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ORIGINAL ARTICLE

Variant *ASGR1* Associated with a Reduced Risk of Coronary Artery Disease

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ABSTRACT

BACKGROUND

Several sequence variants are known to have effects on serum levels of non–high-density lipoprotein (HDL) cholesterol that alter the risk of coronary artery disease.

METHODS

We sequenced the genomes of 2636 Icelanders and found variants that we then imputed into the genomes of approximately 398,000 Icelanders. We tested for association between these imputed variants and non-HDL cholesterol levels in 119,146 samples. We then performed replication testing in two populations of European descent. We assessed the effects of an implicated loss-of-function variant on the risk of coronary artery disease in 42,524 case patients and 249,414 controls from five European ancestry populations. An augmented set of genomes was screened for additional loss-of-function variants in a target gene. We evaluated the effect of an implicated variant on protein stability.

RESULTS

We found a rare noncoding 12-base-pair (bp) deletion (del12) in intron 4 of *ASGR1*, which encodes a subunit of the asialoglycoprotein receptor, a lectin that plays a role in the homeostasis of circulating glycoproteins. The del12 mutation activates a cryptic splice site, leading to a frameshift mutation and a premature stop codon that renders a truncated protein prone to degradation. Heterozygous carriers of the mutation (1 in 120 persons in our study population) had a lower level of non-HDL cholesterol than noncarriers, a difference of 15.3 mg per deciliter (0.40 mmol per liter) ($P=1.0\times10^{-16}$), and a lower risk of coronary artery disease (by 34%; 95% confidence interval, 21 to 45; $P=4.0\times10^{-6}$). In a larger set of sequenced samples from Icelanders, we found another loss-of-function *ASGR1* variant (p.W158X, carried by 1 in 1850 persons) that was also associated with lower levels of non-HDL cholesterol ($P=1.8\times10^{-3}$).

CONCLUSIONS

ASGR1 haploinsufficiency was associated with reduced levels of non-HDL cholesterol and a reduced risk of coronary artery disease. (Funded by the National Institutes of Health and others.)

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Epidemiologic and genetic studies have shown a causal link between levels of non–high-density lipoprotein (non-HDL) cholesterol and of low-density lipoprotein (LDL) cholesterol and the risk of coronary artery disease and myocardial infarction.[1-3] Non-HDL cholesterol has been shown to be a better predictor of cardiovascular risk than LDL cholesterol, since it encompasses all cholesterol-containing proatherogenic lipoproteins, including LDL cholesterol, very-low-density lipoprotein, intermediate-density lipoprotein, lipoprotein(a), and chylomicron.[4] Non-HDL cholesterol levels are calculated by subtracting HDL cholesterol levels from total cholesterol levels.

Through the discovery of sequence variants that affect both cholesterol and the risk of coronary artery disease, genetic studies have provided targets for the development of drugs to treat dyslipidemia and thereby prevent coronary artery disease.[5-10] In our search for new variants that affect non-HDL cholesterol levels, we applied a method[11] of interrogating whole genomes in samples obtained from a large number of Icelanders. We then tested for association between implicated variants and the risk of coronary artery disease.

METHODS

STUDY PARTICIPANTS

Details regarding the population sample sets from Iceland, Denmark, and the Netherlands that were used to measure the various lipid traits (non-HDL cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides), alkaline phosphatase, and vitamin B_{12} are provided in the Methods section and Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org. Details regarding the 10 case–control sample sets are provided in the Methods section and Table S2 in the Supplementary Appendix.

DATA GENERATION AND ANALYSIS

We used Illumina microarrays to genotype the Icelandic samples, as described previously.[11] We sequenced the genomes of 2636 Icelanders using the standard TruSeq methods (Illumina) to a mean depth of at least 10× (median, 20×), an analysis that has also been described previously[11] and is described in the Methods section in the Supplementary Appendix. We generated sequence data from 738 Icelanders using the TruSeq polymerase-chain-reaction (PCR)–free method (Illumina; mean depth, 30×) to improve coverage of the GC-rich sequence of intron 4 in *ASGR1*, which encodes a subunit of the asialoglycoprotein receptor.

To obtain a reliable imputation of a variant with a noncoding 12-base-pair (bp) deletion (del12) that is not easily called from the whole-genome sequence data, we genotyped samples obtained from 3799 Icelanders and used those genotypes as a training set for imputation of del12 in the rest of the Icelandic population. The imputation information for del12 was 0.99. (Details regarding genotype imputation are provided in the Methods section in the Supplementary Appendix.) For a second loss-of-function variant, p.W158X, we performed Sanger sequencing on samples obtained from 345 participants that included 79 carriers and 270 noncarriers of the p.W158X variant and used the genotypes to improve the imputation of the variant in the Icelandic data set. The imputation information for p.W158X was 1.00. (Details are provided in the Methods section in the Supplementary Appendix.)

