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# GENETIC MARKERS OF DEVELOPMENT AND PROGRESSION OF THE ATHEROSCLEROSIS. POSSIBLE ROLE OF VARIANTS THAT CHANGE THE INTERACTIONS WITH THE PROTEOGLYCANS AND LIFETIME EXPOSURE TO LIPID RISK FACTORS IN CARDIOVASCULAR HIGH-RISK PATIENTS


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# INTRODUCTION

## Coronary artery disease (CAD)

Coronary artery disease (CAD), also known as coronary heart disease (CHD), is the leading cause of mortality and morbidity in the world. It is a cardiovascular disease caused by the accumulation of cholesterol on artery walls resulting atherosclerotic occlusion (1). The metabolism of plasma lipoproteins plays a crucial role in the development of the disease, particularly that of low-density-lipoproteins (LDL) (2). The atherosclerotic process begins early in life and is due to a dysfunction of the endothelial cells covering the walls of the coronary arteries that are no longer able to regulate muscle tone. Infiltration by lipoproteins carrying cholesterol into the vessel wall causes an inflammatory response of cholesterol-loaded macrophage foam cells. Underneath the vessel wall, smooth muscle cells (SMCs) proliferate and result in remodeling and narrowing of the vessel which obstructs blood flow (3). The SMCs produce extracellular matrix, in particular collagen and proteoglycans that contribute to the atherosclerotic plaque formation leading the progression of the lesion to fibrous plaque (4). The lesion formed is characterized by a fibrous cap with an overlying lipid-rich core containing necrotic material. The proteoglycans produced by SMCs that persist in the intima of vessel can bind to lipoproteins promoting oxidative modifications and glycation processes thus propagating the inflammatory response (5) (6). The result of atherosclerotic plaque formation in the coronary artery is a lack of balance between the demand and supply of oxygen to the myocardial causing myocardial infarction (7). Epidemiological studies have shown that several factors play a central role in the development of CAD and therefore myocardial infarction (MI), including age, gender, smoking, blood pressure, diabetes, obesity and a sedentary lifestyle and genetic factors are considered predisposing to the disease in 50-60 % of cases (3) (Fig. 1).

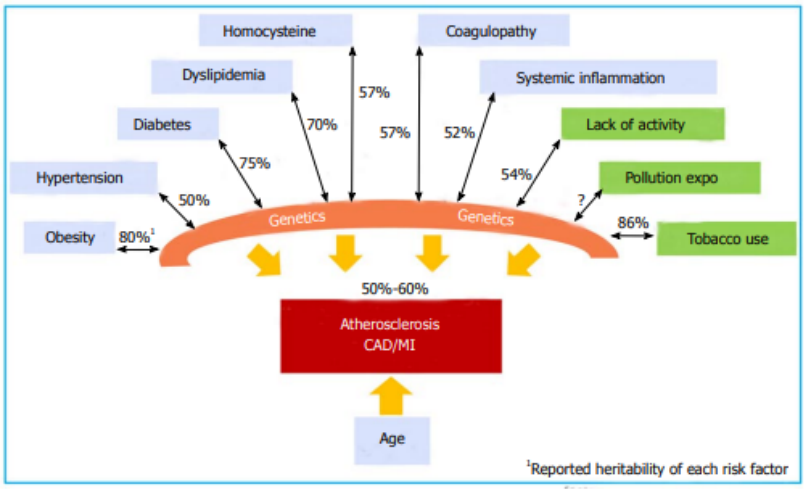


**Fig.1 Environmental and genetics factors in the development of CAD and MI** (figure from Xuming Dai, Szymon Wiernek, James P Evans, Marschall S Runge. Genetics of coronary artery disease and myocardial infarction. World J Cardiol. 2016 January; 26; 8(1): 1-23).

In addition, the increase in circulating low-density lipoproteins cholesterol (LDL-C) and triglyceride (TG)-rich lipoproteins, while the reduction of high-density lipoproteins cholesterol (HDL-C) are associated with an increased or reduced risk of developing CAD. Two monogenic disorders, familial hypercholesterolemia (FH) and familial hypobetalipoproteinemia (FHBL) reinforce this concept. Inheritance of mutations that lead to higher or lower than average levels of LDL-C are associated with increased or reduced CAD risk respectively. All these factors in practice can be combined to identify clusters of populations that have a higher risk of developing CAD and would benefit most from preventive therapies (3).

## Interaction between proteoglycans and LDL and risk of CAD

The interaction between the extracellular matrix (ECM) and LDL in the arterial wall is considered a crucial event for the development of coronary artery disease (CAD) and therefore Ischemic Heart Disease (8) (9). This link mainly occurs between proteoglycans, components of ECM, and apoliproprotein-B100 component of LDL. Proteoglycans (PGs) are biological molecules composed of a protein core covalently bound to linear long chain carbohydrates, called glycosaminoglycans (GAGs). There are different types of GAGs: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin, keratan sulfate and hyaluronan. Different classes of CS and DS containing proteoglycans are found in arteries. These include the versican, a large CS containing proteoglycan, the biglican and the decorin, two small proteoglycans rich in leucine containing respectively DS and CS / DS (8) (10) (Fig.2) The binding of atherogenic lipoproteins to arterial wall proteoglycans is mediated by ionic interactions between clusters of basic apo-B100 amino acids that bind negatively to proteoglycans' glycosaminoglycans. In vitro studies have allowed the identification of specific regions of apo-B100 basic amino acids to negative GAGs (11). Borén et al. in 1998 identified eight clusters of basic amino acids in delipidated apo-B100 that bound the negative chains of proteoglycans. To determine the functional site on the surface of the LDL particles, they analyzed the binding activity of recombinant LDL to proteoglycans isolated from transgenic mice. The replacement of basic amino acids by neutral amino acid residues at site B (residues 3359-3369) abolished both the binding of recombinant LDL to the receptor and the proteoglycans. Chemical changes in the remaining basic residues caused only a marginal further reduction in proteoglycan binding, indicating that site B is the primary binding site of LDL to proteoglycans. Although site B was essential for normal binding of LDL to the receptor and for proteoglycan binding activity, these two mechanisms could be separated into a recombinant LDL containing point mutations (12). In this study, four different types of human apo-B100 were isolated from transgenic mice. The first line of mice expressed control recombinant LDL; whose receptor binding activity was identical to that of LDL isolated from human plasma. The second line of transgenic mice expressed recombinant LDL that had a mutation in a single amino acid, replacing arginine with glutamine at position 3500 in apo-B100 (R3500Q). These lipoproteins normally bound proteoglycans, but interacted defectively with the receptor, which is why they could be an important proatherogenic predictive marker. The third type of LDL transgenic mouse in which the basic amino acids at site B were converted to neutral amino acids by abolishing

both the receptor binding site and the proteoglycans, showed that this was shared. Finally, the fourth transgenic mouse expressed recombinant LDL in which lysine in the amino acid residue 3363 of apo-B100 was replaced with glutamic acid, creating the mutation K3363E. The lipoproteins thus formed normally interacted with the receptor, but not with proteoglycans, thus representing a possible protective marker against CAD (13).

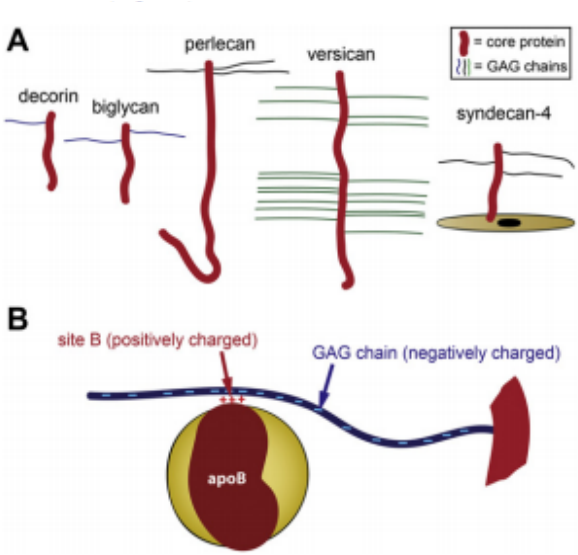


**Fig.2 The most common proteoglycans and the interaction with low-density lipoproteins** (figure from P. Fogelstrand*, J. Bore´n. Retention of atherogenic lipoproteins in the artery wall and its role in atherogenesis. Nutrition, Metabolism & Cardiovascular Disease. 2012; 22, 1-7).

An increasing number of trials have highlighted the importance of the link between proteoglycans and low-density lipoproteins in the development of coronary heart disease and the identification of known site B mutations in subjects with autosomal dominant hypercholesterolemia (ADH) could be a risk or protective factor in patients susceptible or resistant to CHD.

# Familial Hypercholesterolemia (FH) and CAD

## *Monogenic disease*

Monogenic hypercholesterolemia is a group of metabolic disorders in which the defect of a single gene causes an increase in plasma LDL cholesterol levels and a high risk to develop coronary artery disease and stroke. Hypercholesterolemia characterized by levels of LDL-C >190 mg/dl is often considered a phenotypical expression of family hypercholesterolemia (FH) (14). FH is caused by mutations in genes coding for proteins involved in the metabolic pathways that affect the LDL receptor (LDL-R) and its metabolic cycle (LDL-R recycling), resulting in decreased LDL cell uptake and increased plasma concentrations of LDL cholesterol (15).

An autosomal dominant (ADH) and autosomal recessive (ARH) form must be distinguished. ADH is caused by mutations in several genes involved in LDLR pathway: LDLR, APOB and PCSK9. LDLR gene mutations lead to a reduction in plasma LDL cell uptake (Autosomal dominant familial hypercholesterolemia type 1, ADH-1) (16). Apolipoprotein B (APOB) gene (LDL receptor binding region) defects producing a defective apoB-100 which binds poorly to the LDLR (Familial hypercholesterolemia Defective ApoB-100, FDB or ADH-2) (17). Gain of function mutations of proprotein convertase subtilisin/kexin type 9 (PCSK9) gene resulting in abnormal function of the proteolytic enzyme PCSK9 (Autosomal dominant familial hypercholesterolemia type 3, ADH-3) (18). ARH is caused by mutations in the following genes: LDLRAP1, ABCG5/ABCG8 and CYP7A1. Low density lipoprotein receptor adapter protein 1 (LDLRAP1) encodes an adaptor protein involved in clathrin-mediated internalization of the LDLR/LDL complex. (19). ABCG5 (Sterolin 1) and ABCG8 (Sterolin 2) genes belong to transmembrane ATP-binding cassette transporters family (20). CYP7A1 gene, coding for enzyme cholesterol-7a-hydroxylase involved in the first step of cholesterol catabolism and bile acids formation (21). Several studies have been conducted to correlate FH with coronary artery disease. Heterozygous and homozygous mutations in candidate genes are related with an increased LDL cholesterol plasma levels and consequently an increased risk of developing CAD (22). Heterozygous FH (HeFH) are characterized by mutations loss of function in LDLR, heterozygous mutations in APOB and gain of function mutations in PCSK9 (23). LDLR, APOB and PCSK9 mutations are found respectively in >90, 5 and 1% of cases and the frequency various geographically (24). Homozygous FH (HoFH) results from homozygous mutations, compound heterozygous

mutations in either the LDLR or ARH genes and also some rare subjects are 'double heterozygotes', which means they carry mutations in two genes, usually leading to a phenotype that is intermediate between heterozygous and homozygous FH (22). The estimated HeFH prevalence in the general population is 1/500. Recently, in Copenhagen General Population Study, a sample not selected of the general European population which includes 69,016 participants, the heterozygous FH condition was diagnosed according to the criteria of Dutch Lipid Clinic Network (DLCN) (Fig.3). The prevalence of individuals classified with certain FH or probable FH (DLCN criteria with score >5 points) was 0.73% (1/200). On basis of extrapolations of these prevalences estimated between 1/500-1/200, in the world between 14 and 34 million people would be affected by FH (14).

| Family history | Score |
|---|---|
| 1. First-degree relative with premature coronary heart disease | 1 |
| or | |
| 2. First-degree relative with LDL cholesterol >95th percentile by age and gender for country | 1 |
| 3. First-degree relative with xanthoma and/or arcus cornealis | 2 |
| or | |
| 4. Children <18 years with LDL cholesterol >95th percentile by age and gender for country | 2 |
| **Clinical history** | |
| 1. Premature coronary heart disease | 2 |
| 2. Premature cerebral or peripheral vascular disease | 1 |
| **Physical examination** | |
| 1. Tendon xanthoma | 6 |
| 2. Arcus cornealis <45 years | 4 |
| **LDL cholesterol** | |
| 1. >8.5 mmol/l | 8 |
| 2. 6.5–8.4 mmol/l | 5 |
| 3. 5.0–6.4 mmol/l | 3 |
| 4. 4.0–4.9 mmol/l | 1 |
| **DNA analysis** | |
| 1. Causative mutation in *LDLR*, *APOB* or *PCSK9* | 8 |
| **Clinical diagnosis** | |
| Definite | >8 |
| Probable | 6–8 |
| Possible | 3–5 |
| Unlikely | <3 |

**Fig.3 Dutch Lipid Clinic Network criteria for diagnosing familial hypercholesterolemia** (Figure from Alonso R. et al. Familial Hypercholesterolaemia Diagnosis and Management. Eur Cardiol. 2018 Aug;13(1):14-20)

The most severe cases of HeFH are diagnosed because they present severe symptoms such as LDL cholesterol elevation, premature and familial CHD and tendon xanthomas. The understanding of genetic causes associated with the disease has increased but remains a 10-40% of those who have a clinical diagnosis, but not a mutation diagnosis (Fig.4) (25) (26).

