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Potential gene variants influencing FVIII levels: the role of LDL receptor on FVIII clearance

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1. BACKGROUND

1.1 *LDLR* gene and protein

The low density lipoprotein receptor *(LDLR)* is a 45-Kb gene localized on the short arm of chromosome 19 (19p13.1–13.3) consisting in 18 exons and 17 introns.

The protein is encoded as a precursor (pre-LDLR) of 860 aminoacids (90 KDa) with a N-terminal signal sequence of 21 residues. After synthesis, the LDLR protein is folded and partially glycosylated within the endoplasmic reticulum (ER). The glycosylation and the protein maturation are completed in the Golgi.

The mature LDLR is a type I transmembrane protein of 839 aminoacids (130 kDa), structured into functional domains (Figure 1). The ectodomain, encoded by exons 2–15, consists of three functional domains: a ligand-binding domain, an epidermal growth factor (EGF) precursor homology domain and a C-terminal domain.[1] The ligand-binding domain binds the apolipoprotein apoB100 of the low-density lipoproteins (LDL). It contains 7 cysteine-rich repeats of approximately 40 aminoacids, each with three disulphide bridges. A cluster of acidic residues coordinates a Ca2+ ion, useful for the proper folding of the domain. The interaction between the acidic residues in the LDLR-binding domain and the basic residues of apoB100 allows the binding of LDL to the receptor, whereas the LDLR conformational change from an open to a closed conformation allows the intracellular release of the cargo. The EGF precursor homology domain plays a role in the acid-dependent lipoprotein release in the endosome and consists of two EGF-like domains, six YWTD repeats in a six-bladed-propeller, and a third EGF-like repeat. The C-terminal domain, including 58 amino acids rich in threonine and serine residues and O-linked oligosaccharides, contributes to the receptor stabilization. The transmembrane domain, containing 22 hydrophobic amino acids, is necessary for the LDLR anchoring to the cell surface. Last, but not least, the cytoplasmic domain, consisting in 50 aminoacid residues, is necessary for the LDLR internalization.[1]

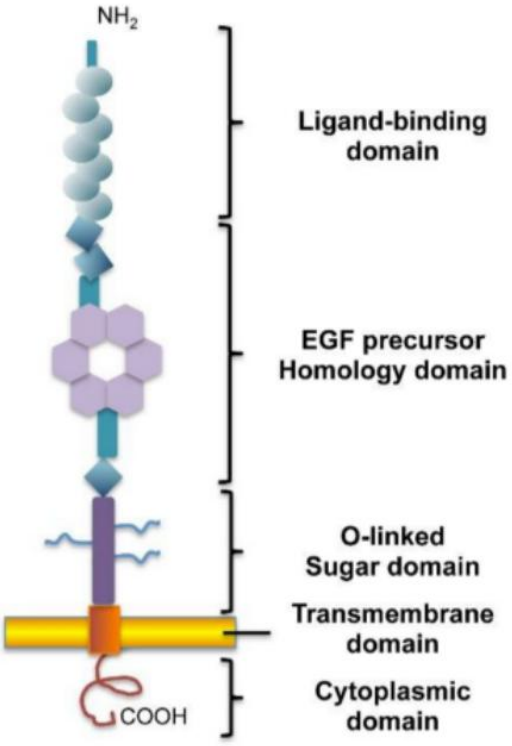


Figure 1
Schematic representation of the LDLR functional domains

The LDLR protein is structured into functional domains: the ectodomain, the transmembrane domain and the cytoplasmic domain. The ectodomain consists of three functional domains: a ligand-binding domain, an EGF precursor homology domain and a C-terminal domain with O-linked oligosaccharides.
From "Validation of LDLr Activity as a Tool to Improve Genetic Diagnosis of Familial Hypercholesterolemia: A Retrospective on Functional Characterization of LDLr Variants",
by A. Benito-Vicente, Int J Mol Sci. 2018.[1]

The LDL receptor plays a key role in the regulation of cholesterol homeostasis in mammalian cells and in the metabolism of LDL and the other apo-B containing lipoproteins.

All the apoB-containing lipoproteins with a diameter less than 70 nm (i.e. very low-density lipoproteins, VLDLs and their remnants, the intermediate-density lipoproteins, IDLs) are atherogenic. Indeed, they are able to cross the endothelial barrier in presence of endothelial dysfunction and remain trapped in the arterial wall by interacting with extracellular components. Among these lipoproteins, LDLs are by far the most prevalent in blood.[2]

The LDL clearance takes place by means of the LDL receptor expressed on the hepatocytes membrane. After LDL binding, the LDLR-LDL complex is internalized by clathrin-coated pits into

clathrin-coated vesicles. After vesicles fusion with endosomes, the endosomal acidification promotes the LDL release. The LDL components are then degraded in lysosomes, whereas the receptor recycles to the cell surface. This cycle is able to maintain a constant cholesterol level in hepatocytes and other cells (Figure 2).[1]

LDLR transcription is regulated by the sterol-responsive element binding protein-2 (SREBP-2) according to variations in intracellular cholesterol concentrations.[3]

Another modulator of the LDLR surface expression is the proprotein convertase subtilisin/kexin type 9 (PCSK9). Extracellularly, PCSK9 is able to bind the first epidermal growth factor-like repeat of LDLR and then the tri-molecular complex (LDLR-LDL-PCSK9) is internalized. After internalization, PCSK9 leads LDLR to lysosomal degradation, impairing the receptor recycling to the cell surface and thus reducing the LDLR surface expression. Intracellularly, PCSK9 plays the same role.[4] Moreover, specific microRNAs (e.g. miR-148a) have been demonstrated to regulate the *LDLR* gene expression at a post-transcriptional level.[5-6]

1.1.1 *LDLR* genetic variants

Genetic variants in the *LDLR* gene have been associated with higher LDL cholesterol levels, premature atherosclerotic cardiovascular disease (ASCVD) and increased cardiovascular risk.[1,7]

More than 90% of the identified mutations in patients with familial hypercholesterolemia (FH), an autosomal dominant disorder burdened by an accelerated atherosclerosis and premature coronary artery disease (CAD), are located in the *LDLR* gene. However, among the 2000 variants identified in *LDLR*, only a minority has been functionally characterized as pathogenic for FH. Therefore, *LDLR* variants are not necessarily associated with a clinical phenotype suggestive for FH.[1]

The most common 48 *LDLR* pathogenic mutations have been identified by the North East Thames Regional Genetics Lab over a 10-year period in patients with a clinical suspicion of FH undergoing DNA analysis.[8]

Usually, null variants (nonsense mutations, out-of-frame indels, most splicing variants and partial gene deletions) are considered pathogenic, whereas the effects on the clinical phenotype of non-null variants (single or multiple nucleotide substitutions and in-frame indels) have to be proven by functional characterization.[7]

LDLR mutations have been classified in six classes based on the phenotypic behavior of the mutant protein, affecting a specific step of the receptor cell cycle (Figure 2):

- class 1: mutations causing a deficit in the synthesis of LDLR, from the precursor to the mature protein;
- class 2: mutations causing an impaired protein migration from the ER to the Golgi or plasma membrane, due to an alteration of the three-dimensional structure, resulting in a complete or partial receptor retention into the ER;
- class 3: mutations causing a deficiency in the LDLR binding activity to apoB100 (2-30% of normal activity), as a result of alterations in the binding ligand domain or of deletions in the EGFP-like domain;
- class 4: mutations causing an impaired endocytosis of the receptor;
- class 5: mutations causing an alteration of the LDLR recycling, leading to the receptor lysosomal degradation;
- class 6: mutations causing a deficient insertion of the receptor into the cell membrane, leading to its secretion.[1]

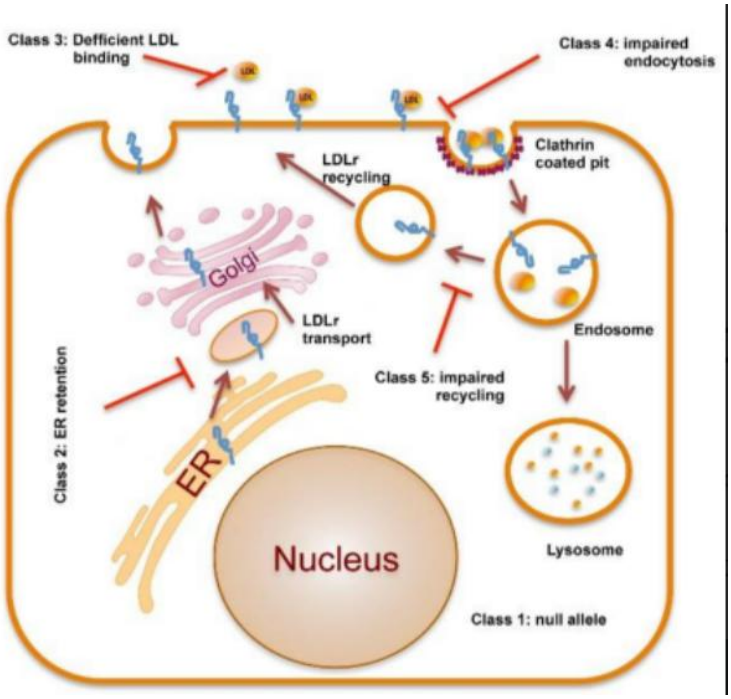


Figure 2
Schematic representation of the LDLR cycle and of the main classes of *LDLR* mutations

LDLR is synthesized in the ER and then transported to the Golgi where the glycosylation and protein maturation are completed. The mature LDLR is transported to the plasma membrane.
After LDL binding, the LDLR-LDL complex undergoes endocytosis. The LDL components are then degraded in lysosomes, whereas the receptor recycles to the cell surface.
LDLR mutations may affect different stages of the LDLR cycle.
From "Validation of LDLr Activity as a Tool to Improve Genetic Diagnosis of Familial Hypercholesterolemia: A Retrospective on Functional Characterization of LDLr Variants",
by A. Benito-Vicente, Int J Mol Sci. 2018.[1]

1.2 LDLR and the lipoproteins-mediated modulation of thrombotic risk

The increased arterial thrombotic risk observed in carriers of *LDLR* variants is mostly the result of a prolonged exposure to high levels of LDL cholesterol (LDL-c).[7]

Indeed, the reduced LDLR expression on hepatocytes membrane causes a lower LDL clearance, leading to increased LDL levels.

Low density lipoproteins, as cholesterol carriers, move cholesterol from the liver to peripheral tissues, including the arterial walls, and are the major determinants of atherosclerosis. When retained in the arterial wall, LDLs lead to lipid deposition and the initiation of atheroma. As result

of endothelial dysfunction, endothelial cells express adhesion molecules, allowing leukocyte transmigration and monocytes translocation in the arterial wall, where monocytes differentiate into macrophages. Macrophages are able to internalize oxidized LDLs, forming the so called ‘‘foam cells’’, which have pro-inflammatory activities and induce vascular smooth muscle cells (SMCs) proliferation, with the consequent production of extracellular matrix molecules. The continuous exposure to LDLs, as well as to other apoB-containing lipoproteins, leads to the progression of the atherosclerotic plaque. Hence, the size of the atherosclerotic plaque burden is determined by both the concentration of LDLs and other apoB-containing lipoproteins and by the total exposure duration to these lipoproteins.[2]

Consistent with this pathogenetic mechanism, a large number of studies showed a log-linear relationship between the absolute changes in plasma LDL levels and the risk of ASCVD.[2]

In addition to consistent experimental findings, these studies provided a strong evidence of a causal association between LDL-c and ASCVD.[9-10] The cumulative effect of LDL-c on the risk of ASCVD is strongly supported by randomization studies showing that a long-term exposure to lower LDL-c levels may induce a much lower cardiovascular risk than a shorter-term exposure.[11-12]

Therefore, current guidelines recommend specific and personalized thresholds for LDL cholesterol levels, as well as for non-HDL cholesterol levels (encompassing all the apoB-containing lipoproteins), with the final goal to prevent cardiovascular events, taking into account the specific risk category of each patient.[13]

In addition to all the aforementioned atherogenic mechanisms in which the low density lipoproteins are the main actors, high LDL levels may induce the activation of different hemostasis pathways. Indeed, the association between high LDL levels and increased oxidative stress leads to the formation of oxidized-LDL (ox-LDL), which play a key role in the inflammation-driven thrombosis, by activating the CD36 and LOX-1 receptors on platelets.[14-17] Activated platelets may also oxidize LDLs, enhancing platelet activation.[18]

Interestingly, the disbalance between circulating LDL and high-density lipoproteins (HDL) levels may contribute to the von Willebrand factor (VWF) self-association. It has been shown that the ability of VWF to induce platelet adhesion strongly depends on the proportion of VWF multimers and on their self-association into larger structures, which is accelerated by shear stress.

HDL and their major apolipoprotein apoA-I are able to prevent VWF self-association into longer strands on the endothelium, leading to an impaired platelet adhesion. Interestingly, in a mouse model of thrombotic microangiopathy, it has been shown a potential role of apoA-I in preventing the microvascular occlusion, which is the result of the interaction between platelets and hyperadhesive VWF.[19]

Moreover, as a result of the reduced LDLR expression, lipoprotein (a) [Lp(a)] levels may increase. Lp(a) is a proatherogenic lipoprotein, with a diameter less than 70 nm, characterized by an apo(a) moiety covalently bound to an apolipoprotein apoB, with a large content of lipids and oxidized phospholipids, determining its proinflammatory properties.[2] Moreover, its apolipoprotein apo(a) contains sequences which are homologous to plasminogen, in particular to kringle IV, kringle V and protease domain. As a consequence of this homology, apo(a) is able to inhibit plasminogen activation within the fibrin clot, leading to an impaired fibrinolysis.[20-21]

1.3 LDLR and the thrombotic risk despite the lipid-lowering treatment

Carriers of *LDLR* variants may have a higher risk of thrombosis, also independently of lipids levels and despite the optimal lipid-lowering treatment (LLT), as observed in several studies.

1.3.1 In patients with familial hypercholesterolemia

Despite the maximum tolerated LLT, FH patients still develop cardiovascular events, with a reported prevalence between 9% and 22%. In a study of 821 FH patients treated with LLT for a median period of 9.5 years, 12% of patients developed a cardiovascular event. Among these, 30%

developed a subsequent cardiovascular event. Traditional modifiable cardiovascular risk factors, such as smoking and hypertension, were the determinant risk factors.[22]

In two Dutch studies, a residual cardiovascular risk of 11 per 1000 patient-years was observed on low-dose statin therapy and of 8.8 per 1000 person-years using moderate-to-high-statin therapy.[23-24] However, only a small percentage of FH patients achieve target LDL-c levels by LLT, as a consequence of statin side effects and low adherence. Therefore, the persistence of higher LDL levels than the recommended thresholds may influence the reported incidence of cardiovascular events during LLT. Moreover, FH patients may have higher Lp(a) levels compared with non-FH subjects, which are not reduced by statins and ezetimibe. Therefore, the increased Lp(a) concentrations may partly explain the residual cardiovascular risk observed in these patients.[22]

1.3.2 In patients with clinically established atherosclerotic cardiovascular disease

In patients with clinically established ASCVD the risk of recurrent cardiovascular events seems to be mainly influenced by the traditional cardiovascular risk factors, as well as vascular disease site and kidney function. In a meta-analysis of 26 studies, authors reported a stroke recurrence rate of 5.7-51.3% with a higher rate of recurrence for large artery atherosclerosis and cardioembolic strokes.[25] Hypertension, diabetes mellitus, atrial fibrillation, a history of transient ischemic attack (TIA) and a higher stroke severity were independent risk factors of recurrence.

In another meta-analysis of 58 studies with a mean follow-up of 3.5 years, the observed risk of myocardial infarction (MI) in patients with a history of ischemic stroke/TIA was 1.7%/year, whereas the risk of recurrent stroke was 4.3%/year.[26] Male sex, hypertension, CAD and peripheral artery disease (PAD) were associated with an increased risk of MI.

In a large study of 7,870 patients who survived a MI, in a median follow-up of 3.9 years, authors observed an incidence of recurrent MI of 2.65%/year for the first year, and 0.91-1.42% thereafter up to 5 years.[27] Diabetes mellitus, history of MI and advanced age were predictors of recurrence.

However, patients with ASCVD show a high risk of recurrent cardiovascular events also despite the maximum tolerated treatment of traditional cardiovascular risk factors, according to the recommended treatment goals. Therefore, in addition to the traditional cardiovascular risk factors, a growing evidence suggests that the risk of recurrent thrombotic episodes may be also influenced by other factors, such as hypercoagulability.

For example, in a prospective study including 120 patients with advanced PAD, authors found that the increased activity of coagulation factors (e.g. coagulation factor VIII), as well as the increased levels of hemostatic markers of endothelial dysfunction (e.g. VWF) were associated with the main PAD complications, such as graft thrombosis, MI, disease progression and restenosis.[28]

1.4 FVIII role in thrombosis

Coagulation factor VIII (FVIII) plays a key role in the amplification and propagation of the coagulation cascade. Its relevance in hemostasis is proven by the effects of its alterations on human diseases. Indeed, its severe deficiency leads to a severe bleeding disorder (hemophilia A), whereas its increased coagulation activity predisposes to thrombosis.

The observed lower cardiovascular mortality rate in hemophilia A patients compared with the general population, despite a comparable prevalence of atherosclerotic disease and of traditional cardiovascular risk factors, has been imputed to the reduced thrombus formation on the plaque and the increased plaque stability, resulting from the hypocoagulability state of these patients.[29-30]

Prospective and case-control studies provided strong evidence on the association between high FVIII levels and the risk of a first or recurrent venous thromboembolism (VTE) episode. In a case-control study of 301 patients under the age of 70 with a first no cancer-related deep venous thrombosis (DVT) and 301 age- and sex-matched healthy controls, blood group, VWF and FVIII levels were associated with DVT. However, by multivariate analysis, only FVIII was confirmed as a risk factor of DVT for levels above 150 IU/dL (adjusted odds ratio=4.8).[31]

In a prospective study, enrolling 360 patients with a history of VTE, a 10.6% rate of VTE recurrence was observed after anticoagulants discontinuation in an average follow-up of 30 months, with a relative risk of recurrence of 1.08 for each increase of 10 IU/dL in plasma FVIII levels. Patients with plasma FVIII levels above the 90th percentile, compared with those with lower FVIII levels, showed an approximately 7 fold higher risk of recurrence, after adjusting for age, sex, factor V Leiden, G20210A prothrombin mutation, and duration of anticoagulation treatment.[32]

Although FVIII could influence the clotting formation on the atherosclerotic plaque, its role in arterial thrombosis is less clear. In a single center retrospective family cohort study involving 1399 subjects, authors found that FVIII level was a mild risk factor of arterial thrombosis.[33]

Other authors reported the association between elevated plasma FVIII levels and increased risk of arterial thrombotic events.[34-36]

Consistently with these findings, in a cohort of patients with and without angiographically confirmed CAD, higher FVIII levels were associated with a progressive increase of CAD risk, independently of all the traditional cardiovascular risk factors.[37]

The hypercoagulable state could play a significant role in ischemic stroke, in particular on the risk of recurrence, as reported by other authors.[38-39]

Moreover, the presence of several clotting factors (e.g. tissue factor, factors VII, X and XII), as well as fibrin and fibrin degradation products in atherosclerotic plaques suggests that the coagulation activation plays a relevant role in atherosclerosis development and progression.[41-44] In mice with an apolipoprotein E deficiency (ApoE(-/-)) a state of hypercoagulability lead to advanced atherosclerosis and the administration of dabigatran and rivaroxaban was able to attenuate atherosclerosis development.[44] Moreover, an association between the state of coagulation activation and the severity of coronary atherosclerosis has been observed.[45]

Interestingly, FVIII may enhance the thrombogenicity of atherosclerotic plaques, as suggested by immunohistochemical studies on human atherectomy specimens, showing the presence of FVIII near macrophages and SMCs. In particular, in mouse models double deficient in factor VIII and

apoE, authors observed a significant lower prevalence of early-stage atherosclerotic lesions, despite the higher plasma cholesterol levels.[46]

Moreover, FVIII is a key determinant of thrombin generation, and thrombin plays several important non-hemostatic activities in the pathogenesis and progression of the atherosclerotic disease, as shown by animal models and immunohistological studies on atherosclerotic lesions. The discovery of protease activated receptors (PARs), activated by thrombin,[47] on platelets, endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and leukocytes supports the thrombin role in the pathophysiology of atherosclerosis.[44] Consistently, increased PAR-1 expression has been shown in human atherosclerotic arteries.[48]

By the PARs axis, thrombin regulates several inflammatory processes, and plays a crucial role in the complex atherosclerosis-related crosstalk between coagulation and inflammation axis (so called immunothrombosis). The atherosclerosis-related pro-inflammatory background (cytokines, neutrophils and neutrophil extracellular traps) leads to an increased thrombin generation and an impaired activated protein C (APC) formation.[44] In this pathophysiological condition, a thrombin conversion from a physiological regulator of hemostasis to a proinflammatory mediator occurs.[44]

Thrombin contributes to the monocytes and T-cells recruitment into the artery wall,[49] modulates the expression of the adhesion molecules on the endothelium, such as E- and P-selectin, vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM), allowing the leukocyte extravasation.[50] Moreover, thombin has been shown to induce the expression of several cytokines, leading to the monocytes adhesion to the endothelium.[51-52] Thrombin is also able to up-regulate vascular endothelial growth factor (VEGF) receptor, transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF) and modulates the migration, proliferation and differentiation of VSMCs.[44] A thrombin role in stimulating the collagen synthesis by SMCs has been demonstrated.[53]

1.5 FVIII clearance

FVIII circulates in plasma with its carrier protein VWF in a tight, non-covalently linked complex.

