Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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Supplementary Appendix for

Inactivating mutations in *NPC1L1* and protection from coronary heart disease

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Supplementary Methods: Sequencing

Exome sequencing was performed in samples from the sequencing phase at the Broad Institute, the Baylor College of Medicine Human Genome Sequencing Center, or the University of Washington. The following is a summary of sequencing procedures followed at the Broad Institute; similar methods were utilized at the other sites. In brief, following DNA quantification using PicoGreen, we confirmed high-molecular weight DNA and performed fingerprint genotyping and gender determination using Illumina iSelect platform. From samples passing these initial quality steps, 3μg of genomic DNA was used to perform library construction and in-solution hybrid selection[1] to target 33Mb of genomic sequence. The resulting exome-enriched DNA was sequenced on either Genome Analyzer II using v3 and v4 Sequencing-by-Synthesis Kits, then analyzed using RTA v1.7.48 or on HiSeq 2,000 using HiSeq 2,000 v2 Sequencing-by-Synthesis Kits, then analyzed using RTA v1.10.15. Sequencing was performed using 76 cycle paired-end runs. Sequencing was considered complete when ≥ 80% of targeted bases were covered with ≥ 20 sequencing reads.

Raw sequence reads were aligned to the human reference genome (HG19) using the Burroughs-Wheeler Alignment tool[2] in paired-end mode. Duplicate reads and reads aligned outside of the exome target were removed.

The Genome Analysis ToolKit[3] (GATK) was then used to locally realign reads, recalibrate base qualities, identify and genotype single nucleotide variants (SNVs) and short insertion and deletion events (indels), and recalibrate the resulting variant quality scores. SnpEff was used to predict the functional consequences of the identified variants[4].

Quality Control for Sequencing

SNVs were flagged for removal if they had low quality (quality per depth score < 5), exhibited strand bias (strand bias ≥ 0.1), or were part of a homopolymer run greater than 4. Indels were flagged for removal if they had low quality (quality score < 30 or quality per depth score < 2) or exhibited strand bias (strand bias > -1.0). We then removed samples that were incompletely sequenced (i.e., did not reach ≥ 80% of targeted bases covered with ≥ 20 reads), those that were discordant between pre- and post-sequencing fingerprints, or were discordant between inferred and reported gender. We also removed samples that were statistical outliers for several sequencing metrics including heterozygosity, the ratio of transitions to transversions, singleton count, total variant count, and missingness. Finally, we removed samples that were statistical outliers in population clustering using principal components analysis and samples with discordance between inferred and self-reported ancestry. We then assessed additional sequencing metrics to refine the variant quality control process to remove those variants likely to be artifacts and removed variants that were statistical outliers for depth of coverage, quality over depth, Hardy Weinberg

equilibrium, and frequency of missing genotypes. SNVs and indels occurring in the protein coding regions of *NPC1L1* were then extracted for this study.

Supplementary Methods: Genotyping

Samples from the genotyping phase cohorts were genotyped for *NPC1L1* p.Arg406X. In brief, after DNA quantification and quality control, samples were processed on the Illumina HumanExome BeadChip array (Illumina, Inc., CA, USA) according to standard protocols suggested by the manufacturer. Genotypes were assigned using GenomeStudio and supplemented with the zCall algorithm[5] when possible. Using these same methods, we also genotyped 2,495 samples from ATVB and 1,597 samples from PROCARDIS that had undergone *NPC1L1* sequencing.

Quality Control for Genotyping

After ensuring genotypes were aligned to the positive strand we removed samples that had an excess of missing genotypes (≥ 5%), were discordant between inferred and reported gender, duplicate samples, statistical outliers for inbreeding coefficient, samples with high proportion of shared genotypes identical by descent, samples with cryptic relatedness, and statistical outliers in principal components analysis. From these samples that passed quality control we extracted genotypes for *NPC1L1* p.R406X (also known as rs145297799 and exm618018).