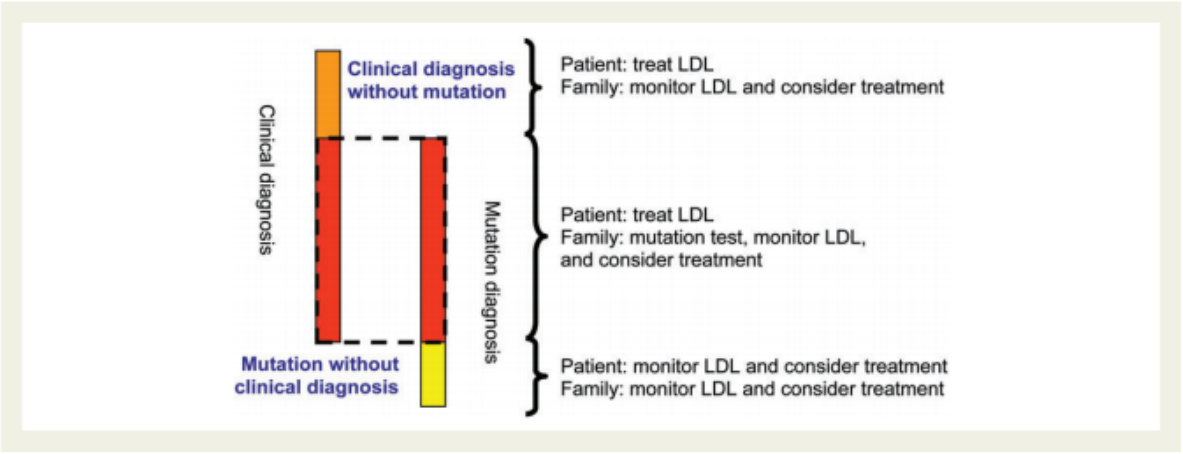


**Fig.4 Comparison between clinical and mutation diagnosis in HeFH** (figure from Børge G. Nordestgaard, M. John Chapman, Steve E. Humphries et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease. European Heart Journal (2013) 34, 3478–3490).

In addition, another factor to consider is the lifetime exposure to high levels of LDL cholesterol in the evaluation of CAD risk. However, whether diagnosed clinically or through a causal mutation, not all FH subjects develop atherosclerosis and CHD (27) (Fig.4). Early treatment is an important factor in assessing the cumulative LDL cholesterol burden. In a 55-year-old person without FH the cumulative LDL cholesterol burden is typically 160 mmol, sufficient to develop CHD. This LDL cholesterol burden for an individual with heterozygous FH, if untreated is achieved by age 35, if treated since 18 is reached by age 48, and by age 53 if treated since age 10. A subject with homozygous FH untreated reach this level at age 12.5. (22) (Fig.5).

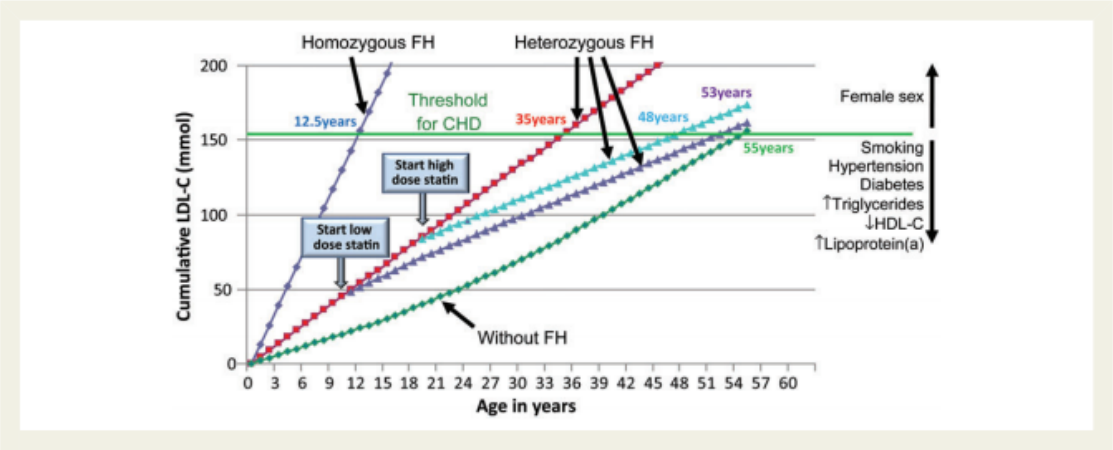


**Fig.5 Cumulative LDL cholesterl burden in subjects with and without FH considering age and treatment** (figure from 22. Børge G. Nordestgaard, M. John Chapman, Steve E. Humphries et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease. European Heart Journal (2013) 34, 3478–3490).

In subjects in which it was not possible to identify a genetic cause, other genes may be involved in the development of the disease, or alternatively, these "orphans" subjects may present a polygenic cause with increased levels of LDL cholesterol regardless of the involvement of the classic FH genes (22).

## *Polygenic disease*

Polygenic hypercholesterolemia is a multifactorial etiology disease caused by environmental and genetic factors. If no pathogenetic variants have been identified in the candidate genes described above, monogenic hypercholesterolemia cannot be diagnosed, and the polygenetic burden must be evaluated. It is not easy to clinically discriminate polygenic hypercholesterolemia from monogenic form. Genome-wide association studies (GWAS) identified 95 loci which include common variants that affects LDL-C levels (28) (29). The variants identified in these loci contribute to the variations of lipid levels and to favor extreme lipid phenotypes. Talmud et al. have demonstrated how the accumulation of allelic variants that influence the increase in LDL-C levels can lead to an increase in cholesterol like patients with diagnosed monogenic FH causing polygenic hypercholesterolemia (30). The study of Philippa Talmud starting from a base of 40% of subjects with clinically familial hypercholesterolemia who presented the mutation to the genetic cascade investigation, imagined calculating a genetic score based on 12 common SNPs (single nucleotide polymorphisms) present on 11 genes (two in ApoE) that condition the levels of LDL-C to

assess the clinical impact on subjects clinically affected by familial hypercholesterolemia with or without an identified mutation (30) (Fig.6).

| | Chromosome number | Gene | Minor allele | Common allele | GLGC weight for score calculation | Minor allele frequency | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | FH with known mutation (n=319) | FH without known mutation (n=321) | WHII controls (n=3020) |
| rs2479409 | 1 | *PCSK9* | G* | A | 0·052 | 0·33 | 0·39 | 0·35 |
| rs629301 | 1 | *CELSR2* | G | T* | 0·15 | 0·19 | 0·12 | 0·21 |
| rs1367117 | 2 | *APOB* | A* | G | 0·10 | 0·35 | 0·37 | 0·33 |
| rs4299376 | 2 | *ABCG8* | G* | T | 0·071 | 0·37 | 0·37 | 0·32 |
| rs1564348 | 6 | *SLC22A1* | C | T* | 0·014 | 0·19 | 0·17 | 0·17 |
| rs1800562 | 6 | *HFE* | A | G* | 0·057 | 0·06 | 0·08 | 0·07 |
| rs3757354 | 6 | *MYLIP* | T | C* | 0·037 | 0·21 | 0·17 | 0·21 |
| rs11220462 | 11 | *ST3GAL4* | A* | G | 0·050 | 0·14 | 0·13 | 0·13 |
| rs8017377 | 14 | *NYNRIN* | A* | G | 0·029 | 0·48 | 0·47 | 0·48 |
| rs6511720 | 19 | *LDLR* | T | G* | 0·18 | 0·10 | 0·08 | 0·13 |
| rs429358 | 19 | *APOE*† | C | T | .. | 0·19 | 0·21 | 0·15 |
| rs7412 | 19 | *APOE*† | T | C | .. | 0·04 | 0·03 | 0·08 |
| ε2ε2 | 19 | *APOE* | .. | .. | −0·9 | .. | .. | .. |
| ε2ε3 | 19 | *APOE* | .. | .. | −0·4 | .. | .. | .. |
| ε2ε4 | 19 | *APOE* | .. | .. | 0·2 | .. | .. | .. |
| ε3ε3 | 19 | *APOE* | .. | .. | 0 | .. | .. | .. |
| ε3ε4 | 19 | *APOE* | .. | .. | 0·1 | .. | .. | .. |
| ε4ε4 | 19 | *APOE* | .. | .. | 0·2 | .. | .. | .. |

LDL-C=low-density lipoprotein cholesterol. FH=familial hypercholesterolaemia. WHII=Whitehall II. G=guanine. A=adenine. T=thymidine. C=cytosine. *Risk alleles (LDL-C-raising). †*APOE* weights were based on haplotypic effects taken from Bennet and colleagues' study,* as described in the Methods section of our study.

*Table 2:* **Global Lipid Genetic Consortium 12-SNP LDL-C gene score calculation**

**Fig.6 The 12 SNPs used to define the LDL-C gene score** (figure from Talmud PJ, Shah S, Whittall R, Futema M, Howard P, Cooper JA, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. Lancet. 2013;381(9874):1293–301)

The polygenic component of the LDL-C increment had a decisive influence on the LDL-C values (each 1 increment of genetic score was associated with a 0.33 mmol/L increase of LDL-C). The percentage of patients above the threshold of 4.9 mmol/L (190 mg/dL) of LDL-C, which represented the diagnostic cut-off of FH, also increased from 17% in the lowest decile of genetic score distribution to 43% in the highest decile. There was also a clear influence on LDL-C values in subjects with familial hypercholesterolemia. Subjects with FH had a higher genetic score than controls and those with negative mutation had a higher score than those with known mutation, suggesting, in addition to a potentially diagnostic role, the possibility that, in these patients, LDL-C levels may be further affected by polygenic components (30) (Fig.7).

| | LDL-C weighted score in WHII controls | | Measured LDL-C (mmol/L) in WHII controls, mean (SD) | WHII controls with LDL-C >4·9 mmol/L | | Risk ratio (95% CI) of LDL-C >4·9 mmol/L* |
|---|---|---|---|---|---|---|
| | Mean (SD) | Range | | Measured | Predicted | |
| Decile 1 | 0·43 (0·14) | −0·5 to 0·58 | 3·76 (0·95) | 36/299 (12%) | 51/302 (17%) | NA |
| Decile 2 | 0·66 (0·04) | 0·58 to 0·73 | 3·99 (0·88) | 43/296 (15%) | 69/302 (23%) | 1·21 (0·80–1·82) |
| Decile 3 | 0·77 (0·03) | 0·73 to 0·81 | 4·21 (0·96) | 71/300 (24%) | 82/302 (27%) | 1·97 (1·36–2·84) |
| Decile 4 | 0·85 (0·02) | 0·81 to 0·88 | 4·34 (0·95) | 85/298 (29%) | 88/303 (29%) | 2·37 (1·66–3·38) |
| Decile 5 | 0·91 (0·02) | 0·88 to 0·93 | 4·36 (0·94) | 80/300 (27%) | 94/302 (31%) | 2·21 (1·55–3·17) |
| Decile 6 | 0·96 (0·01) | 0·94 to 0·98 | 4·48 (0·91) | 96/298 (32%) | 100/302 (33%) | 2·68 (1·89–3·79) |
| Decile 7 | 1·00 (0·01) | 0·98 to 1·02 | 4·50 (1·00) | 102/295 (35%) | 106/302 (35%) | 2·87 (2·04–4·05) |
| Decile 8 | 1·05 (0·02) | 1·02 to 1·08 | 4·56 (0·93) | 96/292 (33%) | 108/301 (36%) | 2·73 (1·93–3·87) |
| Decile 9 | 1·12 (0·02) | 1·08 to 1·16 | 4·68 (1·05) | 120/294 (41%) | 118/302 (39%) | 3·39 (2·42–4·74) |
| Decile 10 | 1·23 (0·06) | 1·16 to 1·46 | 4·90 (0·99) | 148/295 (50%) | 130/302 (43%) | 4·17 (3·01–5·78) |

Please see appendix for details of how we predicted the LDL-C values. LDL-C=low-density lipoprotein cholesterol. WHII=Whitehall II. NA=not applicable. *Decile 1 used as reference.

*Table 3:* **Outcome data in Whitehall II controls according to weighted LDL-C gene score deciles**

**Fig.7 Weighted gene score deciles** (figure from Talmud PJ, Shah S, Whittall R, Futema M, Howard P, Cooper JA, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. Lancet. 2013;381(9874):1293–301)

In 2015 Futema et al. conducted a study on a cohort of patients from 6 countries that included patients carrying mutations in candidate genes (positive FH) and patients who were mutation negative (31). The authors evaluated the performance of the 12 SNPs described in Talmud et al.'s work and classified them according to their allele frequency and the effect they had on LDL-C levels. The first 6 SNPs were selected for the score calculation (30) (31). The sequential removal of SNPs that had minor allelic effects and frequencies highlighted how the score executed on 6 SNPs performed as well as the 12 SNPs score. The SNPs score was based on genotypes of 6 SNPs: rs629301 [CELSR2 (cadherin, EGF LAG 7-pass G-type receptor 2)], rs1367117 [APOB], rs6544713 [proxy of rs4299376, ABCG5/8 (ATPbinding cassette, sub-family G (WHITE), member 5/8)], rs6511720 [LDLR], rs429358 [APOE (apolipoprotein E)], and rs7412 [APOE] (31) (Fig.8).

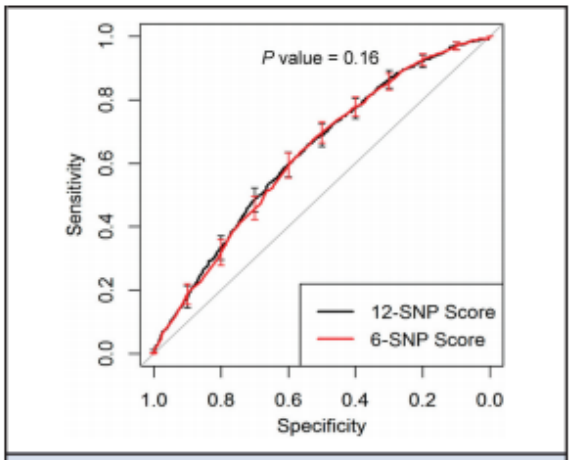


**Fig.8 Discrimination between 12 SNPs score and 6 SNPs score in positive FH and negative FH patients** (figure from 31. Marta Futema et al. Refinement of Variant Selection for the LDL Cholesterol Genetic Risk Score in the Diagnosis of the Polygenic Form of Clinical Familial Hypercholesterolemia and Replication in Samples from 6 Countries. Clinical Chemistry. 2015; 61:1 231–238)

It has been demonstrated that even the 6 SNPs score is able to discriminate between positive FH and control individuals and the use of fewer SNPs certainly has advantages in terms of costs (31). Today these 6 SNPs are genotyped to assess the probability of a patient with suspected FH to develop polygenic hypercholesterolemia. Recent guidelines recommend cascade testing on DNA when the family mutation is known and assessing LDL-C levels where the mutation is not available (32). These data support the hypothesis that in patients with LDL-C>190 mg/dL where no mutation has been identified in known genes associated with FH, the clinical diagnosis of FH is most likely a polygenic cause. If this hypothesis is negative, a mutation should be found in a gene not yet known.