VWF stabilizes the heterodimeric structure of FVIII, prevents its cellular uptake and proteolytic degradation by proteases (e.g. activated protein C and activated FX). Moreover, VWF inhibits FVIII binding to negatively charged phospolipids and to activated FIX. This complex linkage between FVIII and VWF influences the clearance and survival in circulation of both proteins and has recently achieved a growing scientific interest considering its implications in the field of FVIII-replacement therapies and in the pathogenesis of von Willebrand disease (VWD).

A high heritability of FVIII plasma levels has been reported (40–60%).[54-55] However, only a minor proportion of FVIII variance is caused by mutations in *F8* gene.[56-58]

Pharmacokinetic studies in hemophilia A patients treated with different FVIII products have provided relevant informations about FVIII survival and have showed a wide variety of FVIII half-life between patients treated with the same product.[59]

Single nucleotide polymorphisms (SNPs) located in the *ABO* blood group locus have been showed to be involved in the modulation of FVIII levels. In particular, blood group may influence FVIII levels as a result of its effect on the VWF processing and clearance by different glycosylation patterns.[60-61]

The LDL receptor-related protein (LRP1, also named as a2-macrogobulin receptor or CD91) has been the first identified clearance receptor of FVIII, mediating its cellular uptake.[59,62-63]

The relevance of LRP1 in the modulation of FVIII plasma levels has been demonstrated in mouse models.[64] In humans, two *LRP1* polymorphisms have been associated with increased FVIII levels.[65-68]

Heparan-sulfate proteoglycans have been shown to play a role in the cellular internalization of FVIII by favouring its efficient binding to LRP1.[69-70]

However, when LRP1 is absent, FVIII cellular degradation is only partially inhibited, suggesting that LRP1-independent pathways may contribute to the FVIII cellular uptake.[59]

Among the other studied clearance receptors of FVIII, the asialoglycoprotein one is able to bind FVIII. In particular, it has been demonstrated that FVIII B domain is necessary for this interaction.[71] However, the role of the asialoglycoprotein receptor in FVIII clearance in vivo is not clear yet. Indeed, full-length FVIII and B-domainless FVIII showed similar half-lives.[72]

Recently, the low-density lipoprotein receptor (LDLR) has been proposed to influence FVIII plasma levels by modulating FVIII clearance (see paragraph 1.6).

1.6 LDLR role in FVIII clearance

LDLR and LRP are both members of the LDLR family of endocytic receptors. The ligand binding to both receptors is inhibited by the receptor-associated protein (RAP). These two receptors have different peculiarities. Indeed, LRP is able to bind a large variety of structurally and functionally unrelated ligands, including FVIII, whereas LDLR has a ligand specificity only for the apolipoproteins apoE and apoB100. In addition, the region of complement-type repeats CR.2-5 in the extracellular ligand-binding domain of LDLR has been identified as the binding site for FVIII.[73]

The physiologic role of LDLR in FVIII clearance was assessed by mouse models. In mice with hepatic LRP deficiency, LDLR played a dominant role in FVIII clearance, which was accelerated after adenovirus-mediated gene transfer of LDLR. The receptor role in FVIII clearance was not influenced by plasma lipoproteins levels. In *LRP* knockout mice, plasma FVIII levels were increased by 1.6 fold, whereas *LDLR* and *LRP* double knockout mice showed a 4.2 fold increase in FVIII levels, demonstrating a cooperating activity between these two receptors.[74]

Data stemming from clinical studies suggest a direct role of LDLR in modulating FVIII clearance, also independently from LRP. In particular, in a study of 129 heterozygous FH (HeFH) patients, all carriers of *LDLR* mutations and no treated with lipid-lowering drugs, FVIII levels were 9% higher compared with 127 unaffected relatives, after adjustment for family ties, age, LDL and C-reactive protein (CRP) levels.[75] No difference on VWF levels was observed between the two groups, with

consequent higher FVIII/VWF ratio in carriers of *LDLR* variants. A limitation of this study was the wide variety of different types of *LDLR* mutations identified in the analysed population.

Specific *LDLR* variants have been observed to be associated with higher FVIII levels, suggesting their potential role in FVIII clearance (see paragraphs 1.6.1, 1.6.2, 1.6.3).

1.6.1 The rs688 variant

The rs688 is a common synonymous SNP (c.1773 C>T, p.Asn591=), with a minor allele frequency (MAF) of 0.44 in the European population (1000 Genome).

The variant rs688 is located within the *LDLR* exon 12, coding for the epidermal growth factor-like repeat (EGF)-b-propeller region, that plays a key role in displacing the bound lipoproteins and regulating the receptor recycling.[76]

This variant has been associated with a 4–10% increase in plasma cholesterol levels.[76]

The minor frequency allele (T) has been shown to disrupt a splicing enhancer, leading to the reduction in splicing efficiency of about 10% and the generation of a LDLR transcript lacking exon 12, named LDLR12(2). Authors suggested that LDLR12(2) is able to encode a soluble LDL receptor, which may inhibit plasma LDL uptake.[77]

The effect of the minor allele on exon 12 skipping was confirmed in another study, performed in immortalized lymphoblastoid cells. The alternative transcript contains a premature termination codon, leading to a nonsense-mediated decay (NMD).[76]

However, exon 12 is expected to be retained in approximately 90% of LDLR transcripts of T/T homozygotes. In order to understand the effect of this variant on the LDLR protein beyond the alternative splicing-related mechanism, authors produced expression vectors carrying the *LDLR* coding region with the rs688 ‘C’ or ‘T’ allele and performed a transient transfection of HepG2 cells. They quantified the LDLR protein in total cell lysates, cell surface and lysosomes by western blotting and assessed the LDLR intracellular localization by immunofluorescence.[76] Cells expressing T/T LDLR showed a reduced expression of LDLR at the cell surface and a higher

expression in the lysosomes. Moreover, the rs688 variant caused a reduced uptake of LDL and, in presence of exogenous PCSK9, the efficacy of a PCSK9 monoclonal antibody to increase LDL uptake was impaired.[76] By using the Codon Usage Database, authors observed that rs688 caused the convertion from a common codon ACC (36% usage) to a less commonly used codon, ACT (25% usage). The authors suggested that this may alter the LDLR protein folding and function. In particular, the affected EGF region of the receptor may influence the magnitude of the therapeutic response to PCSK9 inhibitors.[76]

Moreover, in a study including 692 patients with angiographically confirmed CAD and 291 controls, the rs688 genotype was predictor of FVIII coagulant activity (FVIII:C) with T alleles associated with higher FVIII levels.[37] However, to date, the effect of this variant on FVIII clearance (i.e. binding and/or uptake) has not been studied *in vitro*.

1.6.2 The rs2228671 variant

The synonymous variant rs2228671 (c.81C>T, p.Cys27=) is a common polymorphism located within the *LDLR* exon 2, with a reported MAF of 0.10 in the European population (1000 Genome).

At the same genomic location, a rare nucleotide substitution (c.81C>A), that causes a nonsense variant (p. Cys27*) was also reported in ClinVar and associated with the FH phenotype.

The variant rs2228671 induces a shift from frequent to rare codons, leading to co-translational folding alterations, which may affect LDLR activity.[76]

The minor allele T of this SNP has been previously reported to be associated with lower LDL plasma levels and consequent reduced cardiovascular risk.[78] In another study, including patients with and without angiographically confirmed CAD, the T/T genotype was not associated with CAD risk.[37] However, the minor allele T resulted to be associated with higher FVIII levels. Therefore, authors interpreted the lack of association with CAD, considering the contrasting effects of this variant on the lipid and coagulation phenotypes. Indeed, rs2228671 was associated with higher and

lower levels of FVIII and LDL, respectively. The preventive role of low LDL cholesterol was likely counterbalanced by the proatherogenic effect of high FVIII:C.

To date, the effect of this variant on FVIII clearance (i.e. binding and/or uptake) has not been studied *in vitro*.

1.6.3 The rs5925 variant

The rs5925 is a common synonymous SNP (c.1959T>C, p.Val653=) located within *LDLR* exon 13, with a reported MAF of 0.45 in the European population (1000 Genome).

In one study, the variant rs5925 was associated with an increased risk of ischemic stroke, independently of LDL levels.[79] However, the authors suggested a linkage disequilibrium between this variant and rs688. Consistent with this hypothesis, no associations between rs5925 and higher LDL levels or higher FVIII levels have been reported. On the contrary, rs688, as aforementioned, has been strongly associated with both higher plasma lipids and FVIII levels. Therefore, among the two polymorphisms, rs688 has been considered the causative one.[76]

To our knowledge, there are no *in vitro* studies concerning the effect of rs5925 on LDLR expression, as well as on the LDLR-mediated FVIII clearance (i.e. binding and/or uptake).

1.7 LDLR and the FVIII-mediated modulation of thrombotic risk

Several studies reported a higher risk of thrombosis in carriers of *LDLR* variants, independently of lipids levels. In a study including 692 patients with angiographically confirmed CAD and 291 with no CAD, rs688 polymorphism was associated with a higher risk of CAD. This association was also confirmed after adjusting for traditional cardiovascular risk factors, including plasma lipids (OR for T carriers: 1.67, CI: 1.10-2.54). The higher FVIII levels observed in subjects carrier of the rs688 variant explained the increased CAD risk.[37]

In another study enrolling 815 ischemic stroke patients and 430 healthy subjects, the haplotype TC of the *LDLR* variants rs688 and rs5925 was associated with a 65% increased risk of overall ischemic stroke, 72% increased risk of atherothrombotic stroke and 70% increased risk of lacunar infarction, compared with the reference CT and regardless of LDL levels. A synergistic effect between these 2 SNPs was suggested.[79]

All these findings support that the increased arterial thrombotic risk observed in *LDLR* carriers is partly independent from the lipoprotein levels and could be related to the LDLR modulation of FVIII levels.

2. AIMS OF THE STUDY AND RESEARCH HYPOTHESIS

Despite the growing evidence about the role of *LDLR* variants in modulating FVIII levels, there is still a relevant gap of knowledge in this field. Indeed, many *LDLR* variants have not been functionally characterized yet, and most of those characterized have been studied only in the hypercholesterolemia field to understand their capability to bind LDL and not as regards their potential "hemostatic pathogenicity" (i.e. role on FVIII clearance). In particular, it is not clear which are the *LDLR* variants involved in the FVIII clearance and the underlying mechanism.

With this background and gap of knowledge this study aims to:

- identify the *LDLR* variants associated with high FVIII coagulant activity (FVIII:C≥150 IU/dL) in a cohort of DVT patients and healthy controls;
- functionally and biochemically characterize the *LDLR* variants (identified in this study and described in literature) associated with high FVIII levels in order to understand their ability to modulate the FVIII clearance, in terms of FVIII binding and/or uptake.

Our hypothesis is that specific *LDLR* variants, by influencing LDLR protein expression and/or LDLR-mediated FVIII binding/uptake, could reduce the FVIII clearance. Therefore, carriers of these variants would have higher FVIII levels, resulting in an increased risk of thrombosis also despite the optimal lipid-lowering treatment.

3. RELEVANCE OF THE STUDY AND CLINICAL IMPLICATIONS

3.1 In the thrombosis field

3.1.1 Genetic risk scores as prediction tools of residual thrombotic risk

The genome-wide association studies (GWAS) identified many gene variants associated with CAD, in particular with premature CAD.

However, single CAD-causing variants are much more rare than polygenic determinants (i.e. multiple variants that contribute to the disease). For example, the prototype of a monogenic disease associated with an increased risk of CAD is familial hypercholesterolemia (FH), an autosomal dominant disorder with an estimated prevalence of 1:200-1:250 in heterozygotes and 1:200,000-1:300,000 in homozygotes. In most of cases, ASCVD is a polygenic disease, arising from the combination of many different SNPs.

With this background, a wide variety of polygenic risk scores (PRS) have been developed. They have been shown to improve the risk prediction of CAD, but with a relatively modest increase in the prediction accuracy.[13]

Scores including a higher number of SNPs have a better predictive power. However, the feasibility of this approach depends on the availability of appropriate statistical modeling tools, able to take into account the complex SNPs correlations (linkage disequilibrium).

Current clinical risk scores do not allow to properly predict CAD in young individuals, because age is one of the most important risk factors included in the available risk prediction tools.[80]

Moreover, differently from non-genetic factors, the impact of genetic risk factors on CAD decreases with age.[81] PRS may provide the opportunity for an early ASCVD risk assessment and prevention at younger ages before the onset of the traditional cardiovascular risk factors.[82-83]

Based on the aforementioned considerations, PRS may provide a benefit when applied early in life, in particular in subjects with a family history of premature ASCVD. Indeed, the early identification of young individuals with a high genetic risk score of CAD should be useful to define personalized

goal treatments for the traditional cardiovascular risk factors and start conventional treatments at earlier stages of the atherosclerotic disease development.[84]

For example, lifestyle modification may provide a higher absolute clinical benefit among subjects with high genetic risk when applied at younger ages.[85]

Genetic risk could be considered a cardiovascular risk-enhancing factor, as well as the other recognized by current guidelines (e.g. chronic inflammatory conditions or family history of premature CAD), because associated with an increased CAD risk regardless of the traditional cardiovascular risk factors.[85] Interestingly, a high PRS was associated with CAD also regardless of a self-reported family history of CAD.[86] Therefore, PRS could be useful in a combined approach with conventional clinical scores in order to identify patients who would have higher benefit by statins in primary cardiovascular prevention.

In two studies, subjects with a high PRS for CAD showed the greater clinical benefit from statins (in terms of number needed to treat to prevent one event), independently of the achieved LDL-c reduction.[87-88] Moreover, patients with very high PRS for CAD could be candidate for an early initiation of statin therapy.[85]

PRS may also be useful to identify the proper age for the assessment of subclinical atherosclerosis by coronary artery calcium (CAC) screening.[84] Indeed, a high PRS for CAD is an independent predictor of premature CAC.[87]

To date, there is poor evidence stemming from genetic studies concerning the PRS prediction accuracy in secondary cardiovascular prevention. In particular, in a study including 2438 patients a strong association between a 27 SNPs-genetic risk score (GRS) and recurrent cardiovascular events was observed.[88] In another study, including 3503 patients admitted with acute coronary syndrome (ACS), a 30 SNPs-GRS was not significantly associated with recurrent cardiovascular events occurring within 1 year after ACS. Authors explained their findings considering that early cardiovascular events post-ACS may have a different etiology than later ones. For example, they could be more procedural-related (e.g. stent restenosis) and less genetically-determined.[89]

Moreover, PRS could be useful to predict the responsiveness to statins and PCSK9 inhibitors.[84-85] In patients already treated with statins a high PRS for CAD was associated with a greater relative and absolute CAD risk reduction with PCSK9 monoclonal antibodies.[90-91]

Although ASCVD is known to have a genetic complex background and family history of premature CAD is a recognized important risk factor of CAD, PRS are not currently used in clinical practice, yet. This could be explained considering that there is no a clear consensus about the genes and SNPs which should be included. Moreover, more and more evidence is necessary to assess the utility of these tools in clinical practice.[13]

Some of the variants/genes, identified to be associated with CAD, target non-traditional pathways of atherothrombosis.

Understanding the *LDLR* variants involved in FVIII clearance could be useful to better stratify thrombotic risk in patients already at risk (e.g. patients with traditional cardiovascular risk factors or in secondary cardiovascular prevention), by improving the prediction accuracy of the pre-existing genetic risk scores.

3.1.2 Genotype characterization for a personalized cardiovascular preventive approach

A better genotype and phenotype characterization (e.g. hemostasis parameters) of patients in primary and secondary cardiovascular prevention could be useful to allow physicians to choose the proper and personalized preventive approach, taking into account the patient thrombosis/bleeding risk balance. In this context, carriers of *LDLR* variants could have higher FVIII levels and thus may have a higher residual thrombotic risk despite the optimal lipid-lowering treatment; these patients could be candidate for additive preventive cardiovascular approaches. Among the approaches targeting pathways other than lipids ones, antiplatelet drugs and anticoagulants could be considered. In patients without established ASCVD no or little benefit with aspirin has been reported with an increased risk of bleeding. However, in selected categories of patients at high cardiovascular risk (e.g. with type 2 diabetes), the benefits could outweigh the risks also in primary cardiovascular

prevention. As concerns secondary prevention, it is known that in patients with previous MI, ischemic stroke, or with lower extremity artery disease (LEAD), antiplatelet drugs benefits on cardiovascular prevention outweigh the risk of bleeding.[13] However, there is still a gap of knowledge in this field. For example, in patients with LEAD, a growing evidence suggests a potential benefit of anticoagulants in cardiovascular prevention, although further studies are necessary. In the COMPASS trial, the combination of aspirin and low-dose rivaroxaban in patients with stable atherosclerotic disease and an ankle brachial index less than 0.90 reduced ASCVD and major adverse limb events, but increased the risk of major bleedings.[92]

3.1.3 Pleiotropic hemostatic effects of lipid-lowering drugs

Statins and PCSK9 inhibitors may show pleiotropic effects. In particular, a cardiovascular protective activity, independent from the LDL-c lowering activity, was observed in several clinical trials; the protection is higher than the expected based on the magnitude of LDL-c reduction.[93-94]

Among the well documented pleiotropic effects, the stabilization of atherosclerotic plaques, the anti-inflammatory properties, the beneficial effect on endothelial dysfunction, the attenuation of platelet activation and coagulation pathways and the increased fibrinolysis deserve to be mentioned.[14]

In this field, a better understanding of the LDLR role on FVIII clearance could be relevant to better predict and explain the hemostatic pleiotropic effects of lipid-lowering drugs.

In particular, more and more evidences support a preventive role of statins on venous thrombosis by reducing FVIII levels, as a result of the LDLR up-regulation and the consequent enhanced FVIII clearance.[95-101]

Two meta-analysis,[102-103] including both randomized controlled trials (RCTs) and observational studies, reported that statins use significantly reduced VTE risk.

These findings were confirmed in a large study of 192,908 patients, wherein an odds ratio of 0.75 for VTE recurrence was found for those patients on statin therapy.[96] Among the different statins, rosuvastatin seems to be the most effective with a reported reduced risk of VTE up to 40%.[97-101]

A role on VTE prevention has also been suggested for PCSK9 inhibitors.[14] This hypothesis is supported by experimental findings showing that PCSK9 $^{-/-}$ mice develop smaller venous thrombi than wild-type mice after ligation of inferior vena cava.[104] A meta-analysis demonstrated a 31% relative risk reduction in VTE with PCSK9 inhibitors. However, a significant interaction between Lp(a) levels and the magnitude of VTE risk reduction was observed. Therefore, the effect on VTE risk was only partly explained by the reduction of FVIII levels.[105]

Interestingly, specific *LDLR* variants could influence the magnitude of the therapeutic response to lipid-lowering drugs, as suggested for the rs688 variant on PCSK9 inhibitors.[76]

3.2 In the bleeding field

As a result of the lowering effect of statins on FVIII levels, authors reported an association between statins use and the risk of hemorrhagic stroke. However, the absolute risk is very small and outweighed by the cardiovascular benefits of these drugs.[106-107] However, there are no data on statins safety in patients already at risk of bleeding (e.g. with congenital bleeding disorders).

Growing evidence supports a potential implication of the LDLR role on FVIII clearance also in the bleeding field. In a study enrolling 447 patients with primary intracerebral hemorrhage (PICH) and 430 healthy control subjects, homozygous T/T subjects for the rs688 variant had a 73% decreased risk of PICH compared with homozygous C/C and heterozygous C/T individuals.[108]

Last but not least, understanding which are the *LDLR* variants involved in FVIII clearance could allow to better predict the pharmacokinetics of FVIII treatments in hemophilia A patients. In a study on 33 hemophilia A patients (FVIII:C≤2IU/dL) without inhibitors, the pharmacokinetics of different FVIII concentrates was influenced by the rs688 and rs2228671 polymorphisms.[109] In particular, the

rs688 polymorphism resulted to be associated with specific pharmacokinetics parameters, suggesting a role on the FVIII initial distribution phase, whereas for rs2228671 an association with FVIII clearance and volume of distribution at steady state was observed.