Technical Validation

We sought to technically validate the presence of inactivating mutations in individuals from the ATVB cohort using the orthogonal method of Sanger sequencing. These mutations were selected for validation: a) to ensure the frameshift indel (p.A296VfsX57) was not an artifact given a higher false discovery rate for indel detection using next-generation sequencing compared with single nucleotide substitutions; and b) to ensure the relatively higher carrier rate in ATVB compared with other sequencing-phase cohorts was not a technical artifact. As shown in Figure S1, the single nucleotide deletion inducing p.A296VfsX57 was also discovered using Sanger sequencing. This deletion and p.R406X were confirmed in all carriers from ATVB.

We also performed technical validation for p.R406X genotyping. Given the low frequency of p.R406X and potential difficulty in identifying carriers through genotyping, we sought to determine the presence of false positives (carriers spuriously identified through genotyping but not confirmed through sequencing) and false negatives (real carriers identified through sequencing but missed by genotyping). In 4,092 samples that underwent both *NPC1L1* sequencing and genotyping we detected zero false positives and zero false negatives.

Association Testing to Account for Confounding

Due to the rarity of inactivating mutations in *NPC1L1*, we had limited statistical power to detect an association with CHD for any single mutation. Therefore, we calculated the summary odds ratio and associated 95% confidence interval of

CHD for carriers of any inactivating mutation across studies using a Mantel-Haenszel meta-analysis. To eliminate the possibility of confounding from population stratification or cryptic relatedness, we performed an additional association test in a subset of samples sequenced at the Broad Institute using a mixed linear model as implemented by EMMAX[6]. The mixed linear model accounts for multiple layers of stratification in the sample sets by incorporating a kinship matrix in the association test to correct for population stratification and sample relatedness. The level of statistical significance for the mixed linear model was similar to the Mantel-Haenszel approach (data not shown), suggesting the association results are not a result of cryptic relatedness or stratification.

Table S1. Sample sets included in the sequencing phase of the study

Study	Study Design	N Cases	N Controls	CHD definition	CHD-free control definition	Ref
ARIC	Prospective cohort	1631	6923	Incident probable or definite MI, silent MI, definite CHD death, or coronary revascularization	Free of CHD during follow-up	7
ATVB	Case-control	1794	1745	MI in men or women ≤ 45 years of age	No history of thromboembolic disease	8
ESP-EOMI	Case-control	178	277	MI in men ≤ 50 years of age or women ≤ 60 years of age	Free of MI, coronary revascularization; men ≥ 50 years of age or women ≥ 60 years of age	9
JHS	Prospective cohort	235	2016	Combination of prevalent CHD (self-reported or electrocardiographic evidence of MI) and incident CHD (MI or coronary revascularization as previously described[10]	Free of CHD during follow-up	11
Munich-MI	Case-control	368	336	MI in men ≤ 40 years of age or women ≤ 55 years of age	Controls without CAD, men ≥ 65 years of age and women ≥ 75 years of age	12
OHS	Case-control	966	987	Angiographically confirmed coronary artery disease (>1 coronary artery with >50% stenosis) without history of diabetes at age ≤ 50 for men or ≤ 60 for women	Asymptomatic, men > 65, women > 70	13
PROCARDIS	Case-control	966	936	Symptomatic CAD before age 66. CAD was defined as clinically documented evidence of myocardial infarction, coronary artery bypass grafting, acute coronary syndrome, coronary angioplasty, or stable angina	No personal or sibling history of CAD before 66 years of age	14
PROMIS	Case-control	844	1107	MI in men and women ≤ 45 years of age	No history of cardiovascular disease	15
REGICOR	Case-control	382	401	MI in men ≤ 50 years of age or women ≤ 60 years of age	Controls from a population-based study; free of MI, coronary revascularization; ≥ 55 and <80 years of age	16