# Familial hypobetalipoproteinemia (FHBL) and CAD

## *Monogenic disease*

The hypobetalipoproteinemia (HBL) is a heterogeneous disease characterized by low levels of LDL-C, total cholesterol and apolipoprotein B (apoB) under the 5$^{th}$ percentile of the general population (33). LDL plays a central role in the transport of cholesterol in the body and apoB is the main apolipoprotein of LDL (34). The main dominant monogenic form of HBL is the Familial hypobetalipoproteinemia (FHBL) (35). The majority FHBL subjects are heterozygotes with a frequency of 1:500-1:1000. The homozygous and compound heterozygous ones are very rare, and they have very low levels of LDL-C and apoB (34) (36) (37). FHBL is caused by loss of function mutation in APOB gene and rarely in PCSK9 gene (38). Most mutations found in APOB gene result in the formation of a premature stop codon in the mRNA of apoB and a truncated form of apoB that is unable to form plasma lipoproteins in liver and/or intestine and export lipids from these organs (33) (39). In heterozygous individuals carrying these mutations, plasma levels of LDL-C and apoB are 30% lower than normal levels (40). This reduction is due not only to low levels of apoB truncated form but also to a 70-75% reduction in apoB-100 production encoded by the normal allele compared to normal subjects (41) (42).
The large number of FHBL subjects in which no mutations were found in the APOB gene suggests that there are other genes involved in the development of the disease, among these recent studies have highlighted loss of function mutations in PCSK9 (43). Loss of function mutations in PCSK9 result in increased degradation of the LDL receptor by reducing the concentration of LDLR on the cellular membrane and thus the cellular uptake of LDL resulting in a reduction in plasma LDL-C levels (44).
Another type of primary HBL is combined familial hypolipidemia characterized by low levels of LDL-C, TG and HDL-C. identified for the first time in two siblings compound heterozygotes for two different nonsense mutations in the ANGPTL3 gene (encoding angiopoietin-like 3 protein), inhibitor of lipoproteins and endothelial lipase that plays a key role in lipid metabolism by increasing triglyceride levels. Loss of function mutations in ANGPTL3 represent a new cause of monogenic HBL (33). Several studies have correlated the presence of causative mutations in candidate genes (APOB, PCSK9 and ANGPT3) and the reduced incidence of developing CAD. A recent study conducted in 57,973 patients in several countries the authors identified 37 protein truncating-variants in APOB gene including 19 nonsense single-nucleotide substitutions, 3 single-nucleotide substitutions and

15 frameshift indels in 56 individuals in heterozygous form. Carriers of these mutations were associated with lower levels of apoB, LDL-C and TG and a lower risk of developing coronary artery disease, less than 72 %, compared to non-carriers (45) (Fig.9).

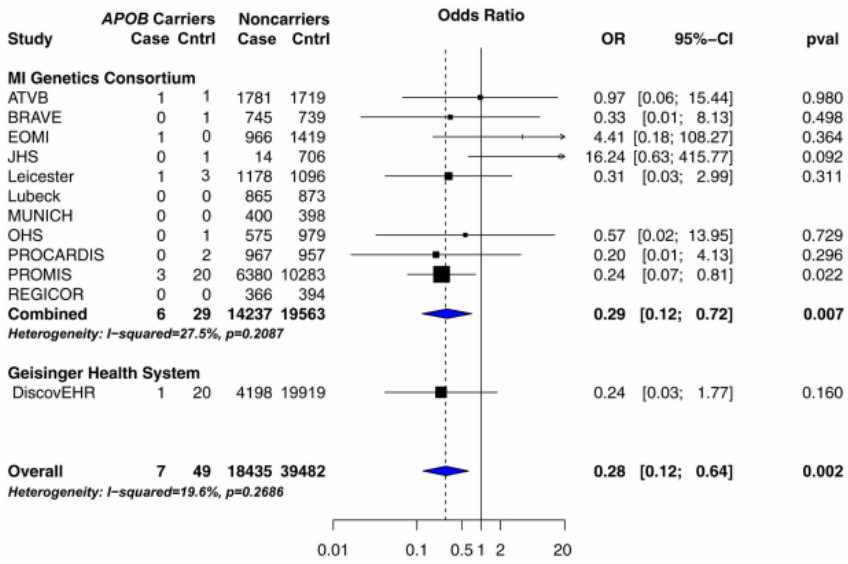


**Fig.9 Association between CHD and APOB truncating variant carrier in 57,973 individuals** (figure from Gina M. Peloso et al. Rare protein-truncating variants in APOB, lower LDL-C, and protection against coronary heart disease: APOB variants with LDL and CHD. Circ Genom Precis Med. 2019 May ; 12(5): e002376)

A study by Coen et al showed that in the African population, carriers of nonsense mutations (Y1423 and C6793) in PCSK9 determined a 28% reduction in LDL-C and 88% of the risk of developing CAD among carriers compared to non-carriers (46) (Fig.10).

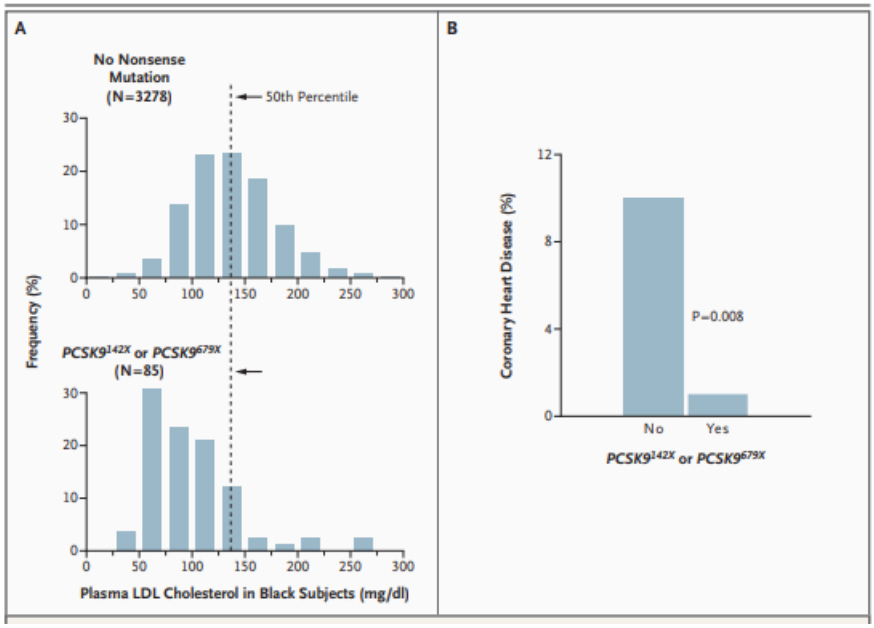


**Fig.10 Plasma LDL-C levels and incidence of coronary heart disease in patients carrier and non-carrier of nonsense PCSK9 mutations** (figure from Jonathan C. Cohen et al. Sequence Variations in PCSK9, Low LDL, and Protection against Coronary Heart Disease. N Engl J Med 2006;354:1264-72)

In the Atherosclerosis Risk in Communities Study, a missense mutation in PCSK9 (R46L), present in 3% of Caucasians, was associated with a 15% reduction in LDL-C and a 46% reduction in CHD (47). The association between R46L loss of function PCSK9 mutation and CHD was replicated in other two case-control studies (48) (49).
A 2017 study showed that the loss of function variants in ANGPTL3 causing protein deficiency leads to a 34% reduction in the risk of CAD among heterozygous carriers. The ANGPTL3 gene was sequenced in 13,914 individuals with CAD and 26,198 controls without CAD. Twenty-one loss of function variants were identified, 7 of which led to the formation of a premature stop codon, 2 splicing variants and 12 frameshift indels. A cohort-based meta-analysis stratified by ancestry was evaluated to establish the correlation between LOF mutations in ANGPTL3 and risk to develop CAD (50) (Fig.11).

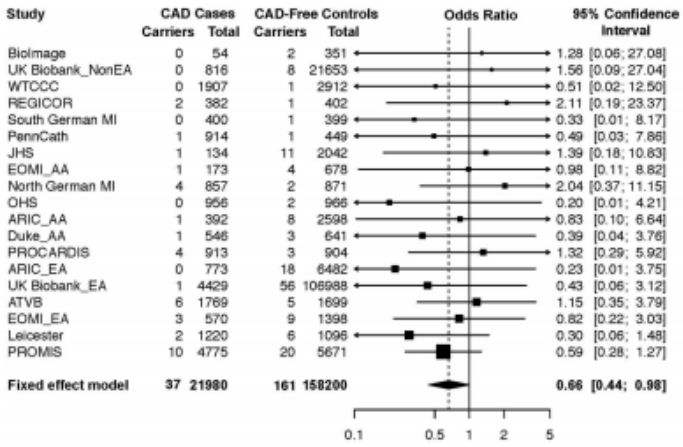


**Fig.11 Correlation between ANGPTL3 mutations and risk to develop CAD** (figure from Nathan O. Stitziel et al. ANGPTL3 Deficiency and Protection Against Coronary Artery Disease. J Am Coll Cardiol. 2017 April 25; 69(16): 2054–2063)

HBL disease may also have a polygenic cause when no pathogenic variants were found in candidate genes described above, in this case more polymorphic variants contribute to determine the clinical phenotype of patients.

## *Polygenic disease*

Several mendelian randomization studies have shown that treatment with statins that leads to lower LDL-C levels is related to a reduction in the risk of developing coronary events (51). Atherosclerosis is a chronic and progressive disease that begins early in an individual's life and develops slowly, more an individual is exposed to low LDL-C levels early more this can prevent or delay the development of the disease and thus improve the benefits of hypolipidemic therapies (52) (53). In these studies, several single nucleotide polymorphisms (SNPs) were associated with LDL-C reductions (54). Inheriting the protective allele associated with low levels of LDL-C is analogous to being treated with hypolipidemic therapy from birth, while inheriting the other allele is analogous to being randomly assigned to a usual treatment. If this hypothesis is true, the association of such polymorphism with low levels of LDL-C should provide an estimate of CHD risk in a similar way to randomized trials that compared lifetime exposure to low levels of LDL-C from early life statin therapy (55) (56). Ference et al. in a 2012 study conducted a meta-analysis to evaluate the association between the lifelong exposure to low LDL-C levels and the risk to develop CHD considering

9 SNPs in different genes previously associated with low LDL-C levels (56). For each SNPs was considered the risk allele exposure associated with low LDL-C levels and CHD risk. In addition, was evaluated the effect of each CHD risk exposure allele adjusted for low LDL-C units (56) (Fig.12).

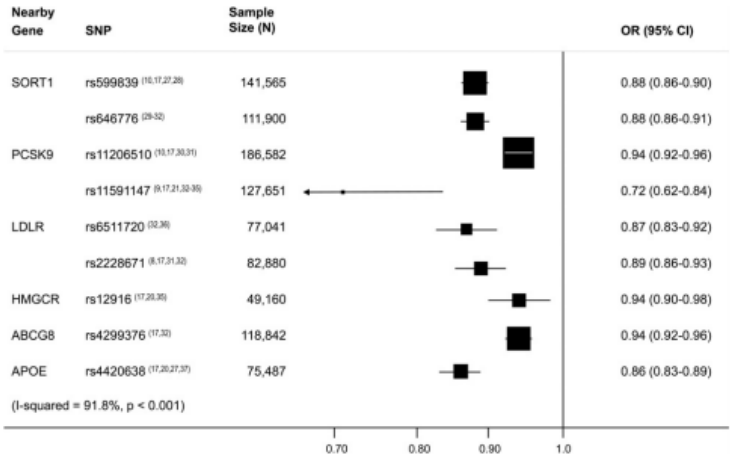


**Fig.12 Association between exposure allele and risk of CHD** (figure from Brian A. Ference et al. Effect of Long-Term Exposure to Lower Low-Density Lipoprotein Cholesterol Beginning Early in Life on the Risk of Coronary Heart Disease. JACC Vol. 60, No. 25, 2012:2631–9)

Inheriting an allele associated with lower LDL-C is therefore analogous to being randomly assigned to a therapy that lowers LDL-C from birth, while inheriting the other allele is analogous to being randomly assigned to normal care or at the placebo. The clinical benefit of lower LDL-C exposure both genetically and pharmacologically appears to be largely determined by the absolute magnitude of lower LDL-C exposure (57). The exposure allele for each SNP was associated to lower LDL-C level between 2,6 and 16,7 mg/dl (58) (59) (Fig.12). Exposure alleles were associated with a reduction of the risk of developing CHD that ranged significantly between 6% and 28%. The graph below shows the proportional risk reduction as a function of the lower LDL-C levels associated with each allele exposure, the ratio of long-term exposure to lower LDL-C to CHD risk was approximately log-linear (56) (Fig.13). In particular long-term exposure for each mmol / L of lower LDL-C is associated with a 55% reduction in the risk of CHD.