4. METHODS

4.1 Study design and patients

We selected 596 Italian subjects (298 DVT patients and 298 controls) enrolled and sequenced in the frame of the DVT-Milan study. DVT-Milan study is a case–control study of 2139 unrelated Italian consecutive patients with a first DVT and 1938 controls, recruited at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center (Milan, Italy) between 1995 and 2010. All subjects underwent a complete thrombophilia screening, including measurement of natural anticoagulant proteins, genotyping of Factor V Leiden (FVL) and prothrombin G20210A, and search for antiphospholipid autoantibodies. FVIII and fibrinogen coagulant activities were also measured. The study was approved by the Institutional Review Board of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and all subjects gave informed consent.

DVT cases were included according to the following criteria: objective diagnosis of DVT of the lower limbs (by compression ultrasonography or venography); idiopathic DVT defined by the absence of cancer, surgery or immobilization; normal levels of the natural anticoagulants (antithrombin, protein C and protein S); absence of FVL or prothrombin G20210A mutations; available DNA and plasma samples. DVT patients were asked to bring to the center the diagnostic documentation of the thrombotic episodes and underwent a clinical interview.

Controls, matched with cases for age (+/- 5 years) and sex, were recruited among partners, friends and non-consanguineous relatives who accompanied patients to the center and agreed to be tested for thrombophilia. They had no FVL and prothrombin G20210A mutations and had normal levels of natural anticoagulants proteins.

All subjects with known congenital or acquired coagulation disorders were not included in the study.

4.2 Power and sample size

We assumed α error = 0.05 and β error = 0.2 to have a power of 80%.

We defined cases and controls individuals that are carriers or not of a *LDLR* variant, respectively. Assuming an expected prevalence of high FVIII:C (≥150 IU/dL) in controls of about 9% (as well as in our sample of healthy controls), in order to have at least a 20% higher prevalence in cases, we estimated a sample size of at least 57 cases and 57 controls.

This sample size allows the identification of common and low-frequency variants associated with high FVIII:C. To increase the power for rare variants a "gene-based analysis" has been considered.

4.3 Study procedures

4.3.1 Data collection and laboratory exams

The following variables have been collected: age (at the DVT episode or at recruitment for controls), sex, Italian region of birth, body mass index and ABO blood group.

FVIII:C and VWF antigen (VWF:Ag) have been measured by one-stage clotting assay and an automated immunoassay on ACL TOP analyzer, respectively.

4.3.2 Next generation sequencing, quality control and bioinformatics analysis

A custom panel of 734 genes involved in thrombosis and hemostasis, including coagulation system, fibrinolysis, platelet function, inflammation, and complement system, was designed.

We sequenced the coding regions plus 10 base pairs flanking the exons in order to cover the splice junctions. For a subset of 48 genes, we additionally sequenced the 3' and 5' untranslated regions (UTR). We performed whole gene sequencing including 10 kilobase pairs (kb) of the promoter region of *F5*, *VWF* and *F8* genes, that are of particular interest for VT risk. We also targeted 179 single nucleotide variants, consisting of 28 variants previously associated with VT and 151

ancestry-informative markers. To facilitate the capture of the selected variants, we introduced in the design 200 base pairs of target region surrounding each variant.

A multiplexed next generation sequencing (NGS, Human Genome Sequencing Center, Baylor College of Medicine, Houston, USA) was performed using unique barcode-sequencing tags, to create library pools of 8–20 samples, which were captured and sequenced in parallel using the Illumina HiSeq 2000 sequencing platform (Illumina, San Diego, USA).

Reads were mapped to reference genome GRCh37/hg19 by using the Burrows–Wheeler aligner, resulting in BAM files per sample. Realignment around insertions and deletions (indels), and recalibration of quality scores was performed with the Genome Analysis Toolkit (GATK). Variant calling was conducted using the Atlas2suite (Atlas-SNP and Atlas-indel), followed by variant annotation as implemented in the Cassandra annotation suite. Individual variant files (vcf) were subsequently merged in a unique file. A smaller dataset was obtained extracting all the identified variants of *LDLR* gene in the Italians patients using bcftools. Variants were reannotated using wANNOVAR (http://wannovar.wglab.org/).

4.3.3 Statistical analysis

In order to select the variants to be characterized *in vitro*, we analyzed the association between *LDLR* variants and high FVIII:C. A case-control association analysis was performed considering as cases subjects with FVIII:C≥150 IU/dL and as controls subjects with FVIII:C less than 150 IU/dL.

Variants have been defined as common, low-frequency or rare variants based on minor allele frequency (MAF) (MAF≥ 5%, 1%≤MAF<5%, MAF<1%, respectively).

For common and low-frequency *LDLR* variants, a logistic regression model was performed. Results have been reported as odds ratios (OR) with 95% confidence intervals (CI). There are no confounders because this is a genetic study performed only in Italian subjects. Potential effect modifiers (e.g. age, sex and blood group) have been taken into account.

Rare *LDLR* variants have been analyzed using cumulative association tests: burden test and Sequence Kernel Association Test (SKAT). The analysis on rare variants has been performed on: i) all identified rare variants in *LDLR*; ii) the variants potentially affecting LDLR expression/activity (no synonymous and intronic variants); iii) the variants predicted as potentially damaging by the Combined Annotation Dependent Depletion (CADD) tool (CADD score>20).

The first endpoint (FVIII:C) has also been considered as continuous variable. In this case, the association has been studied by linear regression model and results have been reported as beta coefficient and 95% CI. Moreover, a combined endpoint (FVIII:C and FVIII:C/VWF:Ag ratio) was also defined with the final goal to select *LDLR* variants associated both to high FVIII:C and increased FVIII:C/VWF:Ag ratio (ratio>1), by logistic regression models.

Statistical analyzes have been performed by PLINK and PLINK/SEQ.

4.3.4 Selection of the *LDLR* variants to be characterized

LDLR variants associated with high FVIII:C (≥150 IU/dL) were selected and characterized *in vitro*. However, because of the limited sample size, we selected not only variants associated with a p value<0.05, but also those with an OR>2, regardless of the p value.

Moreover, we also characterized the *LDLR* variants described in literature to be associated with higher FVIII levels and potentially involved in FVIII clearance.

4.3.5 *LDLR* vectors design and production

We used the pcDNA 3.1± vector, containing the promoter of Cytomegalovirus (CMV), multiple cloning sites and the ampicillin resistance gene (Figure 3). Therefore, cells trasformed with this vector become resistant to ampicillin that has been used as a marker of selection.

We downloaded from the Ensemble Genome Browser the wild-type (WT) cDNA sequence of the *LDLR* gene (2583bp), that was cloned, along with an upstream Kozak sequence (GCCACC), into the NheI/NotI cloning sites of the pcDNA3.1+ vector.

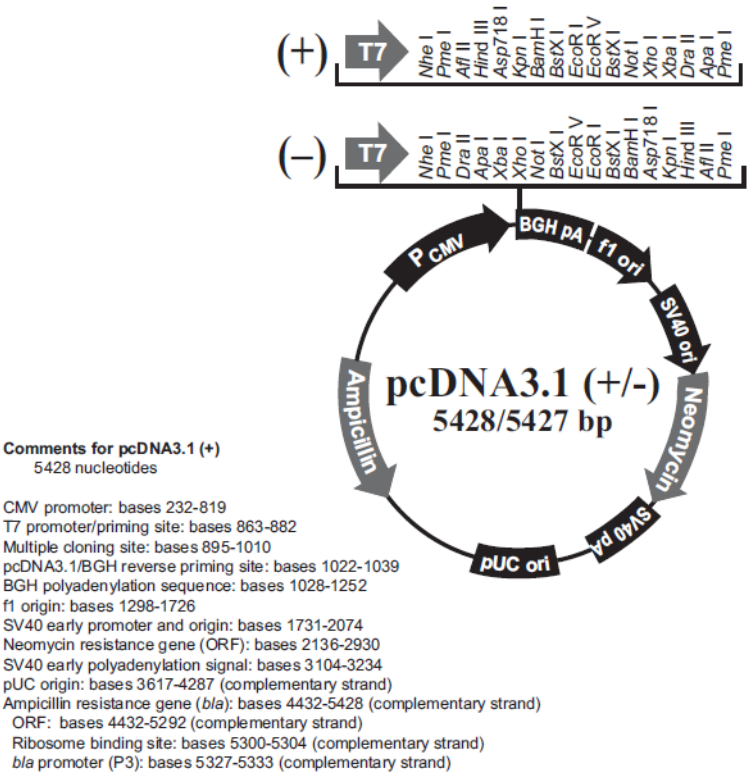


Figure 3
The pcDNA3.1 vector map

The figure summarizes the features of the pcDNA3.1(+) and pcDNA3.1(–) vectors.
The pcDNA3.1(+) (5428 bp) and pcDNA3.1(–) (5427 bp) contain the following elements:
human CMV promoter, T7 promoter/priming site, multiple cloning site in forward or reverse orientation, bovine growth hormone (BGH) polyadenylation signal, f1 origin, SV40 early promoter and origin, neomycin resistance gene, SV40 early polyadenylation signal, pUC origin and ampicillin resistance gene (β-lactamase).

The resulting WT-LDLR vector was used as template to produce mutant vectors carrying each of the identified *LDLR* variants by means of the QuikChange Lightning site-directed mutagenesis kit (Stratagene).

For each mutagenesis, we designed a couple of primers (forward and reverse), based on the following criteria: 25-45 bases in length with the mutation in the middle, a minimum GC content of 40%, one or more C or G bases at the 3', and a melting temperature (T_m)≥78 °C. The T_m, defined as the temperature at which 50% of the primer is annealed to the target, has been calculated by this formula.

$$T_m = 81.5 + 0.41(\%GC) - (675/N) - \%mismatch$$

For calculating T_m:

- N is the primer lenght in bases
- values for %GC and %mismatch are whole numbers.

For each mutagenesis reaction, we prepared a reaction mix, including the WT-LDLR vector (60 ng), the specific couple of mutagenic primers (125 ng each), the QuikChange Lightning enzyme (1Kb/30 sec), without proof reading activity, along with the reaction buffer, the dNTPs and the Quick solution reagent provided with the kit. The mutagenesis reaction program was: initial denaturation step (95°C for 2 minutes), 18 cycles of denaturation (95°C for 2 seconds), annealing (60°C for 10 seconds) and elongation (68°C for 4 minutes), and a final elongation step (68°C for 5 minutes). At the end of the reaction, the WT vector, used as template, was removed by adding the DpnI enzyme, that recognizes the methylated regions. An overview of the site-directed mutagenesis method is shown in Figure 4.

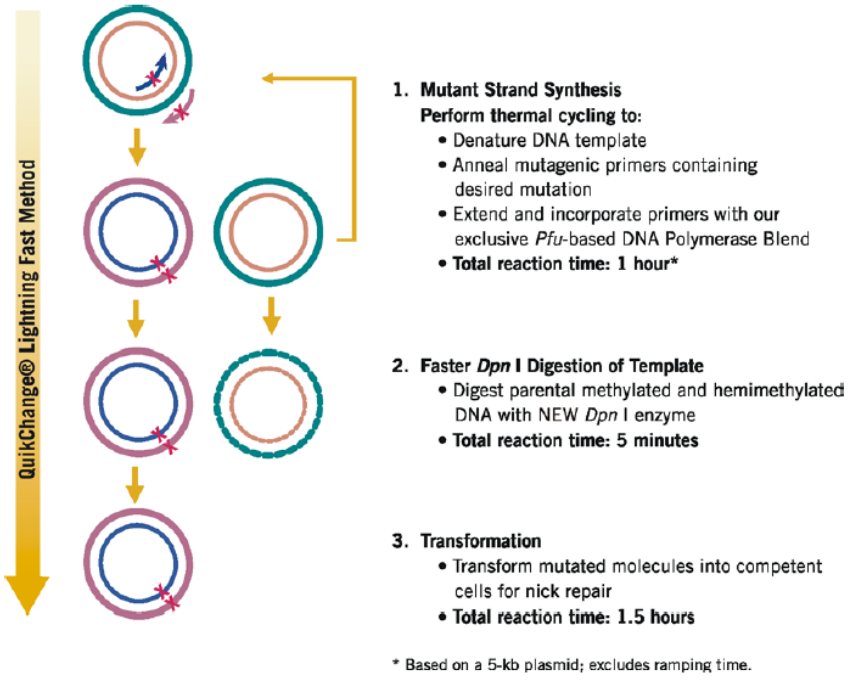


Figure 4
Overview of the site-directed mutagenesis method

The procedure uses a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers with the desired mutation. The oligonucleotide primers are extended during thermal cycling by the DNA polymerase, leading to the generation of a mutated plasmid containing staggered nicks. At the end of the process, the Dpn I, an endonuclease specific for methylated and hemimethylated DNA, digests the DNA template and allows the selection of mutation-containing synthesized DNA. Then, the vector containing the desired mutations can be transformed into XL10-Gold ultracompetent cells.

In order to produce a large amount of each vector (WT and mutant *LDLR*), we transformed XL10-Gold ultracompetent cells (i.e. chemically modified E. Coli strain).

We prepared a Luria Bertani (LB)-AGAR culture medium containing carbenicillin 100 μg/ml, as a marker of selection. Indeed, only trasformed cells became resistant to the antibiotic after transformation. The transformation was performed with the following steps: addition of β-mercaptoetanol (2 μL) to the ultracompetent cells (45 μL), incubation on ice for 2 minutes, prior the addition of the vector (2 ng). After 30 minutes of incubation on ice, the pores of the bacteria membrane were opened by heat shock, i.e. a rapid change of temperature (from ice to 42°C in a water bath for 30 seconds). After 2 minutes of incubation on ice, the transformation was completed by the addition of SOC medium and the bacteria growth with slow agitation at 37°C for one hour.

Bacteria were then seaded on LB-agar medium containing carbenicillin.

After overnight growth, we selected two colonies for each transformation to check the mutagenesis. Small (5 ml) and large (50 ml) bacterial cultures were growth overnight at 37°C to allow small (miniprep) and large (midiprep) vector extraction, respectively.

4.3.6 *LDLR* vectors extraction and check

Miniprep and midiprep plasmid DNAs were isolated by using PureLink™ HiPure Plasmid Kit (Invitrogen). We performed miniprep vector preparation to check by Sanger sequencing the

inserted mutations and midiprep preparation to isolate high-pure and low-endotoxin vectors for mammalian cell transfection.

We performed midiprep preparations according to the procedure in figure 5.

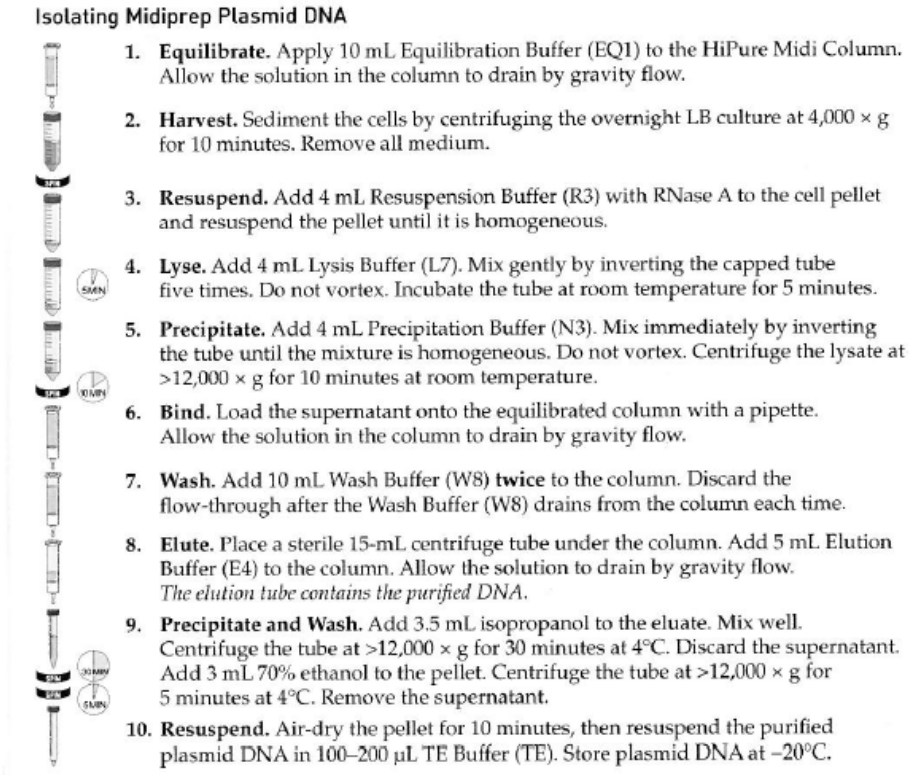


Figure 5
Vector extraction by midiprep procedure

The figure shows the list of 10 steps of the midiprep preparation, which allows the removal of membranes and genomic DNA and the isolation of the purified plasmid.

For each extraction, we performed a qualitative check of the vector by means of agarose gel electrophoresis with the purpose to exclude the presence of genomic DNA, which would have a slower migration than plasmid DNA.

Then, we quantified the DNA by Nanodrop and assessed the degree of DNA purity by measuring two absorbance ratios: 260 nm (DNA)/280 nm (proteins) and 260 nm/230 nm (phenols).

Vectors were directly sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Sequencing steps were: initial denaturation (95ºC for 1 minute) and 25 cycles of denaturation (94ºC for 10 seconds), annealing (50ºC for 5 seconds) and elongation (60ºC for 4 minutes). The sequencing reactions were purified with sephadex (which is able to remove unincorporated fluorescent nucleotides), prior the addition of formamide. Sequences were aligned to the reference *LDLR* sequence by using UGENE and CHROMAS Pro softwares.

4.3.7 Cell transient transfection

We used the LDLR-deficient Chinese hamster ovary (CHO-ldlA7) cell line, kindly provided by Prof. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA, USA). This cell line has been chemically modified and does not express the LDLR protein.

WT and mutant LDLR vectors were independently and transiently transfected in CHO-ldlA7 cells by using Lipofectamine 2000 (Thermo Fisher) transfection reagent with the goal to express the exogenous LDLR proteins.

CHOldlA7 cells were grown in a complete culture medium [HEMS F12, Fetal Bovine Serum (FBS) (10%), glutamine (1%) and antibiotics (1%)]. The day before transfection, cells were counted with the Burker Chamber and 650,000 cells were seeded in 6-wells.

Pilot transfection

At first, we set up the transfection using the WT vector and different concentrations of Lipofectamine 2000. Mock transfected cells (i.e. cells incubated with Lipofectamine without the vector) and no transfected cells (i.e. cells incubated with the culture medium only) were used as negative controls. We prepared the samples, as shown in figure 6. The transfection lasted 5 hours.

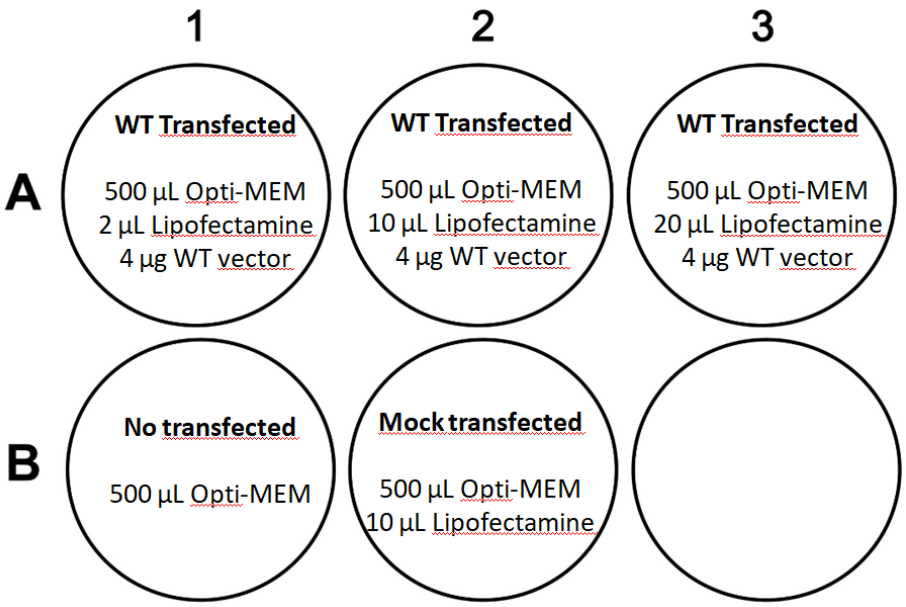


Figure 6
Sample preparation for the pilot test

The figure is a schematic representation of the sample preparation in a 6-well plate.
CHOldlA7 cells were incubated with the wild type (WT) vector, Opti-MEM (a reduced serum medium) and Lipofectamine 2000 at different concentrations (wells A1-3).
As negative controls (wells B1-2) we used: No transfected cells (cells incubated with Opti-MEM only) and Mock transfected cells (cells incubated with Opti-MEM and Lipofectamine 2000, without the vector).

