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PhD in Pharmacological biomolecular sciences, experimental and clinical
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Department of Pharmacological and Biomolecular Sciences

FAMILIAL DYSLIPIDAEMIAS IN ITALY:

SPECTRUM OF MUTATIONS, CLINICAL MANIFESTATIONS AND INFLUENCE OF ENVIRONMENTAL FACTORS

BIO/14

PhD candidate
MARTA GAZZOTTI
Matr. R12355

PhD Tutor: Prof. Paolo Magni
PhD Co-Tutor: Dr. Manuela Casula

PhD Coordinator: Prof. Giuseppe Danilo Norata

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Abstract – English version

Although genetic dyslipidemias are a common cause of cardiovascular morbidity and mortality, they are still underestimated, underdiagnosed and undertreated in the general population. To overcome these issues, disease registries are a useful tool at national and international levels.

The Italian registry has been initiated through the LIPIGEN study, the focus of this PhD thesis, that started by focussing on the most frequent form of genetic dyslipidaemia: familial hypercholesterolemia (FH).

The main aims of my PhD project were to create the first national registry of FH, that could be exported also to other genetic dyslipidemias, and to obtain real-world data of FH Italian population that would allow to identify and overcome obstacles in the detection, diagnosis, and treatments of FH. Subsequent objectives of the thesis were to better describe the discrepancies among clinical and genetic diagnosis, to improve the detection rate of diagnostic algorithm and to adapt them to specific sub-populations as the paediatric one.

Based on these objectives, a full clinical and genetic characterization of both FH adults and children/adolescents was provided through the baseline data that were the starting point for the development of the second part of this thesis. Thus, to fill the gap between clinical and genetic diagnosis, the polygenic aetiology of FH was investigated among genetic negative and positive FH individuals, confirming the role of single nucleotide polymorphisms in the modulation of LDL-cholesterol (LDL-C) level even in monogenic Italian FH subjects, and supporting the use of a polygenic score in the refinement of diagnosis and in the prediction of future cardiovascular risk. Moreover, the Achilles tendon xanthoma detected by ultrasonography was identified as a valuable marker for clinical practice, supporting its introduction in the diagnostic algorithm to help physician in the identification of FH subjects with higher LDL-C burden, who require to be earlier and more aggressively treated. Furthermore, the main differences in the clinical diagnosis of FH between adults and children/adolescents were investigated, confirming the lower prevalence of the typical features of FH, which are associated to a long-life exposure to high levels of LDL-C, that is limited in young subjects. These findings support the need to establish *ad hoc* criteria for their identification, in order to improve and standardize the management of FH in the paediatric population. Finally, case reports of patients affected by other genetic disorders,

setting the first steps for the extension of the LIPIGEN registry to other familial dyslipidemias, are reported.

In summary, the development of the LIPIGEN study contributed to improve the knowledge of genetic dyslipidemias in Italy, to improve the access to the execution and interpretation of genetic results, to promote the process of cascade screening in the family members and to join international collaborations to face the burden of FH at global level.

Abstract – Italian version

Le dislipidemie genetiche rappresentano una delle principali cause di morbilità e mortalità cardiovascolare, tuttavia rimangono ancora sotto-stimate, sotto-diagnosticate e sotto-trattate nella popolazione generale. Negli ultimi anni, i registri di patologia si sono rivelati un utile strumento sia a livello nazionale che internazionale per affrontare queste problematiche. Il registro italiano delle dislipidemie è stato avviato attraverso lo studio LIPIGEN, il focus di questa tesi di dottorato, e ha iniziato la raccolta dei dati della dislipidemia genetica più frequente, rappresentata dall'ipercolesterolemia familiare (FH).

I principali scopi di questo progetto di tesi sono stati quelli di creare il primo registro italiano di FH, il cui modello possa essere esportato anche alle altre forme di dislipidemie genetiche, e di raccogliere dati *real-world* della situazione italiana che aiutassero ad identificare e superare eventuali criticità nell'identificazione e gestione della patologia. Da ciò è derivata la seconda parte della tesi, i cui scopi erano quelli di approfondire le discrepanze tra la diagnosi clinica e genetica, implementare la performance degli attuali algoritmi diagnostici disponibili, cercando anche di adattarli a specifiche sottopopolazioni come quella pediatrica.

Sulla base di questi obiettivi, i dati basali sono stati utilizzati per effettuare una completa caratterizzazione di adulti e bambini/adolescenti affetti da FH sia da un punto di vista clinico che genetico, e identificare i punti critici da sviluppare nella seconda parte della tesi. Per indagare il *gap* tra diagnosi clinica e genetica, è stata approfondita la natura poligenica di FH tra soggetti con diagnosi genetica positiva e negativa di FH. Questo studio ha permesso di confermare il ruolo dei polimorfismi a singolo nucleotide nella modulazione dei livelli di colesterolo LDL (c-LDL) non solo nei soggetti che non presentano una variante causativa di FH ma anche nei soggetti con mutazione monogenica, supportando l'applicazione dello score poligenico nell'implementazione della diagnosi e nella previsione del rischio cardiovascolare futuro.

