruSeq method (Illumina), to a median depth of 20X. We then imputed these variants (assisted by long-range phased haplotypes) into the genomes of 398,000 Icelanders (Supplementary Appendix). Among the samples with imputed genotypes, 119,146 had information on serum non-HDL cholesterol levels that we used to screen for rare variant associations (Table S1). Part of the results from this analysis are reported elsewhere[16,17]. Here we describe the identification of a novel rare signal represented by a set of seven correlated (pairwise r^2>0.7) non-coding SNPs on chromosome 17p13.1 that associate with non-HDL cholesterol levels. The seven variants span 80kb, including the asialoglycoprotein receptor 1 and 2 (*ASGR1* and *ASGR2*) genes. The strongest association was seen with the minor allele of rs186021206 (allelic frequency=0.43%) located 7.3 kb downstream of *ASGR1* that associates with 12.9 mg/dL (95% CI 8.7 to 17.1) lowering of non-HDL cholesterol (P=1.4×10^{-9}) (Table S3).

Although the associated region was rather well covered by the whole-genome sequencing we observed low coverage in intron 4 of *ASGR1.* This intron is 79bp and very GC rich explaining the low sequence depth. To explore this region further we whole-genome sequenced 738 individuals with a TruSeq PCR-free sequencing method (Illumina) that gave better coverage of the intron and led to the identification of a 12bp deletion within the intron, NM_001671.4:c.284-36_283+33delCTGGGGCTGGGG, hereafter referred to as del12. Following direct genotyping of del12 in 3,799 Icelanders and imputation into the Icelandic dataset (imputation info=0.99), we observed that del12 (MAF=0.41%) is highly correlated with rs186021206 (r^2=0.86) and the other six most strongly associated SNPs (Table S3, Table S4). Furthermore, del12 associates more strongly than any of the seven SNPs with lower non-HDL cholesterol levels (decrease of 13.6 mg/dL, 95% CI 9.4 to 17.7, P=2.5×10^{-10}) (Table 1, Figure 1, Table S3 and Table S4). Del12 likewise associates with LDL cholesterol with a similar effect size as seen for non-HDL cholesterol (Table 1). A common variant upstream of

ASGR1 (rs314253; MAF=35.1%) was reported to associate modestly with LDL cholesterol[19]. This association was replicated in our data and is independent of the del12 signal (r^2<0.001, Table S5). After adjusting for del12, neither rs186021206 nor any of the six SNPs remain significantly associated with non-HDL cholesterol (Table S4), indicating that del12 is sufficient to explain the rare non-HDL cholesterol signal at this locus.

Analysis including additional 5,817 WGS individuals, either sequenced with Illumina TruSeq PCR free or TrueSeq Nano methods, which provides greatly improved coverage of the associated region, supports that del12 is the variant with the strongest association with non-HDL in 1Mb region centered on *ASGR1* (Figure S1).

Del12 also associates with increased HDL cholesterol and decreased triglyceride levels, albeit to a much lesser degree than it affects non-HDL cholesterol and LDL cholesterol (Table 1).

We validated the del12 association in samples from The Netherlands[20] and Denmark[21,22] (Table S1, Table 1 and Table S3) and observed a similar effect size to that seen in Iceland for the non-HDL cholesterol association (P_{het}=0.24, Table 1). When all datasets were combined, including the Icelandic discovery data, we found that del12 associates with lowering of non-HDL cholesterol by 15.3 mg/dL (95% CI 11.7 to 18.9 and P=1.0×10^{-16}).

Del12 within intron 4 of *ASGR1* causes a splicing defect resulting in a frameshift and introduction of premature stop codon

Since del12 is located in intron 4 of *ASGR1*, we examined its effect on splicing between exons 4 and 5. We PCR amplified the region between exons 3 and 5 in cDNA generated from blood samples of 12 heterozygous carriers of del12 and 12 non-carriers (Figure 2). We found the expected 239bp band in non-carriers. In del12 carriers, however, a smaller 217bp band was noted in addition to the expected 239bp PCR product (Figure 2B). Upon Sanger sequencing of the 217bp cDNA product we found a 22bp deletion at the end of exon 4 (Figure 2C). The deletion of these 22bp from the *ASGR1* transcript appears to be driven by a pseudo donor-splice site in exon 4 (Figure 2D). To quantify the transcripts with this

splicing defect we used the Illumina TruSeq method for direct digital counting of sequencing reads that were generated by sequencing the two cDNA products detected from del12 heterozygous carriers. All del12 carriers (n=13) generated incorrectly spliced isoform that represents on average an estimated fraction of 30.8% (1st quartile=23.9%, 3rd quartile=34.3%) of the total *ASGR1* transcripts in the blood cells of these carriers (Figure 2E). The estimated fraction of incorrectly spliced isoform is significantly different in carriers from that in non-carriers (P=1.8×10^{-6}, Wilcoxon-Mann-Whitney test). The 22bp deletion is predicted to cause a frameshift in *ASGR1* and introduction of a premature stop codon at amino acid 89 out of the 291 amino acid in the full-length protein (Figure S2).