Transfection of WT and mutant LDLR vectors

Based on the results stemming from the pilot test, we performed transfection of WT and mutant *LDLR* vectors using 10 µl of Lipofectamine 2000. Mock transfected cells were used as negative control. Therefore, we prepared the samples, as shown in figure 7. After 5 hours of incubation, the transfection medium was replaced with fresch medium and cells were grown for additional 72 hours. All transfections were performed in triplicate.

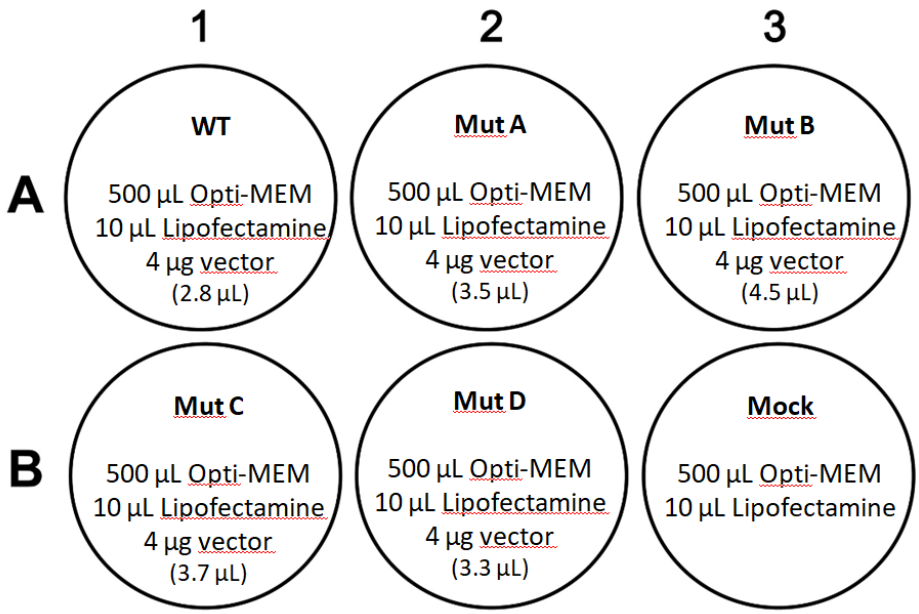


Figure 7
Sample preparation for the transfection of WT and mutant *LDLR* vectors

The figure is a schematic representation of the sample preparation in a 6-well plate.
CHOldlA7 cells were incubated with the specific vector (WT or mutant), Opti-MEM (a reduced serum medium) and Lipofectamine 2000 (wells A1-3 and B1-2).
As negative control (well B3), we used Mock transfected cells (cells incubated with Opti-MEM and Lipofectamine 2000, without the vector).
Mut A, Mut B, Mut C and Mut D are the selected *LDLR* variants (see paragraph "Results").

4.3.8 Western blotting

After 72 hours, we collected the cell lysates: medium was removed and cells were whashed twice with phosphate-buffered saline (PBS) prior the lysis at 4°C for 15 minutes with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1%Nonidet P-40, 0.1%SDS, pH 8) supplemented with phenylmethylsulfonyl fluoride (1%). Cell lysate was centrifuged at 4000 rpm for 5 minutes at 4 °C to remove cell debris. Quantification of protein in cell lysate was performed using the DC protein Assay (Bio-Rad, Hercules, CA, USA). This is a colorimetric method based on a Lowry assay. In order to create a standard curve and a calibration line, we loaded the blank and other 4 standards (bovine serum albumin at different concentrations in lysis buffer+1% PMSF). We prepared

standards and samples (2.5 µL of the specific cellular lysate and 2.5 µL of lysis buffer+1% PMSF). Finally, we added the reagents to allow the colorimetric reaction.

We performed Western Blotting (WB) on total cell lysates, at first as pilot test only for WT to set up the procedure and then for WT and mutant LDLR lysates. Each step is explained in detail below.

Gel preparation

We prepared a 4% stacking gel (30% acrylamide, 0.5 M Tris pH 6.8, 10% SDS, 10% APS and TEMED) and a 8% running gel (30% acrylamide, 1.5 M Tris pH 8.8, 10% SDS, 10% APS and TEMED). We loaded the running gel between the two vertical glasses, we added water on the top to isolate the solution from the air, allowing the gel polymerization (30 minutes). Then we loaded the stacking gel and waited 30 minutes for its polymerization.

We prepared the tubes containing 45 µL of each sample and 15 µL of non-reducing samples buffer (Tris-HCl 0.5 M pH 6.8, glycerol, SDS 10% and bromophenol blue 1% in distilled water).

We boiled the tubes for 5 minutes to denature proteins and then we loaded the samples and the molecular weight marker Precision plus on gels.

Electrophoresis

We prepared the running buffer (Tris 0.025 M and glycine 0.192 M, pH 8.3) and added 10% SDS, useful to provide with negative charges to the linear structure of the protein, allowing the protein migration to the positive pole. We loaded the boiled samples into the assembled electrophoresis chamber. The electrophoretic run was: 100 V (0.07 Amp) for 15 minutes, and 200 V (0.1 Amp) for 45 minutes.

Blotting

We prepared the blotting buffer (running buffer without SDS). On the support, we put in this order: a foam pad; a double filter paper soaked in TB buffer; the gel; the nitrocellulose membrane; a double filter paper soaked in TB buffer; a foam pad. We loaded in the chamber the blotting buffer. Blotting lasted one hour at 100 V (0.27 Amp). During this phase, proteins were transferred from the gel to the membrane.

Blocking

We prepared the blocking solution [TBS pH 7.4 (NaCl 0.02 M, Tris 0.004 M) and milk powder 5%]. Membranes were soaked in the blocking solution for 2 hours at room temperature with gentle agitation. During this phase the saturation of all the potential protein binding sites takes place, preventing from a non-specific antibodies binding.

Immunodetection

We diluted the primary polyclonal antibodies in blocking solution and Tween 20 0.05% as follows: rabbit anti-alpha Tubulin antibody (Invitrogen) (1:2000), rabbit anti-LDLR antibody (ProGene) (1:200). After an overnight incubation of the membranes with the primary antibodies at room temperature in slow shaking, we performed three washes with TTBS, in order to remove the unbound antibodies. Then we added the secondary antibody, an anti-Rabbit IgG HRP-linked from donkey (Amersham) (1:5000) and incubated for 2 hours at room temperature in slow shaking. After other three TTBS washes, we added the opti 4CN staining solution (BioRad) that contains the substrate for the peroxidase enzyme coniugated to the secondary antibody.

We stained the membranes for 30 minutes at room temperature in slow shaking and then we stopped the colorimetric reaction by adding distilled water.

Image acquisition and analysis

Image acquisition was performed with Syngene and the Gene SNAP tool. Image J software was used for the densitometric analysis. The relative expression of each *LDLR* variant has been calculated as the ratio of intensities of LDLR bands compared to tubulin. Then we calculated the ratio between this value (normalized for tubulin) and the WT LDLR density. All WB experiments have been performed in triplicate.

4.3.9 Immunofluorescence

A pilot test has been performed with cells expressing the WT LDLR. Then, immunofluorescence (IF) has been also performed in cells expressing the selected LDLR mutants. The main steps were: sample preparation, staining, image acquisition, data storage, processing and analysis.

Pilot test: sample preparation and staining

CHOldlA7 cells (60,000 cells/well) were seeded in 8-well chamber slides (Ibidi) and transfected the day after. After 72 hours from transfection, cells were fixed with paraformaldehyde 4% for 10 minutes at 4°C and permeabilized with Triton X-100 0.2% for 10 minutes at room temperature (RT). The permeabilization is necessary to allow the detection of both surface and intracellular LDLR protein. Blocking was performed with fetal calf serum 5% and goat serum 2% in PBS for 30 minutes at RT.

For the pilot test, we prepared 1 chamber-slide with these samples: mock transfected cells and WT *LDLR* transfected cells stained with rabbit polyclonal anti-LDLR antibody (ProGene) at two different concentrations (1:20 and 1:50) and with a secondary antibody conjugated with AlexaFluor 647 (1:1000). We included two negative controls: immunolabeling with a pool of rabbit IgGs (instead of primary antibody) as isotype control and with only the secondary antibody. Nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). A scheme of the samples used for the pilot IF is shown in Figure 8.

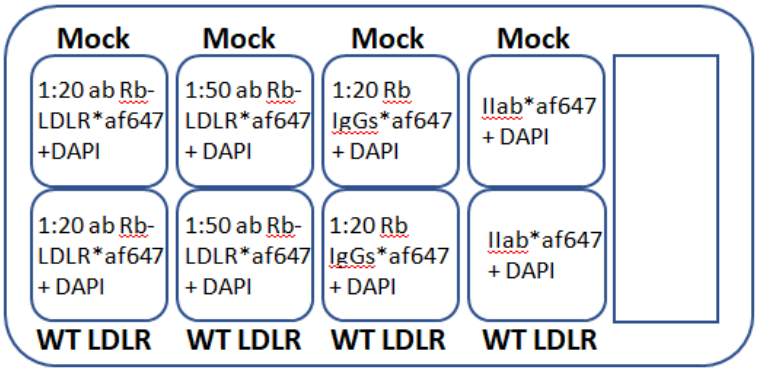


Figure 8
Sample preparation for the pilot IF

We prepared 1 chamber-slide with these samples: mock transfected cells and WT LDLR transfected cells stained with rabbit polyclonal anti-LDLR antibody (ab Rb-LDLR) at two different concentrations (1:20 and 1:50) and with a secondary antibody conjugated with AlexaFluor 647 (*af647). As negative controls we used: immunolabeling with a pool of rabbit IgGs, instead of primary antibody (Rb IgGs) and with only the secondary antibody (IIab*af647). Nuclei were stained by DAPI.

IF performed on cells expressing WT and mutant LDLR proteins: sample preparation and staining

Sample preparation (permeabilization, fixation and blocking) were performed as in the pilot experiment. We prepared the chamber slides with: mock transfected cells, WT and mutant *LDLR* transfected cells. All samples were stained with rabbit polyclonal anti-LDLR antibody (1:50) and with secondary antibody conjugated with AlexaFluor 647 (1:000). We also labeled actin with phalloidin (1:300 in BSA 3%) conjugated with Alexa Fluor 488 with the goal to discriminate between the surface and intracellular LDLR. Two additional mock transfected cells were included: a sample (i.e. the isotype control) stained with a pool of rabbit IgGs (1:50) and the secondary antibody, and a sample stained only with secondary antibody. Nuclei were detected by DAPI. This experiment has been performed in triplicate. A scheme of the samples used is shown in Figure 9.

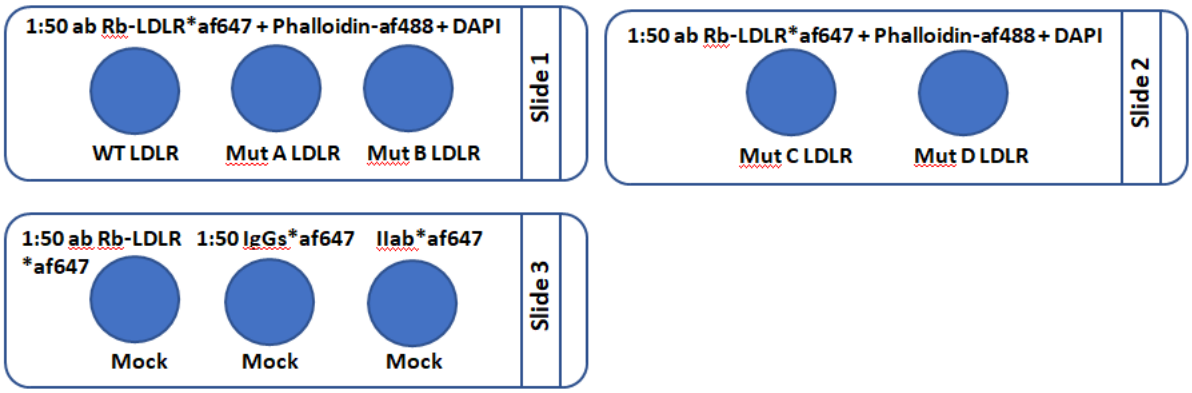


Figure 9
Sample preparation for IF experiments performed on cells expressing WT and mutant LDLR proteins

WT and mutant LDLR transfected cells were stained with rabbit polyclonal anti-LDLR antibody (ab Rb-LDLR) and with a secondary antibody conjugated with AlexaFluor 647 (*af647) (slides 1-2). We labeled actin with phalloidin conjugated with Alexa Fluor 488 (af488).
We used three samples of mock transfected cells (slide 3): stained with rabbit polyclonal anti-LDLR antibody and secondary antibody; stained with a pool of rabbit IgGs and secondary antibody; stained only with secondary antibody. Nuclei were detected by DAPI.
Mut A, Mut B, Mut C and Mut D: selected *LDLR* variants (see the paragraph "Results")

Image acquisition, data storage, processing and analysis

As image acquisition tool, we used a high-resolution spinning disk confocal microscope. Images have been acquired at 4x, 10x and 40x, always by 1 z-plan only (best focal plan) in spinning disk confocal with 180micron pinholes. All images have been processed for sequential background fluorescence suppression as follows: mathematical subtraction of background fluorescence in 647 channel in mock cells positive for the secondary antibody (II-ab*647) only; mathematical subtraction of remaining background fluorescence in 647 channel in mock cells positive for IgG*647; mathematical subtraction of remaining background fluorescence in 647 channel in mock cells positive for ab anti-LDLR*647.

Images were then thresholded, binarized and segmented to quantify the number of cellular objects within each field of vision (FOV) positive for LDLR*647 signal. Moreover, within positive cells, specific combined binary masks were performed to differentiate between high-levels and low-levels of LDLR positivity and, for specific subcellular detections, in order to specify the levels of LDLR

expression in cell surface or cytoplasm, using the GA-intelligent automated analysis module in NIS-Elements v5.30 (Figure 10).

Therefore, the total and the cell surface LDLR expression (fluorescence intensity) have been compared between samples (cells transfected with WT *LDLR* and those transfected with each of the *LDLR* variants of interest), from FOVs acquired from each sample at 10x and 40x, respectively. Non-parametric Kruskal-Wallis test with multiple comparisons have been performed by PRISM-Graphpad. Duplicate biological samples were acquired and analysed simultaneously, using equal setting. All experiments have been performed in triplicate.

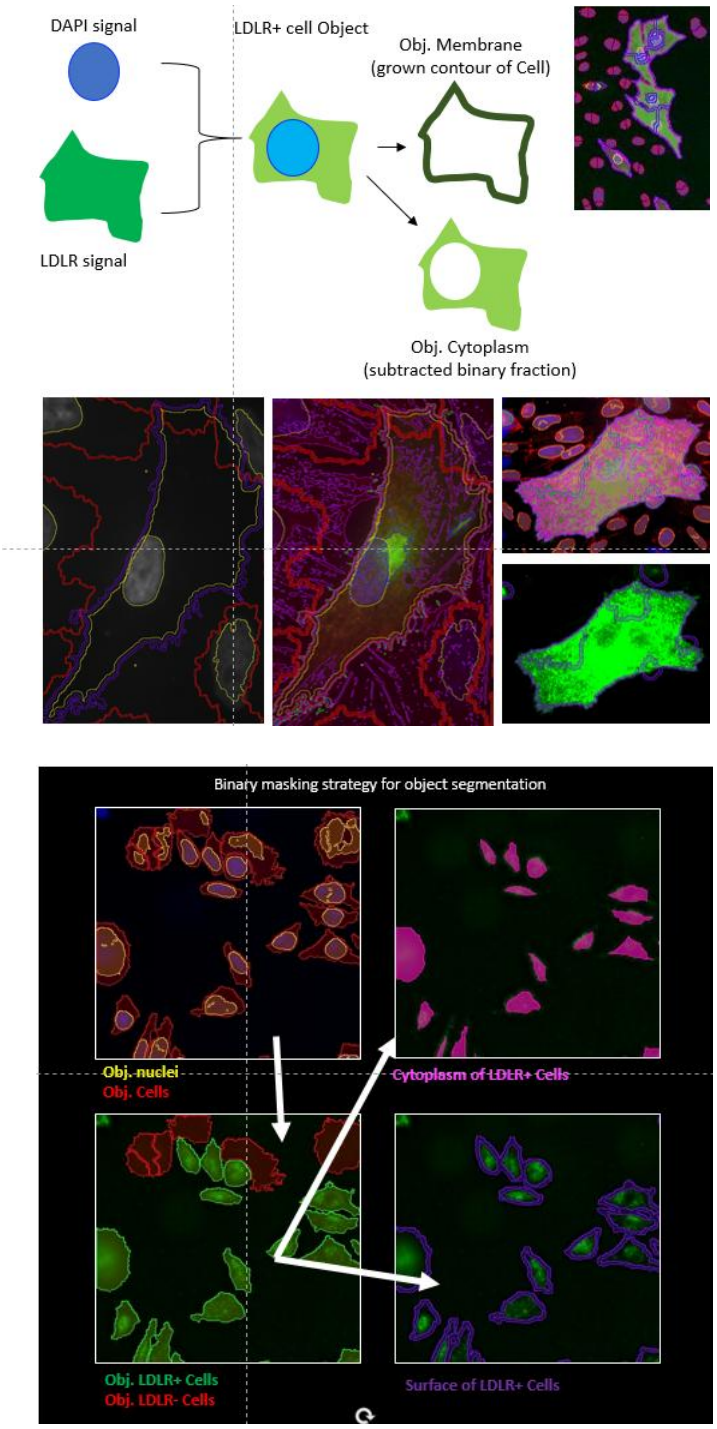


Figure 10
Graphic representation of the digital analysis strategy via sequential channel binarization and object segmentation by binary masking strategy

This method, in an automated way, defines a membrane mask and a cytoplasmic mask and, by means of a cell growing algorithm, is able to discriminate the LDLR signal between surface and cytoplasm.

5. RESULTS

5.1 Main characteristics of the study population

Data on FVIII:C were available on 464/596 subjects (eligible sample).

As expected, in the group of patients with high FVIII:C we observed a higher prevalence of DVT and of no 0 blood group and they were slightly older (Table 1).

Variables	High FVIII:C (≥150 IU/dL) n=111	No High FVIII:C (<150 IU/dL) n=353
DVT, n (%)	91 (82.0%)	159 (45.0%)
Age, median [IQR]	45.8 [34.3-60.7]	40.9 [31.1-52.7]
Male, n (%)	44 (39.6%)	135 (38.2%)
No 0 blood group, n (%) *	47 (83.9%)	136 (56.7%)

Table 1. Descriptive table of eligible sample (n=464)
*available in 296 subjects (56 in "high FVIII:C" group, 240 in "No high FVIII:C" group)

5.2 Allelic association analyses

Because of the limited sample size, we have considered as associated not only the variants with a p value less than 0.05, but also those with an OR more than 2, independently of p value.

Three *LDLR* variants resulted to be associated with high FVIII:C also after adjusting for age and sex in a logistic regression model (Table 2).

***LDLR* variant ID**	**Variant**	**Type of variant**	**OR (95% CI)**	**p**
rs147509697	c.58G>A (p.Gly20Arg)	Missense	2.33 (0.14- 37.97)	0.55
rs72658861	c.1061-8T>A (-)	Intronic	2.53 (0.75- 8.56)	0.14
rs45508991	c.2177C>T (p. Thr726Ile)	Missense	2.44 (0.63-9.40)	0.20

Table 2. *LDLR* variants found to be associated with high FVIII:C (≥150 IU/dL)
Logistic regression model adjusted for age and sex (n=464).

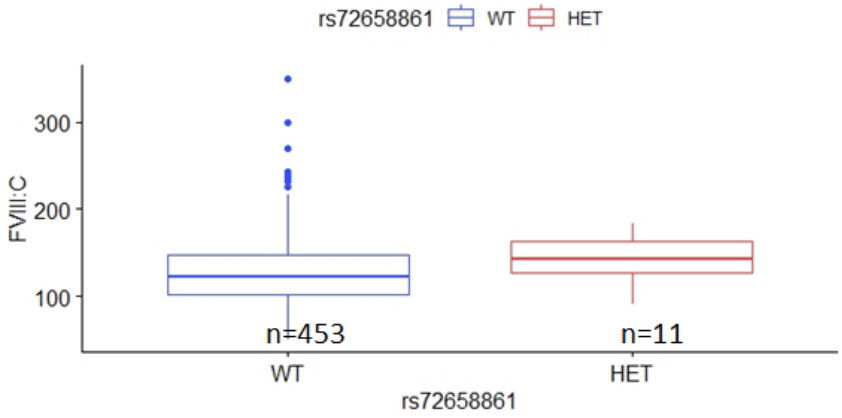
Two of these variants (rs72658861 and rs45508991) were confirmed to be associated with high FVIII:C also after adjusting for blood group (Table 3). These two variants resulted to be associated with FVIII:C also in the subgroup of healthy controls, after adjusting for age and sex (for both OR= 3.00 and p=0.36).