STATISTICAL ANALYSIS

We tested associations between imputed genotypes and levels of serum lipids, alkaline phosphatase, and vitamin B_{12} in the Icelandic data set using generalized linear regression, assuming an additive genetic model, as described previously[11,12] and as outlined in the Methods section in the Supplementary Appendix.

For the Icelandic data set, we used logistic regression to test for association between the del12 variant and coronary artery disease and myocardial infarction, treating disease status as the outcome and the number of copies of del12 in individual carriers as the explanatory variable. We used NEMO software[13] and assumed a multiplicative risk model when testing for an association between del12 and coronary artery disease in the non-Icelandic samples. We combined the results for the Icelandic and the non-Icelandic sample sets using a Mantel–Haenszel fixed-effects model.

To estimate the effect of the del12 variant on myocardial infarction-free survival, we generated Kaplan–Meier curves for the time until the first

myocardial infarction in heterozygous carriers and noncarriers. Details regarding this analysis are provided in the Methods section in the Supplementary Appendix. We corrected for familial relatedness in the Icelandic data sets using the method of genomic control[14] 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 alkaline phosphatase, 1.30 for vitamin B_{12}, 1.71 for coronary artery disease, and 1.48 for myocardial infarction.

RESULTS

ASSOCIATION OF SEQUENCE VARIANTS WITH NON-HDL CHOLESTEROL

We obtained 25.3 million sequence variants by sequencing the genomes of 2636 Icelanders and then imputed those variants into the genomes of approximately 398,000 living and deceased Icelanders. Among the samples with imputed genotypes, 119,146 had information on serum non-HDL cholesterol levels (Table S1 in the Supplementary Appendix). Some analyses that we carried out on these data have been reported previously.[15,16] We identified a set of seven correlated noncoding single-nucleotide polymorphisms (SNPs) on chromosome 17p13.1 (pairwise $r^2>0.70$) that have association with non-HDL cholesterol levels. The seven variants span 80 kb, including *ASGR1* and *ASGR2*, which encode subunits of the asialoglycoprotein receptor. The strongest association was seen with the minor allele of rs186021206 located 7.3 kb downstream from *ASGR1* (minor allele frequency, 0.43%). This allele is associated with a lowering of non-HDL cholesterol by 12.9 mg per deciliter (95% confidence interval [CI], 8.7 to 17.1 [0.33 mmol per liter; 95% CI, 0.22 to 0.44]; $P=1.4\times10^{-9}$) (Table S3 in the Supplementary Appendix).

Although this seven-SNP region was well covered by reads obtained on whole-genome sequencing, we obtained low coverage of intron 4 of *ASGR1*, which is enriched in guanosine (G) and cytosine (C) residues and therefore difficult to sequence. We thus sequenced the whole genomes of 738 participants with a PCR-free method that is better suited to obtaining the sequence of GC-rich tracts and observed a del12 within intron 4 (NCBI reference sequence, NM_001671.4; c.284-36_283+33delCTGGGGCTGGGG).

After direct genotyping of del12 in 3799 Icelanders and imputation in 398,000 Icelanders, we observed that del12 (minor allele frequency, 0.41%; corresponding to a frequency of 1 in 120 heterozygous carriers) was highly correlated with rs186021206 ($r^2=0.86$) and the other six strongly associated SNPs (Tables S3 and S4 in the Supplementary Appendix). Furthermore, del12 was more strongly associated with lower non-HDL cholesterol levels than were any of the seven SNPs, with a level that was lower among heterozygous carriers of del12 than among noncarriers by 13.6 mg per deciliter (95% CI, 9.4 to 17.7 [0.35 mmol per liter; 95% CI, 0.24 to 0.46]; $P=2.5\times10^{-10}$) (Table 1, and Tables S3 and S4 and Fig. S1 in the Supplementary Appendix). The del12 variant was also associated with lower LDL cholesterol levels, with an effect size similar to that observed between del12 and non-HDL cholesterol levels.

We replicated a previously reported association between a common variant upstream of *ASGR1* (rs314253; minor allele frequency, 35.1%) and lowering of LDL cholesterol levels[17]; however, this association was independent of the del12 signal ($r^2<0.001$) (Table S5 in the Supplementary Appendix). After adjustment for the presence of del12, neither rs186021206 nor any of the other six SNPs remained significantly associated with the non-HDL cholesterol level (Table S4 in the Supplementary Appendix).

An analysis of an extended Icelandic data set with 5817 additional genome sequences, which provided increased sequence coverage, indicated that of all the variants within a 1-Mb region centered on *ASGR1*, del12 showed the strongest association with non-HDL levels (Fig. S2 in the Supplementary Appendix). The del12 variant was also associated with increased HDL cholesterol levels and decreased triglyceride levels, although the association was weaker than that with reduced non-HDL cholesterol and LDL cholesterol levels (Table 1).