It is well known that nonsense mediated decay (NMD) is responsible for eliminating aberrant mRNA transcripts with premature stop codons such as the one generated by del12. Since ~30.8% of the *ASGR1* transcripts in the blood of heterozygous carriers have the 22bp frameshift deletion, these transcripts are not fully eliminated by NMD in blood cells, although such an effect cannot be ruled out in other tissues such as the liver which is the main tissue of expression for *ASGR1*[23]. If translated, the mutated *ASGR1* transcript should generate a truncated protein lacking two thirds of the full length protein (Figure S2). To determine whether such a protein is generated in cells we transiently overexpressed the *ASGR1* cDNA harboring the 22bp deletion and wild type *ASGR1* in HeLa cells. Western blot analysis detected high levels of the wild type protein but not the truncated form of ASGR1 (Figure 2F) despite both types of transcripts being highly transcribed (data not shown). However, when the transfected cells were treated with a proteasome inhibitor, which blocks degradation of truncated and misfolded proteins, the truncated protein could be detected (Figure 2F, Figure S3). This demonstrates that the truncated ASGR1 protein is degraded as is commonly observed for such proteins[24,25] and supports a loss-of-function effect of del12 on *ASGR1*.

Association of del12 with serum levels of alkaline phosphatase and vitamin B12

ASGR1 is the major subunit of the hepatic asialoglycoprotein receptor (ASGPR, also known as the Ashwell receptor), known to recognize and mediate the endocytosis and degradation of a wide variety

of desialylated glycoproteins that contain terminal galactose (Gal) or N-acetylgalactosamine (GalNAc) residues on their N-linked carbohydrate chains[26-29]. The Gal or GalNAc residues of glycoproteins are exposed by removal of sialic acid by sialidases, hence the term asialoglycoprotein for the ligands of ASGPR receptors.

To evaluate if serum level of any sialylated glycoprotein were altered in carriers of del12 we tested the association of del12 with serum levels of various substances that are routinely measured at hospitals and clinical laboratories in Iceland (Supplementary Appendix). Apart from the association with blood lipids we observed a highly significant association of del12 with higher levels of circulating alkaline phosphatase (ALP) (50.1% increase that corresponds to 43.6 U/L increase, 95% CI 37.3 to 49.8, $P=3.6\times10^{-63}$) and vitamin B12 (16.6% increase that corresponds to 66.1 pmol/L increase, 95% CI 45.8 to 85.6, $P=3.1\times10^{-12}$) (Table 1 and Table S3). Region plots for the association of ALP and vitamin B12 mirrored that of the non-HDL cholesterol association, with del12 demonstrating the strongest association for all three traits (Figure S4), however, with opposite effects on non-HDL cholesterol (decreased levels) on one hand and ALP and vitamin B12 (increased levels) on the other hand. Furthermore, the common variant previously known to associate with decreased LDL cholesterol has also been reported to associate with moderately increased ALP[18] which is consistent with our results (Table S5).

We replicated, with comparable effect sizes, the del12 association with higher levels of ALP and vitamin B12 in individuals from Denmark (combined $P=5.6\times10^{-69}$ for ALP and $P=8.3\times10^{-14}$ for vitamin B12) (Table 1 and Table S3).

In both the Icelandic and the Danish datasets ALP level is positively correlated with non-HDL cholesterol level whereas no correlation was observed for vitamin B12. Furthermore, after adjusting the association of del12 with non-HDL cholesterol for ALP, the non-HDL cholesterol association becomes stronger indicating that del12 association with non-HDL cholesterol is independent of ALP

(Table S6). Adjusting the association of non-HDL cholesterol for vitamin B12 did not affect the association (Table S6).

An increase in ALP levels may reflect liver disease, however, there was no association of del12 with serum gamma glutamyl transferase (GGT), bilirubin, alanine aminotransferase or other measures of liver function that commonly parallel changes in ALP in liver disease (Table S7). Both ALP and the vitamin B12 transporter, haptocorrin, are sialylated glycoproteins known to bind ASGPR and to be cleared from the circulation by the receptor[30-33]. The increase in ALP and vitamin B12 levels in del12 carriers likely reflects decreased clearance of desialylated forms of those molecules from the circulation as a result of reduced number of functional ASGPR receptors in del12 carriers.

The del12 variant in *ASGR1* and risk of coronary artery disease

Given the effect of del12 on non-HDL cholesterol levels, we assessed its impact on the risk of coronary artery disease in 33,090 cases and 236,254 controls from Iceland and 8,558 cases and 11,120 controls from the USA, UK, New Zealand and Denmark. Del12 associates with lower risk of coronary artery disease in the Icelandic set (odds ratio 0.64; 95% CI 0.51 to 0.80; P=5.8×10^{-5}) and in the non-Icelandic sets (odds ratio 0.69; 95% CI 0.51 to 0.95; P=0.022) for a combined odds ratio of 0.66 (95% CI 0.55 to 0.79 and P of 4.0×10^{-6}) (Figure 3A). There was no evidence of heterogeneity across the eight study populations (P_{het}=0.96). Del12 also decreased risk of myocardial infarction in Iceland (hazard ratio 0.64; 95% CI 0.64 to 0.80; P=8.5×10^{-5}) (Figure 3B). In agreement with the protective effect on coronary artery disease, del12 carriers have a lifespan that is 1.5 years longer than that of non-carriers (95% CI 0.2 to 2.8 years; P=0.020). We did not observe an association of del12 with other known coronary artery disease risk factors such as hypertension, smoking, obesity or type 2 diabetes (Table S8).

