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DOTTORATO DI RICERCA IN
MEDICINA MOLECOLARE

CICLO XXXVI

Exome variants influencing LDL cholesterol in early-onset myocardial infarction

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ABSTRACT

Background: Low-density lipoprotein cholesterol (LDL-C) causes atherosclerotic cardiovascular diseases, including myocardial infarction (MI). The aim of the Italian Genetic Study on Early-onset MI was to evaluate the effect of the rare exome variants influencing LDL-C on the risk of recurrent ischemic events after a MI occurring before the age of 45 years.

Methods*:* The study population consisted of 2,000 patients hospitalised because of a first early-onset MI, who were followed up for a median of 19.9 years for the occurrence of the primary endpoints of cardiovascular death, non-fatal MI or non-fatal stroke. Whole-exome sequencing led to the annotation of deleterious variants of 17 genes known to have a significant impact on LDL-C.

Results: One hundred patients had exome variants influencing hypercholesterolemia (HYPER); 60 had variants influencing hypocholesterolemia (HYPO); and 1,840 had no variants (NV). At the time of the index event, the median plasma LDL-C level was 190 mg/dL (interquartile range [IQR] 136-251) in the HYPER group, 114 mg/dL (IQR 86-131) in the HYPO group, and 140 mg/dL (IQR 112-168) in the NV group (p for trend <0.001). During the long-term follow-up the progression of coronary atherosclerosis and the cumulative incidence of primary endpoints was significantly different among the groups, and trend analysis showed that the incremental risk was HYPO<NV<HYPER (p for trend <0.01 and <0.004, respectively).

Conclusion*:* Exome variants influencing LDL-C have a measurable impact on coronary atherosclerosis progression and the probability of recurrent ischemic events after early-onset MI.

Key words: Early-onset myocardial infarction; cholesterol; genetic variants; prognosis.

INTRODUCTION

Myocardial infarction (MI) is a complex disease caused by environmental and genetic factors. Although the influence of environmental factors has been well established, the influence of genetic factors, which play an especially important role in determining the risk of developing early-onset MI,[1,2] is still being investigated.

A large body of evidence has shown that low-density lipoprotein cholesterol (LDL-C) is a causal risk factor for MI and that its levels are influenced by environmental and genetic factors[3]. It is known that a number of specific genetic variants are associated with increased or decreased LDL-C levels, and these can be identified using exome sequencing, a powerful means of identifying the human genomic protein-coding variants that cause the onset of monogenic disease or affect the biological background of more complex diseases.[4]

The aim of this exome sequencing study was to evaluate the effect of the rare genetic variants that influence LDL-C on the long-term risk of recurrent ischemic events in a population of patients with early-onset MI.

MATERIALS AND METHODS

Patient population

The studied population consisted of the patients enrolled in the Italian Genetic Study on Early-onset Myocardial Infarction, which was carried out in 125 Italian Coronary Care Units and consisted of an initial case-control study followed by a prospective follow-up study of the cases.[5,6] The cases and controls were enrolled consecutively between 1998 and 2002.

The cases were eligible if they had been hospitalised because of a first type I MI occurring before the age of 45 years and had undergone coronary angiography at the time of the index event. The index MI was diagnosed on the basis of a combination of three characteristics: 1) symptoms suggesting myocardial ischemia; 2) ECG changes consistent with acute myocardial ischemia; and 3) an increase in cardiac biomarker levels (CK-MB mass assay and troponin T or I assays) to more than twice the upper limit of normal that followed a rise and fall pattern.

The controls were mainly recruited from among blood donors unrelated to the patients, but individually matched by age, gender and geographic origin.

Study protocol

The original study protocol was approved by the Ethics Committee of the coordinating centre, and written informed consent was given by all of the patients. After identifying consenting patients suitable for enrolment, the investigators completed a standardised case report form (CRF) that collected

detailed information concerning the cardiovascular disease history of the individual patients and all of their first- and second-degree relatives, cardiovascular risk factors, lifestyle, and medications (for further details, see Supplementary Material 1).

The patients were followed up for any subsequent hospital admission due to cardiovascular causes. This was done by means of scheduled outpatient visits and standardised telephone contacts. Follow-up by means of visits was attempted in all patients but, when this was not possible, their primary care physicians were contacted. If this was unsuccessful or not possible, a member of the family was contacted. The relevant medical record(s) of the patients reporting an event were obtained for verification purposes, and source data verification was applied to all meaningful cardiovascular events.

Blood collection, processing and storage

Blood was drawn from the antecubital vein into three tubes containing 0.106 M tri-sodium citrate, and was separated into plasma and red cells by means of centrifugation. The plasma was divided into five separate aliquots, and DNA was isolated from white blood cells using the salting out method. Both the plasma and DNA were stored at -80°C (for further details, see Supplementary Material 2).

Clinical endpoints

The primary endpoint was the composite of cardiovascular death, the recurrence of non-fatal MI, and the occurrence of non-fatal stroke. All deaths

were recorded on the basis of death certificates specifying the cause of death, and all events were investigated by means of source data verification. Cardiovascular death was defined as any death attributed to a cardiovascular cause on the patient's death certificate. The re-occurrence of MI was defined as subsequent hospitalisation ending with a discharge diagnosis of MI. Only ischemic strokes were considered (intra-cerebral bleeds and sub-arachnoid hemorrhages were not counted as stroke). All of the events were adjudicated by a Clinical Event Committee (CEC) consisting of two cardiologists who were unaware of the genotyping results; in the case of disagreement, the opinion of a third cardiologist was required

Exome sequencing and data processing

Whole-exome sequencing was performed at the Broad Institute (Boston, MA). The analysed genes were selected on the basis of the following criteria:

a) they have been associated with familial hypercholesterolemia (FH) in the literature;
b) they have been significantly associated with LDL-C levels in at least two families or population studies;
c) they have been significantly associated with LDL-C levels in only one family, but functional data supporting their role have been published.

Only the genes for which the direction of the effect of the loss-of-function mutations on cholesterol levels could be reasonably predicted were selected. Annotations were made of deleterious loss-of-function variants in 17 genes

(*ABCG5, ABCG8, ANGPTL3, APOB, APOE, CETP, CYP7A1, INSIG2, LDLR, LDLRAP1, LIMA1, LIPA, MYLIP, NPC1L1, PCSK9, PNPLA5, and STAP1*) that are known to have a significant impact on LDL-C levels (for further details, see Supplementary Material 3 and 4). Patients carrying exome variants associated with high LDL-C levels were defined as HYPER; those carrying exome variants associated with low LDL-C levels were defined as HYPO; and those not carrying any variant influencing LDL-C levels were defined as NV.

Cholesterol levels

Total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglyceride levels were measured using standard procedures in fasting venous blood samples. LDL-C levels were derived using Friedewald's formula, or directly measured in subjects whose triglyceride levels exceeded 400 mg/dL (4.5 mmol per litre).

Angiographic coronary artery disease

The total angiographic absence of any narrowing in coronary diameter was considered evidence of normal coronary arteries. A narrowing of <70% (50% in the case of the left main coronary artery) was considered non-significant coronary artery stenosis, and a narrowing of ≥70% (≥50% in the case of the left main coronary artery) was considered significant coronary artery stenosis. Significant coronary artery stenosis involving one of the three major coronary arteries was defined as single-vessel disease; significant stenosis affecting two or three of the major coronary arteries was defined as multi-vessel disease.

The extent and complexity of coronary artery disease were graded by means of the SYNTAX score, which was computed by means of an on-line calculator in accordance with the definitions.[7] To this end, the baseline and follow-up coronary angiograms were reviewed by two trained interventional cardiologists. In the case of disagreement, the judgement of a third was integrated, and the final decision was made by consensus. The delta SYNTAX score was calculated in the patients who underwent at least one repeat coronary angiography examination during the follow-up as the difference between the baseline score and the score at the time of the last examination.

Statistical analysis

Descriptive statistics were used to compare the baseline characteristics of the three groups in order to test for potential imbalances in variables that could influence survival outcomes. LDL-C levels and the SYNTAX score were evaluated using the Kruskal-Wallis test.