Inoltre, il rilevamento della presenza di xantoma a livello del tendine di Achille tramite ecografia è stato identificato come un utile marker nella pratica clinica, supportando la sua introduzione negli algoritmi diagnostici per guidare i clinici nell'identificazione di soggetti FH con maggiore *burden* di c-LDL, che necessitano di essere trattati più precocemente possibile.

Inoltre, sono state indagate le principali differenze dei parametri utilizzati per la diagnosi clinica di FH tra gli adulti e i bambini/adolescenti, confermando la minor prevalenza dei tratti caratteristici di FH in quest'ultimo gruppo a causa dell'esposizione ancora limitata nel tempo

ad elevati livelli di c-LDL. Ciò ha permesso di sottolineare l'importanza di stabilire criteri *ad hoc* per l'identificazione di FH in questa coorte, al fine di migliorare e standardizzare la gestione di FH nella popolazione pediatrica. Infine, sono stati riportati alcuni *case report* di pazienti affetti da altri disordini genetici, che costituiscono il punto di partenza per permettere l'apertura del registro LIPIGEN anche alle altre forme di dislipidemie genetiche.

In conclusione, lo sviluppo e la conduzione dello studio LIPIGEN hanno permesso di aumentare le conoscenze nell'ambito delle dislipidemie genetiche in Italia, di facilitare l'accesso all'esecuzione e all'interpretazione dei risultati genetici, di promuovere lo screening a cascata nei familiari dei soggetti affetti da FH e di partecipare ad iniziative internazionali per promuovere la gestione di FH a livello mondiale.

CHAPTER 1

BACKGROUND

1.1 Introduction

The term 'dyslipidemias' includes a group of disorders of lipoprotein metabolism leading to both abnormally low (hypolipidemias) or high (hyperlipidemias) lipoprotein levels and alteration in the composition of these particles. Dyslipidemias can be divided in primary, caused by inherited genetic defect, and in secondary dyslipidemias, due to other diseases, drugs, or unhealthy lifestyles.

The inherited dyslipidemias are due to genetic defects that could lead to increased low-density lipoprotein cholesterol (LDL-C) or triglycerides (TG) levels or alteration in their removals or to decreased production or excessive removal of high-density lipoprotein cholesterol (HDL-C). The clinical impact of these diseases is mainly due to their contribution to atherogenesis, representing a major risk factor for cardiovascular disease (CVD), even if fatty liver disease and pancreatitis are other significant manifestations of lipid disease.

Familial hyperlipidemias were originally divided in five types according to the Fredrickson classification (**Table 1.1**), based on the pattern of involved lipoproteins (chylomicrons [CMs], low-density lipoprotein [LDL], very-low-density lipoproteins [VLDL]) (Fredrickson 1965, Fredrickson 1971), although this classification does not account for HDL neither for the causative genes that could be responsible for some differences within these conditions.

Table 1.1 – Fredrickson classification for lipid disorders

Classification	Synonyms	Lipoprotein abnormality
Type I	Chylomicronemia syndrome	CMs
Type IIa	Familial hypercholesterolemia	LDL
Type IIb	Familial combined hyperlipidemia	LDL and VLDL
Type III	Familial dysbetalipoproteinemia	Remnants of VLDL and CMs
Type IV	Familial hypertriglyceridemia	VLDL
Type V	Hyperprebetalipoproteinemia	CMs and VLDL

1.1.1 Lipid and lipoproteins

Cholesterol has multiple roles and different functions: it is a structural component of cell membranes, a precursor for the production of steroid hormones, vitamin D, oxysterols and bile acids, and an activator of neuronal signalling molecules. The majority of cholesterol

(about 80%) originates from *de novo* endogenous biosynthesis with the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, as the rate-limiting step, while a small quantity derives from the diet. Most cholesterol circulates as esterified cholesterol; consequently, free cholesterol represents a minor proportion (Hegele 2009).

Triglycerides are a crucial source of energy; they are composed by free fatty acids (FFAs) ester linked to glycerol backbone, and its synthesis is at level of intestine and liver cells. The synthetized TG are transported in the plasma and the lipolysis process at endothelial surface allow to deliver FFA to peripheral cells for β-oxidation or storage.

Because of their insolubility in the plasma, cholesterol and TGs are transported in the lipoproteins, spheroidal macromolecules composted by a hydrophobic central core (including cholesteryl ester, phospholipids (PLs), and triglycerides) surrounded by a hydrophilic coat, containing free cholesterol, phospholipid and apolipoprotein molecules (**Figure 1.1**) (Hegele 2009).