We also tested for associations between del12 and lipid levels in samples from the Netherlands[18] and Denmark[19,20] (Table 1, and Tables S1 and S3 in the Supplementary Appendix) and observed associations between del12 and non-HDL cholesterol with effect sizes similar to those seen in the sample from Iceland ($P=0.24$ for heterogeneity). After combining all the data sets (including the Icelandic discovery data), we

Table 1. Association of del12 with Lipids, Alkaline Phosphatase, and Vitamin B_{12} in Iceland, Denmark, and the Netherlands.*

Variable and Study Population	Participants Evaluated	MAF of del12	Effect (95% CI)†	P Value	Value in General Population‡
	no.	%	*mg/dl*		*mg/dl*
Non-HDL cholesterol					
Iceland	119,146	0.41	−13.6 (−17.7 to −9.4)	2.5×10^{-10}	154.7±45.6
Denmark A§	6,182	0.22	−21.3 (−36.8 to −5.9)	0.007	161.6±44.1
Denmark B¶	9,656	0.32	−22.2 (−32.8 to −11.7)	3.8×10^{-5}	164.7±40.7
The Netherlands‖	5,537	0.50	−17.0 (−28.3 to −5.7)	0.003	170.7±41.3
Combined	140,521		−15.3 (−18.9 to −11.7)	1.0×10^{-16}	
LDL cholesterol					
Iceland	53,841	0.41	−9.5 (−14.0 to −5.1)	2.8×10^{-5}	133.0±41.4
Denmark A	6,098	0.22	−22.1 (−35.5 to −8.7)	0.001	137.2±37.5
Denmark B	8,080	0.32	−19.0 (−29.2 to −8.8)	2.6×10^{-4}	139.3±37.4
The Netherlands	5,523	0.50	−16.0 (−26.1 to −6.0)	0.002	138.6±36.4
Combined	73,542		−12.5 (−16.2 to −8.8)	3.9×10^{-11}	
HDL cholesterol					
Iceland	119,514	0.41	2.4 (0.7 to 4.1)	0.006	54.7±17.0
Denmark A	6,182	0.22	4.6 (−0.8 to 9.9)	0.10	54.2±15.8
Denmark B	9,656	0.32	2.4 (−1.8 to 6.7)	0.26	60.0±16.4
The Netherlands	5,537	0.50	2.4 (−1.3 to 6.0)	0.20	52.6±13.4
Combined	140,889		2.5 (1.1 to 4.0)	3.9×10^{-4}	
			% change		
Triglycerides					
Iceland	80,011	0.41	−6.1 (−10.8 to −1.5)	0.01	133.6 (67.6–190.5)
Denmark A	6,182	0.22	−6.0 (−5.2 to 11.4)	0.53	105.8 (60.8–183.9)
Denmark B	8,163	0.32	−8.9 (−21.0 to 2.3)	0.15	117.4 (73.5–187.3)
The Netherlands	5,537	0.50	−4.4 (−17.9 to 8.2)	0.52	155.8 (94.5–256.8)
Combined	99,893		−6.3 (−10.3 to −2.3)	0.003	
					U/liter
Alkaline phosphatase					
Iceland	126,060	0.41	50.1 (42.9 to 57.2)	3.6×10^{-63}	87.1 (53.5–141.7)
Denmark A	5,829	0.22	29.1 (14.8 to 42.5)	3.1×10^{-6}	41.3 (30.7–55.6)
Combined	131,889		46.5 (40.1 to 52.7)	5.6×10^{-69}	
					pmol/liter
Vitamin B_{12}					
Iceland	97,910	0.41	16.6 (11.5 to 21.5)	3.1×10^{-12}	398 (256–618)
Denmark A	5,826	0.22	18.6 (3.9 to 32.4)	0.005	398 (286–554)
Combined	103,736		16.8 (12.0 to 21.5)	8.3×10^{-14}	

* Plus–minus values are means ±SD. All discovery analyses were performed in the Icelandic data set, with replication analyses performed in the other data sets. To convert the values for non–high-density lipoprotein (HDL), low-density lipoprotein (LDL), and HDL cholesterol to millimoles per liter, multiply by 0.02586. To convert the values for triglycerides to millimoles per liter, multiply by 0.01129. MAF denotes minor allele frequency.

† Effect estimates and 95% confidence intervals are provided in milligrams per deciliter for non-HDL, LDL, and HDL cholesterol and as percentage change for triglycerides, alkaline phosphatase, and vitamin B_{12}.

‡ For triglycerides, alkaline phosphatase, and vitamin B_{12}, the population mean and the standard deviation were calculated for log-transformed values and transformed back to original units, so the range of values is included to represent the distributions with greater accuracy.

§ Data for the Denmark A population are from the Danish Inter99 study by Jørgensen et al.[19]

¶ Data for the Denmark B population are from the Danish Addition study by van den Donk et al.[20]

‖ Data for the Dutch population are from the Nijmegen Biomedical Study by Hoogendoorn et al.[18]

found that del12 was associated with lowering of non-HDL cholesterol by 15.3 mg per deciliter (95% CI, 11.7 to 18.9 [0.40 mmol per liter; 95% CI, 0.30 to 0.49]; $P=1.0\times10^{-16}$).