On the basis of the assumption that carrying a genetic variant increasing LDL-C levels is associated with an increased risk of recurrent primary ischemic events and that carrying a genetic variant reducing LDL-C levels is associated with a reduced risk, we tested the following pre-specified alternative hypothesis of Hazard Ratio $(HR)_{HYPO} < HR_{NV} < HR_{HYPER}$. The analysis of the primary endpoints was made using Cox proportional hazard models adjusted for the main known variables associated with the primary outcome. The proportional hazards assumption for the Cox regression model was confirmed

using Schoenfeld's residuals test. The cumulative incidence of the primary endpoints during the follow-up was graphically represented using Aalen-Johansen curves, and the significance of the differences between the sub-distribution of the hazards was tested by means of the Fine-Gray model.

An Andersen-Gill intensity model analysis was not initially pre-specified, but was made using a time-dependent model in order to account for the repeated occurrence of all of the components of the primary endpoint during the study period.

All of the statistical tests were 2-sided at a significance level of 0.05. The statistical analyses were made using R Statistical software, version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

All 2,000 patients successfully underwent whole-exome sequencing, and 160 carried at least one exome variant influencing LDL-C levels: 100 carried variants associated with high LDL-C levels (HYPER) and 60 carried variants associated with low LDL-C levels (HYPO). One patient was homozygous for the *LDLR* loss-of-function mutation, and one carried two heterozygous mutations in different genes (*LDLR* and *ABCG8*). One thousand eight hundred and forty patients did not carry any of the investigated exome variants influencing LDL-C levels (NV).

Baseline characteristics

Table 1 shows the demographic, clinical, angiographic and treatment characteristics of the study patients by exome variant status. All of the characteristics were well balanced except for a history of hypercholesterolemia, which was more frequently observed in the patients carrying HYPER variants (p for trend <0.01).

Multi-vessel coronary artery disease was more frequently observed in the patients carrying HYPER variants whereas normal or non-significant coronary artery disease was more frequently observed in the patients carrying HYPO variants (p for trend <0.01). There was no significant difference in the drug treatments received at any stage during the follow-up, including statin treatment.

Genotypes, lipid levels, and coronary atherosclerosis

At the time of the index event, the median baseline plasma LDL-C level was 190 mg/dL (IQR: 136-251.4) in the patients carrying an exome variant associated with increased LDL-C levels, 114 mg/dL (IQR: 85.6-131.3) in the patients carrying a genetic variant associated with decreased LDL-C levels, and 139.7 mg/dL (IQR: 112.2-167.8) in the patients not carrying any genetic variant associated with increased or decreased LDL-C levels (p for trend <0.001) (Fig. 1).

The trend of total cholesterol levels was also significant, with higher levels in the HYPER group (262 mg/dL, IQR 211-323), intermediate levels in the NV group (215 mg/dL, IQR 185-250), and lower levels in the HYPO group (182 mg/dL, IQR 161-219.5) (p for trend <0.001) while HDL cholesterol showed the opposite with lower level in HYPER (39 mg/dL IQR: 33-45), intermediate in NV (40 mg/dL IQR: 34-47) and higher in HYPO (45 mg/dL IQR 36-52) (p for trend <0.02) . There was no significant between-group difference in median triglyceride levels.

The burden and complexity of coronary atherosclerosis measured by means of the SYNTAX score at baseline was higher in the HYPER group (12, IQR 6-19) than in the NV (9, IQR 6-14) or HYPO group (9, IQR 4-14) (p for trend=0,002) (for further details, see Supplementary Material 5, Figure 1).

Genotype, atherosclerosis progression and long-term clinical outcomes

The patients were followed up for a median of 19.9 years (IQR 18.1-22.6), a total of 39,535 person-years. The follow-up was completed by 1,984 patients

(16 [0,8%] were lost to follow-up), but the vital status of 1,988 patients (99,4%) was ascertained.

During the follow-up, 549 patients underwent at least one repeat coronary angiography examination. Figure 2 shows the difference in Syntax scores between the baseline and last repeat examination divided by time. The median delta of the Syntax score was 0.61/year in the HYPER group, 0.58/year in the NV group, and 0.21/year in the HYPO group. Trend analysis showed that the incremental risk was HYPO < NV <HYPER (p for trend <0.01).

During the follow-up, 714 patients (36%) reached a primary endpoint: 153 patients died of cardiovascular causes, 479 patients experienced a recurrent MI, and 82 suffered an ischemic stroke.

Carrying a genetic variant causing increased LDL-C levels was associated with an increased risk of the primary endpoint in comparison with the patients not carrying any variants influencing cholesterol levels (HR 1.61, 95% CI 1.2-2.16; p<0.001), whereas carrying a genetic variant causing decreased LDL-C levels was associated with a non-significant reduction in the risk (HR 0.85, 95% CI 0.54-1.35; p=NS) (Tab. 2). Trend analysis showed an incremental risk across the three groups (p for trend <0.004) (Fig. 3).

Carrying a genetic variant causing increased LDL-C levels was associated with an increased risk of all of the recurrent primary endpoints in comparison with the patients not carrying any variants influencing cholesterol levels (HR 1.51, 95% CI 1.18-1.94; p<0.001), whereas carrying a genetic variant causing

decreased LDL-C levels was associated with a non-significant change in the risk of all of the recurrent primary endpoints (HR 1.02, 95% CI 0.70-1.49; p=NS) (Tab.2). Trend analysis showed an incremental risk across the three groups (p for trend <0.03) (for further details, see Supplementary Material 5, Figure 2).

DISCUSSION

The impact of rare genetic variants on cardiovascular outcomes has been previously explored in a number of settings. Of particular relevance to the present study is the finding of a causal association between LDL-C and major cardiovascular events in patients with familial hypercholesterolemia, an inherited autosomal dominant genetic disorder characterised by the defective plasma clearance of LDL-C due to mutations in the *LDLR*, *APOB* and *PCSK9* genes.[8,9,10,11,12] As nearly 5% of the patients who experience an MI before the age of 60 years have heterozygous familial hypercholesterolemia, and nearly 50% of untreated patients with heterozygous familial hypercholesterolemia experience an MI by the age of 60, the identification of patients with the rare hypercholesterolemic variants should be considered particularly important in the context of early-onset MI[13].

The findings of this study demonstrate the usefulness of whole-exome sequencing in the risk stratification of patients who have experienced an early-onset MI, which is critically important in order to enable the early and aggressive treatment of patients at higher risk.

In our study, exome sequencing identified variants influencing LDL-C in 8% of the patients with early-onset MI and, as expected, LDL-C levels were significantly higher in the patients with hypercholesterolemic variants and lower in those with hypocholesterolemic variants. A genetic predisposition to increased circulating LDL-C levels implies cumulative exposure to high plasma

LDL levels from birth, a greater risk of developing coronary atherosclerosis at a younger age, and possibly a greater risk of subsequent ischemic events after an initial MI.[14,15] This is consistent with our finding that increased baseline LDL-C levels in patients carrying hypercholesterolemic variants are associated with a higher burden and the more rapid progression of coronary atherosclerosis and, ultimately, a higher risk of one or more recurrences of major cardiovascular events than in patients with no mutations or hypocholesterolemic mutations. Our most important finding is that analysing genetic variants in addition to cholesterol levels identifies a small but not negligible 'niche' group of patients at even higher risk of events among a population that is already at risk *per se*. This underlines the importance of distinguishing genetically determined hypercholesterolemia from conditions that have phenotypically similar presentations in order to ensure appropriate therapeutic management and targeted genetic and family counselling,[16] although further studies are needed to determine whether more aggressive treatment has significant impact on long-term prognosis.