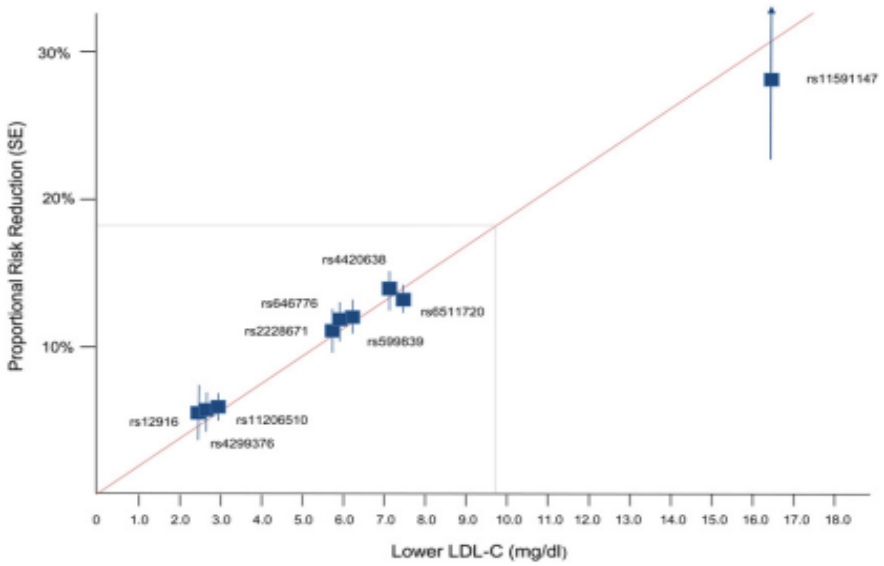


**Fig.13 Log-linear relationship between proportional risk reduction and lower LDL-C associated with each exposure allele** (figure from Brian A. Ference et al. Effect of Long-Term Exposure to Lower Low-Density Lipoprotein Cholesterol Beginning Early in Life on the Risk of Coronary Heart Disease. JACC Vol. 60, No. 25, 2012:2631–9).

Extending this log-linear relationship of this study to general population suggests that prolonged exposure to very low levels of LDL-C could potentially result in a reduced risk of developing CHD (60). In the future it may be possible to extend this strategy by evaluating the heredity of one or more protective alleles against CHD of known polymorphisms associated with low levels of LDL-C in determining the possibility of building a polygenic risk burden and evaluating polygenic hypocholesterolemia.

## Association between TG levels and CAD

Unlike individuals characterized by high levels of LDL-C with FH, many individuals with high levels of triglycerides do not necessarily develop coronary artery disease and atherosclerosis (61). This initial idea led to conflicting interpretations on the role of triglyceride concentration in the development of CAD, but was resolved by the fact that the ability of TG-rich lipoproteins to penetrate the subendothelium is inversely related to their size, therefore, while VLDL and chylomicrons, due to their considerable size, are not able to cross the basal membrane, IDL and LDL penetrate more easily and accumulate. Recent data seem to indicate a direct relationship between small VLDL/IDL and atherogenesis (62) (63) (64). TG-rich lipoproteins contain both exogenous and endogenous TG. Triglycerides originating from dietary fats are absorbed by enterocytes and combined with apoB-48 to form chylomicrons (larger lipoprotein particles) which are transported to the mesenteric lymphatic vessels before entering the bloodstream where they acquire apoC-II, apoC-III and apoE. In the blood, chylomicrons are rapidly hydrolyzed by lipoprotein lipase (LPL) along the luminal surface of the capillaries, resulting in the production of acid free fats and chylomicron remnants. The activity of LPL is highly regulated both positively and negatively by various proteins such as apoC-III, apoA-V, ANGPTL3 and ANGPTL4. Triglycerides are also synthesized in free fatty acids and glycerol from hepatocytes and then, with apoB, form VLDL particles. After secretion, the VLDL particles are hydrolyzed by LPL in the plasma, producing progressively smaller VLDL particles and possibly intermediate density lipoproteins (IDL) (65) (Fig.14).

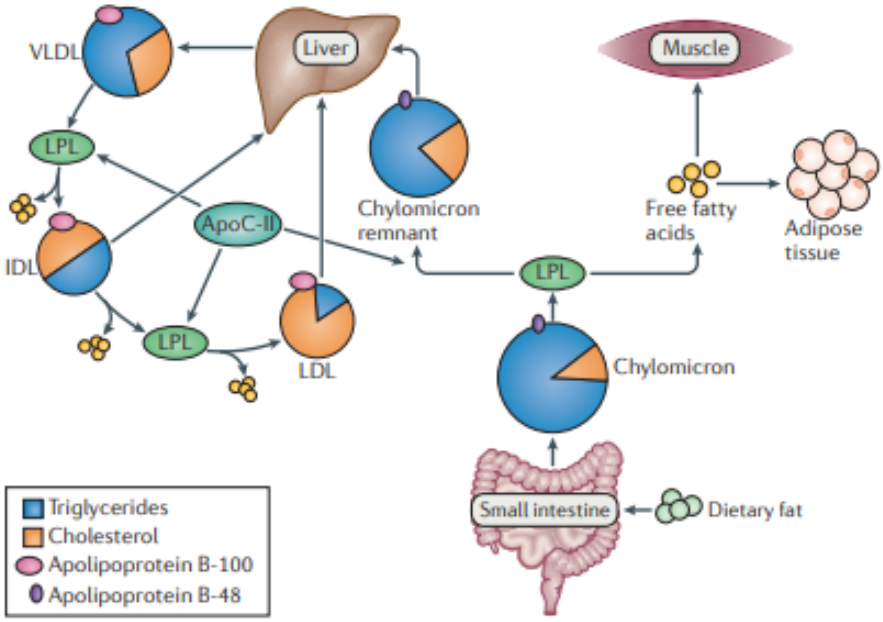


**Fig.14 Triglycerides metabolism** (figure from 65. Željko Reiner. Hypertriglyceridaemia and risk of coronary artery disease. Nat Rev Cardiol 2017 Jul;14(7):401-411).

Pathogenic mutations which determined loss of function in genes encoding for proteins involved in triglyceride metabolism that led to increased plasma triglyceride levels cause metabolic disorders known as hypertriglyceridemia (HTG). It is possible to distinguish a severe form characterized by TG levels>10 mmol/L (885 mg/dL) and a mild-moderate form characterized by levels between 2 to 10 mmol/L (175–885 mg/dL) (66). Increased triglyceride levels can be caused by mutations in single genes involved in triglyceride metabolism (monogenic hypertriglyceridemia) but also by the effect of multiple variants in multiple genes affecting VLDL production and removal (polygenic hypertriglyceridemia). The main genes involved in monogenic hypertriglyceridemia are: APOA5, APOC2, APOC3, GPD1, GPIHBP1, LMF1, and LPL (67). LPL (lipoprotein lipase) gene mutations characterize most patients with the monogenic form. Variants in this gene are positively associated with TG levels leading to an increase and with the development of coronary artery disease. Epidemiological and GWAS studies suggest a correlation between plasma triglyceride levels and coronary artery disease (57). Following this line, recent scientific evidence suggests the important role of variants in genes coding for ApoC-III and ApoA-V (two regulators of lipoprotein-lipase). Heterozygous carriers of loss of function (LOF) variants APOC3 gene have lower plasma levels of TG and apoC-III and a 40% risk reduction of developing CAD. More, rare loss of function variants of the APOA5 gene

contribute to higher plasma TG levels and an increased risk of developing CAD (64). ANGPTL4 is a negative regulator of LPL activity, for example the E40K variant is associated with lower levels of triglycerides and higher levels of HDL cholesterol. Exome sequencing studies have found that mutations with loss of function in ANGPTL4 are associated with substantially lower triglyceride levels without significant differences in LDL or HDL cholesterol levels, and that these variants correlate with a risk less than 53% of coronary heart disease (68).

However, most cases of hypertriglyceridemia are caused by multigenic or polygenic factors determined by small or large effect size, in terms of allelic frequency, of SNPs associated with the disease (69). Although prospective studies have identified high levels of triglycerides as a cardiovascular risk factor, the uncertainty of the role of triglyceride-rich lipoproteins in atherogenesis remains (70) (71) (72). GWAS studies on hypertriglyceridemia have shown that common variants in different genes such as APOA5, GCKR, LPL and APOB were strongly associated with the susceptibility to develop HTG (73) (74). Common variants in triglyceride-associated genes were evaluated in healthy and affected subjects showing that these were strongly associated with hypertriglyceridemia. A genetic risk score built by evaluating the carrier status of alleles in 32 triglyceride-associated loci showed which was higher in affected patients than in healthy patients (Fig. 15) (75) (76).

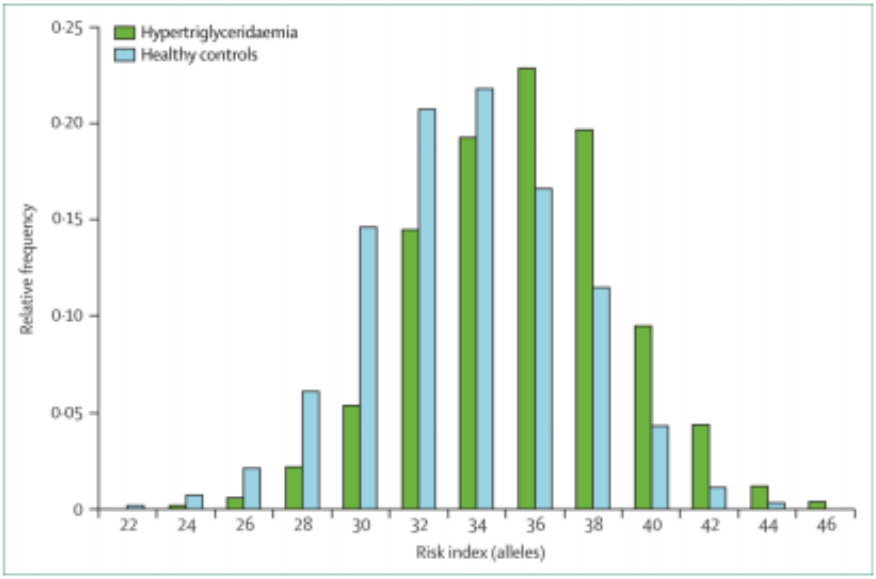


**Fig.15 Genetic risk score for tryglicerides associated risk alleles in patients with and without HTG** (figure from Hegele, R. A. et al. The polygenic nature of hypertriglyceridaemia: implications for definition, diagnosis, and management. Lancet Diabetes Endocrinol. 2, 655–666 2014).

Mendelian randomization analyses suggest a direct association between triglycerides, triglyceride-rich lipoproteins and coronary artery disease. Epidemiological, genetic and clinical evidence has laid the basis for reducing the diagnosis of hypertriglyceridemia in severe, more likely related to a monogenic cause and mild-to moderate more likely related to a polygenic cause with the addition of secondary factors (67).

## Association between HDL levels and CAD

Observational studies have highlighted the opposite association between LDL cholesterol and HDL cholesterol and the possibility of developing coronary heart disease and myocardial infarction. While high levels of LDL cholesterol are associated with an increased risk of developing CAD, high levels of HDL cholesterol are associated with a reduced risk. But while for LDL cholesterol levels the results of all large scale-randomized trials suggest a positive relation between LDL and CAD, mendelian randomization study to HDL cholesterol are inconsistent and ambiguous (77) (78) (79). The main apolipoprotein of HDL is ApoAI that involved the regulation of plasma HDL levels, acts as a cofactor of LCAT (responsible for the formation of cholesterol esters) and promotes the efflux of cholesterol from cells. Mutations in ApoA1 gene caused the familial hypoalphalipoproteinemia. Patients with homozygous mutations in ApoA1 gene have HDL-C< 5 mg/dL while heterozygous loss of function mutations are associated with HDL levels less than 50% of normal ones (80). Prospective studies have made it possible to evaluate the plasma concentration of ApoAI by comparing with that of HDL in predicting cardiovascular events and it seems that the evaluation of ApoAI gives greater benefits in predicting risk than HDL (81). Heterozygous mutations in the ApoA1 gene appear to be associated with a lower atherosclerotic risk compared to low HDL concentrations (82). Another gene involved in HDL metabolism is ABCA1 (ATP Binding Cassette A1) expressed by hepatocytes and enterocytes to acquire lipids, thus generating a rising HDL particle (83). The rarity of homozygosity mutations involving both alleles and causing Tangier's disease has not yet allowed to define the risk of developing coronary heart disease (84) (85). In the case of loss of function mutations in heterozygosity in ABCA1, subjects have HDL values of about half of normal levels, while some studies have suggested an increase in atherosclerosis, larger population-based studies show that there is no increased risk of developing CAD (86). The enzyme lecithin cholesteryl acyl transferase (LCAT) acts on nascent HDL particles to determine the formation of cholesterol esters contained in the lipoprotein core (87). Patients carriers of homozygous mutations have low levels of HDL but premature coronary artery disease is not a common manifestation while loss of function heterozygous mutations are associated with a lower reduction of HDL cholesterol but increased risk of CAD (88) (89). CETP (cholesteryl ester transfer protein) deficiency is causing raised HDL levels, but it is not yet clear whether this protects against coronary heart disease (90). Added to this is recent data on common and low frequency genetic variants associated with changes in HDL levels. Loss-of function variants

in LIPG (endothelial lipase) gene are associated with high HDL concentrations (91). A 2012 study tested the LIPG Asn396Ser variant in 20 studies (20.913 myocardial infarction cases, 95.407 controls). The Asn396Ser carriers have high HDL levels compared to non-carriers, based on association between SNP and HDL cholesterol and HDL and myocardial infarction the authors estimated that the SNP should decrease myocardial infraction risk of 13% (77) (Fig.16) (Fig.17).

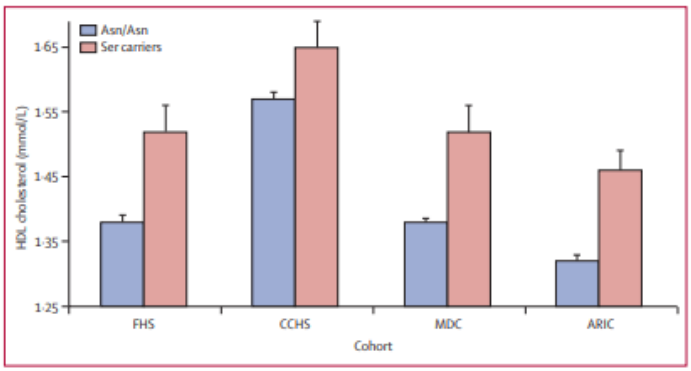


**Fig.16 Plasma HDL concentration among carriers and non-carriers of the Ser allele in LIPG Asn396Ser variant** (figure from Benjamin F Voight et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomization study. Lancet 2012; 380: 572–80).