***LDLR* variant ID**	**Variant**	**Type of variant**	**OR (95% CI)**	**p**
rs72658861	c.1061-8T>A (-)	Intronic	5.47 (0.92-32.7)	0.06
rs45508991	c.2177C>T (p. Thr726Ile)	Missense	5.47 (0.92-32.7)	0.06

Table 3. *LDLR* variants found to be associated with high FVIII:C (≥150 IU/dL)
Logistic regression model adjusted for age, sex and ABO blood group (n=296).

The graph 1A shows the median FVIII levels observed in our study population in subjects heterozygous for the rs72658861 variant allele (A) (n=11) compared with subjects homozygous for the WT allele (T) (n=453).

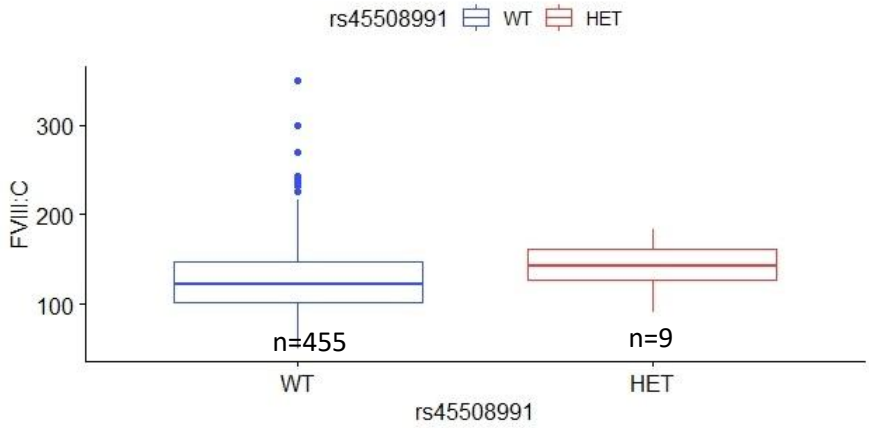
Heterozygous subjects for the rs72658861 variant showed higher median FVIII levels than no carriers of this variant (143 IU/dL and 122 IU/dL, respectively).



Graph 1A
Median FVIII levels in subjects heterozygous for the rs72658861 variant allele and subjects homozygous for the WT allele
WT:wild type; HET:heterozygous subjects.

The graph 1B shows the median FVIII levels observed in our study population in subjects heterozygous for the rs45508991 variant allele (T) (n=9) compared with subjects homozygous for the WT allele (C) (n=455).

Heterozygous subjects for the rs45508991 variant showed higher median FVIII levels than subjects without this variant (143 IU/dL and 122 IU/dL, respectively).



Graph 1B
Median FVIII levels in heterozygous subjects for the rs45508991 variant allele and in homozygous subjects for the WT allele
WT:wild type; HET:heterozygous subjects.

VWF:Ag was available only in 71 patients. Therefore, the combined endpoint (high FVIII:C and increased FVIII:C/VWF:Ag ratio) was not evaluable. However, the association between *LDLR* variants and FVIII:C/VWF:Ag ratio has been considered. By logistic regression model adjusted for age and sex (n=71), the intronic variant rs72658861 and two synonymous variants (rs688 and rs5925) resulted to be associated with an increased ratio (Table 4). The two synonymous variants were confirmed to be associated also after adjusting for ABO blood group (n=41; for both OR=3.58 [1.06-12.07], p=0.04).

LDLR variant ID	Variant	Type of variant	OR (95% CI)	p
rs688	c.1773C>T (p.Asn591=)	Synonymous	3.15 (1.19-8.35)	0.02
rs5925	c.1959T>C (p.Val653=)	Synonymous	3.04 (1.16-7.97)	0.02
rs72658867	c.2140+5G>A (-)	Intronic	6.98 (0.34-145)	0.21

Table 4. *LDLR* variants found to be associated with increased FVIII:C/VWF:Ag ratio (>1)
Logistic regression model adjusted for age and sex (n=71).

By linear regression model, we found two *LDLR* variants statistically significant associated with FVIII:C (Table 5). These two variants (rs750363970 and rs879255217) were confirmed to be associated also adjusting for blood group (n=296, β=69.6 and p=0.04, β=78.4 and p=0.02, respectively). However, these variants were very rare in our population with only one patient carrier in heterozygosity. Therefore, these results stemming from the linear model were interpreted as an effect by chance.

LDLR variant ID	Variant	Type of variant	beta	P
rs750363970	c.1384G>A (p.Val462Ile)	Missense	73.3	0.05
rs879255217	c.2476C>A (p.Pro826Thr)	Missense	81.3	0.03

Table 5. *LDLR* variants found to be associated with higher FVIII:C
Linear regression model adjusted for age and sex (n=464).

For all the 39 identified rare variants in the *LDLR* gene, a burden testing was performed (p=0.10). The same analysis was made also after excluding synonymous and intronic rare variants (n. of selected variants=28, p=0.09) and after selection by CADD score (n. of selected variants=19, p= 0.11).

5.3 Selected *LDLR* variants

We selected 4 *LDLR* variants to be characterized in vitro: 3 synonymous (rs688, rs2228671 and rs5925), previously reported to be associated with higher FVIII levels or increased risk of ischemic

stroke/CAD, and 1 missense variant (rs45508991), which resulted to be associated with high FVIII:C in our population. The intronic variant (rs72658861), associated with high FVIII:C in our cohort, was not selected since the expression vector for *in vitro* characterization contains only the cDNA and do not allow the analysis of intronic variants.

The main characteristics of the selected variants are shown in Table 6.

***LDLR* variant ID**	**Variant**	**Type of variant**	**MAF ***	**Pathogenic ****	**Disease *****
rs2228671	c.81C>T p.Cys27=	Synonymous	0.10	Benign	LDL cholesterol levels
rs688	c.1773C>T p.Asn591=	Synonymous	0.44	benign/likely benign	LDL cholesterol levels
rs5925	c.1959T>C p.Val653=	Synonymous	0.45	benign/likely benign	no diseases
rs45508991	c.2177C>T p. Thr726Ile	Missense	0.007	not reported in ClinVar	no diseases

Table 6. Main characteristics of the *LDLR* variants selected to be expressed *in vitro*
*based on the Europe 1000 Genome; ** based on Clin Var; ***based on GWAS Catalog.

The localization of the 4 selected *LDLR* variants within the protein is shown in Figure 11.

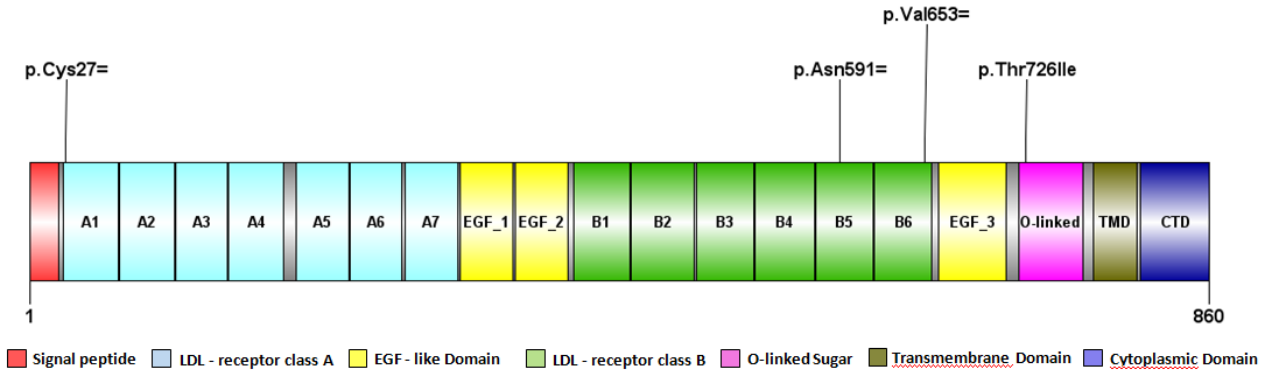


Figure 11
The pre-LDLR protein domains and the localization of the selected variants

The figure shows a schematic representation of the precursor of LDLR protein (pre-LDLR) and the localization of the 4 variants. The protein pre-LDLR (860 amino acids) includes a 21-residues N terminal signal peptide. The mature LDLR protein (the remaining 839 amino acids) is structured in functional domains. The ligand-binding domain (named in the figure LDL-receptor class A) contains 7 cysteine-rich repeats (A1-A7). The EGF precursor homology domain consists of 2 EGF-like

domains, 6 YWTD repeats (named in the figure LDL-receptor class B, B1-B6) and a third EGF-like repeat. The C-terminal domain includes the O-linked oligosaccharides. The other 2 domains are: the transmembrane domain (TMD) and the cytoplasmic domain (CTD).
Protein informations were obtained from UniProt (P01130) and the figure was made using GPS 2.1 (*http://gps.biocuckoo.cn/*).

Therefore, we designed and produced the WT *LDLR* vector and the 4 vectors carrying the selected *LDLR* variants and performed transient transfection as explained in the "Methods" paragraph.

5.4 LDLR expression assessed by western blotting

We performed WB on total cell lysates of WT *LDLR* and of each of the 4 selected *LDLR* variants in triplicate.

A representative result of one WB is shown in Figure 12. A 130 kDa band corresponding to the LDLR protein was found in WT and all mutant samples. As expected, the LDLR band was not found in mock transfected cells that do not express the protein, whereas tubulin was observed in all analyzed samples.

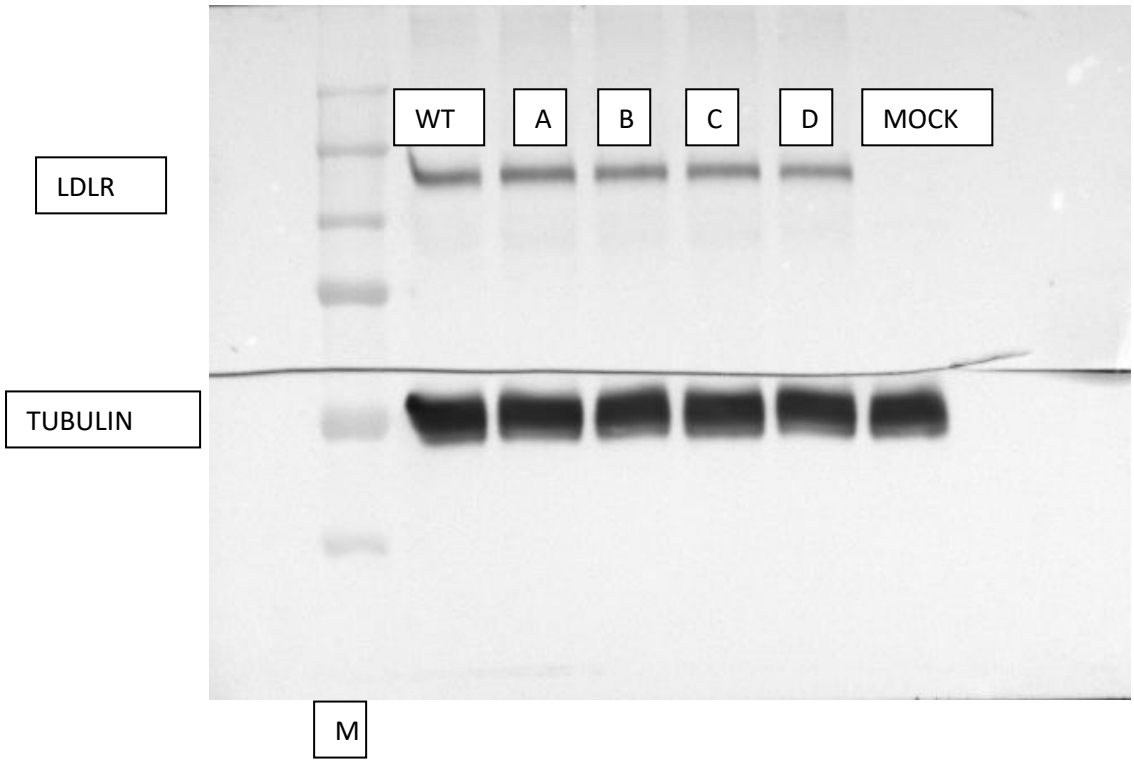


Figure 12

WB performed on total cell lysates from the following samples: cells transfected with WT *LDLR*, cells transfected with *LDLR* variants (A,B,C,D) and mock transfected cells

M: molecular weight marker (precision plus);
WT: wild type *LDLR;* A: rs2228671; B: rs688; C: rs5925; D: rs45508991.

Table 7 shows the results of the three densitometric analyses (1,2,3) performed on total cell lysates of cells transfected with WT and mutant vectors. For each experiment, the LDLR density of each sample was normalized for the corresponding tubulin and then reported as percentage of WT sample (Table 7).

A reduced LDLR expression of about 30% was observed in cells transfected with vectors expressing the rs5925 and rs45508991 variants.

Variant	Normalized*/WT (1)	Normalized*/WT (2)	Normalized*/WT (3)	Mean ± SD
Wild type *LDLR*	1.00	1.00	1.00	1.00 ± 0
rs2228671	0.82	0.99	0.93	0.91 ± 0.09
rs688	0.72	0.94	0.95	0.87 ± 0.13
rs5925	0.58	0.83	0.77	0.73 ± 0.13
rs45508991	0.58	0.76	0.76	0.70 ± 0.10

Table 7. Densitometric analysis of the WB on total cell lysates performed in triplicate
* Normalized LDLR density for tubulin; SD: standard deviation.

5.5 LDLR expression assessed by immunofluorescence

We performed immunofluorescence (IF) to evaluate total, surface and intracellular expression of LDLR proteins. All IF experiments have been performed in triplicate.

Representative field of vision (FOVs) acquired at 10x and 40x are shown in Figures 13A and 13B, respectively. As expected, the LDLR protein (in green) has been observed only in WT and all mutant tranfected cells. LDLR was not found in mock transfected cells, except for a background signal, which was mathematically subtracted. Phalloidin (in red) was observed in all samples, as well as DAPI (in blu).

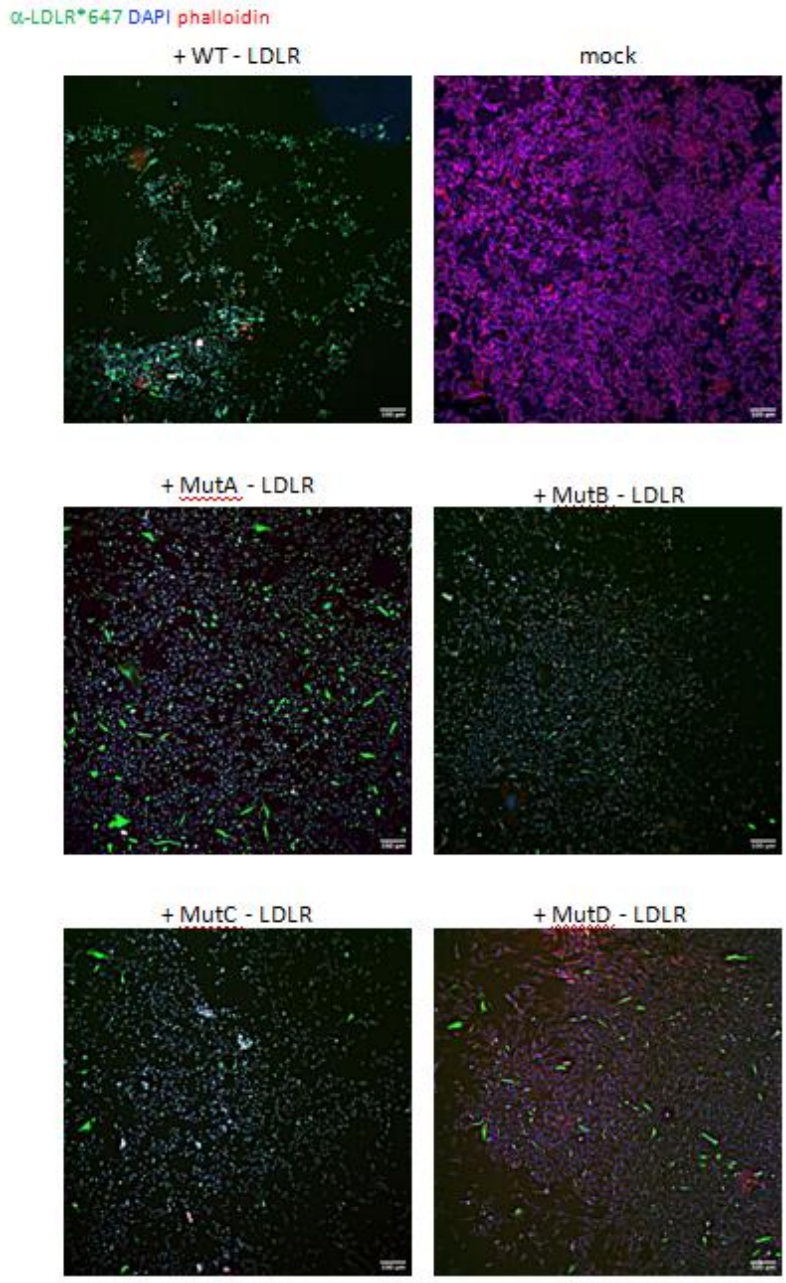


Figure 13A
Images acquired from representative FOVs at 10x

As shown in the figure, the LDLR protein (in green) was observed in WT and all mutant tranfected cells and was not found in mock transfected cells, except for a background signal. Phalloidin (in red) and DAPI (in blu) were observed in all samples.
WT: wild type *LDLR;* MutA: rs2228671; MutB: rs688; MutC: rs5925; MutD: rs45508991.