The primary outcome risk analysis also showed that hypocholesterolemic mutations do not protect against the recurrence of ischemic events after early-onset MI. It is possible that this was because the number of patients with hypocholesterolemic mutations was too small to detect a difference or because the potentially protective effect of hypocholesterolaemic variants was obscured by the 'bias' of selecting patients who had already suffered an event. All of the

study patients had experienced an early-onset MI, and it is likely that those with hypocholesterolemic mutations were exposed to unmeasured environmental risk factors or were subject to different biological pathways leading to ischemic events that may have increased the risk sufficiently to offset the possible protective impact of the hypocholesterolemic mutations themselves. Whatever the reason, the finding remains important because it shows that the presence of a hypocholesterolemic genetic variant should not be considered reassuring after an early-onset MI as it does not indicate a reduced individual risk of recurrent ischemic events.

Overall, the study provides new information concerning the significance of the effects of genetic variants influencing cholesterol levels on long-term prognosis after an early-onset MI. It remains to be determined whether the genetic information coming from the Italian Study on Early-onset Myocardial Infarction can be transferred to the general population of patients with MI, including those experiencing a first event after the age of 45 years.

In conclusion, the results of this exome sequencing study aimed at exploring the effects of hyper- and hypocholesterolemic genetic variants on the risk of recurrence in the context of early-onset MI show that the patients who carried a hypercholesterolemic variant experienced significantly higher rates of a recurrent ischemic event than those carrying no variants or hypocholesterolemic variants. On the other hand, the patients with hypocholesterolemic variants were not protected against the risk of

recurrences probably because of the presence of unmeasured, risk-increasing factors that offset the potentially protective effect of the variants themselves.

TABLES

Table 1. Baseline demographic, clinical, angiographic and treatment characteristics of the study population by genetic variant status.

Characteristics		HYPO Carriers of genetic variants influencing hypocholesterolemia (n=60)	NV Patients not carrying genetic variants influencing hypocholesterolemia or hypercholesterolemia (n=1,840)	HYPER Carriers of genetic variants influencing hypercholesterolemia (n=100)
Demographic data				
Median age (IQR), years		39 (35.7-43)	41 (37-43)	41 (35-44)
Gender	Male	54 (90.0%)	1637 (89.0%)	87 (87.0%)
	Female	6 (10.0%)	203 (11.0%)	13 (13.0%)
Median weight (IQR), kg		80 (72-90)	78 (70-88)	75 (68-85)
Median height (IQR), cm		171 (167.8-178)	172 (168-177)	170 (167-175.2)
Clinical data				
Index event	STEMI	50 (83.3%)	1572 (85.4%)	85 (85.0%)
	NSTEMI	10 (16.7%)	268 (14.6%)	15 (15.0%)
Family history of CVD		48 (80.0%)	1,492 (81.1%)	87 (87.0%)
Hypertension		14 (23.3%)	504/1,839 (27.4%)	23 (23.0%)
Hypercholesterolemia		22 (36.6%)	1,082/1,776 (60.9%)	79 (79%)
Smoking		30 (50.0%)	853 (46.4%)	38 (38.0%)
Median body mass index (IQR)		26.21 (24.70-30.21)	26.30 (24.04-29.05)	26.07 (23.98-28.09)
Diabetes		5 (8.3%)	141 (7.6%)	6 (6.0%)

Exercise 0 1 2	 38 (63.3%) 10 (16.7%) 12 (20.0%)	 960/1,835 (52.3%) 395/1,835 (21.5%) 480/1,835 (26.2%)	 57 (57.0%) 17 (17.0%) 26 (26.0%)
Alcohol consumers	35 (58.3%)	1,124/1,837 (61.2%)	62 (62.0%)
Cocaine users	10 (16.7%)	43 (2.3%)	3 (3.0%)
Coronary angiography data			
Normal or non-significant stenosis	11 (18.3%)	247 (13.4%)	8 (8.0%)
Single-vessel disease	30 (50.0%)	807 (43.9%)	33 (33.0%)
Multi-vessel disease	18 (30.0%)	760 (41.3%)	55 (55.0%)
Left main coronary artery involvement	1 (1.7%)	26 (1.4%)	4 (4.0%)
Treatment data*			
Beta blocker	45 (75.0%)	1,503 (81.7%)	81 (81.0%)
Aspirin	56 (93.3%)	1,713 (93.1%)	91 (91.0%)
P2Y12 inhibitor	29 (48.3%)	911 (49.5%)	69 (69.0%)
ACE-inhibitor or ARB	32 (53.3%)	783 (42.5%)	50 (50.0%)
Statin	58 (96.7%)	1,791 (97.3%)	100 (100%)

IQR: inter-quartile range; STEMI: ST segment elevation myocardial infarction; NSTEMI: non-ST segment elevation myocardial infarction. CVD: cardiovascular disease; Exercise: 0 = no exercise, 1 = occasional exercise, 2 = habitual exercise; *Treatment received at any time during the follow-up. P2Y12 inhibitors: clopidogrel, ticlopidine, ticagrelor, prasugrel. ACE: angiotensin-converting enzyme; ARB angiotensin receptor blockade. The denominator is shown in the case of missing data

Table 2 Incidence of the primary endpoint and recurrences of all endpoints by genetic variant status

Endpoints	HYPO Carriers of genetic variants influencing hypocholesterolemia (n=60)	NV Patients not carrying genetic variants influencing hypocholesterolemia or hypercholesterolemia (n=1,840)	HYPER Carriers of genetic variants influencing hypercholesterolemia (n=100)	HR [HYPER *vs* NV]	95% CI	HR [HYPO *vs* NV]	95% CI
First occurrence of primary endpoint							
Composite primary endpoint	19	647	48	1.61	1.2-2.16	0.85	0.54-1.35
Cardiovascular death	4	144	5				
Non-fatal myocardial re-infarction	14	423	42				
Non-fatal ischemic stroke	1	80	1				
Reoccurrences of all primary endpoints							
All primary endpoints	29	872	72	1.51	1.18-1.94	1.02	0.70-1.49
Cardiovascular death	7	222	17				
Non-fatal myocardial re-infarction	19	545	53				
Non-fatal ischemic stroke	3	105	2				