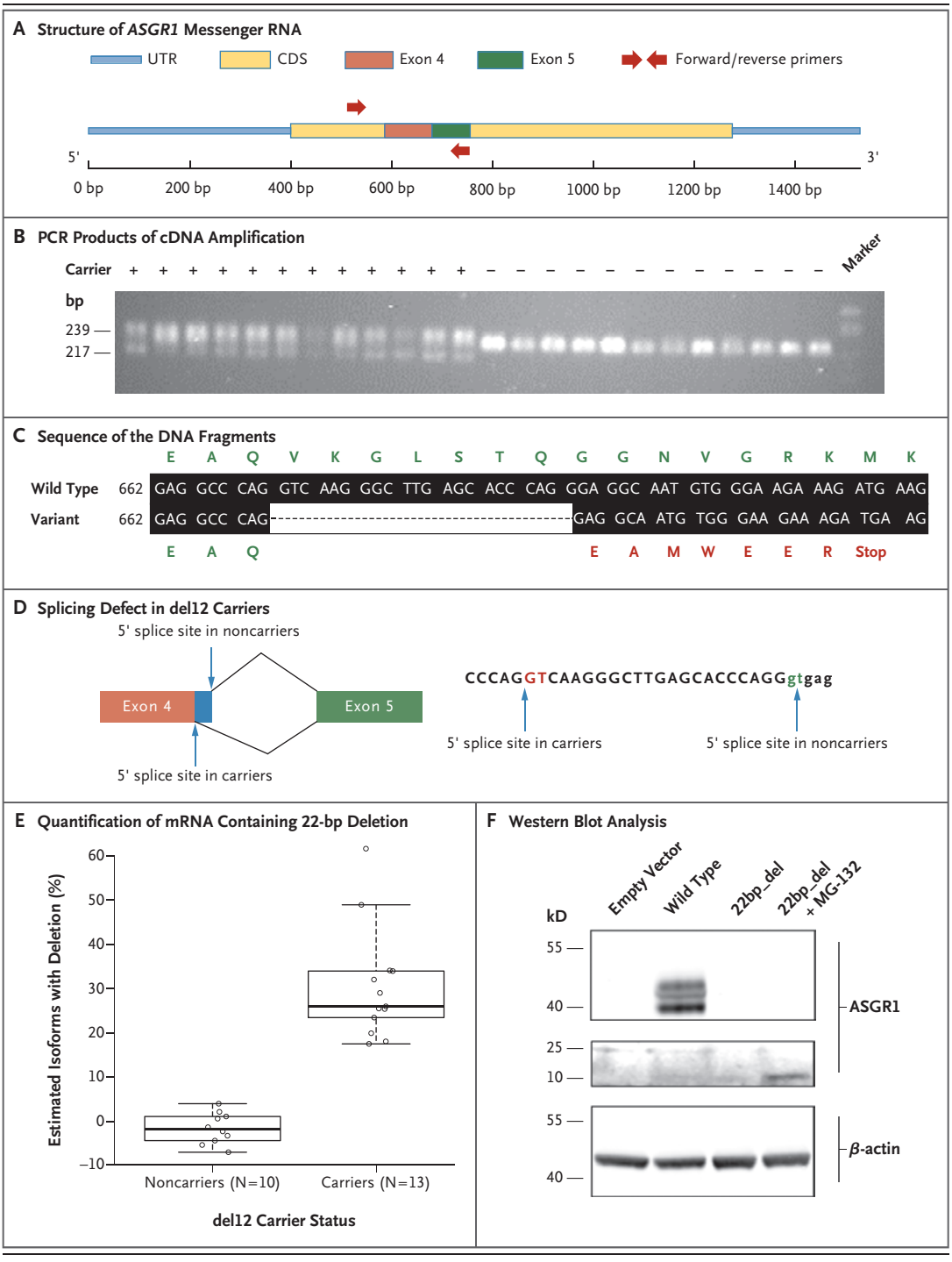
EFFECT OF DEL12 ON *ASGR1* MRNA SPLICING

We examined the effect of del12 on the splicing of exons 4 and 5 of *ASGR1* on PCR assays (Fig. 1A) and observed two PCR products generated from messenger RNA (mRNA) in blood samples obtained from del12 carriers, whereas only the larger product was detected in noncarriers (Fig. 1B). The sequence of the smaller product had a 22-bp deletion at the end of exon 4 (Fig. 1C), which was probably the result of a pseudo donor splice site in exon 4 (Fig. 1D). Direct digital counting of sequencing reads provided an approximate estimate of the fraction of *ASGR1* mRNA transcripts in the blood cells of the 13 del12 carriers that were incorrectly spliced. This analysis showed that the variant mRNA transcripts represented about one third of *ASGR1* transcripts (Fig. 1E), a fraction that was significantly different from that in noncarriers ($P=1.8\times10^{-6}$ by the Wilcoxon–Mann–Whitney test). The 22-bp deletion is predicted to cause a frameshift mutation in *ASGR1* and thereby introduce a premature stop codon at amino acid 89 in the 291-amino-acid full-length protein (Fig. S3 in the Supplementary Appendix).