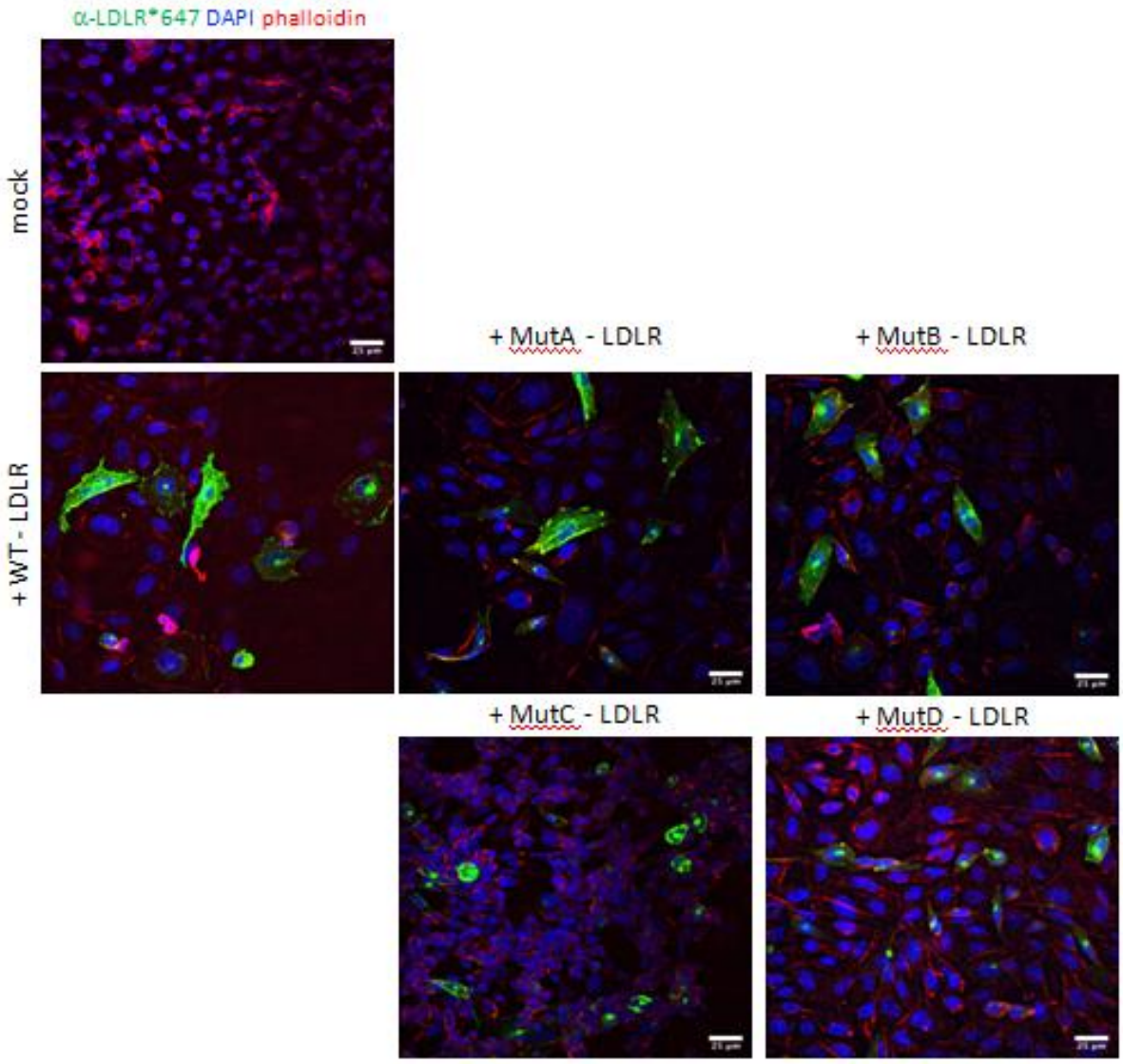


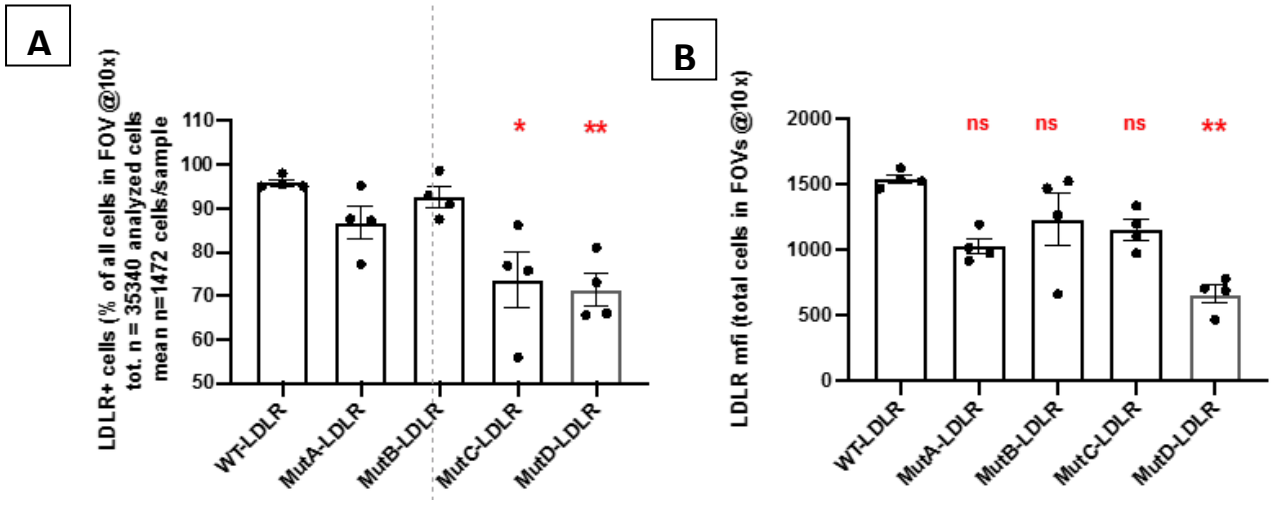
Figure 13B
Images acquired from representative FOVs at 40x

As shown in the figure, the LDLR protein (in green) was observed in WT and all mutant tranfected cells and was not found in mock transfected cells, except for a background signal. Phalloidin (in red) and DAPI (in blu) were observed in all samples.
WT: wild type *LDLR;* MutA: rs2228671; MutB: rs688; MutC: rs5925; MutD: rs45508991.

The results of one of the three IF experiments we performed are shown in graphs 2A-B and 3A-C.

The graphs 2A-B show the proportion of cells stained with the anti-LDLR antibody (2A) and the total LDLR fluorescence intensity (2B) of each analyzed sample.

In this experiment, the proportion of stained cells was significantly reduced in samples expressing rs5925 and rs45508991 variants (graph 2A). However, total LDLR expression (i.e. fluorescence intensity) was significantly reduced only in cells expressing the rs45508991 variant (graph 2B).



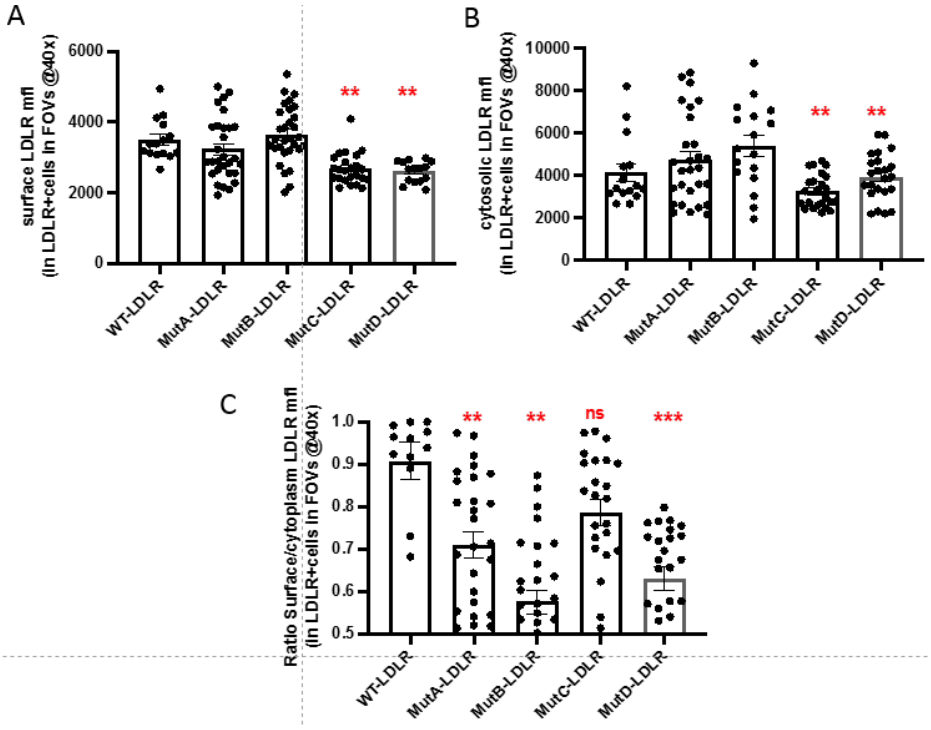
Graphs 2A-B

Graph A shows the percentages of LDLR+ cells in analysed samples at 10x.
Graph B shows the mean fluorescence of LDLR signal from cells in FOVs acquired at 10x, as quantitative measurement of LDLR protein levels (as recognized by immuno-fluorescence technique and acquired at 16-bit).
Duplicate biological samples were acquired simultaneously, using equal settings, and analysed simultaneously with an ad-hoc created segmentation pipeline in NIS-Elements V.5.30.
Statistics was performed via PRISM-GraphPad: non-parametric Kruskal-Wallis test with multiple comparisons; ns: not significant, (*) ≤0.05, (**)≤ 0.01.
WT: wild type *LDLR;* MutA: rs2228671; MutB: rs688; MutC: rs5925; MutD: rs45508991.

The graphs 3A and 3B show respectively the surface and the cytoplasmic LDLR fluorescence intensity in each analysed sample (FOVs acquired at 40x). Graph 3C shows the ratio between the surface and cytosolic LDLR signals.

In this experiment, the LDLR surface and cytosolic expression was significantly reduced in cells transfected with rs5925 and rs45508991 variants. As concern the ratio between surface and

cytoplasm LDLR signal, it was significantly reduced for all variants, except rs5925, although a trend was observed.



Graphs 3A-C

Graphs A and B show the fluorescence intensity of LDLR signal calculated specifically within cellular external membrane mask (A) and the cytoplasmic mask (B) of LDLR+cells from FOVs acquired at 40x for each sample. Data here shown have been corrected for mfi channel signals in surface and cytoplasm mask binaries over mock transfected cells.
Graph C shows the ratio of the LDLR fluorescence intensity between cell surface and cytoplasm. Data have been generated cell-wise (i.e. ratioing every single cell signals) and FOV-wise (i.e. averaging for the number of measured cells/FOVs and showing data from at least n=12 FOVs/sample) in all LDLR+cells.
Duplicate biological samples were acquired simultaneously, using equal settings, and n=2210 cells were analysed simultaneously with an ad-hoc created segmentation pipeline in NIS-Elements V.5.30. Statistics was performed via PRISM-GraphPad: non-parametric Kruskal-Wallis test with multiple comparisons; ns: not significant, (*)≤0.05, (**)≤0.01, (***)≤0.001.
WT: wild type *LDLR;* MutA: rs2228671; MutB: rs688; MutC: rs5925; MutD: rs45508991.

The results of the IF experiments performed in triplicate are reported in Table 8.

In comparison with WT LDLR, we found a total LDLR expression (i.e. fluorescence intensity) reduced of about 20-25% in cells transfected with rs2228671, rs688 and rs5925 variants, and of about 50% in cells transfected with the rs45508991 variant.

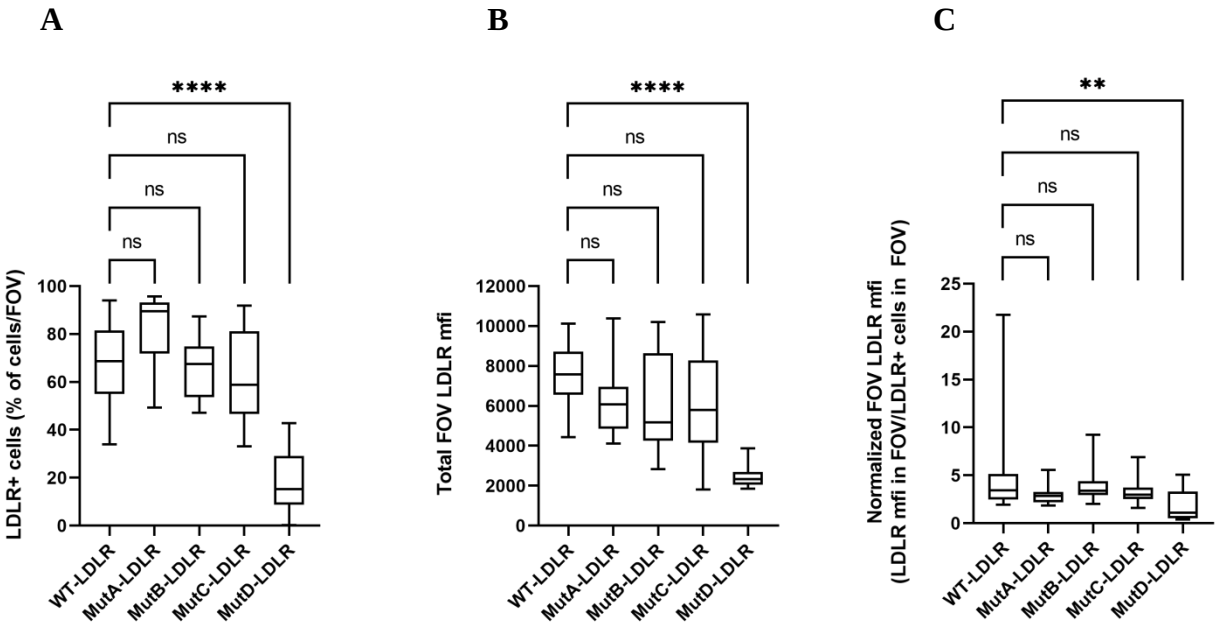
The surface LDLR signal was reduced of about 20% and 30% in cells expressing rs5925 and rs45508991 variants, respectively. As concern the ratio between the surface and the cytosolic LDLR fluorescence intensity, the low data reproducibility limited our findings.

Variant	**Total LDLR mfi** **Mean ± SD**	**Cell surface LDLR mfi** **Mean ± SD**	**Surface/cytoplasm LDLR mfi** **Mean ± SD**
Wild type *LDLR*	1.00 ± 0	1.00 ± 0	1.00 ± 0
rs2228671	0.75 ± 0.08	0.94 ± 0.01	0.67 ± 0.39
rs688	0.73 ± 0.16	0.93 ± 0.13	0.62 ± 0.25
rs5925	0.72 ± 0.14	0.78 ± 0.06	0.81 ± 0.45
rs45508991	0.47 ± 0.22	0.68 ± 0.18	0.78 ± 0.52

Table 8. LDLR fluorescence intensities (total LDLR mfi, surface LDLR mfi and surface/cytoplasm LDLR mfi) normalized for WT.
IF experiments performed in triplicate: mean and standard deviations (SD) are reported.

To overcome the aforementioned low data reproducibility, we also performed a cumulative data analysis excluding the outlier values.

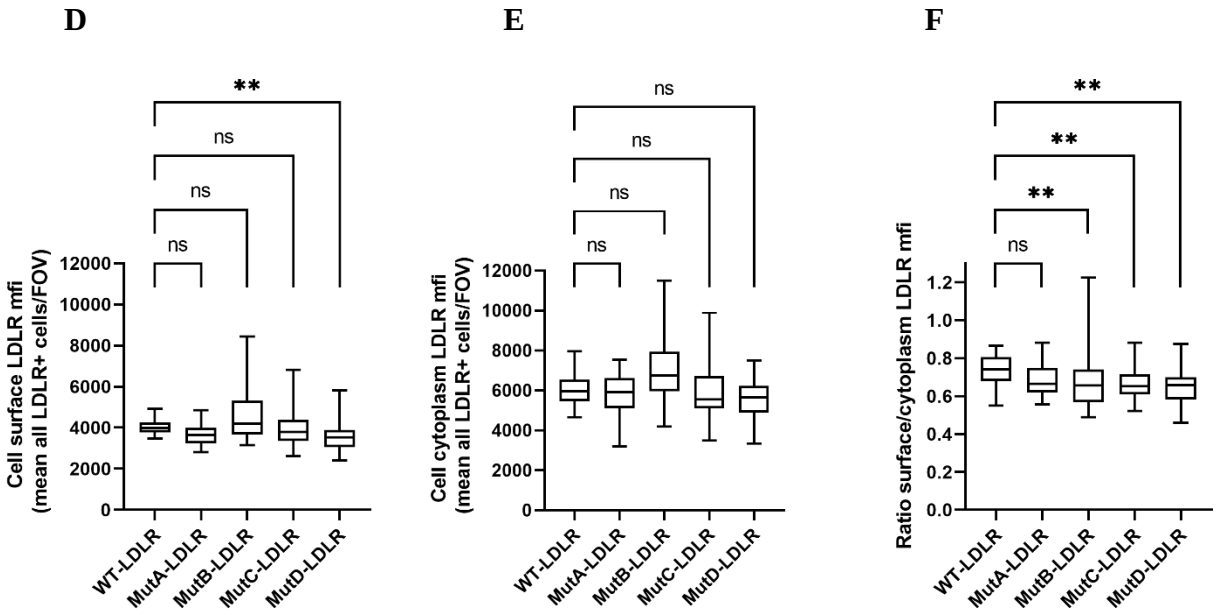
The proportion of cells expressing LDLR (LDLR+ cells) was significantly reduced (about 50%) only in the sample expressing the variant rs45508991 (graph 4A). Consistently, the densitometric analysis of fluorescence showed a significant decrease in total LDLR expression only in the same sample (graph 4B). We also normalized the total LDLR fluorescence signal for the number of LDLR+ cells (graph 4C). Also in this case, the significant reduction of protein expression was confirmed in cells expressing the variant rs45508991.



Graphs 4 A-C

Box-plot chart in A shows the percentages of LDLR+ cells in analysed samples at 10x.
Box-plot chart in B shows the mean fluorescence of LDLR signal from cells in FOVs at 10x, as quantitative measurement of LDLR protein levels (as recognized by immuno-fluorescence technique and acquired at 16-bit).
Box-plot chart in C shows the normalized fluorescence signal of LDLR+ cells (calculated as the mean fluorescence intensity of LDLR in FOV divided by the number of LDLR+ cells in FOV).
All graphs derived from the cumulative data from n=3 independent biological experiments, each with n=2 technical cellular replicates for each sample condition, with a total of n=34 FOVs acquired at 10x magnification for correct statistical sampling and image quantification for cell population analysis. A total of n=400185 cells were analysed with an ad-hoc created segmentation pipeline in NIS-Elements V.5.30, with a mean of 26678 cells/sample condition. Statistical analysis was conducted via PRISM-GraphPad, employing the non parametric Kruskal-Wallis test: not significant (ns); $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).
Outlier values, different for more than 5 times, respect to the mean value of the data-sample were removed from the analysis of each sample condition.
WT: wild type *LDLR;* MutA: rs2228671; MutB: rs688; MutC: rs5925; MutD: rs45508991.

As concern the cellular distribution of LDLR protein, at the surface the expression was significantly reduced only in cells transfected with the variant rs45508991 (graph 4D), whereas the ratio between the surface and cytosolic LDLR expression was significantly reduced for all variants except for rs2228671 (graph 4F).



Graphs 4 D-F

Box-plot charts in D-E refer to sub-cellular compartmentalization and specific evaluation of LDLR mfi on cell surface (D) and in cytoplasm (E). Box-plot chart in F shows the ratio between the two mfi (surface/cytoplasm) as measure of relative predominance of LDLR protein on cell surface or in cytoplasm.

Plotted data in each box-plot are mean per each analysed FOV. All graphs derive from cumulative data from n=3 independent biological experiments, each with n=2 technical cellular replicates for each sample condition, with a total of n=402 FOVs acquired at 40x magnification for correct statistical sampling and image quantification. A total of n=48619 cells were analysed with a mean of 3241 cells/sample condition.

Statistical analysis was conducted via PRISM-GraphPad, employing the non parametric Kruskal-Wallis test: not significant (ns); $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

Outlier values, different for more than 5 times, respect to the mean value of the data-sample were removed from the analysis of each sample condition.

WT: wild type *LDLR;* MutA: rs2228671; MutB: rs688; MutC: rs5925; MutD: rs45508991.

6. DISCUSSION

In a cohort of DVT patients and healthy control subjects we identified the missense *LDLR* variant (rs45508991) associated with high FVIII levels, although this association was not statistically significant, because of the limited sample size. To our knowledge, this association was not previously reported and the variant rs45508991 was not previously functionally characterized.

We have not found the previously reported association of variants rs688 and rs2228671 with high FVIII levels,[37] perhaps because of differences in sample characteristics and variants prevalence between study populations.

Interestingly, in our study, two *LDLR* variants (rs688 and rs5925), previously reported to be associated with high FVIII levels and/or CAD/stroke, were associated with an increased FVIII:C/VWF:Ag ratio, although in a very limited sample (n=71). This increased ratio could be an indicator of a reduced FVIII clearance, although several factors may influence both FVIII and VWF levels and thus the ratio.

Among the 4 *LDLR* variants expressed in vitro, we observed, by both WB and IF, a significant reduced expression of total LDLR protein carrying the rs45508991 variant, compared to the wild type LDLR. Findings about the total expression of LDLR proteins carrying the other three variants were only partly consistent between IF and WB, probably because of the different characteristics of the two laboratory tecniques, partly because of the intrametodic variability which affected both results.

Consistently with a previous study,[76] we found that the variant rs688 altered the receptor intracellular distribution, leading to a reduced ratio between the surface and cytosolic protein expression. However, in our study, the LDLR surface signal was not significantly reduced in cells expressing rs688, although a trend was observed.

Our study has several limitations.

At first, the criterion we used for variants selection. We selected *LDLR* variants associated with high FVIII:C (≥150 IU/dL) in our cohort. However, increased FVIII levels could be not associated with a deficient FVIII clearance. Indeed, FVIII could be increased as a result of higher VWF levels. This is the reason why we wanted to evaluate also the combined endpoint (high FVIII:C and increased FVIII:C/VWF:Ag ratio). Unfortunatelly, VWF levels were available only in 71 patients and thus the association between variants and the combined endpoint was not feasible.

The limited sample size has not allowed us to evaluate other potential associations with rare *LDLR* variants.

In the present study, we performed an allelic association between *LDLR* variants and FVIII:C.

A genotype association was not taken into account. However, it has to be considered that the homozygous genotype for the identified variant (rs45508991) is very rare. Indeed, in our cohort, no homozygous subjects for this variant were identified.

The intramethodic variability for both WB and IF affected our results and limited also the consistency between the two methods.

The low data reproducibility for IF experiments could be explained considering that our cell transfection was transient. Therefore, the different proportion of LDLR expressing cells observed between samples could be caused by differences in the transfection efficiency between samples.

The different proportion of LDLR+ cells between samples could have affected the total LDLR signal we observed. In order to overcome this limit, the mean LDLR fluorescence intensity (mfi) in each FOV has been considered and we normalized the LDLR mfi for the number of LDLR+ cells.