HR: adjusted hazard ratio; CI: confidence interval

FIGURES

Figure 1

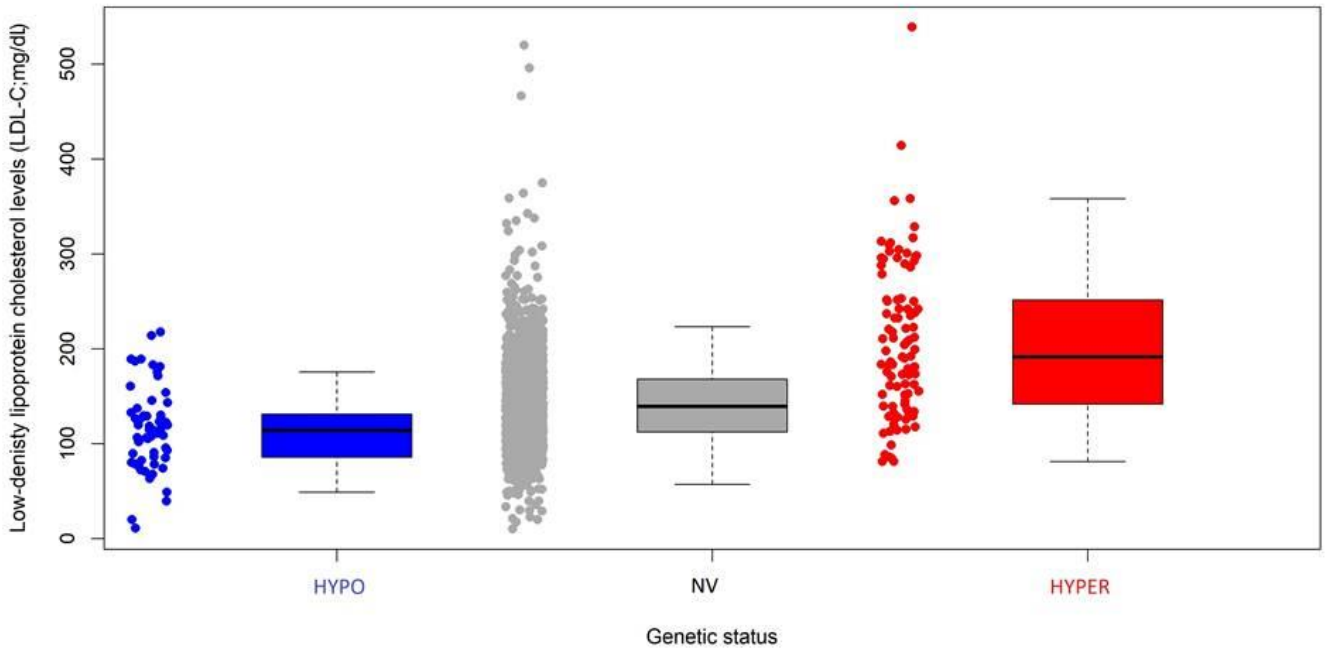


Figure 2

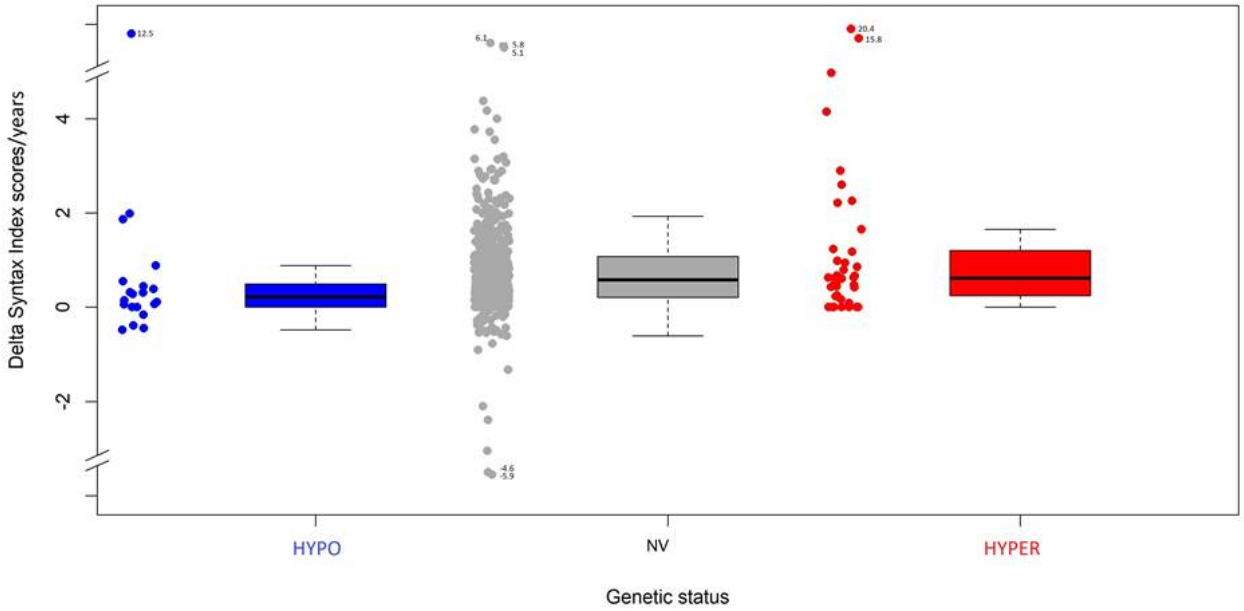
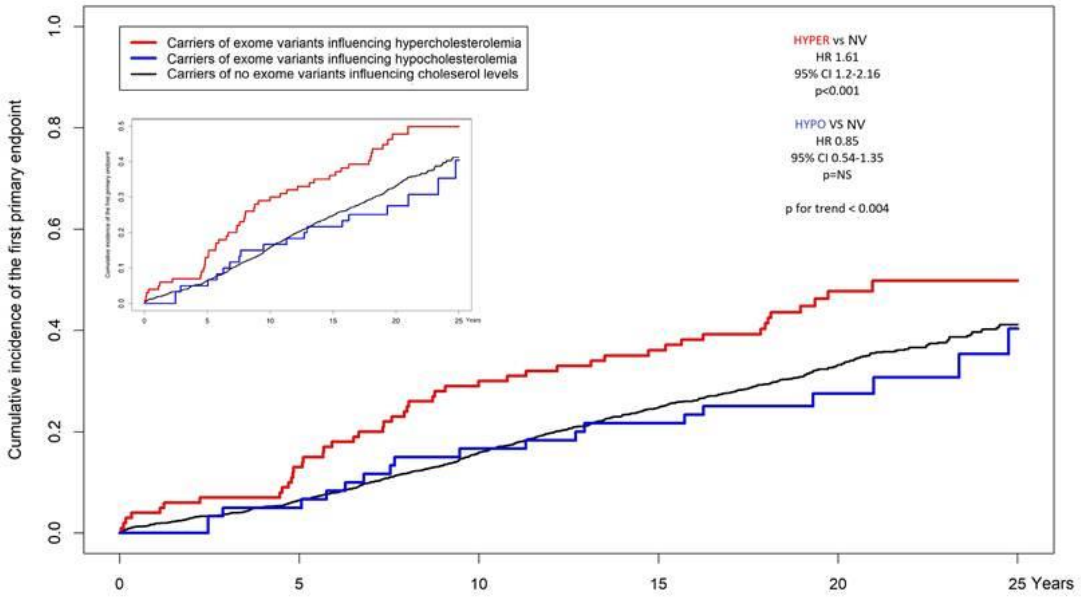

Delta Syntax Index scores/years
4
2
0
-2
12.5
6.1
5.8
5.1
-4.6
-5.9
20.4
15.8
HYPO
NV
HYPER
Genetic status

Figure 3



No.at risk for the primary endpoints:

NV	1840	1702	1499	1273	629	157
HYPER	100	86	68	59	30	5
HYPO	60	57	50	45	24	9

FIGURE LEGENDS

Figure 1: Individual values and distribution of LDL-C levels by genetic variant status.

HYPO = Carriers of genetic variants influencing hypocholesterolemia; NV = Carriers of no genetic variants influencing hypocholesterolemia or hypercholesterolemia; HYPER = Carriers of genetis variants influencing hypercholesterolemia.

Figure 2: Individual values and distribution of Delta SYNTAX scores/year by genetic variant status

.HYPO = Carriers of genetic variants influencing hypocholesterolemia; NV = Carriers of no, genetic variants influencing hypocholesterolemia or hypercholesterolemia; HYPER = Carriers of genetics variants influencing hypercholesterolemia.

Figure 3: Cumulative incidence of the first primary endpoint during follow-up by genetic variant status.

HYPO = Carriers of genetic variants influencing hypocholesterolemia; NV = Carriers of no genetic variants influencing hypocholesterolemia or hypercholesterolemia; HYPER = Carriers of genetic variants influencing hypercholesterolemia.

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SUPPLEMENTARY APPENDIX

SUPPLEMENTARY MATERIAL 1

Definitions and end-points

Family history

A positive family history was defined as the presence of at least one first-degree relative (parent, offspring, or sibling) who developed coronary artery disease before the age of 55 years for men and 65 years for women.

Hypertension

The subjects were considered to have hypertension if they had been diagnosed as hypertensive or were taking antihypertensive medication.

Obesity

The body mass index (BMI) values were categorised as normal weight (18.5-25 kg/m^2), pre-obese (25-30 kg/m^2), or obese (30-35 kg/m^2), the last including WHO classes I, II, and III; underweight subjects were excluded from the analysis because of their small number.

Smoking habits

The subjects were classified as current, former, or never smokers on the basis of self-reports: current smokers were those who reported smoking regularly during the three years preceding the myocardial infarction; former smokers were those who had smoked regularly for at least three years but not during the year preceding the infarction; and never smokers were those who had never smoked regularly or had smoked regularly for less than three years. Never and former smokers were aggregated in the single category of non-smokers.

Diabetes

The subjects were considered to have diabetes if they had ever been diagnosed as having type I or II diabetes by a physician.

Hypercholesterolemia

Hypercholesterolemia was defined as a fasting total serum cholesterol level of 200 mg/dL or the intake of anti-hypercholesterolemia medications.