A process called nonsense-mediated decay is responsible for eliminating aberrant mRNA transcripts with premature stop codons, such as the one generated by del12.[21] However, since one third of the *ASGR1* transcripts in heterozygous carriers had the 22-bp frameshift deletion, these transcripts are not fully eliminated by this process. If translated, the variant *ASGR1* transcript would be predicted to generate a truncated protein (Fig. S3 in the Supplementary Appendix). To determine whether such a truncated protein is produced in cells, we transiently overexpressed *ASGR1* complementary DNA harboring the 22-bp deletion and wild-type *ASGR1* in HeLa cells. Using Western-blot analysis, we detected only wild-type ASGR1 protein (Fig. 1F), even though both types of the mRNA transcript were highly expressed (data not shown). However, when the transfected cells were treated with a proteasome inhibitor, which blocks the degradation of truncated and misfolded proteins,[22,23] we detected the truncated protein (Fig. 1F, and Fig. S4 in the Supplementary Appendix). This finding showed that the truncated ASGR1 protein was synthesized and then degraded.

ALKALINE PHOSPHATASE, VITAMIN B_{12}, AND DEL12

ASGR1 is the major subunit of the hepatic asialoglycoprotein receptor (ASGPR) known to recognize and mediate the endocytosis and degradation of a variety of desialylated glycoproteins.[24-27] We wondered whether the serum levels of any sialylated glycoproteins were altered in del12 carriers, so we tested the association of del12 with serum levels of various substances that are routinely measured at hospitals and clinical laboratories in Iceland. Apart from the associations with blood lipids, we observed a highly significant association of del12 with levels of circulating alkaline phosphatase and vitamin B_{12}. The alkaline phosphatase levels were higher among carriers of del12 than among noncarriers, with a difference of 43.6 U per liter (95% CI, 37.3 to 49.8; $P=3.6\times10^{-63}$), representing levels that were 50.1% higher in carriers than in noncarriers. The vitamin B_{12} levels were also higher among carriers, with a difference of 66.1 pmol per liter (95% CI, 45.8 to 85.6; $P=3.1\times10^{-12}$), representing levels that were 16.6% higher (Table 1, and Table S3 in the Supplementary Appendix). Regional plots, which were based on the significance levels for the association of markers centered on *ASGR1* with levels of alkaline phosphatase and vitamin B_{12}, mirrored the plot of the association with non-HDL cholesterol (Fig. S5 in the Supplementary Appendix). However, the effects of the associations were in opposite directions: carriers of del12 had lower non-HDL cholesterol levels but higher levels of alkaline phosphatase and vitamin B_{12} than noncarriers. In Denmark, we replicated these associations with carriers of del12 who had higher levels of alkaline phosphatase and vitamin B_{12} than noncarriers ($P=3.1\times10^{-6}$ and $P=0.005$, respectively) (Table 1, and Table S3 in the Supplementary Appendix). Moreover, the common variant at the *ASGR1* locus (rs314253) that was previously reported to be associated with decreased levels of LDL cholesterol was also reported to be associated with increased levels of alkaline phosphatase,[28] two associations that we verified for this variant (Table S5 in the Supplementary Appendix).



In both the Icelandic and the Danish data sets, non-HDL cholesterol levels were correlated with alkaline phosphatase levels but not with vitamin B_{12} levels. Furthermore, there was a stronger association of del12 with non-HDL cholesterol levels after adjustment for alkaline phosphatase levels but not after adjustment for vitamin B_{12} levels (Table S6 in the Supplementary Appendix). This finding indicates that the association of del12 with non-HDL cholesterol was

Figure 1 (facing page). Splicing Error and Frameshift in *ASGR1* Created by del12.