Table S2. Sample sets included in the genotyping phase of the study

Study	Study Design	N Cases	N Controls	CHD definition	CHD-free control definition	Ref
ARIC	Prospective cohort	1142	4095	Incident probable or definite MI, silent MI, definite CHD death, or coronary revascularization	Free of CHD during follow-up	[7]
BioVU	Case-control	4587	16556	Cases with were ascertained from the Vanderbilt University Medical Center (VUMC) Biorepository by searching the electronic medical record for ≥ 2 instances of ICD-9 codes 410.x – 414.x	Individuals from the VUMC Biorepository without any record of ICD-9 codes 410.x – 414.x	[17]
German North	Case-control	4464	2886	The German North cohort includes individuals from GerMIFS4, PopGen, and HNR with MI or CAD	Controls from population-based studies in Germany	[18,19,20]
German South	Case-control	5255	2921	The German South cohort includes samples from GerMIFS3 and Munich-MI with MI or CAD	Controls from population-based studies in Germany	[21,22]
GoDARTS	Case-control	997	2768	The GoDARTS (Genetics of Diabetes Audit and Research in Tayside Scotland) study is a joint initiative of the Department of Medicine and the Medicines Monitoring Unit (MEMO) at the University of Dundee, the diabetes units at three Tayside healthcare trusts (Ninewells Hospital and Medical School, Dundee; Perth Royal Infirmary; and Stracathro Hospital, Brechin), and Tayside general practitioners with an interest in diabetes care. Cases were defined as fatal and non-fatal myocardial infarction.	Controls were free of CAD, stroke, and peripheral vascular disease	[23]
Mayo	Case-control	1177	1492	History of MI, coronary revascularization, angina with positive stress test, or >50% stenosis of an epicardial coronary artery	No history of MI or atherosclerotic vascular disease	[24]
PROCARDIS	Case-control	1132	1095	Symptomatic CAD before age 66. CAD was defined as clinically documented evidence of myocardial infarction, coronary artery bypass grafting, acute coronary syndrome, coronary angioplasty, or stable angina	No personal or sibling history of CAD before 66 years of age	[14]
WGHS	Prospective clinical trial	976	21641	Prospectively ascertained MI, coronary revascularization (PCTA or CABG), and cardiovascular death	Free of CHD events during follow-up	[25,26]
WHI	Prospective cohort	2860	14958	MI, coronary revascularization, hospitalized angina, or death due to CHD	Free of CHD during follow-up	[27]

Table S3. Clinical characteristics of *NPC1L1* inactivating mutation carriers compared with non-carriers