Cocaine use

Cocaine use was classified as chronic, occasional, or absent at the time of the index infarction or enrolment. Cocaine users includes chronic and occasional users.

Physical activity

Physical activity was considered habitual if the subjects engaged in moderately intense exercise for 30 minutes every day or vigorous exercise for 45 minutes twice a week or 20 minutes three times a week. Any other level of physical activity was considered occasional, and no exercise was also considered as a separate category.

Alcohol consumption

Alcohol consumption was quantified on the basis of self-reports, with moderate consumption being defined as the intake of 10-30 g of ethanol/day, and high consumption as >30 g of ethanol/day. In the statistical analysis, alcohol consumption was considered a dichotomous variable (yes/no), with moderate and high consumers being aggregated in the same category.

End-points

The primary end-point was the composite of cardiovascular death, the first reoccurrence of a non-fatal myocardial infarction, and the occurrence of a non-fatal stroke.

Cardiovascular death and sudden cardiac death

All reported deaths were recorded and adjudicated by means of death certificates. Cardiovascular death was defined as death due to cardiovascular causes and included sudden cardiac death, death due to acute myocardial infarction, death due to heart failure, death due to a cerebrovascular event, death due to other cardiovascular causes (i.e. pulmonary embolism, aortic disease, cardiovascular intervention)

Sudden cardiac death refers to a death that occurs unexpectedly, not following an acute MI, and includes:

Death witnessed and occurring without new or worsening symptoms

Death witnessed within 60 minutes of the onset of new or worsening cardiac symptoms, unless the symptoms suggest acute MI

Death witnessed and attributed to an identified arrhythmia (e.g. captured on an electrocardiographic (ECG) recording, witnessed on a monitor, or unwitnessed but found on implantable cardioverter-defibrillator review)

Death after unsuccessful resuscitation from cardiac arrest (e.g. implantable cardioverter defibrillator(ICD)-unresponsive sudden cardiac death, pulseless electrical activity arrest)

Death after successful resuscitation from cardiac arrest and without identification of a specific cardiac or non-cardiac etiology

Unwitnessed death in a subject seen alive and clinically stable ≤24 hours prior to being found dead without any evidence supporting a specific non-cardiovascular cause of death.

Non-fatal myocardial re-infarction

Myocardial re-infarction was defined in accordance with the universal definition[1]. Any one of the following criteria justifies a diagnosis of myocardial infarction.

1. Detection of rise and/or fall in cardiac biomarkers (preferably troponin) with at least one value above the 99th percentile of the upper reference limit (URL) together with evidence of myocardial ischemia with at least one of the following:

• Symptoms of ischemia;

• ECG changes indicative of new ischemia (new ST-T changes or new left bundle branch block [LBBB]);

• Development of pathological ECG Q waves;

• Imaging evidence of a new loss of viable myocardium or a new regional wall motion abnormality.

2. Coronary intervention-related MI is arbitrarily defined as an increase in cTn values to more than five times the 99th percentile of the URL in patients with normal baseline values. In patients with high pre-procedural cTn levels in whom the cTn level is stable (≤20% variation) or falling, the post-procedural cTn level must increase by >20%. However, the absolute post-procedural value must still be at least five times the 99th percentile of the URL.

In addition, one of the following elements is required:

• New ischemic ECG changes;

• The development of new pathological Q waves;

• Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality in a pattern consistent with an ischemic etiology;

• Angiographic findings consistent with a procedural flow-limiting complication such as coronary dissection, the occlusion of a major epicardial artery or a side branch occlusion/thrombus, the disruption of collateral flow, or distal embolisation.

3. CABG-related MI is arbitrarily defined as an increase in cTn levels to >10 times the 99th percentile of the URL in patients with normal baseline cTn values. In patients with high pre-procedural cTn levels in whom the cTn level is stable (≤20% variation) or falling, the post-procedural cTn level must increase by >20%. However, the absolute post-procedural value must still be >10 times the 99th percentile of the URL. In addition, one of the following elements is required:

• The development of new pathological Q waves

• An angiographically documented new graft occlusion or new native coronary artery

occlusion

• Imaging evidence of a new loss of viable myocardium or a new regional wall motion abnormality in a pattern consistent with an ischemic etiology.

4. Pathological findings of an acute myocardial infarction.

Stroke

Stroke[2] was defined as an acute episode of neurological dysfunction attributable to a central nervous system vascular cause. Stroke had to be documented by imaging (computed tomography [CT] scan or magnetic resonance imaging [MRI] scan) or autoptic evidence. For the primary endpoint, we only considered ischemic stroke, defined as an acute episode of focal brain, spinal, or retinal dysfunction caused by an infarction of central nervous system tissue and documented by imaging.

Unstable angina

Unstable angina was defined as ischemic symptoms (angina, or symptoms thought to be equivalent) lasting ≥10 minutes and occurring at rest, or an accelerating pattern with frequent episodes associated with progressively decreased exercise capacity, negative cardiac biomarkers, no evidence of an acute MI, and at least one of the following:

a. New or worsening resting ECG ST or T wave changes (in the absence of confounders such as LBBB or LVH). Transient ST elevation lasting <20 minutes. New ST elevation at the J point in two contiguous leads: ≥0.1 mV in all leads other than V2-V3, which the increase must be ≥0.2 mV in men aged ≥40 years (≥0.25 mV in men aged <40 years) or ≥0.15 mV in women. ST depression and T-wave changes: new horizontal or down-sloping ST depression of ≥0.05 mV in two contiguous leads and/or new T inversion of ≥0.3 mV in two contiguous leads with prominent R wave or an R/S ratio of >1.

b. Definite evidence of inducible myocardial ischemia as demonstrated by:

• An early positive exercise stress test, defined as an ST elevation or a ≥2 mm ST depression prior to 5 METS or stress echocardiography (reversible wall motion abnormality)

• Myocardial scintigraphy (reversible perfusion defect)

• MRI findings (myocardial perfusion deficit under pharmacological stress) believed to be responsible for the myocardial ischemic symptoms/signs.

c. Angiographic evidence of new or worse ≥70% lesion (≥50% for left main lesion) and/or thrombus in an epicardial coronary artery that is believed to be responsible for the myocardial ischemic symptoms/signs.

References

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2). Adams HP Jr, del Zoppo G, Alberts MJ et al. Guidelines for the early management of adults with ischemic stroke. Stroke 2007; 38 (5): 1655-1711.

SUPPLEMENTARY MATERIAL 2

Laboratory methods

DNA extraction and quality control

Genomic DNA was extracted from peripheral blood using a Microlab STAR Liquid Handler (Hamilton, Bonaduz, Switzerland) integrated with a Chemagen automated DNA extraction system (Chemagen AG, Baesweiler, Germany). The DNA samples were quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and checked for integrity using standard agarose gel electrophoresis.

Library preparation and sequencing

Libraries were prepared using the Nextera DNA Exome kit (Illumina, San Diego, CA, USA) and the xGen Lockdown Exome Panel (IDT, San Jose, CA, USA) in accordance with the manufacturers' instructions. The libraries were quality controlled using a TapeStation 2200 System (Agilent, Santa Clara, CA, USA) and quantified using a Qubit fluorometer (Thermo Fisher Scientific). Sequencing was carried out on a NextSeq 500 machine (Illumina) using a 75-bp paired-end strategy.

Readout mapping and variant analysis

The readouts were aligned and the variants called using BWA1, GATK2, and the hg19 release of the genome. The variants were then processed and annotated using bcftools3, and Ensembl Variant Effector Predictor.4

Only the variants passing quality filters (FS<60, QD>2, PL<20, GQ>20, MQ>40, DP>5) were retained for further analysis.

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4) McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GR, Thormann A, Flicek P, Cunningham F. The Ensembl Variant Effect Predictor. Genome Biol. 2016 Jun 6;17(1):122. doi: 10.1186/s13059-016-0974-4. PubMed PMID: 27268795; PubMed Central PMCID: PMC4893825.