Panel A shows an overview of the structure of the *ASGR1* messenger RNA (mRNA) with highlighting of exons 4 and 5. A rare noncoding 12-base-pair (bp) deletion (del12) lies within intron 4, which sits between exons 4 and 5. Also shown are the positions of the primers that were used to amplify the complementary DNA (cDNA) on polymerase-chain-reaction (PCR) assay. CDS denotes coding sequence, and UTR untranslated region. Panel B shows the PCR products produced by amplifying cDNA generated from RNA isolated from the blood of heterozygous carriers (plus signs) and noncarriers (minus signs) of the del12 variant. Labels indicate the size of the expected PCR product (239 bp) and the size of the truncated band (217 bp) observed only in del12 heterozygous carriers. Panel C shows the differences between the full-length (239-bp) and truncated (217-bp) cDNA fragments on Sanger sequencing. The truncated fragment in carriers of the del12 variant lacks 22 bp at the end of exon 4, which results in a frameshift and an introduction of a stop codon. Panel D shows the splicing defect observed in del12 carriers. The sequence around the boundary between exon 4 (in uppercase letters) and intron 4 (in lowercase letters) is shown, along with the 5′ splice site in noncarriers and the cryptic 5′ splice site activated in del12 carriers. Panel E shows the quantification of the cDNA fragments with the 22-bp deletion in heterozygous del12 carriers and noncarriers, which 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-bp fragment at the end of exon 4 and the median coverage over exon 5. There was a significant difference between noncarriers and carriers in the estimated fraction of incorrectly spliced isoforms ($P=1.8\times10^{-6}$ by the Wilcoxon–Mann–Whitney test). The horizontal lines indicate the median fraction, and the top and bottom of the boxes indicate the first and third quartiles. The top of the I bar indicates the smaller of the maximum fraction or the 75th percentile plus 1.5 times the interquartile range, and the bottom of the I bar indicates the larger of the minimum fraction or the 25th percentile minus 1.5 times the interquartile range. Panel F shows Western blot analysis of HeLa cells that transiently overexpress wild-type *ASGR1* cDNA or mutated *ASGR1* cDNA lacking the 22-bp fragment 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 that was used recognizes amino acids 1 to 41 of the ASGR1 protein. The blot in the center panel had a longer exposure time to detect the truncated ASGR1 protein after treatment with MG-132 than did those in the top and bottom panels.

independent of both alkaline phosphatase and vitamin B_{12} levels. The increase in levels of alkaline phosphatase associated with del12 is unlikely to reflect the presence of liver disease since we observed no association between del12 and serum levels of gamma-glutamyltransferase, bilirubin, alanine aminotransferase, or other measures of liver function that commonly accompany changes in alkaline phosphatase levels during the course of liver disease (Table S7 in the Supplementary Appendix).

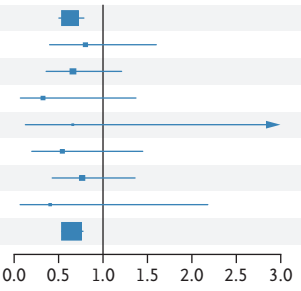
CORONARY RISK AND DEL12

Given the effect of del12 on non-HDL cholesterol levels, we assessed the effect of the variant on the risk of coronary artery disease in 33,090 case patients and 236,254 controls from Iceland and 9434 case patients and 13,160 controls from the United States (Emory University, Duke University, and University of Pennsylvania), the United Kingdom (Leicester Myocardial Infarction Study and British Heart Foundation Family Heart Study), New Zealand, and Denmark. The risk of coronary artery disease was lower among carriers of the del12 variant than among noncarriers in both the Icelandic set (odds ratio, 0.64; 95% CI, 0.51 to 0.80; $P=5.8\times10^{-5}$) and the non-Icelandic sets (odds ratio, 0.69; 95% CI 0.51 to 0.95; P=0.02), for a combined odds ratio of 0.66 (95% CI, 0.55 to 0.79; $P=4.0\times10^{-6}$) (Fig. 2A). There was no evidence of heterogeneity across the eight study populations (P=0.96). Among Icelanders, the risk of myocardial infarction was lower among carriers of the del12 variant than among noncarriers (hazard ratio, 0.64; 95% CI, 0.64 to 0.80; $P=8.5\times10^{-5}$) (Fig. 2B). In agreement with the protective effect against coronary artery disease, Icelandic del12 carriers had a life span that was 1.5 years longer than that of noncarriers (95% CI, 0.2 to 2.8; P=0.02). We did not observe an association (inverse or otherwise) of del12 with other known risk factors for coronary artery disease, such as hypertension, smoking status, obesity, or type 2 diabetes (Table S8 in the Supplementary Appendix).

Several studies have shown a strong positive correlation between the effects of lipid-associated sequence variants on non-HDL cholesterol levels and the risk of coronary artery disease.[7-10,29] However, in our data, several published variants deviated from the overall trend (Fig. 3, and Table S9 in the Supplementary Appendix). The del12 mutation in *ASGR1* is another example of a variant for which the effect on coronary artery disease is

A Coronary Risk Associated with del12 Variant

Study	No. of Cases/No. of Controls	MAF %	Odds Ratio (95% CI)	P Value
Iceland	33,090/236,254	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 populations	42,524/249,414		0.66 (0.55–0.79)	4.0×10^{-6}