Cohort		Number	Male gender (%)	Age, mean (SD), years	BMI, mean (SD) kg/m2	Current or Former Smoker (%)	Diabetes (%)	Systolic blood pressure, mean (SD), mmHg
ARIC AA	C	8	12.5%	54.1 (5.9)	26.9 (3.7)	50%	12.5%	129.9 (25.2)
	NC	2828	36.2%	53.2 (5.8)	29.8 (6.3)	52.5%	17.2%	127.2 (19.0)
ARIC EA	C	10	50%	53.4 (4.4)	25.1 (5.7)	60%	0%	112.2 (14.2)
	NC	10945	46.9%	54.3 (5.7)	27.0 (4.9)	59.7%	8.8%	118.4 (17.0)
ATVB	C	7	86%	37.4 (6.4)	24.5 (1.9)	71%	0%	120.0 (10)
	NC	3532	89%	39.6 (4.9)	25.9 (3.9)	68%	3.2%	129.1 (18.7)
BioVU	C	12	50%	60.7 (18.0)	30.0 (9.2)	50%	33.3%	126.1 (10.4)
	NC	21131	46%	65.1 (17.0)	27.8 (6.0)	26.7%	14.1%	126.1 (13.0)
ESP-EOMI	C	1	100%	72.6 (NA)	28.8 (NA)	0%	0%	101.5 (NA)
	NC	454	26%	62.5 (13.5)	31.6 (7.2)	60%	31.7%	138.0 (31.8)
German North	C	1	100%	53.0 (NA)	28.2 (NA)	100%	NA	NA
	NC	7349	60%	54.7 (10.3)	27.0 (4.4)	55%	NA	NA
German South	C	3	67%	64.3 (7.1)	29.3 (3.7)	66%	33%	NA
	NC	8173	62%	58.8 (11.7)	27.35 (4.6)	33.6%	12.4%	NA
GoDARTS	C	4	25%	67.5 (5.6)	31.7 (9.8)	25%	75%	151.2 (10.4)
	NC	3761	60%	61.4 (11.1)	30.9 (5.7)	61%	45%	140.4 (17.2)
JHS	C	6	67%	56.8 (17.6)	32.3 (7.7)	33%	33%	120.4 (18.1)
	NC	2245	37%	50.8 (12.5)	32.6 (4.1)	30.5%	21.3%	124.8 (17.6)
Mayo	C	2	0%	63.3 (8.7)	29.7 (0.1)	0%	0%	132.5 (16.3)
	NC	2667	59.6%	64.5 (9,3)	29.3 (5.6)	60.5%	19.9%	123.9 (17.3)
Munich-MI	C	1	100%	71 (NA)	26.04 (NA)	100%	0%	142 (NA)
	NC	703	64.3%	56.9 (16.6)	27.5 (7.5)	52.2%	12.5%	136.1 (27.2)
OHS	C	1	0%	78.1 (NA)	24.9 (NA)	58.56%	0%	106 (NA)
	NC	1952	67%	64.4 (16.3)	27.3 (4.4)	0%	0%	135.2 (18.8)
PROCARDIS	C	2	100%	65.5 (0.71)	29.4 (5.4)	50%	50%	142.5 (17.7)
	NC	4127	59.9%	56.6 (11.9)	27.8 (4.6)	61.96%	10.2%	134.7 (18.8)
PROMIS	C	1	100%	44 (NA)	25.8 (NA)	100%	0%	140 (NA)
	NC	1950	87.8%	49.5 (10.6)	25.1 (3.9)	45.6%	14.5%	128.8 (19.5)
REGICOR	C	2	100%	68 (5.7)	27.5 (1.1)	50%	0%	152.5 (9.2)
	NC	781	78.4%	57.1 (12.1)	27.6 (3.9)	41.4%	21.5%	139.4 (18.5)
WGHS	C	11	0%	55.1 (5.9)	23.7 (3.5)	54.5%	0%	123.2 (11.7)
	NC	22606	0%	54.7 (7.1)	25.9 (5.0)	49.1%	2.5%	123.6 (13.7)
WHI	C	10	0%	64.8 (6.6)	27.1 (5.1)	0%	11.1%	130.1 (20.7)
	NC	17808	0%	66.4 (6.7)	28.3 (5.9)	7.7%	6.8%	129.9 (17.9)

C=Carrier of NPC1L1 inactivating mutation; NC=Noncarrier; BMI=Body mass index; NA=Not available; SD=Standard deviation; AA=African ancestry; EA=European ancestry

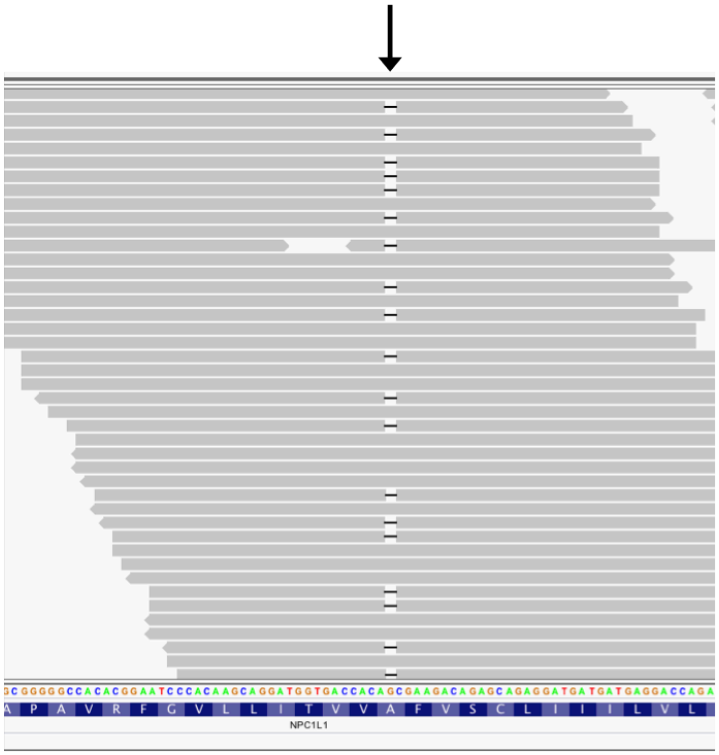
Table S4. Association of NPC1L1 inactivating mutations with risk of coronary heart disease by cohort