There is a strong positive correlation between the effects of lipid associated sequence variants on non-HDL cholesterol levels and risk of coronary artery disease[7-10,34] (Figure 4 and Table S9). However, several published variants deviate from the overall trend. For example, *LPA* and *ANGPTL4* variants have a substantially greater effect on coronary artery disease than their non-HDL cholesterol effects would

predict, while the effect of the *APOE* variants is weaker than predicted by the non-HDL cholesterol effect (Figure 4, Table S9). Del12 in *ASGR1* is still another example of a variant whose effect on coronary artery disease is stronger than predicted by the effect on non-HDL cholesterol (Figure 4). When adjusting the coronary artery disease and myocardial infarction associations for ALP the associations become stronger whereas adjusting for vitamin B12 has no effect (Table S10). This demonstrates that the additional artheroprotective effects of del12 is not mediated through increase in either ALP or vitamin B12.

An additional rare loss of function sequence variant in *ASGR1*

To look for additional loss of function variants in *ASGR1* we screened an extended dataset based on sequence variants identified through WGS of additional group of 5,817 WGS Icelanders on top of the 2,636 described above (Supplementary Appendix). In this dataset we identified an additional rare loss of function variant, a four bp insertion (MAF=0.027%; NM_001671.4:c.469_472dupAACT) that introduces a stop codon at amino acid 158 out of the 291 amino acid full length protein (NP_001662.1:p.W158X). We directly genotyped potential carriers and non-carriers by Sanger sequencing, and used those genotypes to re-impute p.W158X into 150,656 Icelandic chip-typed individuals and their first and second degree relatives (imputation info = 1.0). The p.W158X variant associates with decrease in non-HDL cholesterol (-24.9mg/dL; 95% CI -40.6 to -9.3; P=1.8×10^{-3}) and increase in ALP (45.3% increase, 95% CI 20.4 to 68.2, P=7.9×10^{-6}) (Table S11). The direction of the effects of p.W158X and the effect sizes are similar to that of del12 (Table S11). Given that we are performing a single test these results provide a replication of the *ASGR1* loss of function effect on non-HDL and ALP. For coronary artery disease in Iceland the odds ratio for p.W158X is 0.65 (95% CI 0.26 to 1.40; P=0.24). The p.W158X variant is independent of del12 as none of the 79 carriers found in Iceland carried del12. The variant also appears to be specific to the Icelandic population as it is not reported in large population databases such as (Exact Aggregation Consortium (ExAC,

http://exac.broadinstitute.org), Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS), Genomes of the Netherlands (GoNL, http://nlgenome.nl) and dbSNP (http:// ncbi.nlm.nih.gov/SNP).

DISCUSSION

Through whole-genome sequencing of the Icelandic population we found a rare 12 bp deletion, del12, in intron 4 of the *ASGR1* gene, a deletion that is also present in other European ancestry populations. Del12 associates with lower non-HDL cholesterol levels and protection against coronary artery disease in European populations and in line with the protection against coronary artery disease, heterozygote carriers of del12 live on average 1.5 years longer than non-carriers. Del12 activates a cryptic splice site that leads to a 22bp deletion from the mature mRNA at the end of exon 4 that causes a frameshift introducing a premature stop codon at amino acid 89 out of the 291 amino acid full length protein. We show that the truncated protein is degraded in cells underscoring that del12 is a loss of function mutation. ASGR1 oligomerizes with ASGR2 to form ASGPR that mediates the binding and endocytosis of a wide range of asialoglycoproteins. In line with a loss of function effect of del12, the variant associates with increased levels of the asialoglycoprotein ALP in the circulation. A further support for the loss of function effect of ASGR1 comes from identification of a second rare (0.027%) four bp insert that introduces a stop mutation p.W158X This variant has comparable effects on non-HDL cholesterol and ALP as del12.

Although the ASGPR and its ability to mediate endocytosis and degradation of desialylated glycoproteins has been known for nearly 4 decades, the endogenous ligands and the physiological function of the receptor has been difficult to establish[35]. *Asgr1*$^{-/-}$ mice (which lack any Asgpr activity) thrive normally and do not accumulate desialylated glycoproteins in their circulation although they are unable to clear exogenously added asialoglycoproteins This suggests that under normal physiological conditions, Asgpr is not essential for homeostasis of circulating asialoglycoproteins in the mouse[32] while our results indicate that it may be so in man. We established a clear role for human ASGR1 in the control of non-HDL cholesterol levels and risk of coronary artery disease and myocardial infarction. We also demonstrated that the ASGPR receptor regulates the endogenous levels of at least some asialoglycoproteins as *ASGR1* del12 loss of function mutation increases the levels of ALP and vitamin

B12; both ALP and the vitamin B12 transporter, haptocorrin, are asialylated glycoproteins known to bind ASGPR and to be cleared from the circulation by the receptor[30-33]. It is unlikely that the ALP increase mediated by del12 reflects an underlying liver disease since other measures of liver function are not affected in del12 carriers. The more likely explanation for the increase, is decreased clearance of desialylated molecules from the circulation due to reduced number of functional ASGPR receptors in del12 carriers.