The low reproducibility of some IF findings could be explained also considering the heterogeneity in fluorescence intensity observed within the LDLR+ cells. Indeed, we noticed different cell populations, with a high or a low signal intensity. Therefore, a stratified analysis was performed, but it was biased by the selection of a limited number of FOVs.

Moreover, the use of a polyclonal anti-LDLR antibody in both WB and IF could have affected the specificity in LDLR detection. However, the background signal observed in mock transfected cells by IF has been taken into account by mathematical subtraction.

As concerns our IF results about the surface/cytoplasm LDLR distribution, we had to analyze only the FOVs acquired at 40x. Therefore, for these results, a selection bias has to be considered, as well as a sample size reduction, leading to a reduced data reproducibility.

Moreover, the transient transfection mimics a non-physiological situation. Indeed, it may induce cells to produce a large amount of the protein in a non physiological way. Therefore, the large amount of LDLR may alter its intracellular physiological distribution.

Last, but not least, it has to be considered that our study models (WB and IF) are only quantitative assessments of LDLR protein expression and do not allow us to evaluate qualitative or functional features of the protein, as well as the causing mechanism leading to the reduced expression and the class type of mutation. Three of the 4 selected variants are synonymous variants and thus they induce codon substitutions altering the mRNA coding sequence but they preserve the encoded amninoacid sequence. Synonymous variants have been traditionally considered to be phenotypically silent. However, recent studies have showed that several mechanisms may be induced by synonymous variants, such as alternative splicing leading to non-sense mediated decay of trascripts, alterations of the translational accuracy and secretion efficiency, as well as post-translational modifications. Among the most fascinating mechanisms in which synonymous variants may be involved, the perturbations in the cotraslational folding mechanism has to be mentioned, able to impair the elongation rate of the protein, as showed for rs688.[76] Indeed, synonymous variants may induce shift from frequent to rare codons, which are translated more slowly, requiring more time to adopt a stable tertiary structure.[110] Consistenly, a direct proportionality between the frequency of codon usage and the amount of cognate tRNA has been observed.[76] The resulting alteration of the protein conformation may induce also a change in its intracellular distribution.

7. CONCLUSIONS AND PERSPECTIVES

Our hypothesis is that a reduced expression of LDLR, total and on cell surface, caused by the identified variant rs45508991, could impair FVIII binding and clearance, leading to an increase in FVIII levels.

In order to test this hypothesis, at first we want to replicate the IF findings regarding both the total LDLR expression and surface/cytoplasm distribution for a more complete and accurate results interpretation, also considering the aforementioned limits.

Then, we will perform the fluorescence-activated cell sorting (FACS) in order to confirm IF findings concerning the cell surface LDLR expression in all samples, and to evaluate the LDLR-mediated binding of FVIII. In particular, to determine LDLR cell surface expression by FACS, transfected CHO-ldlA7 cells will be incubated with an anti-LDLR primary antibody and then with Alexa Fluor 488-conjugated secondary antibody. To determine FVIII binding, transfected CHO-ldlA7 cells will be incubated with fluorescein isothiocyanate (FITC)-FVIII. Fluorescence intensities will be measured and results of each LDLR mutant will be expressed as relative fluorescence compared with the wild type LDLR.

In order to understand the causing mechanism leading to the reduced LDLR expression that we observed with the variant rs45508991, transcription analyses could be taken into account, as well as colocalization studies by IF or confocal laser scanning microscopy (CLSM). These studies could allow us to co-localize the protein with ER and lysosomes by means of specific antibodies.

Moreover, within this research line, it would be very interesting to understand the effect of the identified *LDLR* variant rs45508991 on the LDLR-mediated FVIII uptake by IF.

8. REFERENCES

1. Benito-Vicente A, Uribe KB, Jebari S, Galicia-Garcia U, Ostolaza H, Martin C. Validation of LDLr Activity as a Tool to Improve Genetic Diagnosis of Familial Hypercholesterolemia: A Retrospective on Functional Characterization of LDLr Variants. Int J Mol Sci. 2018 Jun 5;19(6):1676. doi: 10.3390/ijms19061676.

2. Authors/Task Force Members; ESC Committee for Practice Guidelines (CPG); ESC National Cardiac Societies. 2019 ESC/EAS guidelines for the management of dyslipidaemias: Lipid modification to reduce cardiovascular risk. Atherosclerosis. 2019 Nov;290:140-205. doi: 10.1016/j.atherosclerosis.2019.08.014.

3. Dawson PA, Hofmann SL, van der Westhuyzen DR, Südhof TC, Brown MS, Goldstein JL. Sterol-dependent repression of low density lipoprotein receptor promoter mediated by 16-base pair sequence adjacent to binding site for transcription factor Sp1. J Biol Chem. 1988 Mar 5;263(7):3372-9. PMID: 3277969.

4. Zhang DW, Garuti R, Tang WJ, Cohen JC, Hobbs HH. Structural requirements for PCSK9-mediated degradation of the low-density lipoprotein receptor. Proc Natl Acad Sci U S A. 2008 Sep 2;105(35):13045-50. doi: 10.1073/pnas.0806312105.

5. Ambros V. The functions of animal microRNAs. Nature. 2004 Sep 16;431(7006):350-5. doi: 10.1038/nature02871.

6. Goedeke L, Rotllan N, Canfrán-Duque A, Aranda JF, Ramírez CM, Araldi E, Lin CS, Anderson NN, Wagschal A, de Cabo R, Horton JD, Lasunción MA, Näär AM, Suárez Y, Fernández-Hernando C. MicroRNA-148a regulates LDL receptor and ABCA1 expression to control circulating lipoprotein levels. Nat Med. 2015 Nov;21(11):1280-9. doi: 10.1038/nm.3949.

7. Lee S, Akioyamen LE, Aljenedil S, Rivière JB, Ruel I, Genest J. Genetic testing for familial hypercholesterolemia: Impact on diagnosis, treatment and cardiovascular risk. Eur J Prev Cardiol. 2019 Aug;26(12):1262-1270. doi: 10.1177/2047487319829746.

8. Wald DS, Bestwick JP, Morris JK, Whyte K, Jenkins L, Wald NJ. Child-Parent Familial Hypercholesterolemia Screening in Primary Care. N Engl J Med. 2016 Oct 27;375(17):1628-1637. doi: 10.1056/NEJMoa1602777.

9. Ference BA, Ginsberg HN, Graham I, Ray KK, Packard CJ, Bruckert E, Hegele RA, Krauss RM, Raal FJ, Schunkert H, Watts GF, Borén J, Fazio S, Horton JD, Masana L, Nicholls SJ, Nordestgaard BG, van de Sluis B, Taskinen MR, Tokgözoglu L, Landmesser U, Laufs U, Wiklund O, Stock JK, Chapman MJ, Catapano AL. Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. Eur Heart J. 2017 Aug 21;38(32):2459-2472. doi: 10.1093/eurheartj/ehx144.

10. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R, Simes R; Cholesterol Treatment Trialists' (CTT) Collaborators. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet. 2005 Oct 8;366(9493):1267-78. doi: 10.1016/S0140-6736(05)67394-1.

11. Ference BA, Yoo W, Alesh I, Mahajan N, Mirowska KK, Mewada A, Kahn J, Afonso L, Williams KA Sr, Flack JM. Effect of long-term exposure to lower low-density lipoprotein cholesterol beginning early in life on the risk of coronary heart disease: a Mendelian randomization analysis. J Am Coll Cardiol. 2012 Dec 25;60(25):2631-9. doi: 10.1016/j.jacc.2012.09.017.

12. Cohen JC, Boerwinkle E, Mosley TH Jr, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med. 2006 Mar 23;354(12):1264-72. doi: 10.1056/NEJMoa054013.

13. Visseren FLJ, Mach F, Smulders YM, Carballo D, Koskinas KC, Bäck M, Benetos A, Biffi A, Boavida JM, Capodanno D, Cosyns B, Crawford C, Davos CH, Desormais I, Di Angelantonio E, Franco OH, Halvorsen S, Hobbs FDR, Hollander M, Jankowska EA, Michal M, Sacco S, Sattar N, Tokgozoglu L, Tonstad S, Tsioufis KP, van Dis I, van Gelder IC, Wanner C, Williams B; ESC

Scientific Document Group. 2021 ESC Guidelines on cardiovascular disease prevention in clinical practice. Eur J Prev Cardiol. 2021 Sep 24:zwab154. doi: 10.1093/eurjpc/zwab154.

14. Paciullo F, Momi S, Gresele P. PCSK9 in Haemostasis and Thrombosis: Possible Pleiotropic Effects of PCSK9 Inhibitors in Cardiovascular Prevention. Thromb Haemost. 2019 Mar;119(3):359-367. doi: 10.1055/s-0038-1676863.

15. Obermayer G, Afonyushkin T, Binder CJ. Oxidized low-density lipoprotein in inflammation-driven thrombosis. J Thromb Haemost. 2018 Mar;16(3):418-428. doi: 10.1111/jth.13925.

16. Chen K, Febbraio M, Li W, Silverstein RL. A specific CD36-dependent signaling pathway is required for platelet activation by oxidized low-density lipoprotein. Circ Res. 2008 Jun 20;102(12):1512-9. doi: 10.1161/CIRCRESAHA.108.172064.

17. Hofmann A, Brunssen C, Morawietz H. Contribution of lectin-like oxidized low-density lipoprotein receptor-1 and LOX-1 modulating compounds to vascular diseases. Vascul Pharmacol. 2017 Oct 19:S1537-1891(17)30171-4. doi: 10.1016/j.vph.2017.10.002.

18. Carnevale R, Bartimoccia S, Nocella C, Di Santo S, Loffredo L, Illuminati G, Lombardi E, Boz V, Del Ben M, De Marco L, Pignatelli P, Violi F. LDL oxidation by platelets propagates platelet activation via an oxidative stress-mediated mechanism. Atherosclerosis. 2014 Nov;237(1):108-16. doi: 10.1016/j.atherosclerosis.2014.08.041.

19. Chung DW, Chen J, Ling M, Fu X, Blevins T, Parsons S, Le J, Harris J, Martin TR, Konkle BA, Zheng Y, López JA. High-density lipoprotein modulates thrombosis by preventing von Willebrand factor self-association and subsequent platelet adhesion. Blood. 2016 Feb 4;127(5):637-45. doi: 10.1182/blood-2014-09-599530.

20. Romagnuolo R, Scipione CA, Bazzi ZA, Boffa MB, Koschinsky ML. Inhibition of pericellular plasminogen activation by apolipoprotein(a): Roles of urokinase plasminogen activator receptor and integrins $\alpha_M\beta_2$ and $\alpha_V\beta_3$. Atherosclerosis. 2018 Aug;275:11-21. doi: 10.1016/j.atherosclerosis.2018.05.029.

21. Hancock MA, Boffa MB, Marcovina SM, Nesheim ME, Koschinsky ML. Inhibition of plasminogen activation by lipoprotein(a): critical domains in apolipoprotein(a) and mechanism of inhibition on fibrin and degraded fibrin surfaces. J Biol Chem. 2003 Jun 27;278(26):23260-9. doi: 10.1074/jbc.M302780200.

22. Galema-Boers AM, Lenzen MJ, Engelkes SR, Sijbrands EJ, Roeters van Lennep JE. Cardiovascular risk in patients with familial hypercholesterolemia using optimal lipid-lowering therapy. J Clin Lipidol. 2018 Mar-Apr;12(2):409-416. doi: 10.1016/j.jacl.2017.12.014.

23. Versmissen J, Oosterveer DM, Yazdanpanah M, Defesche JC, Basart DC, Liem AH, Heeringa J, Witteman JC, Lansberg PJ, Kastelein JJ, Sijbrands EJ. Efficacy of statins in familial hypercholesterolaemia: a long term cohort study. BMJ. 2008 Nov 11;337:a2423. doi: 10.1136/bmj.a2423.

24. Besseling J, Kindt I, Hof M, Kastelein JJ, Hutten BA, Hovingh GK. Severe heterozygous familial hypercholesterolemia and risk for cardiovascular disease: a study of a cohort of 14,000 mutation carriers. Atherosclerosis. 2014 Mar;233(1):219-23. doi: 10.1016/j.atherosclerosis.2013.12.020.

25. Kolmos M, Christoffersen L, Kruuse C. Recurrent Ischemic Stroke - A Systematic Review and Meta-Analysis. J Stroke Cerebrovasc Dis. 2021 Aug;30(8):105935. doi: 10.1016/j.jstrokecerebrovasdis.2021.105935.

26. Boulanger M, Béjot Y, Rothwell PM, Touzé E. Long-Term Risk of Myocardial Infarction Compared to Recurrent Stroke After Transient Ischemic Attack and Ischemic Stroke: Systematic Review and Meta-Analysis. J Am Heart Assoc. 2018 Jan 18;7(2):e007267. doi: 10.1161/JAHA.117.007267.

27. Nakatani D, Sakata Y, Suna S, Usami M, Matsumoto S, Shimizu M, Sumitsuji S, Kawano S, Ueda Y, Hamasaki T, Sato H, Nanto S, Hori M, Komuro I; Osaka Acute Coronary Insufficiency Study (OACIS) Investigators. Incidence, predictors, and subsequent mortality risk of recurrent

myocardial infarction in patients following discharge for acute myocardial infarction. Circ J. 2013;77(2):439-46. doi: 10.1253/circj.cj-11-1059.

28. Kalinin RE, Suchkov IA, Mzhavanadze ND, Zhurina ON, Klimentova EA, Povarov VO. Coagulation Factor Activity and Hemostatic Markers of Endothelial Dysfunction in Patients with Peripheral Arterial Disease. Vasc Specialist Int. 2021 Aug 5;37:26. doi: 10.5758/vsi.210010.

29. Kamphuisen PW, ten Cate H. Cardiovascular risk in patients with hemophilia. Blood. 2014 Feb 27;123(9):1297-301. doi: 10.1182/blood-2013-11-453159.

30. Biere-Rafi S, Zwiers M, Peters M, van der Meer J, Rosendaal FR, Büller HR, Kamphuisen PW. The effect of haemophilia and von Willebrand disease on arterial thrombosis: a systematic review. Neth J Med. 2010 May;68(5):207-14. PMID: 20508269.

31. Koster T, Blann AD, Briët E, Vandenbroucke JP, Rosendaal FR. Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. Lancet. 1995 Jan 21;345(8943):152-5. doi: 10.1016/s0140-6736(95)90166-3.

32. Kyrle PA, Minar E, Hirschl M, Bialonczyk C, Stain M, Schneider B, Weltermann A, Speiser W, Lechner K, Eichinger S. High plasma levels of factor VIII and the risk of recurrent venous thromboembolism. N Engl J Med. 2000 Aug 17;343(7):457-62. doi: 10.1056/NEJM200008173430702.

33. Mulder R, van Schouwenburg IM, Mahmoodi BK, Veeger NJ, Mulder AB, Middeldorp S, Kluin-Nelemans HC, Lijfering WM. Associations between high factor VIII and low free protein S levels with traditional arterial thrombotic risk factors and their risk on arterial thrombosis: results from a retrospective family cohort study. Thromb Res. 2010 Oct;126(4):e249-54. doi: 10.1016/j.thromres.2010.07.013.

34. Tanis B, Algra A, van der Graaf Y, Helmerhorst F, Rosendaal F. Procoagulant factors and the risk of myocardial infarction in young women. Eur J Haematol. 2006 Jul;77(1):67-73. doi: 10.1111/j.1600-0609.2006.00656.x.

35. Meade TW, Cooper JA, Stirling Y, Howarth DJ, Ruddock V, Miller GJ. Factor VIII, ABO blood group and the incidence of ischaemic heart disease. Br J Haematol. 1994 Nov;88(3):601-7. doi: 10.1111/j.1365-2141.1994.tb05079.x.

36. Folsom AR, Rosamond WD, Shahar E, Cooper LS, Aleksic N, Nieto FJ, Rasmussen ML, Wu KK. Prospective study of markers of hemostatic function with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. Circulation. 1999 Aug 17;100(7):736-42. doi: 10.1161/01.cir.100.7.736.

37. Martinelli N, Girelli D, Lunghi B, Pinotti M, Marchetti G, Malerba G, Pignatti PF, Corrocher R, Olivieri O, Bernardi F. Polymorphisms at LDLR locus may be associated with coronary artery disease through modulation of coagulation factor VIII activity and independently from lipid profile. Blood. 2010 Dec 16;116(25):5688-97. doi: 10.1182/blood-2010-03-277079.

38. Chang TR, Albright KC, Boehme AK, Dorsey A, Sartor EA, Kruse-Jarres R, Leissinger C, Martin-Schild S. Factor VIII in the setting of acute ischemic stroke among patients with suspected hypercoagulable state. Clin Appl Thromb Hemost. 2014 Mar;20(2):124-8. doi: 10.1177/1076029613488936.

39. Gouse BM, Boehme AK, Monlezun DJ, Siegler JE, George AJ, Brag K, Albright KC, Beasley TM, Leissinger C, El Khoury R, Martin-Schild S. New Thrombotic Events in Ischemic Stroke Patients with Elevated Factor VIII. Thrombosis. 2014;2014:302861. doi: 10.1155/2014/302861.

40. Ananyeva NM, Kouiavskaia DV, Shima M, Saenko EL. Intrinsic pathway of blood coagulation contributes to thrombogenicity of atherosclerotic plaque. Blood. 2002 Jun 15;99(12):4475-85. doi: 10.1182/blood-2001-11-0140.

41. Wilcox JN, Noguchi S, Casanova JR, Rasmussen ME. Extrahepatic synthesis of FVII in human atheroma and smooth muscle cells in vitro. Ann N Y Acad Sci. 2001 Dec;947:433-8. doi: 10.1111/j.1749-6632.2001.tb03980.x.

42. Konings J, Govers-Riemslag JW, Philippou H, Mutch NJ, Borissoff JI, Allan P, Mohan S, Tans G, Ten Cate H, Ariëns RA. Factor XIIa regulates the structure of the fibrin clot independently of

thrombin generation through direct interaction with fibrin. Blood. 2011 Oct 6;118(14):3942-51. doi: 10.1182/blood-2011-03-339572.

43. Borissoff JI, Heeneman S, Kilinç E, Kassák P, Van Oerle R, Winckers K, Govers-Riemslag JW, Hamulyák K, Hackeng TM, Daemen MJ, ten Cate H, Spronk HM. Early atherosclerosis exhibits an enhanced procoagulant state. Circulation. 2010 Aug 24;122(8):821-30. doi: 10.1161/CIRCULATIONAHA.109.907121.

44. Kalz J, ten Cate H, Spronk HM. Thrombin generation and atherosclerosis. J Thromb Thrombolysis. 2014 Jan;37(1):45-55. doi: 10.1007/s11239-013-1026-5.

45. Borissoff JI, Joosen IA, Versteylen MO, Spronk HM, ten Cate H, Hofstra L. Accelerated in vivo thrombin formation independently predicts the presence and severity of CT angiographic coronary atherosclerosis. JACC Cardiovasc Imaging. 2012 Dec;5(12):1201-10. doi: 10.1016/j.jcmg.2012.01.023.

46. Khallou-Laschet J, Caligiuri G, Tupin E, Gaston AT, Poirier B, Groyer E, Urbain D, Maisnier-Patin S, Sarkar R, Kaveri SV, Lacroix-Desmazes S, Nicoletti A. Role of the intrinsic coagulation pathway in atherogenesis assessed in hemophilic apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol. 2005 Aug;25(8):e123-6. doi: 10.1161/01.ATV.0000171995.22284.9a.

47. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. J Thromb Haemost. 2005 Aug;3(8):1800-14. doi: 10.1111/j.1538-7836.2005.01377.x.

48. Nelken NA, Soifer SJ, O'Keefe J, Vu TK, Charo IF, Coughlin SR. Thrombin receptor expression in normal and atherosclerotic human arteries. J Clin Invest. 1992 Oct;90(4):1614-21. doi: 10.1172/JCI116031.

49. Colotta F, Sciacca FL, Sironi M, Luini W, Rabiet MJ, Mantovani A. Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. Am J Pathol. 1994 May;144(5):975-85. PMID: 8178946; PMCID: PMC1887349.

50. Minami T, Sugiyama A, Wu SQ, Abid R, Kodama T, Aird WC. Thrombin and phenotypic modulation of the endothelium. Arterioscler Thromb Vasc Biol. 2004 Jan;24(1):41-53. doi: 10.1161/01.ATV.0000099880.09014.7D.

51. Naldini A, Carney DH, Pucci A, Pasquali A, Carraro F. Thrombin regulates the expression of proangiogenic cytokines via proteolytic activation of protease-activated receptor-1. Gen Pharmacol. 2000 Nov;35(5):255-9. doi: 10.1016/s0306-3623(01)00113-6.