B Survival Free of Myocardial Infarction

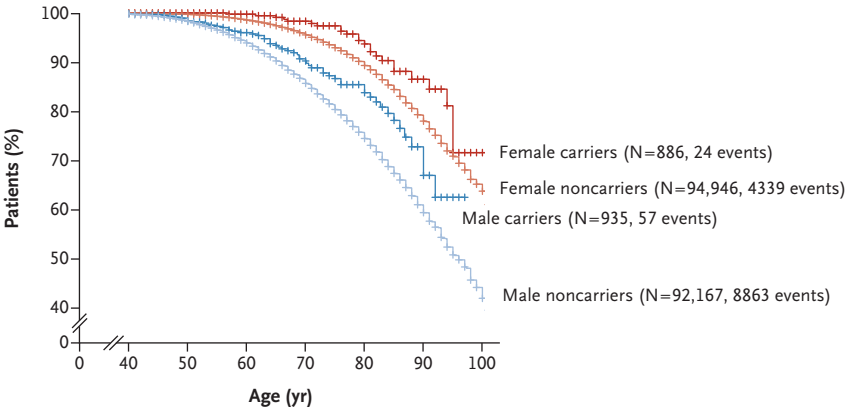


Figure 2. Association of del12 in *ASGR1* with a Reduced Risk of Coronary Artery Disease and Myocardial Infarction.

Panel A shows odds ratios for coronary artery disease associated with the del12 variant in *ASGR1* among a total of 42,524 participants with coronary artery disease and 249,414 controls in Iceland, Denmark, the United States (Emory University, Duke University, and the University of Pennsylvania [UPenn]), the United Kingdom (UK-I and UK-II), and New Zealand. For each sample set, the size of the square for the odds ratio is proportional to the number of participants who were evaluated. The del12 variant was associated with a significantly lower risk of coronary artery disease in both the Icelandic set ($P=5.8\times10^{-5}$) and the non-Icelandic sets ($P=0.02$), for a combined odds ratio of 0.66 ($P=4.0\times10^{-6}$). There was no evidence of heterogeneity across the eight study populations ($P=0.96$). MAF denotes minor allele frequency. Panel B shows Kaplan–Meier curves for survival free of a first myocardial infarction among heterozygous carriers and noncarriers of the del12 variant, stratified according to sex. The hatch marks indicate censoring of data.

stronger than that predicted by its effect on non-HDL cholesterol.

The associations of del12 with both the risk of coronary artery disease and the risk of myocardial infarction became stronger after adjustment for the association between del12 and alkaline phosphatase levels and was unchanged after adjustment for the association with vitamin B_{12} levels (Table S10 in the Supplementary Appendix). Thus, it is implausible that the unexplained atheroprotective effects of del12 (i.e., those not associated with lowering of non-HDL levels) are mediated through modulating levels of alkaline phosphatase or vitamin B_{12}.

SECOND LOSS-OF-FUNCTION VARIANT IN *ASGR1*

In addition to samples obtained from 2636 Icelanders that we had previously sequenced, we screened an extended data set that included variants of the genomes of 5817 Icelanders and observed another rare loss-of-function variant, a 4-bp insertion (minor allele frequency, 0.027%; NM_001671.4; c.469_472dupAACT) that introduces a stop codon at position 158 (NP_001662.1; p.W158X) in the ASGR1 protein (Fig. S3 in the Supplementary Appendix). We directly genotyped predicted carriers and noncarriers by means of Sanger sequencing and used those genotypes to improve the imputation of p.W158X. The

p.W158X variant was associated with a lower non-HDL cholesterol level in carriers than in noncarriers (–24.9 mg per deciliter; 95% CI, –40.6 to –9.3; $P=1.8\times10^{-3}$) and a higher level of alkaline phosphatase (by 45.3%; 95% CI, 20.4 to 68.2; $P=7.9\times10^{-6}$) (Table S11 in the Supplementary Appendix). The direction and size of the effects of p.W158X on non-HDL cholesterol and alkaline phosphatase are similar to those of del12. For coronary artery disease in Iceland, the odds ratio for p.W158X was 0.65 (95% CI, 0.26 to 1.40; P=0.24). None of the 79 Icelandic carriers of p.W158X also carried del12. We did not observe p.W158X in large population databases such as the Exome Aggregation Consortium, the Exome Variant Server, or Genomes of the Netherlands.

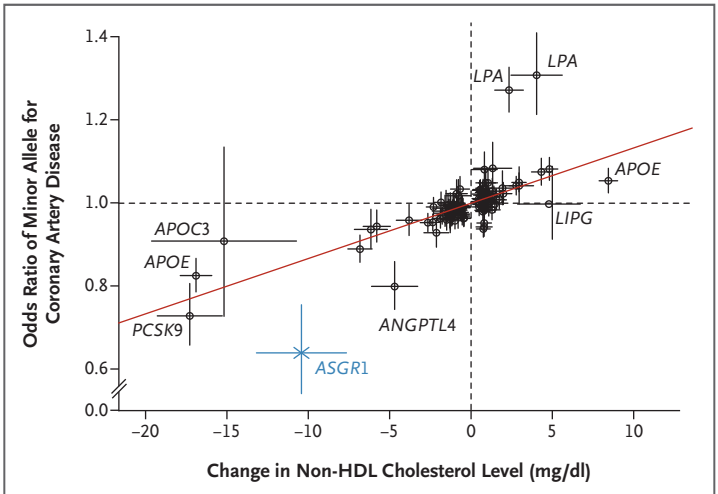


Figure 3. Relationship between the Effect of Sequence Variants on Non-HDL Cholesterol and on the Risk of Coronary Artery Disease.