The del12 and the p.W158X mutations have effects on non-HDL cholesterol levels that are opposite to their effects on ALP and vitamin B12 levels; decreasing non-HDL cholesterol and increasing ALP and vitamin B12. It is important to note that the common variant previously described that associates with ALP and LDL cholesterol also has opposing effects on these serum components[18,19]; hence ASGR1 likely affects the circulating level of these molecules through different mechanisms. The decreased levels of non-HDL cholesterol in del12 and p.W158X carriers in the face of reduced *ASGR1* function suggest that ASGR1 affects non-HDL cholesterol levels by mechanisms other than direct binding and endocytosis of cholesterol particles. Some insight into the mechanism through which ASGR1 may regulate non-HDL cholesterol levels has come from mice expressing a hypomorphic form of neuraminidase 1 (Neu1), a sialidase that cleaves the sialic acid residues thereby generating substrates for ASGR1. In the hypomorphic mouse, LDL receptor (Ldlr) is sialylated and this form of the receptor is more stable and takes up LDL cholesterol more avidly (LDL levels were decreased in these mice) than the asialylated form of Ldlr in the wild type mouse [37]. Reduced expression of Neu1 thus reduces Ldlr recycling leading to enhanced uptake by the liver of LDL cholesterol. Both ASGPR and LDLR are located in clathrin-coated pits on hepatocytes and may be ASGPR interacts with the asialylated form of the LDLR leading to LDLR uptake by hepatocytes through endocytosis.

We demonstrated that the *ASGR1* del12 variant, like variants in *ANGPTL4* and *LPA*, has a larger impact on coronary artery disease risk than is predicted by the non-HDL cholesterol effect, suggesting atheroprotective effects of del12 in addition to lowering serum cholesterol levels. We show that this

additional artheroprotective effect is neither mediated through increase in ALP nor vitamin B12, as adjusting the coronary artery disease and myocardial infarction association for ALP strengthens del12 association and vitamin B12 has no effect. Studies have shown that sialylation can play a key role in the recruitment of inflammatory cells to atherosclerotic plaques via effects on chemokines or their receptors[38-40]. The recruitment of inflammatory cells could thus be affected in del12 carriers and mediate atheroprotective effect.

In conclusion, we have identified novel rare loss of function variants in *ASGR1* that lower non-HDL cholesterol levels and protects against coronary artery disease. These variants disrupts ASGR1 function and highlights a link between the sialylation pathway and atherosclerotic diseases. Our findings suggest that targeting ASGR1 may be a feasible atheroprotective strategy.

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Table 1. Association of del12 with Non-HDL Cholesterol, LDL Cholesterol, HDL Cholesterol, Triglyceride, ALP and Vitamin B12 in Iceland, Denmark and The Netherlands

	Study population (n)		Effect (95% CI)[a]	*P* value	Population mean value[e] (± 1SD)
	Non-HDL cholesterol	**del12 freq. (%)**	**mg/dL**		**mg/dL**
Discovery	Iceland (119,146)	0.41	-13.6 (-17.7,-9.4)	2.5×10^{-10}	154.7 (109.1-200.3)
Replication	Denmark A[b] (6,182)	0.22	-21.3 (-36.8,-5.9)	0.0069	161.6 (117.5-205.7)
Replication	Denmark B[c] (9,656)	0.32	-22.2 (-32.8,-11.7)	3.8×10^{-5}	164.7 (124.0-205.4)
Replication	The Netherlands[d] (5,537)	0.50	-17.0 (-28.3,-5.7)	0.0032	170.7 (129.4-212.0)
	Combined		-15.3 (-18.9,-11.7)	1.0×10^{-16}	
	LDL cholesterol		**mg/dL**		
Discovery	Iceland (53,841)	0.41	-9.5 (-14.0,-5.1)	2.8×10^{-5}	133.0 (91.6-174.4
Replication	Denmark A (6,098)	0.22	-22.1 (-35.5,-8.7)	0.0012	137.2 (99.7-174.7)
Replication	Denmark B (8,080)	0.32	-19.0 (-29.2,-8.8)	0.00026	139.3 (101.9-176.1)
Replication	The Netherlands (5,523)	0.50	-16.0 (-26.1,-6.0)	0.0018	138.6 (102.2-175.0)
	Combined		-12.5 (-16.2,-8.8)	3.9×10^{-11}	
	HDL cholesterol		**mg/dL**		**mg/dL**
Discovery	Iceland (119,514)	0.41	2.4 (0.7,4.1)	0.0058	54.7 (37.7-71.7)
Replication	Denmark A (6,182)	0.22	4.6 (-0.8,9.9)	0.096	54.2 (38.4-70.0)
Replication	Denmark B (9,656)	0.32	2.4 (-1.8,6.7)	0.26	60.0 (43.6-76.4)
Replication	The Netherlands (5,537)	0.50	2.4 (-1.3,6.0)	0.20	52.6 (39.2-66.0)
	Combined		2.5 (1.1,4.0)	0.00039	
	Triglyceride		**% change**		**mg/dL**
Discovery	Iceland (80,011)	0.41	-6.1 (-10.8,-1.5)	0.012	133.6 (67.6-190.5)
Replication	Denmark A (6,182)	0.22	-6.0 (-25.2,11.4)	0.53	105.8 (60.8-183.9)
Replication	Denmark B (8,163)	0.32	-8.9 (-21.0,2.3)	0.15	117.4 (73.5-187.3)
Replication	The Netherlands (5,537)	0.50	-4.4 (-17.9,8.2)	0.52	155.8 (94.5-256.8)
	Combined		-6.3 (-10.3,-2.3)	0.0032	
	ALP		**% change**		**U/L**
Discovery	Iceland (126,060)	0.41	50.1 (42.9,57.2)	3.6×10^{-63}	87.1 (53.5-141.7)
Replication	Denmark A[c] (5,829)	0.22	29.1 (14.8,42.5)	3.1×10^{-6}	41.3 (30.7-55.6)
	Combined		46.5 (40.1,52.7)	5.6×10^{-69}	
	Vitamin B12		**% change**		**pmol/L**
Discovery	Iceland (97,910)	0.41	16.6 (11.5,21.5)	3.1×10^{-12}	398 (256-618)
Replication	Denmark A[c] (5,826)	0.22	18.6 (3.9,32.4)	0.0053	398 (286-554)
	Combined		16.8 (12.0,21.5)	8.3×10^{-14}	