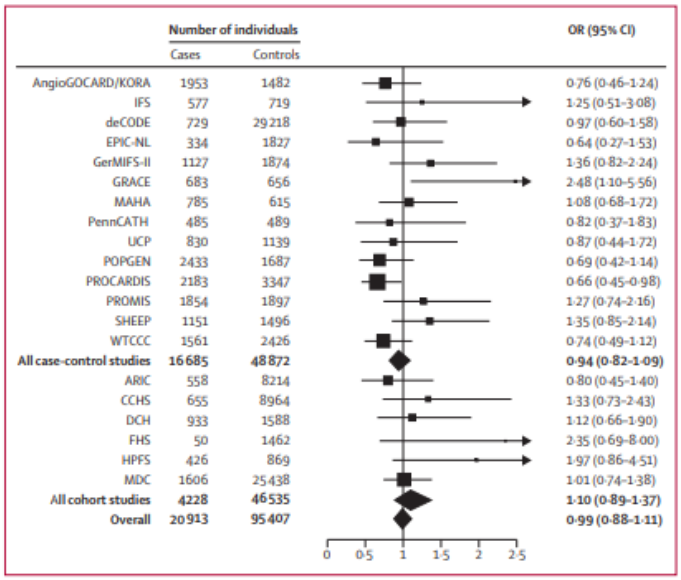


**Fig.17 Association between LIPG Asn396Ser and myocardial infarction** (figure from Benjamin F Voight et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomization study. Lancet 2012; 380: 572–80).

The answer to the question whether HDL cholesterol concentrations are uniquely correlated with the risk of cardiovascular disease is not unidirectional. Further studies may clarify the role in HDL in the predisposition of CAD.

# OBJECTIVES

Coronary artery disease being the main cause of mortality in the world is at the center of scientific debate and the discovery of new genetic risk markers could provide valuable innovative tools of prediction in the development of atherosclerosis. To improve the prediction of "susceptibility" or "resistance" on a genetic basis to CAD, it is necessary to identify new genetic variants that alone or through gene-gene interactions have a substantial CAD risk effect. In carrying out this objective the research project follows two distinct methodological approaches.

The first approach is to identify subjects with genetically determined ADH through the analysis of candidate genes in order to identify mutations in the site B of apo-B100, a region binding with proteoglycans, and to identify susceptibility/resistance to Coronary Heart Disease.

The second approach aims to assess, in a cohort of subjects at high risk of CAD, long-term exposure to high/low levels of LDL-C through the construction of a genetic score and correlation with the risk of CAD. In addition, the effect of rare genetic variants on extreme lipid phenotypes characterized by high or low levels of LDL, HDL, TG and ApoC-III will be evaluated.

# METHODS AND MATERIALS

## *Cohort studies*

Two different studies cohort were selected to evaluate the objectives described above. For the first approach were identified patients with genetically determined autosomal dominant hypercholesterolemia (ADH) selected from databases of patients / families with ADH already available in our laboratory and characterized clinically and genetically. In total 240 samples were sequenced by using Sanger sequencing to research two mutations in the site B of apoB-100.

The second approach includes patients with angiographically documented CAD (positive CAD) and subjects with a completely normal coronary tree (negative CAD), were selected 1295 between positive CAD (n.955) and negative CAD (n.340). To evaluate long-term exposure to high/low LDL-C levels Real-time PCR genotyping were performed. To identified rare genetic variants on extreme lipid phenotypes positive and negative CAD cohort were sequenced by Next Generation Sequencing (NGS).

# First approach

## *Genomic DNA extraction*

Genomic DNA has been extracted from whole blood by standard purification procedures from circulating leukocytes using a commercial kit (Wizard® Genomic DNA purification Kit, PROMEGA) which is based on 4 step process: lysis of red blood cells, lysis of white blood cells and their nuclei, protein precipitation, concentration and washing of DNA. For the isolation of DNA from white blood cells, this kit involves the lysis of red blood cells in 900 µl of Cell Lysis Solution transferred into sterile 1.5 ml eppendorf to which 300 µl of previously mixed blood are added. The tubes are gently inverted and incubated at room temperature for 10-15 minutes. At the end of the incubation the tubes were centrifuged at 14,000 x g for 4 minutes at room temperature. After centrifugation, the supernatant is removed using a disposable Pasteur pipette, avoiding touching the visible pellet. For the next phase of lysis of white blood cells and their respective nuclei, 300 µl of Lysis Solution Nuclei are added to the pellet, making up and down with the pipette until a viscous solution free of cell aggregates is obtained. In the third phase of protein saline precipitation, 100 µl of Protein

Precipitation Solution were added to the cell lysate and then vortexed vigorously for 10-20 seconds. The resulting samples were centrifuged at 14,000 x g for 3 minutes at room temperature, after which a dark brown protein pellet can be observed at the bottom of the eppendorf. In the fourth and final phase of DNA concentration and washing, the supernatant was transferred into clean eppendorf containing 300 µl of 100% isopropanol by gently inverting until white DNA strands formed a visible mass. The samples are then centrifuged at 14,000 x g for 1 minute. At the end, a small white DNA pellet can be observed. The supernatant is removed and then 300 µl of 70% ethanol is added and gently inverted to wash the pellet and the eppendorf walls. The tubes are then centrifuged at 14,000 x g for 1 minute to remove the supernatant and invert the tubes on absorbent carat so that the ethanol evaporates completely. Finally, 100 µl of DNA Rehydration Solution was added to rehydrate the DNA at room temperature over-night. The DNA is then stored at 4°C for further analysis.

## *Genomic DNA amplification*

Genomic DNA binding region in the site B of apo-B100 was subjected to PCR amplification with suitable primers and conditions. The amplification profile involves a first step of activation of the polymerase at a temperature of 95°C for 5 min (hot start then the polymerase is inactive at low temperatures). It follows the step of denaturation at 95°C for 1 minute, after which it will have the single-stranded target molecules. At this point the annealing step follows, for 1 minute to different annealing temperatures of the primers, allowing the base-pairing to target specific primers. Finally, there is an extension phase at 72°C for 1 minute during which the polymerase is placed close to the target, recognizes the 3'-OH free of specific primer and begins to polymerize. These steps are repeated for 35 cycles and then switch to a stage of final extension at 72°C for 7 minutes to complete all the amplification products. The PCR reaction was performed in a final volume of 30 µl containing dNTPs (each at a final concentration of 2.5 uM), 3 uL of 10X buffer, 1.5 mM MgCl2, 20 pmol of each primer, 2U of Taq polymerase (Taq Invitrogen) and 100 ng of genomic DNA. Each amplifier has been evaluated in a standard 1.5% agarose gel using the Sybr Safe, a non-specific DNA intercalating double stranded.

## *PCR products purification*

The fragments obtained by amplification reactions were purified by electrophoresis on agarose gel 1% Low Melting Point in 1X TAE (Tris-acetate 0,04M, 0,001M EDTA) and eluted through purification on column (Wizard® PCR Preps DNA Purification System - Promega). The samples were resuspended in mQ water and quantified by spectrophotometric reading (260 nm); the integrity of the products was evaluated visually on agarose gel 1.5% in TAE.

## *Sanger sequencing*

The DNA sequencing method used is the enzymatic method created by Fred Sanger in the mid-70s, modified over time using Taq DNA polymerase that has favored the development of the cyclic sequencing. The method is based on using a single primer and double-stranded DNA, which is subjected to a series of cycles, each consisting of denaturing, annealing and elongation, in the presence of Taq polymerase, deoxyribonucleotides triphosphate (dNTP) and dideossiribonucleotides triphosphate (ddNTP). A ddNTP is very similar to its counterpart dNTP, but it is devoid of hydroxy group in the carbon atom in position 3' and the carbon atom in position 2'. At each cycle DNA fragments will be synthesized which may be interrupted if they have incorporated a ddNTP, not being provided the 3'-OH group for the addition of further nucleotides, and which can be separated on the basis of their length. The substrate for sequencing DNA is produced by PCR amplification, it converts it into single-stranded form and using it as a template for sequencing. The four ddNTP are distinguishable among them as labeled with different fluorochromes that emit fluorescence at different wavelength. The fluorescent DNA fragments are analyzed by an automatic sequencer, which is based capillary electrophoresis, 3500 Genetic Analyzer (Applied Biosystems). The electrophoretic run occurs within 8 capillaries containing a polymer similar to a polyacrylamide gel (POP 7 that allows to separate more or less long fragments). A laser beam hits the capillary exciting the fluorochrome markers fragments of different length. The fluorescence emission is read by a CCD (charge-coupled device) camera. The fluorescence emitted by the excited molecules is collected as a band of a particular wavelength and stored as a digital signal. To analyze the raw data is used a dedicated program that is able to determine which base corresponds to a given fluorescence intensity (Sequencing Analysis 6) and showing data in a colored graph peaks, each corresponding to

a particular base, said electropherogram. The amplified and purified products, quantified by spectrophotometric reading, are used as a template for the direct sequence reaction. A quantity of 10 ng of each template DNA to be sequenced every 100 bp is used, in the presence of 3.2 pM of one of the primers specific for the exon in the analysis (usually the forward primer) and 1 ul of BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem) that contains the deoxynucleotide, the dideoxynucleotides marked, the reaction buffer and Taq specification in a final volume of 10 ul. The Taq specification has a point mutation in the active site to give good affinity for the ddNTP and a point mutation in the N-terminal domain which eliminates the nuclease activity 5 '→ 3'.

The profile of the reaction sequence involves:

- First step at 96°C for 10 seconds in which the DNA is denatured.
- Second step at 50°C for 5 seconds to allow annealing of the primers.
- Third step at 60°C for 4 minutes during which the Taq lengthens the chain until it eventually meets a ddNTP.

This is repeated for 25 cycles and with a rise of temperature of 1 ° C per second. These reactions were purified on plates using BigDye XTerminator® Purification Kit (Applied Biosystems) by adding only two reactants:

- Xterminator ™ Solution eliminates what is not incorporated and free salts after the sequencing reaction.
- SAM ™ Solution improves the performance of the first reagent and also stabilizes the post-purification reactions.

The purified reaction was subjected to capillary electrophoresis for 56 minutes [10 minutes of the capillary with the polymer filler, 10-minute pre-travel to 6-10 mA (constant current) and 36 minutes electrophoresis in 6-10 mA]. The analysis of the raw data was performed using the Sequencing Analysis program 6; the subsequent comparison with the sequences in the database was performed with the program SeqScape 3.

# Second approach

## *High/low LDL-C associated SNPs to genetic risk score construction*

To evaluate long-term exposure to high and low LDL-C levels, were chosen two sets of SNPs already described in the literature to evaluate the quantitative polygenic effect on LDL-C plasma levels. To evaluate exposure to LDL-C levels above average (high exposure), 6 SNPs were chosen in 5 genes that have a documented effect on the increase in plasma levels of LDL cholesterol: rs629301 of the CELSR2 gene, rs1367117 gene APOB, rs6544713 of the ABCG8 gene, rs6511720 of the LDLR gene, rs429358 and rs7412 of the APOE gene (genotype determination). To evaluate the exposure to lower than average LDL-C levels (low exposure) a panel of 9 SNPs will be used in 6 genes that have a documented effect on the reduction of plasma LDL cholesterol levels. This panel of SNPs has been used in a recent meta-analysis of Mendelian randomization studies, demonstrating that long-term exposure to low levels of LDL-C is associated with a greater reduction in the risk of cardiovascular disease (56). For this purpose, validated and commercially available TaqMan assays have been identified C____972962_10 and C___3160062_10 for the gene SORT1, C__32221221_10 and C___2018188_10 for the PCSK9 gene, C__34514854_10 and C__27208873_10 for the LDLR gene, C___7445046_10 for the HMGCR gene, C__26135637_10 for the gene ABCG8 gene. For the molecular typing of the rs4420638 polymorphism in APOE gene, a custom assay (Applied Biosystems) already validated and available in the literature will be used.

## *Real-time PCR genotyping assay*

All SNPs were detected by genotyping assay using the ViiA™ 7 Real-Time PCR System. A genotyping assay detects variants of a single nucleic acid sequence without target quantification. The presence of two probes in each reaction allows the genotyping of the two possible variants: the mutated allele and the wild type allele. Each genotyping assay contains two sequence-specific primers for amplification of the region of interest and two TaqMan allele-specific MGB probes for allele detection for the region of interest. Each TaqMan allele-specific MGB probe presents:

- a reporter dye at its 5' end
    - VIC® dye is bound to the 5' end of the wild type allele probe (allele 1).

- FAM™ dye is bound to the 5' end of the mutated allele probe (allele 2).

- a Minor Groove Binder (MGB), which increases the Melting Temperature for a given probe length allowing for shorter probe designs and therefore more robust genotyping.
- a Non-Fluorescent Quencher (NFQ) at its 3' end, allowing a higher sensitivity for fluorescence detection of the reporter dye than a fluorescent quencher.