52. Shpacovitch VM, Brzoska T, Buddenkotte J, Stroh C, Sommerhoff CP, Ansel JC, Schulze-Osthoff K, Bunnett NW, Luger TA, Steinhoff M. Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor kappaB in human dermal microvascular endothelial cells. J Invest Dermatol. 2002 Feb;118(2):380-5. doi: 10.1046/j.0022-202x.2001.01658.x.

53. Dabbagh K, Laurent GJ, McAnulty RJ, Chambers RC. Thrombin stimulates smooth muscle cell procollagen synthesis and mRNA levels via a PAR-1 mediated mechanism. Thromb Haemost. 1998 Feb;79(2):405-9. PMID: 9493599.

54. Souto JC, Almasy L, Borrell M, Garí M, Martínez E, Mateo J, Stone WH, Blangero J, Fontcuberta J. Genetic determinants of hemostasis phenotypes in Spanish families. Circulation. 2000 Apr 4;101(13):1546-51. doi: 10.1161/01.cir.101.13.1546.

55. de Lange M, Snieder H, Ariëns RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. Lancet. 2001 Jan 13;357(9250):101-5. doi: 10.1016/S0140-6736(00)03541-8.

56. Scanavini D, Legnani C, Lunghi B, Mingozzi F, Palareti G, Bernardi F. The factor VIII D1241E polymorphism is associated with decreased factor VIII activity and not with activated protein C resistance levels. Thromb Haemost. 2005 Mar;93(3):453-6. doi: 10.1160/TH04-09-0629.

57. Nossent AY, Eikenboom JC, Vos HL, Bakker E, Tanis BC, Doggen CJ, Bertina RM, Rosendaal FR. Haplotypes encoding the factor VIII 1241 Glu variation, factor VIII levels and the risk of venous thrombosis. Thromb Haemost. 2006 Jun;95(6):942-8. doi: 10.1160/TH06-01-0024.

58. Viel KR, Machiah DK, Warren DM, Khachidze M, Buil A, Fernstrom K, Souto JC, Peralta JM, Smith T, Blangero J, Porter S, Warren ST, Fontcuberta J, Soria JM, Flanders WD, Almasy L, Howard TE. A sequence variation scan of the coagulation factor VIII (FVIII) structural gene and associations with plasma FVIII activity levels. Blood. 2007 May 1;109(9):3713-24. doi: 10.1182/blood-2006-06-026104.

59. Lenting PJ, VAN Schooten CJ, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. J Thromb Haemost. 2007 Jul;5(7):1353-60. doi: 10.1111/j.1538-7836.2007.02572.x.

60. Matsui T, Titani K, Mizuochi T. Structures of the asparagine-linked oligosaccharide chains of human von Willebrand factor. Occurrence of blood group A, B, and H(O) structures. J Biol Chem. 1992 May 5;267(13):8723-31. PMID: 1577715.

61. Jenkins PV, O'Donnell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? Transfusion. 2006 Oct;46(10):1836-44. doi: 10.1111/j.1537-2995.2006.00975.x.

62. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. J Clin Invest. 2001 Sep;108(6):779-84. doi: 10.1172/JCI13992.

63. Saenko EL, Yakhyaev AV, Mikhailenko I, Strickland DK, Sarafanov AG. Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. J Biol Chem. 1999 Dec 31;274(53):37685-92. doi: 10.1074/jbc.274.53.37685.

64. Bovenschen N, Herz J, Grimbergen JM, Lenting PJ, Havekes LM, Mertens K, van Vlijmen BJ. Elevated plasma factor VIII in a mouse model of low-density lipoprotein receptor-related protein deficiency. Blood. 2003 May 15;101(10):3933-9. doi: 10.1182/blood-2002-07-2081.

65. Marchetti G, Lunghi B, Legnani C, Cini M, Pinotti M, Mascoli F, Bernard F. Contribution of low density lipoprotein receptor-related protein genotypes to coagulation factor VIII levels in thrombotic women. Haematologica. 2006 Sep;91(9):1261-3. PMID: 16956829.

66. Vormittag R, Bencur P, Ay C, Tengler T, Vukovich T, Quehenberger P, Mannhalter C, Pabinger I. Low-density lipoprotein receptor-related protein 1 polymorphism 663 C > T affects

clotting factor VIII activity and increases the risk of venous thromboembolism. J Thromb Haemost. 2007 Mar;5(3):497-502. doi: 10.1111/j.1538-7836.2007.02337.x.

67. Cunningham N, Laffan MA, Manning RA, O'Donnell JS. Low-density lipoprotein receptor-related protein polymorphisms in patients with elevated factor VIII coagulant activity and venous thrombosis. Blood Coagul Fibrinolysis. 2005 Oct;16(7):465-8. doi: 10.1097/01.mbc.0000178831.45049.aa.

68. Morange PE, Tregouet DA, Frere C, Saut N, Pellegrina L, Alessi MC, Visvikis S, Tiret L, Juhan-Vague I. Biological and genetic factors influencing plasma factor VIII levels in a healthy family population: results from the Stanislas cohort. Br J Haematol. 2005 Jan;128(1):91-9. doi: 10.1111/j.1365-2141.2004.05275.x.

69. Sarafanov AG, Ananyeva NM, Shima M, Saenko EL. Cell surface heparan sulfate proteoglycans participate in factor VIII catabolism mediated by low density lipoprotein receptor-related protein. J Biol Chem. 2001 Apr 13;276(15):11970-9. doi: 10.1074/jbc.M008046200.

70. MacArthur JM, Bishop JR, Stanford KI, Wang L, Bensadoun A, Witztum JL, Esko JD. Liver heparan sulfate proteoglycans mediate clearance of triglyceride-rich lipoproteins independently of LDL receptor family members. J Clin Invest. 2007 Jan;117(1):153-64. doi: 10.1172/JCI29154.

71. Bovenschen N, Rijken DC, Havekes LM, van Vlijmen BJ, Mertens K. The B domain of coagulation factor VIII interacts with the asialoglycoprotein receptor. J Thromb Haemost. 2005 Jun;3(6):1257-65. doi: 10.1111/j.1538-7836.2005.01389.x.

72. Fijnvandraat K, Berntorp E, ten Cate JW, Johnsson H, Peters M, Savidge G, Tengborn L, Spira J, Stahl C. Recombinant, B-domain deleted factor VIII (r-VIII SQ): pharmacokinetics and initial safety aspects in hemophilia A patients. Thromb Haemost. 1997 Feb;77(2):298-302. PMID: 9157585.

73. Kurasawa JH, Shestopal SA, Karnaukhova E, Struble EB, Lee TK, Sarafanov AG. Mapping the binding region on the low density lipoprotein receptor for blood coagulation factor VIII. J Biol Chem. 2013 Jul 26;288(30):22033-41. doi: 10.1074/jbc.M113.468108.

74. Bovenschen N, Mertens K, Hu L, Havekes LM, van Vlijmen BJ. LDL receptor cooperates with LDL receptor-related protein in regulating plasma levels of coagulation factor VIII in vivo. Blood. 2005 Aug 1;106(3):906-12. doi: 10.1182/blood-2004-11-4230.

75. Huijgen R, Kastelein JJ, Meijers JC. Increased coagulation factor VIII activity in patients with familial hypercholesterolemia. Blood. 2011 Dec 22;118(26):6990-1. doi: 10.1182/blood-2011-10-386227.

76. Gao F, Ihn HE, Medina MW, Krauss RM. A common polymorphism in the LDL receptor gene has multiple effects on LDL receptor function. Hum Mol Genet. 2013 Apr 1;22(7):1424-31. doi: 10.1093/hmg/dds559.

77. Zhu H, Tucker HM, Grear KE, Simpson JF, Manning AK, Cupples LA, Estus S. A common polymorphism decreases low-density lipoprotein receptor exon 12 splicing efficiency and associates with increased cholesterol. Hum Mol Genet. 2007 Jul 15;16(14):1765-72. doi: 10.1093/hmg/ddm124.

78. Linsel-Nitschke P, Götz A, Erdmann J, Braenne I, Braund P, Hengstenberg C, Stark K, Fischer M, Schreiber S, El Mokhtari NE, Schaefer A, Schrezenmeir J, Rubin D, Hinney A, Reinehr T, Roth C, Ortlepp J, Hanrath P, Hall AS, Mangino M, Lieb W, Lamina C, Heid IM, Doering A, Gieger C, Peters A, Meitinger T, Wichmann HE, König IR, Ziegler A, Kronenberg F, Samani NJ, Schunkert H; Wellcome Trust Case Control Consortium (WTCCC); Cardiogenics Consortium. Lifelong reduction of LDL-cholesterol related to a common variant in the LDL-receptor gene decreases the risk of coronary artery disease--a Mendelian Randomisation study. PLoS One. 2008 Aug 20;3(8):e2986. doi: 10.1371/journal.pone.0002986. Erratum in: PLoS ONE. 2008;3(9). doi: 10.1371/annotation/9f64c41a-8cf6-40f2-8988-0d48b04dd8cb.

79. Lee JD, Lee TH, Kuo YW, Huang YC, Hsu HL, Lin YH, Wu CY, Huang YC, Lee M, Hsiao KM. Polymorphisms at the LDLR locus may be associated with ischemic cerebrovascular disease independent of lipid profile. Curr Neurovasc Res. 2012 Aug;9(3):200-6. doi: 10.2174/156720212801618965.

80. Karmali KN, Goff DC Jr, Ning H, Lloyd-Jones DM. A systematic examination of the 2013 ACC/AHA pooled cohort risk assessment tool for atherosclerotic cardiovascular disease. J Am Coll Cardiol. 2014 Sep 9;64(10):959-68. doi: 10.1016/j.jacc.2014.06.1186.

81. Jiang X, Holmes C, McVean G. The impact of age on genetic risk for common diseases. PLoS Genet. 2021 Aug 26;17(8):e1009723. doi: 10.1371/journal.pgen.1009723.

82. Aragam KG, Natarajan P. Polygenic Scores to Assess Atherosclerotic Cardiovascular Disease Risk: Clinical Perspectives and Basic Implications. Circ Res. 2020 Apr 24;126(9):1159-1177. doi: 10.1161/CIRCRESAHA.120.315928.

83. Natarajan P. Polygenic Risk Scoring for Coronary Heart Disease: The First Risk Factor. J Am Coll Cardiol. 2018 Oct 16;72(16):1894-1897. doi: 10.1016/j.jacc.2018.08.1041.

84. German CA, Shapiro MD. Polygenic Risk Scores to Identify CVD Risk and Tailor Therapy: Hope or Hype? Curr Atheroscler Rep. 2021 Jun 28;23(9):47. doi: 10.1007/s11883-021-00950-3.

85. Klarin D, Natarajan P. Clinical utility of polygenic risk scores for coronary artery disease. Nat Rev Cardiol. 2021 Nov 22. doi: 10.1038/s41569-021-00638-w.

86. Tada H, Melander O, Louie JZ, Catanese JJ, Rowland CM, Devlin JJ, Kathiresan S, Shiffman D. Risk prediction by genetic risk scores for coronary heart disease is independent of self-reported family history. Eur Heart J. 2016 Feb 7;37(6):561-7. doi: 10.1093/eurheartj/ehv462. Epub 2015 Sep 20.

87. Natarajan P, Young R, Stitziel NO, Padmanabhan S, Baber U, Mehran R, Sartori S, Fuster V, Reilly DF, Butterworth A, Rader DJ, Ford I, Sattar N, Kathiresan S. Polygenic Risk Score Identifies Subgroup With Higher Burden of Atherosclerosis and Greater Relative Benefit From Statin Therapy in the Primary Prevention Setting. Circulation. 2017 May 30;135(22):2091-2101. doi: 10.1161/CIRCULATIONAHA.116.024436.

88. Mega JL, Stitziel NO, Smith JG, Chasman DI, Caulfield M, Devlin JJ, Nordio F, Hyde C, Cannon CP, Sacks F, Poulter N, Sever P, Ridker PM, Braunwald E, Melander O, Kathiresan S, Sabatine MS. Genetic risk, coronary heart disease events, and the clinical benefit of statin therapy:

an analysis of primary and secondary prevention trials. Lancet. 2015 Jun 6;385(9984):2264-2271. doi: 10.1016/S0140-6736(14)61730-X.

89. Labos C, Martinez SC, Leo Wang RH, Lenzini PA, Pilote L, Bogaty P, Brophy JM, Engert JC, Cresci S, Thanassoulis G. Utility of a genetic risk score to predict recurrent cardiovascular events 1 year after an acute coronary syndrome: A pooled analysis of the RISCA, PRAXY, and TRIUMPH cohorts. Atherosclerosis. 2015 Sep;242(1):261-7. doi: 10.1016/j.atherosclerosis.2015.07.029.

90. Marston NA, Patel PN, Kamanu FK, Nordio F, Melloni GM, Roselli C, Gurmu Y, Weng LC, Bonaca MP, Giugliano RP, Scirica BM, O'Donoghue ML, Cannon CP, Anderson CD, Bhatt DL, Gabriel Steg P, Cohen M, Storey RF, Sever P, Keech AC, Raz I, Mosenzon O, Antman EM, Braunwald E, Ellinor PT, Lubitz SA, Sabatine MS, Ruff CT. Clinical Application of a Novel Genetic Risk Score for Ischemic Stroke in Patients With Cardiometabolic Disease. Circulation. 2021 Feb 2;143(5):470-478. doi: 10.1161/CIRCULATIONAHA.120.051927.

91. Damask A, Steg PG, Schwartz GG, Szarek M, Hagström E, Badimon L, Chapman MJ, Boileau C, Tsimikas S, Ginsberg HN, Banerjee P, Manvelian G, Pordy R, Hess S, Overton JD, Lotta LA, Yancopoulos GD, Abecasis GR, Baras A, Paulding C; Regeneron Genetics Center and the ODYSSEY OUTCOMES Investigators. Patients With High Genome-Wide Polygenic Risk Scores for Coronary Artery Disease May Receive Greater Clinical Benefit From Alirocumab Treatment in the ODYSSEY OUTCOMES Trial. Circulation. 2020 Feb 25;141(8):624-636. doi: 10.1161/CIRCULATIONAHA.119.044434.

92. Anand SS, Bosch J, Eikelboom JW, Connolly SJ, Diaz R, Widimsky P, Aboyans V, Alings M, Kakkar AK, Keltai K, Maggioni AP, Lewis BS, Störk S, Zhu J, Lopez-Jaramillo P, O'Donnell M, Commerford PJ, Vinereanu D, Pogosova N, Ryden L, Fox KAA, Bhatt DL, Misselwitz F, Varigos JD, Vanassche T, Avezum AA, Chen E, Branch K, Leong DP, Bangdiwala SI, Hart RG, Yusuf S; COMPASS Investigators. Rivaroxaban with or without aspirin in patients with stable peripheral or carotid artery disease: an international, randomised, double-blind, placebo-controlled trial. Lancet. 2018 Jan 20;391(10117):219-229. doi: 10.1016/S0140-6736(17)32409-1.

93. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R, Simes R; Cholesterol Treatment Trialists' (CTT) Collaborators. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet. 2005 Oct 8;366(9493):1267-78. doi: 10.1016/S0140-6736(05)67394-1.

94. Ludman A, Venugopal V, Yellon DM, Hausenloy DJ. Statins and cardioprotection--more than just lipid lowering? Pharmacol Ther. 2009 Apr;122(1):30-43. doi: 10.1016/j.pharmthera.2009.01.002.

95. Kunutsor SK, Seidu S, Khunti K. Statins and secondary prevention of venous thromboembolism: pooled analysis of published observational cohort studies. Eur Heart J. 2017 May 21;38(20):1608-1612. doi: 10.1093/eurheartj/ehx107.

96. Stewart LK, Sarmiento EJ, Kline JA. Statin Use is Associated with Reduced Risk of Recurrence in Patients with Venous Thromboembolism. Am J Med. 2020 Aug;133(8):930-935.e8. doi: 10.1016/j.amjmed.2019.12.032.

97. Lijfering WM, Biedermann JS, Kruip MJ, Leebeek FW, Rosendaal FR, Cannegieter SC. Can we prevent venous thrombosis with statins: an epidemiologic review into mechanism and clinical utility. Expert Rev Hematol. 2016 Nov;9(11):1023-1030. doi: 10.1080/17474086.2016.1245137.

98. Kunutsor SK, Seidu S, Khunti K. Statins and primary prevention of venous thromboembolism: a systematic review and meta-analysis. Lancet Haematol. 2017 Feb;4(2):e83-e93. doi: 10.1016/S2352-3026(16)30184-3.

99. Rahimi K, Bhala N, Kamphuisen P, Emberson J, Biere-Rafi S, Krane V, Robertson M, Wikstrand J, McMurray J. Effect of statins on venous thromboembolic events: a meta-analysis of published and unpublished evidence from randomised controlled trials. PLoS Med. 2012;9(9):e1001310. doi: 10.1371/journal.pmed.1001310.

100. Glynn RJ, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Ridker PM. A

randomized trial of rosuvastatin in the prevention of venous thromboembolism. N Engl J Med. 2009 Apr 30;360(18):1851-61. doi: 10.1056/NEJMoa0900241.

101. Biedermann JS, Kruip MJHA, van der Meer FJ, Rosendaal FR, Leebeek FWG, Cannegieter SC, Lijfering WM. Rosuvastatin use improves measures of coagulation in patients with venous thrombosis. Eur Heart J. 2018 May 14;39(19):1740-1747. doi: 10.1093/eurheartj/ehy014.

102. Squizzato A, Galli M, Romualdi E, Dentali F, Kamphuisen PW, Guasti L, Venco A, Ageno W. Statins, fibrates, and venous thromboembolism: a meta-analysis. Eur Heart J. 2010 May;31(10):1248-56. doi: 10.1093/eurheartj/ehp556.

103. Agarwal V, Phung OJ, Tongbram V, Bhardwaj A, Coleman CI. Statin use and the prevention of venous thromboembolism: a meta-analysis. Int J Clin Pract. 2010 Sep;64(10):1375-83. doi: 10.1111/j.1742-1241.2010.02439.x.

104. Wang H, Wang Q, Wang J, Guo C, Kleiman K, Meng H, Knight JS, Eitzman DT. Proprotein convertase subtilisin/kexin type 9 (PCSK9) Deficiency is Protective Against Venous Thrombosis in Mice. Sci Rep. 2017 Oct 30;7(1):14360. doi: 10.1038/s41598-017-14307-x.

105. Marston NA, Gurmu Y, Melloni GEM, Bonaca M, Gencer B, Sever PS, Pedersen TR, Keech AC, Roselli C, Lubitz SA, Ellinor PT, O'Donoghue ML, Giugliano RP, Ruff CT, Sabatine MS. The Effect of PCSK9 (Proprotein Convertase Subtilisin/Kexin Type 9) Inhibition on the Risk of Venous Thromboembolism. Circulation. 2020 May 19;141(20):1600-1607. doi: 10.1161/CIRCULATIONAHA.120.046397.

106. Hackam DG, Woodward M, Newby LK, Bhatt DL, Shao M, Smith EE, Donner A, Mamdani M, Douketis JD, Arima H, Chalmers J, MacMahon S, Tirschwell DL, Psaty BM, Bushnell CD, Aguilar MI, Capampangan DJ, Werring DJ, De Rango P, Viswanathan A, Danchin N, Cheng CL, Yang YH, Verdel BM, Lai MS, Kennedy J, Uchiyama S, Yamaguchi T, Ikeda Y, Mrkobrada M. Statins and intracerebral hemorrhage: collaborative systematic review and meta-analysis. Circulation. 2011 Nov 15;124(20):2233-42. doi: 10.1161/CIRCULATIONAHA.111.055269.

107. McKinney JS, Kostis WJ. Statin therapy and the risk of intracerebral hemorrhage: a meta-analysis of 31 randomized controlled trials. Stroke. 2012 Aug;43(8):2149-56. doi: 10.1161/STROKEAHA.112.655894.

108. Lee JD, Hsiao KM, Lee TH, Kuo YW, Huang YC, Hsu HL, Lin YH, Wu CY, Huang YC, Lee M, Yang HT, Hsu CY, Pan YT. Genetic polymorphism of LDLR (rs688) is associated with primary intracerebral hemorrhage. Curr Neurovasc Res. 2014 Feb;11(1):10-5. doi: 10.2174/1567202610666131202115038.

109. Lunghi B, Bernardi F, Martinelli N, Frusconi S, Branchini A, Linari S, Marchetti G, Castaman G, Morfini M. Functional polymorphisms in the LDLR and pharmacokinetics of Factor VIII concentrates. J Thromb Haemost. 2019 Aug;17(8):1288-1296. doi: 10.1111/jth.14473.

110. Walsh IM, Bowman MA, Soto Santarriaga IF, Rodriguez A, Clark PL. Synonymous codon substitutions perturb cotranslational protein folding in vivo and impair cell fitness. Proc Natl Acad Sci U S A. 2020 Feb 18;117(7):3528-3534. doi: 10.1073/pnas.1907126117.