Shown are estimated odds ratios of the minor allele of sequence variants for coronary artery disease in Iceland among 33,090 case patients and 236,254 controls as a function of the estimated effect of the minor allele on non-HDL cholesterol levels among 119,146 participants for whom data were available. In addition to the effect of del12 in *ASGR1*, the effects of variants that have an association with non-HDL cholesterol levels (as derived from Do et al.[29]) are shown for comparison (Table S9 in the Supplementary Appendix). For the sequence variants with the largest effects, the affected gene is shown. Two genes — *LPA* and *APOE* — are each plotted at two points in the graph because there was more than one independent signal at those loci. The del12 variant in *ASGR1* (in blue) is shown to have a stronger effect on coronary artery disease than predicted by its effect on non-HDL cholesterol levels. The error bars represent 95% confidence intervals. The red line indicates the best linear-regression fit through the origin. To convert the values for non-HDL cholesterol to millimoles per liter, multiply by 0.02586.

DISCUSSION

In our study, we found an associations between *ASGR1* variants and non-HDL cholesterol levels and the risk of coronary artery disease and myocardial infarction. Although it has been long established that ASGPR (in which ASGR1 is the major subunit) mediates endocytosis and degradation of desialylated glycoproteins, the endogenous ligands and the physiologic function of the receptor have been difficult to establish.[30] Mice lacking *Asgr1* thrive normally and do not accumulate desialylated glycoproteins in the circulation, although they are unable to clear exogenously added asialoglycoproteins.[31] Through analysis of loss-of-function variants in *ASGR1*, we have established a role for human ASGR1 in the control of non-HDL cholesterol levels and in regulation of the endogenous levels of at least some asialoglycoproteins.

We found that the two loss-of-function variants identified in *ASGR1*, del12 and p.W158X, are associated with decreased non-HDL cholesterol levels and increased levels of alkaline phosphatase and vitamin B_{12}. The previously described common variant close to *ASGR1* is also associated with increased levels of alkaline phosphatase and low levels of LDL cholesterol.[17,28] We propose that the loss-of-function *ASGR1* variants probably exert these "opposite" effects (i.e., increased alkaline phosphatase and vitamin B_{12} levels and decreased non-HDL cholesterol levels) through different mechanisms. Both alkaline phosphatase and haptocorrin, a vitamin B_{12} transporter, are asialylated glycoproteins that are known to bind ASGPR and thus be cleared from the circulation.[31-34] The likely explanation for the increased levels of alkaline phosphatase among carriers of loss-of-function *ASGR1* variants is that clearance of desialylated molecules from the circulation is compromised.

The mechanism by which the variants bring about a reduction in non-HDL cholesterol levels is probably a different one. Some insight into this mechanism may be provided by analyses of mice that express a hypomorphic form of neuraminidase 1 (Neu1), a sialidase that cleaves the sialic acid residues and thereby generates substrates for ASGPR. In the hypomorphic mouse, the LDL receptor is sialylated. Since this form of the receptor is more stable than that in wild-type mice, more particles of LDL cholesterol would be

expected to collect on the surface of the hepatocyte, which would result in increased uptake of LDL cholesterol.[35] Thus, reduced expression of Neu1 decreases the recycling of the LDL receptor, which leads to enhanced uptake of LDL cholesterol by the liver. Both ASGPR and the LDL receptor are located in clathrin-coated pits (which often fold inward to form endosomes) on the surface of hepatocytes. Perhaps ASGPR interacts with the asialylated form of the LDL receptor, which leads to recycling of the receptor by hepatocytes through endocytosis. If this is the case, a paucity of functional ASGPR would be predicted to diminish the rate at which asialylated LDL receptors are removed from the cell surface.

In our study, we found that the *ASGR1* del12 variant, like variants in *ANGPTL4* and *LPA*, has a larger effect on the risk of coronary artery disease than is predicted by its effect on levels of non-HDL cholesterol, which suggests that the atheroprotective effects of del12 go beyond the lowering of serum cholesterol levels. This additional atheroprotective effect would not appear to be mediated through an increase in levels of alkaline phosphatase or vitamin B_{12}. Sialylation of chemokines or their receptors can influence the recruitment of inflammatory cells to atherosclerotic plaques,[36-38] a finding that may be relevant to the atheroprotective effect of del12.

In conclusion, we have identified rare loss-of-function variants in *ASGR1* that are associated with lowering of non-HDL cholesterol levels and a reduced risk of coronary artery disease. These variants disrupt ASGR1 function and represent a link between the sialylation pathway and atherosclerotic diseases.

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