During PCR, each TaqMan MGB probe performs annealing specifically to its complementary sequence between the forward and reverse primer regions. When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye prevents the signal from being emitted by the reporter. The polymerase of the TaqMan Genotyping Master Mix II extends the primers bound to the genomic DNA template and separates the probes that are hybridized to the target sequence. The splitting of the probes hybridized to the target sequence separates the quencher dye from the reporter dye, which increases the reporter's fluorescence. The fluorescence signal generated by PCR amplification indicates which alleles are present in the sample (Fig.18)

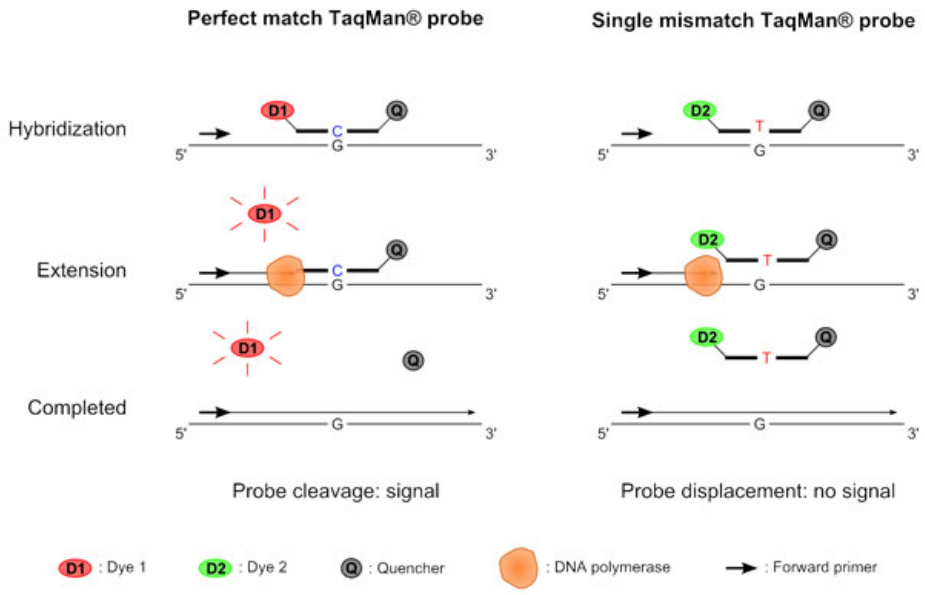


**Fig.18 Real-time PCR genotyping reaction**

The amplification protocol for genotyping involves using approximately 10 ng of template DNA in the presence of 10 µl of TaqMan® Genotyping Master Mix (Applied Biosystems) 2X, which contains AmpliTaq Gold DNA polymerase UP (Ultra Pure), dNTP, ROX as passive reference and reaction-optimizing buffers, and 0.5 µl of custom 40X probe in a final volume of 20 µl (Table 1):

| REAGENTE | VOLUME IMPIEGATO | CONCENTRAZIONE FINALE |
|---|---|---|
| TaqMan Genotipyng Master Mix II (2X) | 10 µl | 1 X |
| Custom TaqMan Assay (40X) | 0,5 µl | 1 X |
| Template (genomic DNA sample) | 1 µl | 1 – 20 ng |
| $H_2O$ Nuclease free | 8,5 µl | |

**Table 1. Optimization of genotyping reaction**

The reaction mix is prepared, containing the indicated volumes, for 96 samples. The prepared reaction mix is added to each well of the reaction plate to which the template of each sample to be analyzed is added. The preparation of the reaction mix and its addition to the wells of the reaction plate was done taking care not to expose the reagents to light to avoid deterioration. The plate is then sealed with MicroAmp® Optical Adhesive Film and loaded into the ViiA™ 7 Real-Time PCR System. The ViiA™ 7 software genotypes 92 DNA samples simultaneously from the MicroAmp® Optical 96-Well Reaction Plate in which is also placed a Negative Control with Nuclease free water and three Positive Controls: a homozygous control for allele 1, a homozygous control for allele 2 and a heterozygous control (allele 1/allele 2).

The reaction is expected:

- Incubation at 60°C for 30" (pre-PCR Read).
- Activation of the polymerase of TaqMan Genotyping Master Mix II at 95°C for 10'.
- 40 amplification cycles according to the following parameters

| | | |
|---|---|---|
| **DENATURATION** | 15 sec. | 92°C |
| **ANNEALING** | 1 min. | 60°C |
| **EXTENSION** | 1 min. | 60°C |

- A post-PCR Read phase at 60°C for 30".

After amplification by PCR, an end-point plate reading was performed using the Applied Biosystems ViiA™ 7 Real-Time PCR System. First, the software normalizes the fluorescence of the reporter dyes to the fluorescence of the passive reference dye in each well. Next, the software displays the normalized intensities (Rn) of the reporter dyes in each well of the sample on the allelic discrimination plot, which contrasts the intensities of the reporter dyes of the allele-specific probes. The Allelic Discrimination Plot contrasts the normalized fluorescence of the reporter dye (Rn) for the allele-specific probes of the assay. It can vary along the horizontal axis (allele 1), the vertical axis (allele 2) or diagonally (allele 1/allele 2) (Fig.19) This variation results in differences in the fluorescent intensity of the reporter dye after PCR amplification. A substantial increase in fluorescence only of the probe labeled with VIC indicates homozygosity for allele 1; increase in fluorescence only of the probe labeled with FAM dye indicates homozygosity for allele 2; increase in fluorescence of both the probe labeled with VIC and the probe labeled with FAM indicates heterozygosity of allele 1/allele 2.

Finally, in order to verify the correctness of the assignment of genotypes to the samples carried out by the software, the amplification plots of each sample were analyzed, which allows to visualize the amplification of the sample following the acquisition of fluorescence data during the analysis. The plot shows the fluorescence of the normalized dye (ΔRn) and the cycle number (Fig. 20, 21, 22).

## *Next Generation Sequencing*

## *Design of customized panel for analysis of extreme lipid phenotypes*

A NGS custom panel was designed to analyze candidate genes related to LDL, HDL and triglycerides metabolism:

- High LDL-related genes. Were chosen sets of genes affecting: the LDL receptor pathway (LDLR, APOB, APOE, PCSK9, LDLRAP1 and IDOL); the regulation of cholesterol and LDL receptor genes (SCAP, INSIG2, SREBP1 and SREBP2); intestinal cholesterol absorption (ABCG5, ABCG8); intracellular metabolism of cholesteryl esters (LIPA).
- Low LDL-related genes. Were chosen sets of genes affecting: the VLDL-LDL secretion (APOB, MTTP, SORT1 and PLTP); the LDL receptor degradation (PCSK9); inhibitor of Lipoprotein Lipase (ANGPTL3, ANGPTL4 and APOC3).

- HDL-related genes. Were chosen sets of genes affecting: structure and formation of HDL (APOA1, APOA2, APOC3, APOF and ABCA1); HDL remodeling in plasma (LCAT, CETP); HDL receptor (SCARB1); lipases acting on HDL (LIPC and LIPG); removal of HDL by the kidney (CUBILIN); Glycosyltransferase (GALANT2).
- Triglyceride-related genes. Were chosen sets of genes affecting: TG intravascular lipolysis (LPL, LIPG, LIPC, APOA5, APOC2, GPIHBP1 and LMF1); apoCIII plasma level (APOC3 and GALNT2); transcription factors of TG related genes (CREB3L3); inhibitors of lipolysis (ANGPTL3, ANGPTL4, and ANGPTL8); factors affecting TG and carbohydrate metabolism (GCKR, MLXIPL, TRIB1 and GPD1).

Primers for each gene were designed using the Webbased Ion AmpliSeqTM Designer software (https://www.ampliseq.com). This tool generates different primer design solutions, which differ for specificity, sample type and application. It was chosen the design pipeline able to give the higher specificity for target genes with an expected coverage of about 97%.

## *Library preparation*

Genomic DNA from all subjects were extracted from EDTA treated whole blood samples using the Wizard DNA Purification System (Promega, Italy). DNA quantification was performed using Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Italy). Targeted regions were amplified in a Veriti Dx Thermal Cycler (Thermo Fisher Scientific, Italy) by using 10 ng of genomic DNA for each pool, Ion AmpliSeq$^{TM}$ Library Kit Plus and 2× Ion AmpliSeq Primers Pool in a total volume of 20 μL. The PCR conditions were 2 min at 99°C for polymerase activation and 20 cycles of 15 sec at 99°C for denaturation and 4 min at 60°C for annealing/extension. The obtained amplicons were treated with FuPa Reagent in a total volume of 22 to partially digest the primers and phosphorylate the amplicons. Ion Xpress Barcode adapters were ligated to the amplicons by DNA ligase. The adapters function is to permit the binding on a magnetic bead to make the future sequence, while the barcode is used to distinguish the samples. Barcoded libraries were purified with Agencourt AMPure XP Reagent (Beckman Coulter, CA, USA) and quantified using Ion Library TaqMan Quantitation Kit on the Viia7 Dx Real-Time PCR System (Thermo Fisher).

## *Clonal amplification*

The emulsion PCR was carried out with the Ion One Touch System and Ion 540™ Kit-OT2 (Thermo Fisher Scientific). Each micro droplet of the emulsion oil/water represents a microreactor, within the amplification occurs. In every single droplet there is a bead, an adapter and (theoretically) a single DNA molecule (monoclonal) or more DNA molecule (polyclonal). The sequencing of the DNA molecule coming from monoclonal spheres will produce sequence data that will be analyzed and processed; all polyclonal spheres instead, will produce sequence data that will not be examined. Then, the template-positive Ion Sphere particles were enriched with Dynabeads MyOne Streptavidin C1 beads on the Ion One touch ES system (Thermo Fisher).

## *Sequencing and bioinformatics analysis*

Sequencing was performed on an Ion GeneStudio™ S5 Plus System using the Ion 540™ Sequencing Kit and the Ion 540 Chip. At the end of the run, data were elaborated by Ion Torrent Suite 5.12.2 version and two different plug-ins were applied for a preliminary analysis. Coverage Analysis plug-in (5.12.0.32 version) was used to verify coverage of amplicons, missed bases and a well-adjusted distribution between forward and reverse reads. Variant Calling from the sequencing data was performed with a "variant caller" plug-in (5.12 version) using the human genome hg19 as reference and germline-low stringency parameter settings. Only reads with a "mapping quality" >4 and a "base minimum quality score" >10 were included. Heterozygous variants were called if the minor allele was present in at least 10% of the reads at a given position. Variants found were annotated in Ion Reporter (version 5.16) from VCFs generated by Ion Torrent through the Annotate Variants Single Sample workflow. The analysis output shows all detected and filtered variants as a list in excel format; for each variant in silico functionality output generated from multiple databases interrogation (SIFT, Poliphen-2, db-SNP, 1000 genomes). In addition, Genome Aggregation Database (gnomAD) Browser (https://gnomad.broadinstitute.org) was used; potentially and probably deleterious SNP and INDEL were also visualized by using the Broad Institute Integrative Genomic Viewer (version 2.3.40, www.broadinstitute.org/igv).

## *Variants confirmation by Sanger Sequencing*

To confirm Genomic DNA regions encompassing suspected pathogenetic mutations in candidate genes analyzed by Ion GeneStudio™ S5 Plus sequencing system PCR amplification and Sanger sequencing were performed as previously described.

# RESULTS AND DISCUSSION

## First approach

Were selected 240 samples of patients affected by ADH and were determined the susceptibility and resistance of CHD status through the clinical data analysis from several database already available in our laboratory (85 from CAL list, 42 from VHS list, 76 from Elders FH and 37 from UNILEVER).

Susceptible subjects will be those patients with familial hypercholesterolemia with total cholesterol > 290 mg/dl and a diagnosis of CHD:

- By 41 years for nonsmoking males.
- By 36 years for male smokers or former smokers (with smoking history at the time of the cardiovascular event).
- By 61 years for nonsmoking females.
- By 44 years for smoking or former smoking females.

All others will be classified as CHD-resistant patients.

We analyzed by Sanger Sequencing two known mutations in the ApoB-100 site B (exon 26, 3359-3369 amino acids residues) binding LDL to the matrix of proteoglycans, R3500Q and K3363E. Carriers of the R3500Q variant have LDL normal binding activity to proteoglycans but interacted defensively with the receptor; they might represent an important pro-atherogenic predictive marker. Carriers of the K3363E mutation have LDL lipoproteins that interact normally with the receptor but not with proteoglycans, thus representing a possible protective marker against CAD. All analyzed samples were negative for both variants.

# Second approach

## Long-term exposure to high and low levels of LDL-C

### *High exposure polygenic score*

We performed the genotyping of 1197 subjects out of a total of 1295 between positive CAD (n.955) and negative CAD (n.340) in order to evaluate long-term exposure to high LDL-C levels. It was not possible to perform the analysis on 95 samples due to insufficiency or poor quality of DNA. We chose 6 SNPs in 5 genes that have a documented effect on the increase in plasma levels of LDL cholesterol (31). This panel of SNPs was used to evaluate the polygenic burden to justify the FH clinical phenotype in the absence of mutations in candidate genes. Genotyping experiments were performed by PCR Real Time on ViiA7 PCR System (Life Technologies) using TaqMan assays (Applied Biosystems). For each subject and for each SNP studied, a score of 0 was assigned indicating the absence of the risk allele, 1 indicating the presence of a single allele and 2 the presence of both. The score assigned to each allele of SNPs associated to high levels of LDL-C (range from 0 to 12 alleles) will allow to stratify the subjects in low, medium and high exposure. In particular those who are carriers of the 12 alleles of susceptibility to the increase in LDL-C levels will have the highest genetically determined exposure (Table 2).

| | Risk allele | Other allele | Score homozygous risk allele | Score Heterozygous | Score homozygous other allele |
|---|---|---|---|---|---|
| **CELSR2 rs629301** | T | G | 2 | 1 | 0 |
| **APOB rs1367117** | A | G | 2 | 1 | 0 |
| **LDLR rs6511720** | G | T | 2 | 1 | 0 |
| **ABCG8 rs6544713** | G | T | 2 | 1 | 0 |
| **APOE rs429358** | C | T | 2 | 1 | 0 |
| **APOE rs7412** | C | T | 2 | 1 | 0 |

**Table 2. High exposure to LDL-C levels and risk allele**

## *Low exposure polygenic score*

To evaluate the exposure to lower than average LDL-C levels (low exposure) a panel of 9 SNPs will be used in 6 genes that have a documented effect on the reduction of plasma LDL cholesterol levels. This panel of SNPs has been used in a recent meta-analysis of Mendelian randomization studies, demonstrating that long-term exposure to low levels of LDL-C is associated with a greater reduction in the risk of cardiovascular disease (56). The genetic analysis was performed in 1197 subjects out of a total of 1295 between positive CAD (n.955) and negative CAD (n.340). It was not possible to perform the analysis on 95 samples due to insufficiency or poor quality of DNA. Genotyping experiments were performed by PCR Real Time on ViiA7 PCR System (Life Technologies) using TaqMan assays (Applied Biosystems). For each subject and for each SNP studied, a score of 0 was assigned indicating the absence of protective allele, 1 indicating the presence of a single allele and 2 the presence of both. The score assigned to each allele of SNPs associated to low levels of LDL-C (range from 0 to 18 alleles) will allow to stratify the subjects to the lower genetically determined exposure (Table 3).

| | **Protective allele** | **Other allele** | **Score homozygous protective allele** | **Score heterozygous** | **Score homozygous other allele** |
|---|---|---|---|---|---|
| **PSRC1-SORT1 rs599839** | G | A | 2 | 1 | 0 |
| **CELSR2-SORT1 rs646776** | G | T | 2 | 1 | 0 |
| **PCSK9 rs11206510** | C | T | 2 | 1 | 0 |
| **PCSK9 rs11591147** | T | G | 2 | 1 | 0 |
| **LDLR rs6511720** | T | G | 2 | 1 | 0 |
| **LDLR rs2228671** | T | C | 2 | 1 | 0 |
| **HMGCR rs12916** | T | C | 2 | 1 | 0 |
| **ABCG8 rs4299376** | T | G | 2 | 1 | 0 |
| **APOE-C1-C2 rs4420638** | A | G | 2 | 1 | 0 |

**Table 3. Low exposure to LDL-C levels and protective allele**

## *Evaluation long-term exposure to high/low levels of LDL-C*

To assess long-term exposure to high and low LDL-C levels, we considered the distribution of the mean polygenic burden in LDL-C tails (the 10th percentile, between the 10th and 90th percentiles, and the 90th percentile) in subjects not on hypolipidemic treatment (Fig.19).