SUPPLEMENTARY MATERIAL 3

Table 1. Loss-of-function (LOF) and missense mutations predicted to be deleterious by five out of five software programs and identified within the analysed genes. The table shows the genomic position of each variant, the corresponding amino-acid change, the expected effect, the type of variation, the number of cases carrying each variant, and the references.

Gene	Position[a]	Effect on the protein	Direction[b]	Type[c]	Carriers	Ref.
ABCG5	2:44041643_C/A	p.Glu579Ter	↑	LOF	1	
	2:44047211_G/A	p.Arg498Ter	↑	LOF	1	
	2:44052127_C/T	p.Gly269Arg	↑	missense	1	1
	2:44052144_A/G	p.Ile263Thr	↑	missense	1	
	2:44053567_C/T	p.Arg243Gln	↑	missense	1	
	2:44055146_C/T	p.Ala204Thr	↑	missense	1	
	2:44055163_C/T	p.Arg198Gln	↑	missense	5	2
	2:44055181_C/A	p.Gly192Val	↑	missense	1	
	2:44055202_A/T	p.Ile185Asn	↑	missense	1	
	2:44058985_G/A	p.Leu142Phe	↑	missense	1	
	2:44059096_T/C	p.Tyr131Cys	↑	missense	1	
	2:44059097_A/G	p.Tyr131His	↑	missense	1	
	2:44059195_G/C	p.Ala98Gly	↑	missense	25	
	2:44064975_G/C	p.Ser88Ter	↑	LOF	1	
	2:44065003_C/T	p.Gly79Arg	↑	missense	2	3
	2:44065755_G/A	p.Gln22Ter	↑	LOF	3	
ABCG8	2:44078846_C/T	p.Ala149Val	↑	missense	1	
	2:44078890_C/T	p.Arg164Ter	↑	LOF	1	
	2:44079524_G/T	p.Arg198Met	↑	missense	1	
	2:44079529_T/C	p.Cys200Arg	↑	missense	1	

	2:44079575_G/T	p.Gly215Val	↑	missense	1	
	2:44079755_G/A	p.Glu238Lys	↑	missense	1	
ABCG8	2:44079765_C/T	p.Ser241Phe	↑	missense	3	
	2:44079992_C/A	p.Pro317Thr	↑	missense	1	
	2:44099118_A/T	p.Asp323Val	↑	missense	1	
	2:44099233_G/A	p.Trp361Ter	↑	LOF	3	
	2:44100939_A/G	p.Asn409Asp	↑	missense	1	4
	2:44100948_C/T	p.Arg412Ter	↑	LOF	1	
	2:44101102_T/A	p.Val463Asp	↑	missense	1	
	2:44101577_AC/A	p.Leu482TrpfsTer40	↑	LOF	2	
	2:44101610_T/A	p.Tyr492Ter	↑	LOF	2	
ANGPTL3	1:63063411_A/C	p.Lys58Asn	↓	missense	1	
	1:63063616_C/T	p.Leu127Phe	↓	missense	15	
	1:63063667_CAACT/C	p.Asn147Ter	↓	LOF	4	
	1:63067475_G/T	p.Gly253Cys	↓	missense	1	
	1:63068005_C/A	p.Phe295Leu	↓	missense	1	5
	1:63068052_G/GT	-[d]	↓	LOF	2	
	1:63069652_T/C	p.Leu315Ser	↓	missense	1	
	1:63069706_T/G	p.Ile333Ser	↓	missense	2	
	1:63069787_T/A	p.Leu360Gln	↓	missense	1	
	1:63069855_A/T	p.Thr383Ser	↓	missense	3	6
	1:63069856_C/T	p.Thr383Ile	↓	missense	1	
APOB	2:21224973_CA/C	p.Glu4441SerfsTer36	↓	LOF	2	
	2:21225090_C/A	p.Glu4402Ter	↓	LOF	1	
	2:21225680_G/A	p.Pro4205Leu	↑	missense	1	
	2:21225765_G/A	p.Arg4177Ter	↓	LOF	1	

	2:21229068_G/A	p.Arg3558Cys	↑	missense	2	7
APOB	2:21229355_T/C	p.Tyr3462Cys	↑	missense	1	
	2:21229860_A/G	p.Ser3294Pro	↑	missense	3	
	2:21230249_G/A	p.Thr3164Met	↑	missense	2	
	2:21230270_C/T	p.Gly3157Asp	↑	missense	1	
	2:21231482_G/C	p.Pro2753Arg	↑	missense	1	
	2:21232943_A/C	p.Leu2266Arg	↑	missense	1	
	2:21234674_C/T	p.Arg1689His	↑	missense	6	
	2:21234731_T/C	p.Glu1670Gly	↑	missense	1	
	2:21234944_C/T	p.Arg1599His	↑	missense	1	
	2:21236221_G/A	p.Pro1343Ser	↑	missense	1	
	2:21238350_T/C	p.Arg1134Gly	↑	missense	1	
	2:21245889_G/A	p.Pro877Leu	↑	missense	3	
	2:21249734_C/A	p.Gly724Cys	↑	missense	1	
	2:21249763_C/A	p.Ser714Ile	↑	missense	2	
	2:21250764_G/A	p.Pro668Leu	↑	missense	1	
	2:21251367_G/A	p.Pro554Leu	↑	missense	2	
	2:21252534_G/A	p.Arg532Trp	↑	missense	3	
	2:21255394_A/G	p.Leu395Pro	↑	missense	1	
	2:21265336_C/T	p.Arg45Gln	↑	missense	1	
APOE	19:45411858_C/G	p.Pro102Arg	↑	missense	1	
CETP	16:57004961_C/T	p.Gln182Ter	↓	LOF	1	
	16:57016107_C/T	p.Gln427Ter	↓	LOF	1	
CYP7A1	8:59404192_C/T	p.Glu453Lys	↑	missense	1	
	8:59404257_TTTAAC/T	p.Leu430ValfsTer35	↑	LOF	1	
	8:59405036_C/T	p.Arg364Gln	↑	missense	2	
	8:59407065_C/T	p.Asp347Asn	↑	missense	2	

CYP7A1	8:59409428_CTT/C	p.Lys214SerfsTer51	↑	LOF	1	
INSIG2	2:118864724_G/T	p.Gly199Ter	↑	LOF	1	
LDLR	19:11210912_C/G	p.Cys27Trp	↑	missense	1	8
	19:11210928_C/T	p.Gln33Ter	↑	LOF	1	
	19:11211016_C/T	p.Thr62Met	↑	missense	3	
	19:11213359_CG/C	p.Asp72ThrfsTer134	↑	LOF	1	
	19:11213390_C/T	p.Arg81Cys	↑	missense	1	9
	19:11213453_C/T	p.Gln102Ter	↑	LOF	1	
	19:11213463_G/A	-[d]	↑	LOF	5	
	19:11215974_A/G	p.Asp131Gly	↑	missense	1	
	19:11215992_G/T	p.Gly137Val	↑	missense	1	
	19:11216000_G/T	p.Glu140Ter	↑	LOF	2	
	19:11216047_C/A	p.Cys155Ter	↑	LOF	1	
	19:11216112_C/T	p.Ser177Leu	↑	missense	1	10
	19:11216171_T/C	p.Cys197Arg	↑	missense	2	11
	19:11216243_G/A	p.Asp221Asn	↑	missense	1	12
	19:11216244_A/G	p.Asp221Gly	↑	missense	8	13
	19:11216247_G/A	p.Cys222Tyr	↑	missense	1	13
	19:11218077_G/C	p.Cys276Ser	↑	missense	1	14
	19:11218096_C/A	p.Phe282Leu	↑	missense	1	15
	19:11221334_A/G	p.Asn316Ser	↑	missense	2	16
	19:11221390_G/A	p.Gly335Ser	↑	missense	1	17
	19:11221414_G/A	p.Gly343Ser	↑	missense	2	18
	19:11221435_C/T	p.Arg350Ter	↑	LOF	1	
	19:11223962_G/A	p.Ala399Thr	↑	missense	1	19
	19:11224013_C/T	p.Arg416Trp	↑	missense	1	20
	19:11224024_C/G	p.Tyr419Ter	↑	LOF	1	