Cohort	Ancestry	Carriers		Total Number		Carrier frequency		OR* (95% CI)‡	P value
		Cases	Controls	Cases	Controls	Cases	Controls		
ARIC AA	AA	1	7	474	2362	0.21%	0.30%		
ARIC EA†	EA	3	7	2299	8656	0.13%	0.08%		
ATVB	EA	1	6	1794	1745	0.06%	0.34%		
ESP EOMI	AA	0	1	178	277	0%	0.36%		
OHS	EA	0	1	966	987	0%	0.10%		
PROCARDIS†	EA	0	2	2098	2031	0%	0.10%		
JHS	AA	1	5	235	2016	0.43%	0.25%		
Munich-MI	EA	0	1	368	336	0%	0.30%		
REGICOR	EA	0	2	382	401	0%	0.50%		
PROMIS	SA	0	1	844	1107	0%	0.09%		
BioVU	EA	1	11	4587	16556	0.022%	0.066%		
German North	EA	0	1	4464	2886	0%	0.03%		
German South	EA	1	2	5255	2921	0.02%	0.07%		
GoDARTS	EA	0	4	997	2768	0%	0.14%		
Mayo	EA	0	2	1177	1492	0%	0.13%		
WGHS	EA	2	9	976	21641	0.21%	0.042%		
WHI	EA	1	9	2860	14958	0.03%	0.06%		
Total		11	71	29954	83140	0.037%	0.085%	0.47 (0.25-0.87)	8.0×10^{-3}

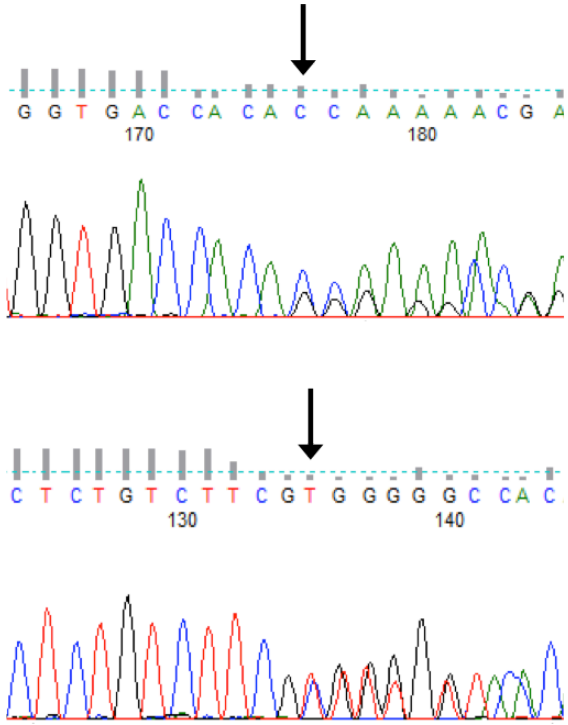
*OR=Odds Ratio; CI=Confidence interval; †Independent samples from sequencing and genotyping combined; ‡Test for heterogeneity P=0.43; AA=African ancestry; EA=European ancestry; SA=South Asian ancestry

Figure S1. Technical validation of frameshift indel observed in ATVB. A) Integrated genomics viewer screenshot of next-generation sequencing data supporting the p.A296VfsX57 frameshift in one individual. Horizontal gray bars represent individual sequencing reads aligned to the human reference genome below. The arrow indicates the alignment gap induced by the heterozygous single base-pair deletion. B) Chromatograms from Sanger confirmation sequencing of the forward (top) and reverse (bottom) strands. Arrows indicate the position of the deletion and the beginning of the frameshift.

A)



B)



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