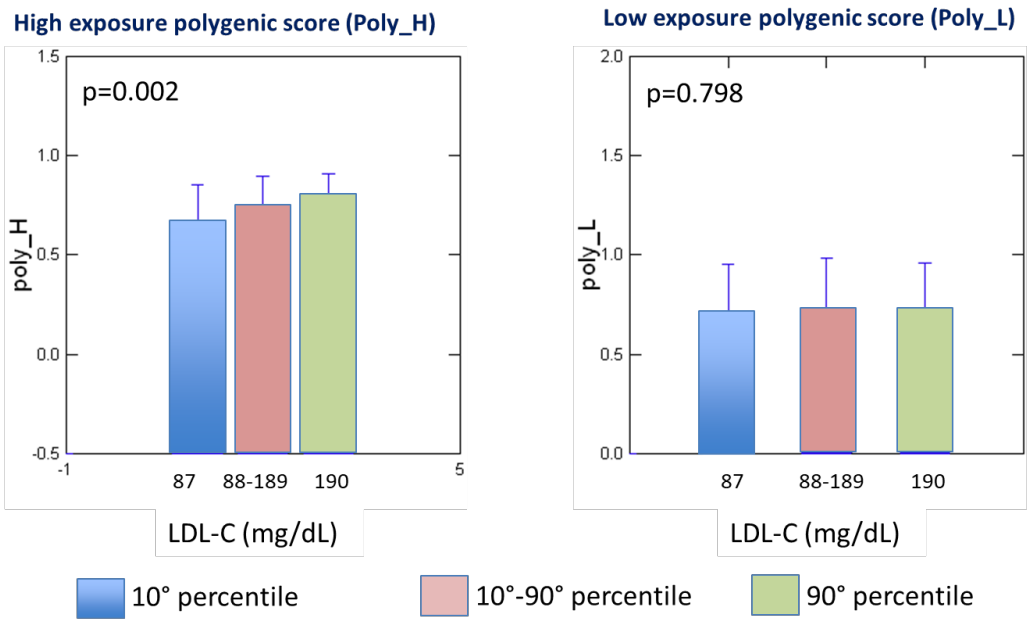


**Fig.19 Correlation between high/low exposure polygenic score and LDL-C levels**

High exposure polygenic score values increase as LDL-C rise, but LDL-C levels seems to be unaffected to low exposure polygenic score.

# Correlation between long-term exposure score and CAD

Statistical analysis for correlation of high and low exposure polygenic score with CAD was performed using SYSTAT software comparing the scores obtained from calculation of polygenic scores with the cohort of positive and negative CAD subjects (Fig.20).

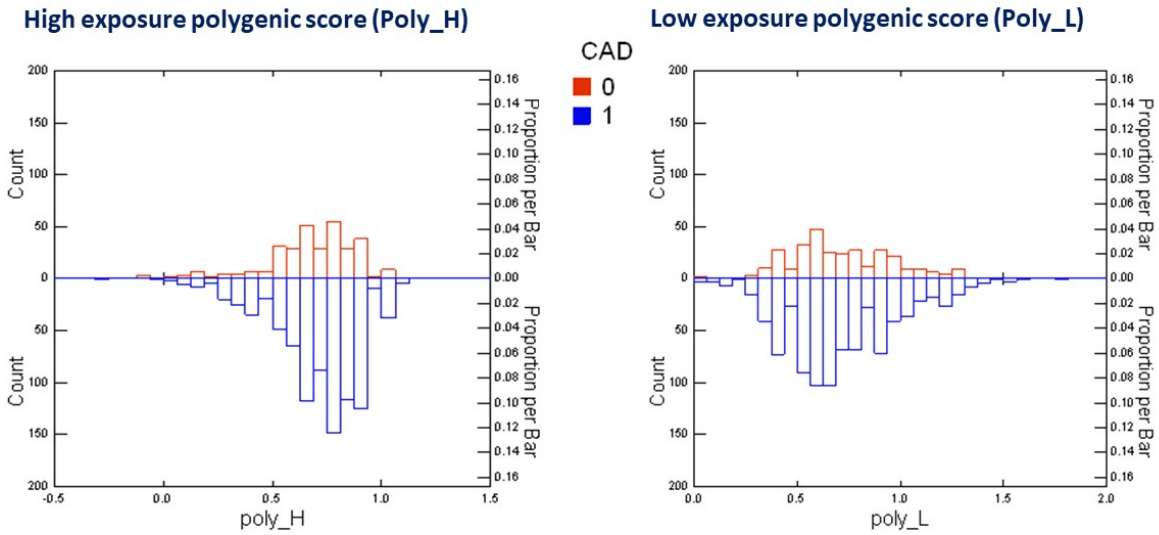


**Fig.20 High/low exposure polygenic score and CAD**

Positive CAD were shown in red and negative CAD in blue. Statistical analysis shows no correlation between exposure score and coronary artery disease in either the high-exposure or low-exposure polygenic burden of LDL-C levels.

In addition, was assessed the number of compromised blood vessels, characterized by occlusion as a result of atherosclerotic plaque formation, in the cohort of CAD-positive subjects (Fig.21).

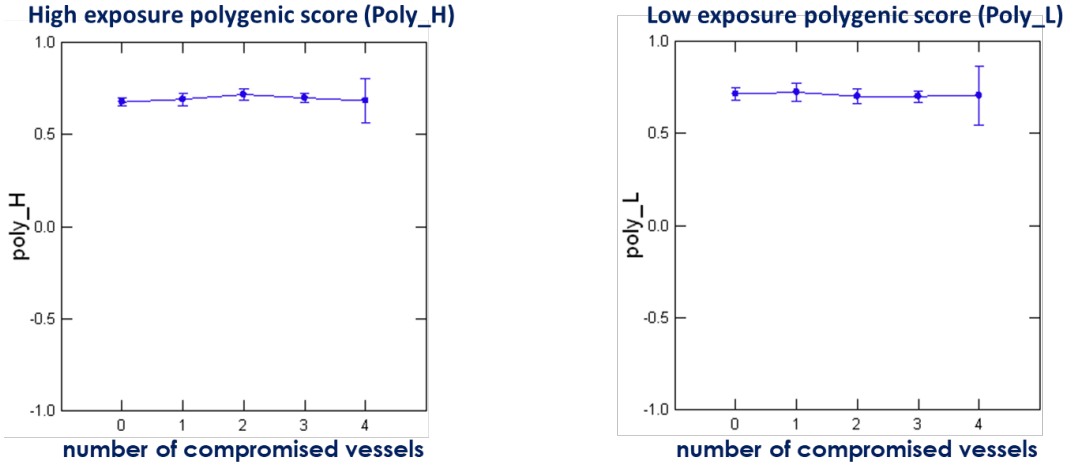


**Fig.21 High/low exposure polygenic score and number of compromised vessels positive and negative CAD patients.**

CAD-negative subjects in whom no vessel was compromised by atherosclerotic plaque were indicated by 0. 1,2,3,4 respectively indicated the number of compromised vessels in CAD-positive patients.

The result obtained is that the high/low exposure score does not show a correlation with the degree of severity of coronary artery disease.

## Genetic characterization of subjects with and without CAD carrying "extreme lipid phenotypes"

Genetic analysis was conducted by next generation sequencing on a selection of patients with extreme LDL-C, HDL-C, TG, and apoC-III phenotypes, including both CAD-positive and CAD-negative subjects. In particular, the tails of LDL-C, HDL-C, and TG (<2nd and >98th percentile) and of apoC-III (<5th and >95th percentile) were selected with reference to the lipid phenotype of each patient. Bioinformatic analysis of VCF files obtained after sequencing was performed using Ion Reporter software in particular Annotate Variants Single Sample plug-in that allows to generate a list of variants in excel format for each sample. In order to assign a possible pathogenic role to the non-synonymous variants, the MAF (minor allele frequency) was considered and the information reported in the HGMD (Human Gene Mutation Database) was consulted. For variants not described in the literature, the prediction of the effect on the protein was evaluated based on the scores obtained by the PolyPhen-2 algorithm.

### *Low LDL-C phenotype*

Among subjects with low LDL-C phenotype, those with LDL-C cut-off <2nd percentile (66 mg/dl) were selected regardless of plasma HDL-C and TG levels. There were 16 subjects available for inclusion in the study (6 negative CAD and 10 positive CAD), the analysis was conducted on 12 patients (6 negative CAD and 6 positive CAD). Within this group, large-scale molecular genetic analysis of candidate genes allowed the identification of 2 subjects carrying causative mutations of the observed dyslipidemic phenotype. Subject #445 (negative CAD, LDL-C 44 mg/dl) was found to be a heterozygous carrier of the nonsense mutation in the APOB gene c.6943G>T p.Glu2315Ter. In subject #91 (negative CAD, LDL-C 46 mg/dl) the frameshift mutation of APOB gene c.3422delGTCinsTGTGG p.1141fs43X was identified in heterozygosity. Both mutations result in a premature stop codon and the formation of a truncated apoB protein that is not fully functional justifying the hypocholesterolemic phenotype observed in both individuals. Subject #1063 (positive CAD, LDL-C 59 mg/dl) was found to be a carrier in heterozygosity of the c.137G>T p.Arg46Leu missense variant of the PCSK9 gene. A 2006 study (46), performed on two different populations, showed the correlation of this PCSK9 allele with low levels of LDL-C and a reduction in the incidence of coronary heart disease. Subject #666 was double heterozygous

for FGFR4 (c.1585C>T, p.Arg529Ter) and PCSK9 (c.1681G>A, p.Gly561Ser) gene variants. The variant in FGFR4 is known on Exac with MAF <0.005 and consulting the Uniprot database, position 467-755 falls in the cytoplasmic domain with kinase activity. The variant in the PCSK9 gene is known on Exac with MAF <0.005, from analysis on Uniprot it was assessed that this variant falls in the 450 to 692 amino acid position that corresponds to the C-terminal domain essential for LDLR binding and degradation activity (92).

| | **LDL-C (mg/dl)** | **CAD** | **Gene** | **Mutation** |
|---|---|---|---|---|
| **#445** | 44 | No | APOB | c.6943G>T (p.Glu2315Ter) in heterozygous |
| **#91** | 46 | No | APOB | c.3422delGTCinsTGTGG (p.1141fs43X) in heterozygous |
| **#1063** | 59 | Yes | PCSK9 | c.137G>T (p.Arg46Leu) in heterozygous |
| **#666** | 65 | No | PCSK9 | c.1681G>A (p.Gly561Ser) in heterozygous |
| | | | FGFR4 | c.1585C>T (p.Arg529Ter) in heterozygous |

In subjects in whom no pathogenic mutations were identified, the risk of polygenic hypocholesterolemia was assessed by genotyping analysis of the 9 associated SNPs, the results of which are shown in the following table:

| | **LDL-C (mg/dl)** | **CAD diagnosis** | **Low LDL-C Genetic Score** | **High polygenic hypocholesterolemia probability (>0,88)** |
|---|---|---|---|---|
| **#977** | 48 | Yes | 0,57 | Low |
| **#1063** | 59 | Yes | 1,24 | High |
| **#2209** | 60 | Yes | 0,73 | Low |
| **#280** | 60 | Yes | 0,32 | Low |
| **#2033** | 63 | Yes | 0,39 | Low |
| **#1402** | 65 | Yes | 0,39 | Low |
| **#1689** | 31 | No | 0,58 | Low |
| **#670** | 63 | No | 0,90 | High |
| **#2347** | 64 | No | 0,51 | Low |
| **#666** | 65 | No | 0,8 | Low |

The calculation of polygenic score associated with low LDL-C levels was obtained considering the scores assigned in the work of Ference et al (56) in association with the presence or absence of the protective allele in the individual genotypes (homozygous for the

protective allele, heterozygous and homozygous for the other allele) of each SNP. The sum of all scores obtained will determine the genetic score associated with low LDL-C levels of each individual subject. If the score is >0,88 there is a high probability of developing polygenic hypocholesterolemia.