LDLR	19:11224044_T/C	p.Leu426Pro	↑	missense	1	21
	19:11224061_C/G	p.Leu432Val	↑	missense	2	22
	19:11224126_G/A	-[d]	↑	LOF	1	
	19:11224326_G/A	p.Asp492Asn	↑	missense	1	21
	19:11224419_G/A	p.Val523Met	↑	missense	1	23
	19:11224422_G/A	p.Val524Met	↑	missense	1	24
	19:11224428_C/T	p.Pro526Ser	↑	missense	1	17
	19:11224437_G/C	p.Gly529Arg	↑	missense	1	
	19:11224439_G/A	-[d]	↑	LOF	1	
	19:11226781_G/A	p.Trp533Ter	↑	LOF	1	
	19:11227549_C/T	p.Arg574Cys	↑	missense	1	25
	19:11227568_C/T	p.Ser580Phe	↑	missense	1	26
	19:11227576_C/G	p.His583Asp	↑	missense	1	27
	19:11227604_G/A	p.Gly592Glu	↑	missense	2	17
	19:11227613_G/A	p.Arg595Gln	↑	missense	2	28
	19:11227676_T/C	-[d]	↑	LOF	1	
	19:11230858_C/A	p.Leu646Ile	↑	missense	1	29
	19:11230873_G/A	p.Asp651Asn	↑	missense	1	30
	19:11231112_C/T	p.Pro685Leu	↑	missense	2	31
	19:11231159_G/A	p.Gly701Ser	↑	missense	1	18
	19:11234017_C/T	p.Gln770Ter	↑	LOF	1	
	19:11240210_T/TG	p.Val806GlyfsTer11	↑	LOF	2	
LDLRAP1	1:25880427_T/C	p.Trp35Arg	↑	missense	1	
	1:25883729_C/CA	p.His144GlnfsTer27	↑	LOF	2	
	1:25883750_C/T	p.Arg151Trp	↑	missense	2	
	1:25883751_G/A	p.Arg151Gln	↑	missense	2	
	1:25893340_C/T	p.Leu262Phe	↑	missense	1	

LIMA1	12:50571458_G/A	p.Pro558Ser	↓	missense	1	
	12:50575756_C/T	p.Arg403His	↓	missense	1	
	12:50598446_AT/A	p.Asn251MetfsTer17	↓	LOF	1	
	12:50615856_G/A	p.Pro193Leu	↓	missense	1	
LIPA	10:90988006_G/A	p.Arg127Trp	↑	missense	1	
	10:90988074_A/G	p.Leu104Pro	↑	missense	1	
	10:91005469_G/A	p.Arg65Ter	↑	LOF	1	
MYLIP	6:16130851_G/A	p.Gly51Ser	↓	missense	5	
	6:16141888_T/G	p.Leu104Arg	↓	missense	1	
	6:16144084_G/A	p.Ala273Thr	↓	missense	2	
NPC1L1	7:44555468_G/C	p.Leu1271Val	↓	missense	3	
	7:44555477_G/A	p.Arg1268Cys	↓	missense	2	
	7:44555503_G/A	p.Ala1259Val	↓	missense	1	
	7:44555739_T/G	p.Thr1220Pro	↓	missense	2	
	7:44555756_C/T	p.Arg1214His	↓	missense	3	32
	7:44561410_A/C	p.Ser952Ala	↓	missense	1	
	7:44561793_C/T	p.Val896Met	↓	missense	1	
	7:44571352_A/C	-[d]	↓	LOF	2	
	7:44573143_T/C	p.Met766Val	↓	missense	2	
	7:44574135_G/A	p.Arg693Cys	↓	missense	1	33
	7:44575543_G/A	p.Arg627Cys	↓	missense	1	
	7:44578575_G/A	p.Pro474Leu	↓	missense	1	
	7:44578579_C/T	p.Ala473Thr	↓	missense	1	
	7:44578642_G/A	p.Arg452Trp	↓	missense	1	

NPC1L1	7:44578780_G/A	p.Arg406Ter	↓	LOF	1	
	7:44578861_G/T	p.Pro379Thr	↓	missense	1	
	7:44578870_T/A	p.Thr376Ser	↓	missense	2	
	7:44579394_C/T	p.Arg201His	↓	missense	1	
	7:44579506_A/G	p.Phe164Leu	↓	missense	1	
	7:44579575_G/A	p.Arg141Cys	↓	missense	1	
	7:44579583_T/C	p.Asn138Ser	↓	missense	1	
	7:44579613_G/A	p.Thr128Met	↓	missense	1	
PCSK9	1:55505647_G/T	p.Arg46Leu	↓	missense	35	34
	1:55512275_G/A	p.Arg160Gln	↓	missense	2	
	1:55518007_C/T	p.Arg194Trp	↓	missense	1	
	1:55523187_G/A	p.Gly394Ser	↓	missense	1	
	1:55523831_G/T	-[d]	↓	LOF	1	
PNPLA5	22:44286992_C/A	p.Gly126Ter	↑	LOF	1	
	22:44287049_CG/C	p.Asp107ThrfsTer14	↑	LOF	1	
	22:44287073_G/A	p.Gln99Ter	↑	LOF	3	
	22:44287109_G/T	p.His87Asn	↑	missense	1	
STAP1	4:68447185_C/T	p.Pro176Ser	↑	missense	1	35
	4:68458976_A/G	-[d]	↑	LOF	1	

[a]Nucleotide position according to the human genome release GRCh37/hg19, February 2009; [b]↑ = the variant increases cholesterol level; ↓ = the variant decreases cholesterol level; [c]LOF mutations defined as: nonsense mutations, insertions or deletions, and splicing mutations; [d]variants with an impact donor/acceptor splice sites.

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SUPPLEMENTARY MATERIAL 4

Table 1. Genes selected for the analysis, and published evidence of their association with the phenotype.

Gene	Position (hg19)	Associated with total or LDL cholesterol	Identified in exome sequencing analyses	Functional studies support role in LDL metabolism	Effect of loss-of-function on LDL cholesterol levels	References
ABCG5	chr2:44039611-44065958	X	X	X	↑	1-4
ABCG8	chr2:44066103-44105605	X	X	X	↑	1-4
ANGPTL3	chr1:63063158-63071976	X	X	X	↓	5-9
APOB	chr2:21224301-21266945	X	X	X	↑/↓	5,10-12
APOE	chr19:45409006-45412652	X	X	X	↑	5,12,13
CETP	chr16:56995835-57017756	X	X	X	↓	14-16
CYP7A1	chr8:59402737-59412720	X	-	X	↑	5,12
INSIG2	chr2:118846050-118867597	X	X	X	↑	5,17,18
LDLR	chr19:11200038-11244505	X	X	X	↑	5,12,19
LDLRAP1	chr1:25870076-25895377	X	X	X	↑	6,20,21
LIMA1	chr12:50569563-50677353	X	X	X	↓	22
LIPA	chr10:90973326-91011796	X	X	X	↑	12,23,24
MYLIP	chr6:16129317-16148478	X	-	X	↓	5,25
NPC1L1	chr7:44552135-44580914	X	X	X	↓	5,26
PCSK9	chr1:55505149-55530526	X	X	X	↓	5,11,12
PNPLA5	chr22:44275558-44287893	X	X	X	↑	11,27
STAP1	chr4:68424415-68473059	X	X	-	↑	28,29