[a]Effect estimates and 95% confidence intervals (95% CI) in mg/dL for the non-HDL cholesterol and HDL cholesterol and as percentage change for triglyceride, ALP and vitamin B12. [b]The Danish Inter99 study (Jørgensen et al. 2003). [c]The Danish Addition study (van den Donk et al. 2011). [d]The Nijmegen Biomedical Study (Hoogendoorn et al. 2006). [e]For triglyceride, ALP and vitamin B12, the population mean and the SD are calculated for log-transformed values and transformed back to original units. To convert the values for non-HDL and HDL cholesterol to mmol/L, multiply by 0.02586. To convert triglyceride to mmol/L, multiply by 0.01129.

FIGURE LEGENDS

Figure 1. Regional association plot for a 1 Mb region on chromosome 17 centered on del12.

The figure shows the -$\log_{10}$ P-values (left vertical axis) for variant association with non-HDL in the Icelandic samples against their position. The purple circle indicates del12 and circles corresponding to other SNPs are color coded to reflect their LD with del12. The red line indicates recombination rates (right axis), based on the Icelandic recombination map for males and females[41] combined with the peaks indicating recombination hotspots defining LD blocks. Known genes in the region are shown underneath the plot, taken from the UCSC genome browser. All positions are in NCBI Build 36 coordinates. The plot was created using a standalone version of the LocusZoom software (http://csg.sph.umich.edu/locuszoom/)[42].

Figure 2. The del12 Variant Creates a Splicing Error and Frameshift in *ASGR1*

(A) Overview of the structure of the *ASGR1* mRNA. Exons 4 (red) and 5 (green) are highlighted (the del12 variant lies within intron 4 between exons 4 and 5 in the unspliced RNA) along with the positions of the PCR primers (red arrows) used to amplify the cDNA. (B) Agarose gel showing the PCR products produced by amplifying cDNA generated from RNA isolated from the blood of del12 heterozygous carriers and non-carriers. Arrows indicate the size of the expected PCR product (239bp) and the size of the truncated band (217bp) observed only in del12 heterozygote carriers. (C) Shown is the sequence difference, based on Sanger sequencing, between the full-length (239bp) and truncated (217bp) cDNA fragments. The truncated fragment in del12 carriers lacks 22bp at the end of exon 4 that results in a frameshift and an introduction of a stop codon. (D) Diagrammatic representation of the splicing defect observed in del12 carriers. The sequence around the exon 4-intron 4 boundary (exon 4 sequence in capital letters and intron 4 sequence in small letters) is shown along with the 5´splice site in non-carriers (green) and the cryptic 5' splice site activated in del12 carriers (red). (E) Quantification of the cDNA fragments with the 22bp deletion in heterozygote del12 carriers and non-carriers was done by

direct digital counting of sequencing reads using the Illumina TruSeq method. The estimate of the fraction of incorrectly spliced isoforms out of the total *ASGR1* transcripts was based on the ratio of read coverage over the 22 base pairs at the end of exon 4 and the median coverage over exon 5. The estimated fraction of incorrectly spliced isoforms was significantly different between non-carriers and carriers (P=1.8×10^{-6}, Wilcoxon-Mann-Whitney test). (F) Western blot analysis of HeLa cells transiently overexpressing wild type ASGR1 cDNA (WT) or mutant ASGR1 cDNA lacking the 22bp at the end of exon 4 (22bp_del). In the last lane HeLa cells transfected with the 22bp_del cDNA were treated with the proteosome inhibitor MG-132 for 4.5 hours. The ASGR1 antibody used recognizes amino acids 1-41 of the ASGR1. The middle panel has a longer exposure time than the two other to detect the truncated ASGR1 protein following treatment with MG-132.

Figure 3. The *ASGR1* del12 Protects against Coronary Artery Disease and Delays Myocardial Infarction Onset.

(A) The del12 variant was typed in the indicated populations for a total of 41,648 coronary artery disease cases and 247,374 controls. For each sample set, the square indicates the estimated odds ratio and the line shows the 95% confidence interval. There was no evidence of heterogeneity across the eight study populations (P_{het} = 0.96). (B) Kaplan–Meier curves for survival to first myocardial infarction in heterozygous carriers and non-carriers of del12 stratified by sex. The proportion of individuals that have not had a myocardial infarction is shown on the y-axis and plotted against age on the x-axis. Males and females are represented separately and a distinction is made between del12 carriers and non-carriers in each case.

Figure 4. The Relationship Between the Effect of Sequence Variants on Non-HDL-Cholesterol and their Effect on Coronary Artery Disease Risk

Based on the Icelandic population, the estimated odds ratio (OR) of the minor allele for coronary artery disease (coronary artery disease, 41,648 cases and 247,374 controls) as a function of the estimated effect of the minor allele on non-HDL cholesterol levels (N=119,146). A full list of the sequence variants included is provided in Table S7 in the Supplementary Appendix and they were derived from Do *et al*[34]. The error bars represent 95% confidence intervals. The del12 variant in *ASGR1* is shown in blue. The red line indicates the best linear regression fit through the origin.