## *High LDL-C phenotype*

Among subjects with high LDL-C phenotype, those with LDL-C cut-off >98th percentile (222 mg/dl) were selected regardless of plasma HDL-C and TG levels. The available subjects to be included in the study were 23 (6 negative CAD and 17 positive CAD), the analysis was conducted on 19 patients (6 negative CAD and 13 positive CAD). Within this group, subject #791 (positive CAD, LDL-C 236 mg/dl) was found to be the carrier of the pathogenetic mutation of the LDLR gene c.662A>G p.Asp221Gly in heterozygosity described in the literature as a pathogenetic variant and responsible for Familial Hypercholesterolemia (93). It was not possible to perform the genetic analysis in 4 patients because DNA was degraded.

| | **LDL-C (mg/dl)** | **CAD** | **Gene** | **Mutation** |
|---|---|---|---|---|
| **#791** | 236 | Yes | LDLR | c.662A>G p.Asp221Gly in heterozygous (FH-Padova 1) |

In subjects in whom no pathogenic mutations were identified, the risk of polygenic hypercholesterolemia was assessed by genotyping analysis of the 6 associated SNPs, the results of which are shown in the following table:

| | LDL-C (mg/dl) | CAD diagnosis | Genetic Risk Score | High polygenic hypercholesterolemia probability (>0,73) |
|---|---|---|---|---|
| **#140** | 225 | Yes | 0,931 | High |
| **#783** | 232 | Yes | 0,731 | High |
| **#273** | 233 | Yes | 0,902 | High |
| **#122** | 234 | Yes | 0,731 | High |
| **#18** | 247 | Yes | 0,952 | High |
| **#539** | 251 | Yes | 0,4 | Low |
| **#104** | 266 | Yes | 0,551 | Low |
| **#197** | 269 | Yes | 0,902 | High |
| **#231** | 225 | No | 0,902 | High |
| **#467** | 249 | No | 0,731 | High |
| **#356** | 270 | No | 0,931 | High |
| **#1059** | 272 | No | 0,751 | High |
| **#135** | 263 | Yes | 0,76 | High |
| **#173** | 236 | No | 0,902 | High |
| **#294** | 234 | Yes | 0,731 | High |
| **#496** | 232 | No | 0,831 | High |
| **#651** | 286 | Yes | 0,731 | High |
| **#848** | 223 | Yes | 0,551 | Low |

The calculation of polygenic score associated with high LDL-C levels was obtained considering the scores assigned in the work of Futema et al (31) in association with the presence or absence of the risk allele in the individual genotypes (homozygous for the risk allele, heterozygous and homozygous for the other allele) of each SNP. The sum of all scores obtained will determine the genetic score associated with high LDL-C levels of each individual subject. If the score is >0,73 there is a high probability of developing polygenic hypercholesterolemia.

## *Low HDL-C phenotype*

Among subjects with low HDL-C phenotype, those with HDL-C levels <2nd percentile (29 mg/dL) were selected regardless of plasma LDL-C and TG levels. The estimated number of

available subjects to be included in the study was 38 (32 positive CAD and 6 negative CAD). Genetic analysis was conducted in 30 patients (25 positive CAD and 5 negative CAD). Subject #868 (positive CAD, HDL-C 27 mg/dL) was found to be a carrier in heterozygosity of the ANGPTL3 gene mutation c.55delA (p.Ile19fs) described in the literature and responsible of a form of familial hypobetalipoproteinemia (FHBL-2). Affected subjects are characterized by very low LDL-C, HDL-C, TG and total cholesterol levels, but this subject presents a different lipid profile: CT 173 mg/dL, TG 119 mg/dL, LDL-C 127 mg/dL. In subject #1316 (positive CAD, HDL-C 28 mg/dL) the ABCA1 gene mutation c.5398A>C (p.Asn1800His) was identified in heterozygosity, which in homozygosity is responsible for Tangier disease. Two subjects were identified, #249 (positive CAD, HDL-C 27 mg/dL) and #534 (positive CAD, HDL-C 25 mg/dL), both carrying the LCAT gene variant c.694T>A (p. Ser232Thr) in heterozygosity, which is associated with low HDL-C levels as reported by data in the literature (94).

| | **HDL-C (mg/dl)** | **CAD** | **Gene** | **Mutation** |
|---|---|---|---|---|
| **#868** | 27 | Yes | ANGPTL3 | c.55delA (p.Ile19fs) in heterozygous |
| **#1316** | 28 | Yes | ABCA1 | c.5398A>C (p.Asn1800His) in heterozygous |
| **#249** | 27 | Yes | LCAT | c.694T>A (p. Ser232Thr) in heterozygous |
| **#534** | 25 | Yes | LCAT | c.694T>A (p. Ser232Thr) in heterozygous |

## *High HDL-C phenotype*

Among subjects with high HDL-C phenotype, those with HDL-C levels >98th percentile (99 mg/dl) were selected, regardless of plasma LDL-C and TG levels. There were 6 subjects available for inclusion in the study (6 negative CAD and 0 positive CAD). From the analysis conducted on 5 patients, no pathogenic variants were identified. It was not possible to perform the analysis in 1 patient because the DNA was degraded.

## *Low TG phenotype*

Among subjects with low TG phenotype, those with TG cut-off <2nd percentile (57 mg/dl) were selected, regardless of plasma HDL-C and LDL-C levels. There were 18 patients (8

positive CAD, 10 negative CAD) available for inclusion in the study. The analysis was conducted in 9 patients (5 negative CAD and 4 positive CAD). Within this group, large-scale molecular genetic analysis of candidate genes allowed to identify in patient #91 (negative CAD, TG 26 mg/dL) the APOA5 gene variant c.817G>C p.Asp273His in heterozygosity, which is not reported on HGMD. In silico analysis using the PolyPhen-2 bioinformatics tool indicated a prediction score of 1 with a deleterious effect on protein function. Two compound heterozygous subjects were identified for ANGPTL4 gene variants c.797C>T (p.Thr266Met) and c.118G>A (p.Glu40Lys). These were patients #1425 (negative CAD, TG 46 mg/dL) and #1998 (positive CAD, TG 50 mg/dL). In subjects #1378 (negative CAD, TG 39 mg/dL) and #2356 (positive CAD, TG 53 mg/dL) only the c.797C>T (p.Thr266Met) variant of ANGPTL4 was detected. The p.Thr266Met and p.Glu40Lys variants of ANGPTL4, have been associated with low plasma TG levels (95). Analysis of the p.Glu40Lys variant using PolyPhen-2 results in a score of 0.999 with a deleterious effect on the protein. Subjects #977 (positive CAD, TG 52 mg/dL) and #2359 (negative CAD, 53 mg/dL) were found to be compound heterozygotes for the MLXIPL gene variants c.723G>C p.Gln241His, and c.1073C>T p.Ala358Val, the first is associated with low plasma TG levels (96) while for the second variant PolyPhen-2 algorithm predicted a benign effect.

| | **TG (mg/dl)** | **CAD** | **Gene** | **Mutation** |
|---|---|---|---|---|
| **#91** | 26 | No | APOA5 | c.817G>C (p.Asp273His) in heterozygous |
| **#1378** | 39 | No | ANGPTL4 | c.797C>T (p.Thr266Met) in heterozygous |
| **#1425** | 46 | No | ANGPTL4 | c.797C>T (p.Thr266Met) in heterozygous |
| | | | ANGPTL4 | c.118G>A (p.Glu40Lys) in heterozygous |
| **#1998** | 50 | Yes | ANGPTL4 | c.797C>T (p.Thr266Met) in heterozygous |
| | | | ANGPTL4 | c.118G>A (p.Glu40Lys) in heterozygous |
| **#2356** | 53 | Yes | ANGPTL4 | c.797C>T (p.Thr266Met) in heterozygous |
| **#2359** | 53 | No | MLXIPL | c.723G>C (p.Gln241His) in heterozygous |
| | | | MLXIPL | c.1073C>T (p.Ala358Val) in heterozygous |
| **#977** | 52 | Yes | MLXIPL | c.723G>C (p.Gln241His) in heterozygous |
| | | | MLXIPL | c.1073C>T (p.358Val) in heterozygous |

## *High TG phenotype*

Among the subjects with high TG phenotype, those with TG cut-off >98th percentile (298 mg/dl) were selected, regardless of plasma HDL-C and LDL-C levels. There were 19 subjects available for inclusion in the study (16 positive CAD, 3 negative CAD), of whom 8 patients (6 positive CAD, 2 negative CAD) were analyzed. Within this group, subject #145 (positive CAD, TG 44° mg/dL) and #302 (positive CAD, TG 464 mg/dL) were found to be carriers of the c.106G>A variant (p.Asp36Asn) in the LPL gene in heterozygosity, which is associated with hyperlipidemia as reported in the literature (97). Patient #405 was double heterozygous for the variants: c.56C>G (p.Ser19Trp) in the APOA5 gene and c.41G>T (p.Cys14Phe) in the GPIHBP1 gene. The variant in APOA5 is associated with increased plasma TG levels as reported in the literature (98), while the variant in GPIHBP1 causes reduced expression of the protein (99).

| | **TG (mg/dl)** | **CAD** | **Gene** | **Mutation** |
|---|---|---|---|---|
| **#145** | 440 | Yes | LPL | c.106G>A (p.Asp36Asn) in heterozygous |
| **#302** | 464 | Yes | LPL | c.106G>A (p.Asp36Asn) in heterozygous |
| **#405** | 476 | No | APOA5 | c.56C>G (p.Ser19Trp) in heterozygous |
| | | | GPIHBP1 | c.41G>T (p.Cys14Phe) in heterozygous |

## *Low apoC-III phenotype*

Among subjects with low apoC-III phenotype with apoC-III levels <5th percentile (6.1 mg/dl), independent of plasma LDL-C, HDL-C, and TG levels, 25 subjects were selected (11 positive CAD, 14 negative CAD). Within this group, large-scale molecular genetic analysis of candidate genes allowed the identification of a pathogenic mutation in patient #980 (negative CAD, ApoC-III 4.84 mg/dL) of the APOC3 gene c.55C>T (p.Arg19X) in heterozygosity. This variant has already been described in the literature and determines the formation of a premature stop codon that prevents the translation and maturation of the protein, also seems to confer a cardioprotective effect (100).

| | ApoC-III (mg/dl) | CAD | Gene | Mutation |
|---|---|---|---|---|
| **#980** | 4.84 | No | APOC3 | c.55C>T (p.Arg19X) in heterozygous |

## *High apoC-III phenotype*

Among subjects with high apoC-III phenotype with apoc-III levels >95th percentile (17.3 mg/dl) regardless of plasma LDL-C, HDL-C, and TG levels. There were 68 subjects available for inclusion in the study (55 positive CAD, 13 negative CAD). No pathogenic variants were identified from the genetic-molecular study.

# CONCLUSIONS

In this study, the possibility of developing novel genetic markers that may determine susceptibility or resistance to the development and progression of atherosclerosis was highlighted.

Two methodological approaches have been followed, the first to evaluate the role of variants that modify the interactions of LDL with proteoglycans resulting in possible pro-atherogenic or protective markers against CAD that unfortunately did not lead to satisfactory results.

In the second approach a cohort of 1197 CAD-negative and CAD-positive subjects was genotypically characterized to assess long-term exposure to high and low levels of LDL-C. A panel of 6 SNPs was used for the high exposure score and a panel of 9 SNPs was used for the low exposure score. A statistical analysis was performed to correlate the high and low exposure score with LDL-C levels, which showed that LDL-C levels are modulated by the polygenic burden of high exposure and not by the polygenic burden of low exposure.

In the evaluation of the correlation between high/low exposure score and CAD, statistical analysis revealed that both high and low polygenic scores were not associated with CAD and did not appear to vary by severity of coronary artery disease.

Moreover, genetic analysis was conducted by NGS on a selection of patients with extreme LDL-C, HDL-C, TG, and apoC-III phenotypes.

Among subjects with LDL-C<2nd percentile were identified two negative CAD patients carriers of APOB pathogenic heterozygous mutations that justify the hypocholesterolemic phenotype observed in both individuals. For all other subjects, calculation of the polygenic score for low LDL-C levels identified 3 subjects with a high probability of polygenic hypocholesterolemia. These data indicate that: 17% of subjects with LDL-C levels< 2nd percentile were found to have monogenic FHBL (1/1000-3000 in the general population) while in 17% of cases the clinical phenotype can be explained by polygenic FHBL. The remaining 66% of cases can be explained by other causes.

Among subjects with LDL-C>98th percentile one patient was found to be the carrier of the pathogenetic mutation in LDLR gene already described in the literature responsible for familial hypercholesterolemia. Among these patients, 5% of them had monogenic hypercholesterolemia (1/250 in the general population), whereas 79% had polygenic hypercholesterolemia. The remaining 16% of cases can be explained by other causes.

The molecular genetic study conducted on subjects with HDL-C < 2nd percentile allowed to identify: a CAD positive subject carrier of the ANGPTL3 variant and a CAD positive subject heterozygous carrier of the variant in the ABCA1 gene that alone do not explain the lipid phenotype of patients.

Among subjects with TG >98th percentile have been identified two CAD positive subjects heterozygous carriers of the variant in the LPL gene associated with hyperlipidemia and one CAD negative subject, double heterozygous for the variants in APOA5 and GPIHBP1 that determine an increase in TG levels and a reduced expression of GPIHBP1 protein.

The molecular genetic study performed on subjects with ApoC-III <5° percentile allowed to identify a CAD negative subject carrying a pathogenic mutation that would seem to confer a cardioprotective effect.

Among subjects with high HDL phenotype, low TG phenotype and high ApoC-III phenotype NGS genetic analysis has not allowed to identify pathogenic variants already described in the literature that can explain the correlation between biochemical values and the presence of disease and therefore the risk or protection against coronary artery disease.

For "orphan" patients in whom no pathogenic mutations were found in candidate genes and for those in whom mutations have been identified that alone do not appear to explain the clinical phenotype, exome sequencing analysis will allow the identification of new genetic determinants that may contribute to the development of the disease.

Clinical assessment of high risk of CAD in the measure of high and low exposure to LDL-C levels by genetic testing will provide strong evidence on the need to start earlier cardiovascular preventive approach. The discovery of carriers of some gene mutations predisposing to CAD is a prerequisite for appropriate treatment with drugs affecting primarily either TG metabolism or plasma HDL levels.

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