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Supplementary material 5

Figure 1

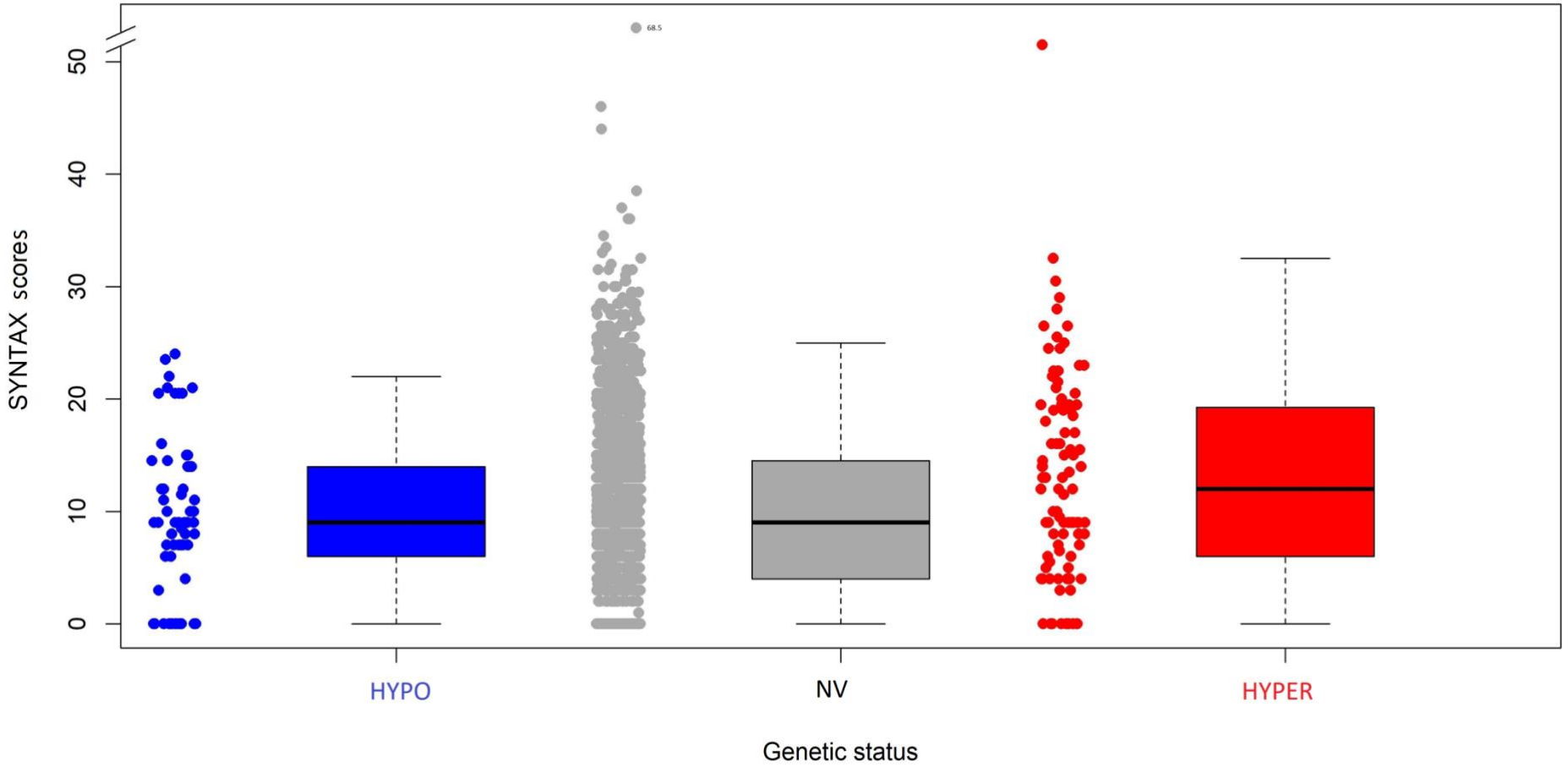
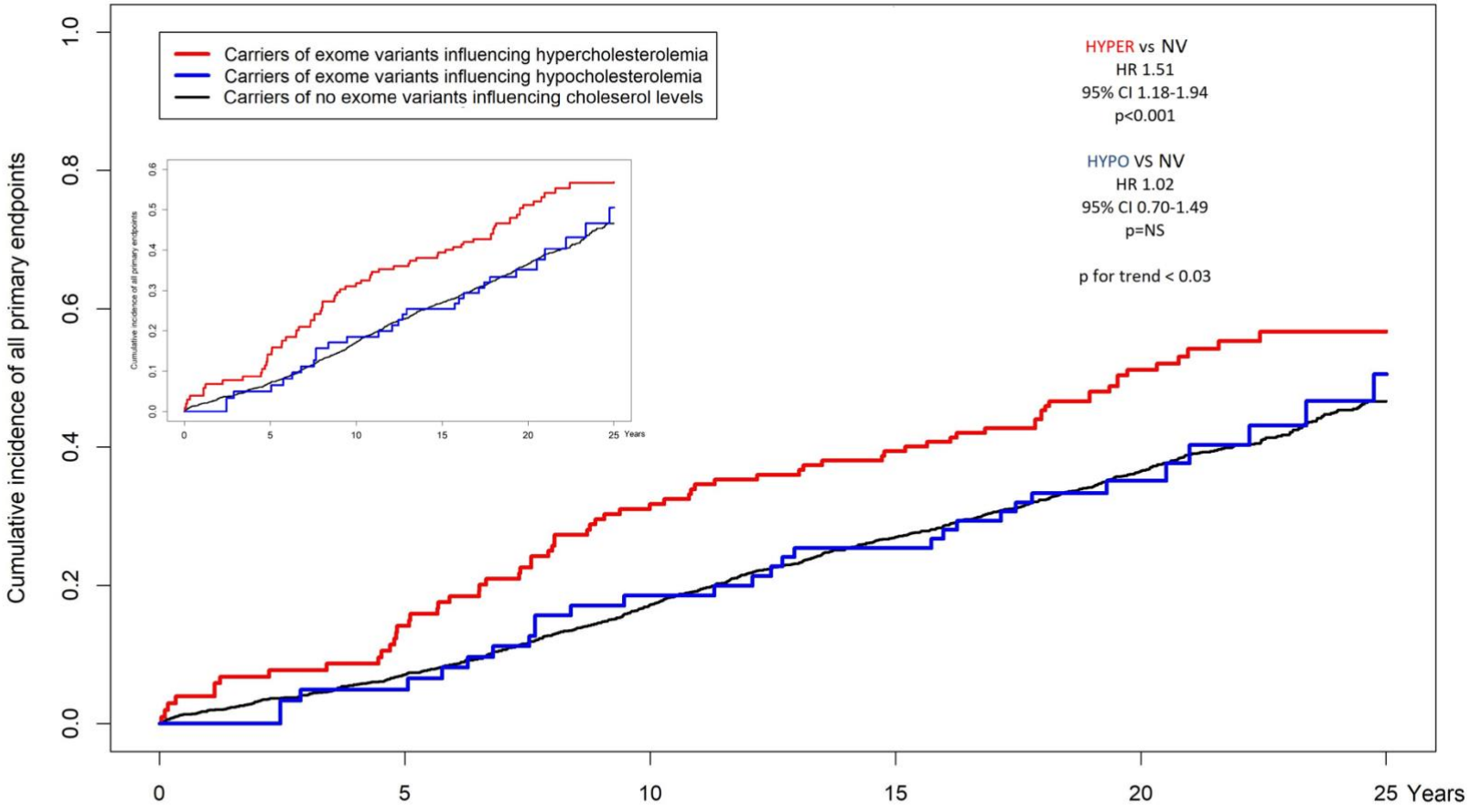


Figure 2



No.at risk for the primary endpoints:

NV	1840	1813	1748	1582	855	228
HYPER	100	97	91	86	54	18
HYPO	60	60	57	55	28	10

Figure 1: Individual values and distribution of SYNTAX scores by genetic variant status.

HYPO = Carriers of genetic variants influencing hypocholesterolemia; NV = Carriers of no genetic variants influencing hypocholesterolemia or hypercholesterolemia; HYPER = Carriers of genetic variants influencing hypercholesterolemia.

Figure 2: Cumulative incidence of all primary endpoints during follow-up by genetic variant status.

HYPO = Carriers of genetic variants influencing hypocholesterolemia; NV = Carriers of no genetic variants influencing hypocholesterolemia or hypercholesterolemia; HYPER = Carriers of genetics variants influencing hypercholesterolemia.