Figure 1

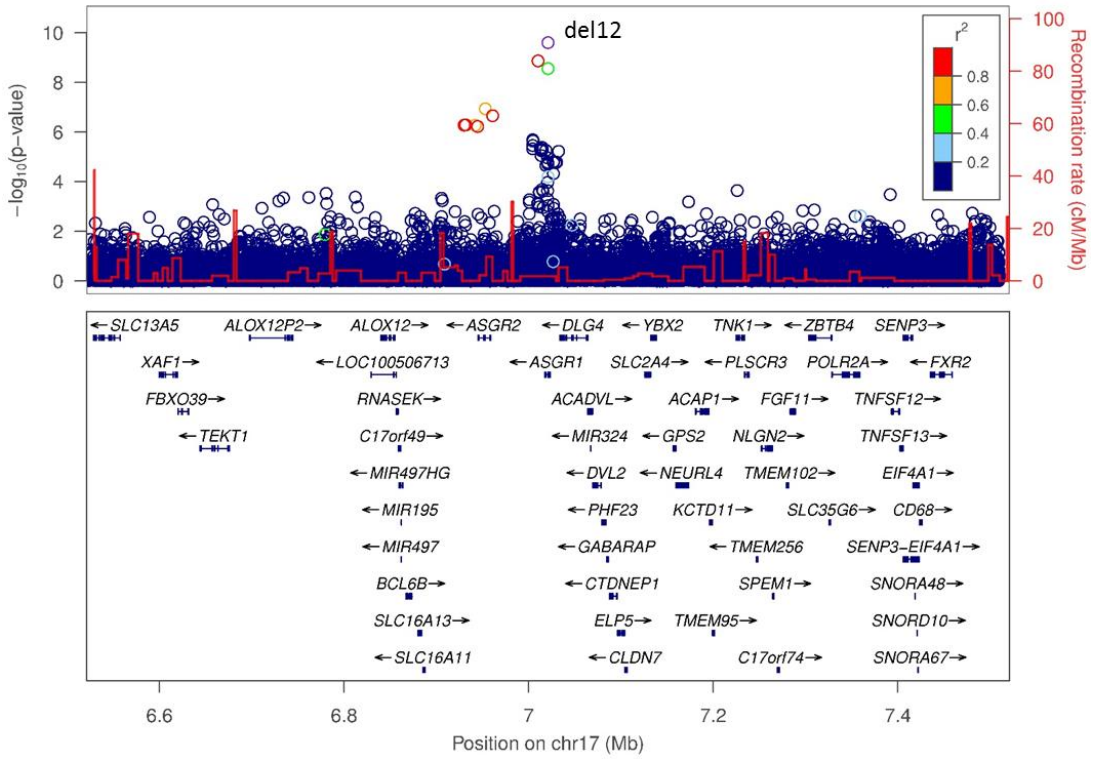
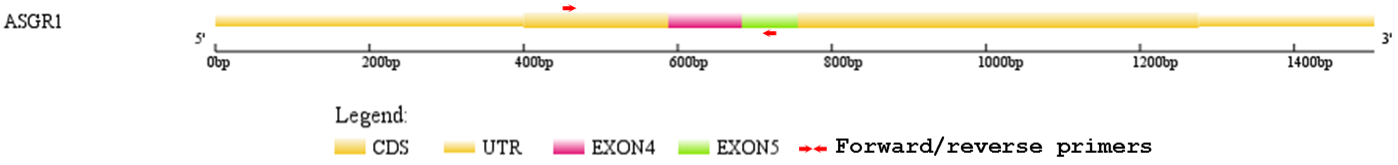
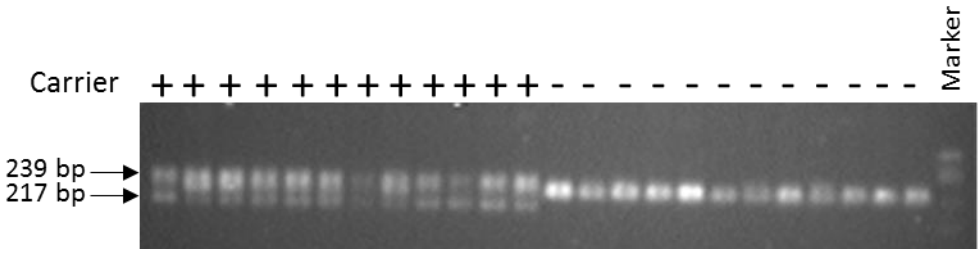

del12
r²
0.8
0.6
0.4
0.2
−log10(p−value)
Recombination rate (cM/Mb)
SLC13A5 ALOX12P2 ALOX12 ASGR2 DLG4 YBX2 TNK1 ZBTB4 SENP3
XAF1 LOC100506713 ASGR1 SLC2A4 PLSCR3 POLR2A FXR2
FBXO39 RNASEK ACADVL ACAP1 FGF11 TNFSF12
TEKT1 C17orf49 MIR324 GPS2 NLGN2 TNFSF13
MIR497HG DVL2 NEURL4 TMEM102 EIF4A1
MIR195 PHF23 KCTD11 SLC35G6 CD68
MIR497 GABARAP TMEM256 SENP3-EIF4A1
BCL6B CTDNEP1 SPEM1 SNORA48
SLC16A13 ELP5 TMEM95 SNORD10
SLC16A11 CLDN7 C17orf74 SNORA67
6.6
6.8
7
7.2
7.4
Position on chr17 (Mb)

Figure 2

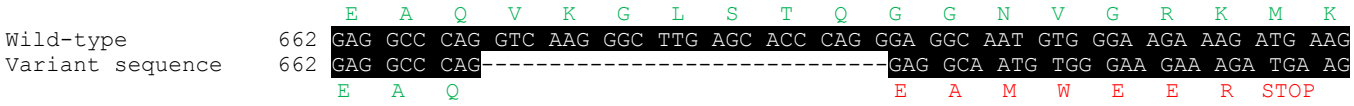
A.



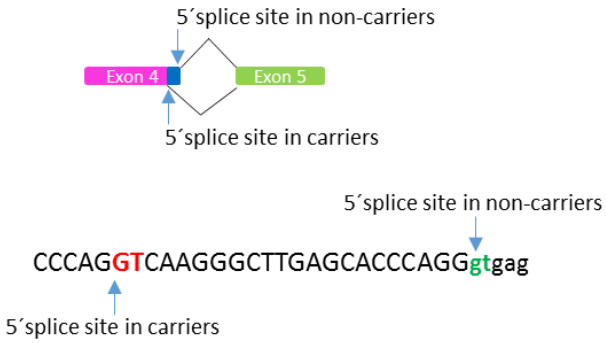
B.



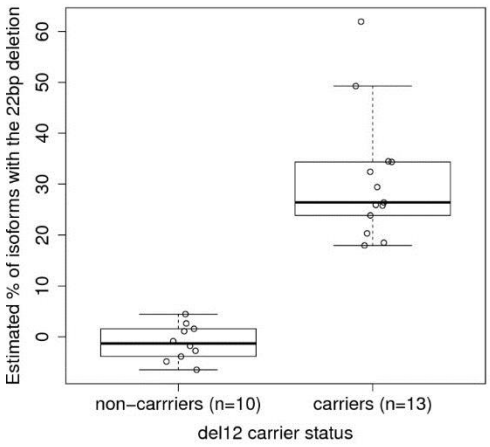
C.



D.



E.



F.

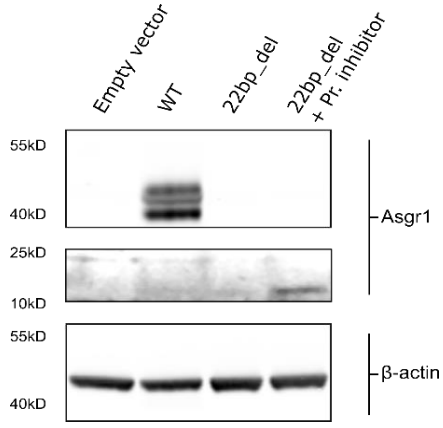
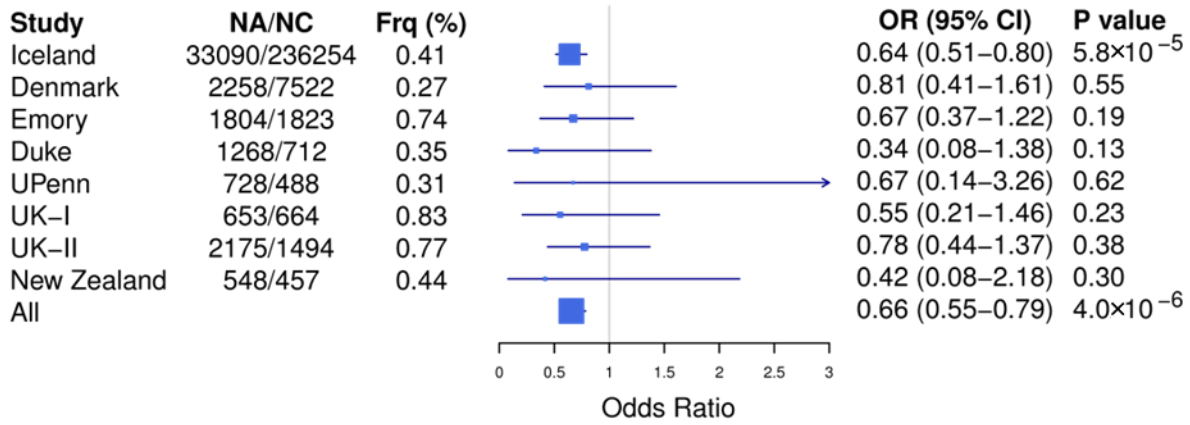


Figure 3

A.


Study
NA/NC
Frq (%)
OR (95% CI)
P value
Iceland
33090/236254
0.41
0.64 (0.51–0.80)
5.8×10 −5
Denmark
2258/7522
0.27
0.81 (0.41–1.61)
0.55
Emory
1804/1823
0.74
0.67 (0.37–1.22)
0.19
Duke
1268/712
0.35
0.34 (0.08–1.38)
0.13
UPenn
728/488
0.31
0.67 (0.14–3.26)
0.62
UK–I
653/664
0.83
0.55 (0.21–1.46)
0.23
UK–II
2175/1494
0.77
0.78 (0.44–1.37)
0.38
New Zealand
548/457
0.44
0.42 (0.08–2.18)
0.30
All
0.66 (0.55–0.79)
4.0×10 −6
0
0.5
1
1.5
2
2.5
3
Odds Ratio

B.

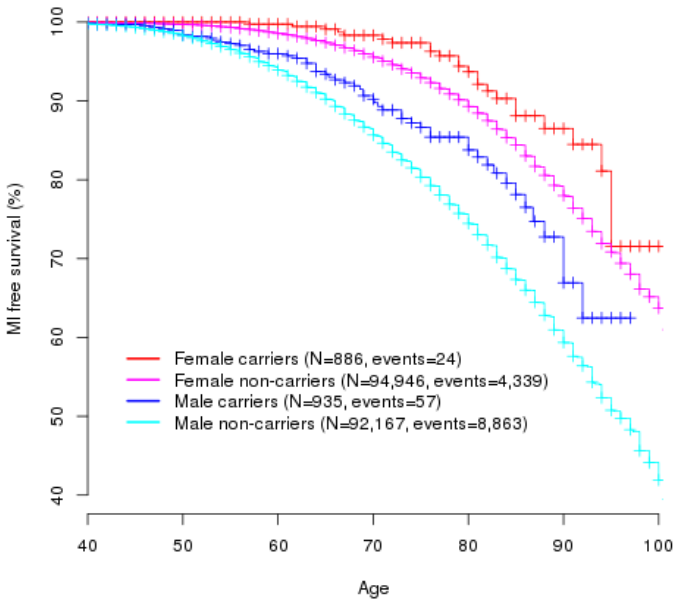
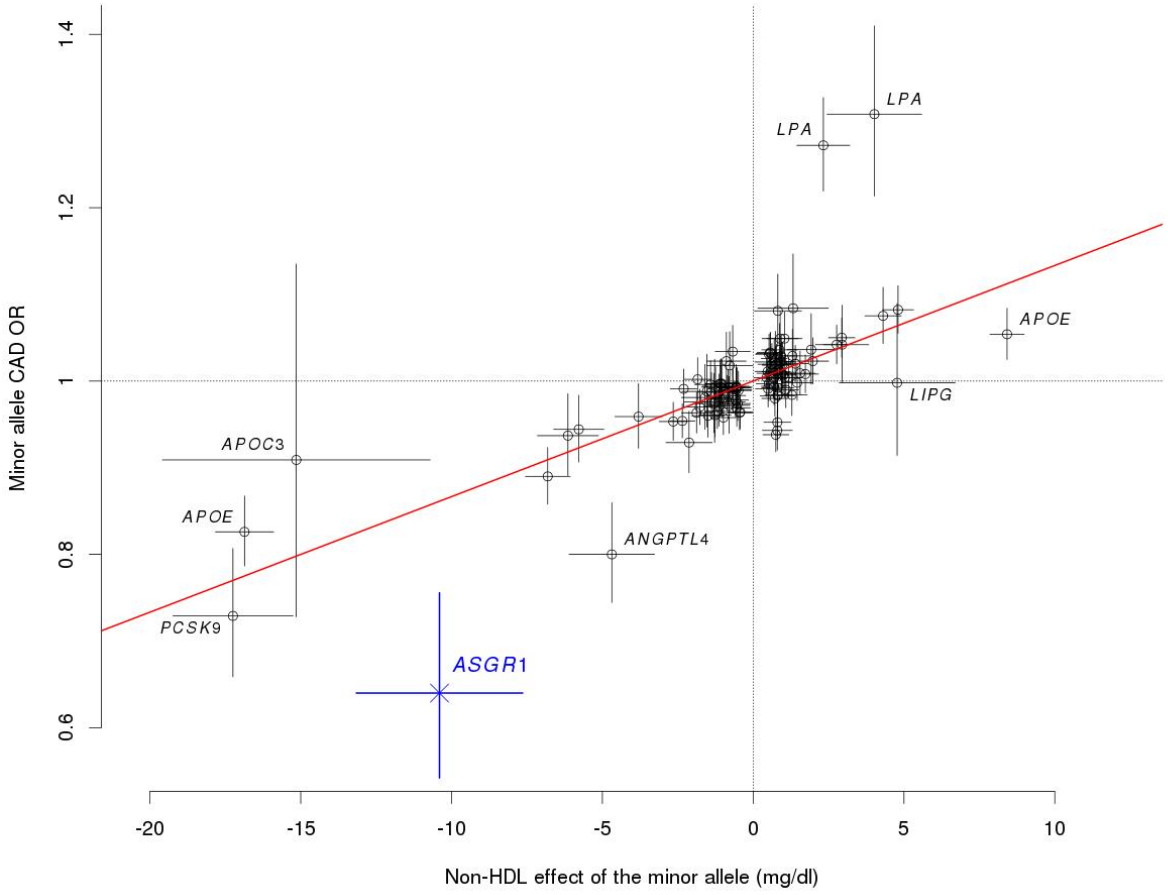

MI free survival (%)
100
90
80
70
60
50
40
Female carriers (N=886, events=24)
Female non-carriers (N=94,946, events=4,339)
Male carriers (N=935, events=57)
Male non-carriers (N=92,167, events=8,863)
40
50
60
70
80
90
100
Age

Figure 4


Minor allele CAD OR
1.4
1.2
1
0.8
0.6
LPA
LPA
APOE
LIPG
APOC3
APOE
ANGPTL4
PCSK9
ASGR1
-20
-15
-10
-5
0
5
10
Non-HDL effect of the minor allele (mg/dl)