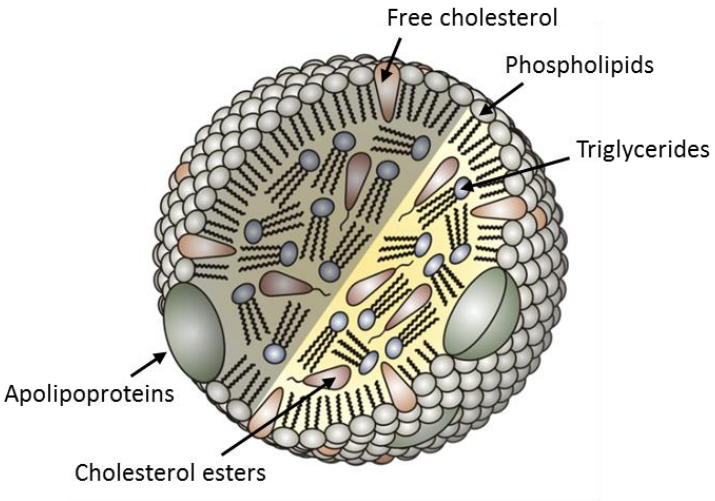


Figure 1.1 – General structure of lipoprotein

The different functions of apolipoproteins are related to provide a structural role, serve as ligands for the receptors of lipoproteins, guide lipoproteins formation and act as activators or inhibitors of enzymes involved in the lipid metabolic pathways. There are several apolipoproteins with different structure and functions (**Table 1.2**) (Dominiczak 2011).

Table 1.2 – Structure and some of the main functions of apolipoproteins. Adapted from Dominiczak et al 2011 (Dominiczak 2011).

Apo	Synthesis	Structure	Function
A1	Liver, intestine	243aa; 28,000 Da	Structural component in HDL, activator of LCAT enzyme
A2	Liver, intestine	77aa, 17,400 Da	Structural component in HDL, TG and fatty acid (FA) metabolism, link with apoE in HDL
A4	Liver, intestine	46,000 Da	Metabolism of TG-rich particles, modulator of lipoprotein lipase activity (LPL)
A5	Liver	39,000 Da	Assembler of CM and VLDL, activator of LPL
C1	Liver, intestine	57aa	Activator of LCAT, inhibitor of LPL, CETP, apoE-LDL receptor-like protein (LRP)
C2	Liver, intestine	79aa, 8,900 Da	Activator of LPL
C3	Liver, intestine	79aa, 8,800 Da	Inhibitor of LPL
B100	Liver	4536aa, 550,000Da	Structural component of VLDL, IDL, LDL and ligand of LDL receptor (LDLR)
B48	Intestine	2152 N-terminal aa of B100, 264,000 Da	Structural component of CMs and CM remnants
E	Liver but also intestine, brain, kidney, spleen, adrenals and other	299 aa, 34,200 Da	Multifunction protein, LDLR ligand for LDL and CM remnants, LRP. Modulator of LDL, CETP, LCAT. Regulator of inflammatory response.
(a)	Liver	Variable molecular mass: 187,000 – 800,000 Da	Pro-atherogenic effects due to its similar structure to plasminogen

Based on the size, density, lipid composition, electrophoretic mobility and apolipoproteins, plasma lipoproteins can be distinguished in six major classes: chylomicrons, VLDL, intermediate-density lipoproteins (IDL), LDL, lipoprotein (a) [Lp(a)], and HDL (**Table 1.3**). Cholesterol is mainly transported within low-density lipoprotein (LDL) and high-density lipoprotein (HDL) while triglycerides within chylomicron (CM) and very low-density lipoprotein (VLDL) (Mach 2020).

Table 1.3– Physical and chemical features of the major classes of lipoproteins. Adapted from Mach et al 2020 (Mach 2020).

	Density (g/mL)	Diameter (nm)	TGs (%)	Cholesteryl esters (%)	PLs (%)	Cholesterol (%)	Apolipoprotein, major and others
CM	<0.95	80-100	90-95	2-4	2-6	1	ApoB48, ApoA1, A2, A4, A5
VLDL	0.95-1.006	30-80	50-65	8-14	12-16	4-7	ApoB100, ApoA1, C2, C3, E, A5
IDL	1.006-1.019	25-30	25-40	20-35	16-24	7-11	ApoB100, ApoC2, C3, E
LDL	1.019-1.063	20-25	4-6	34-35	22-26	6-15	ApoB100
HDL	1.063-1.210	8-13	7	10-20	55	5	ApoAI, ApoA2, C3, E, M
Lp(a)	1.006-1.125	25-30	4-8	35-46	17-24	6-9	Apo(a), ApoB100

1.1.1.1 Lipoprotein metabolism

The lipid metabolism of triglycerides and cholesterol is complex and required several actors. The main steps are summarized in the **Figure 1.2**.

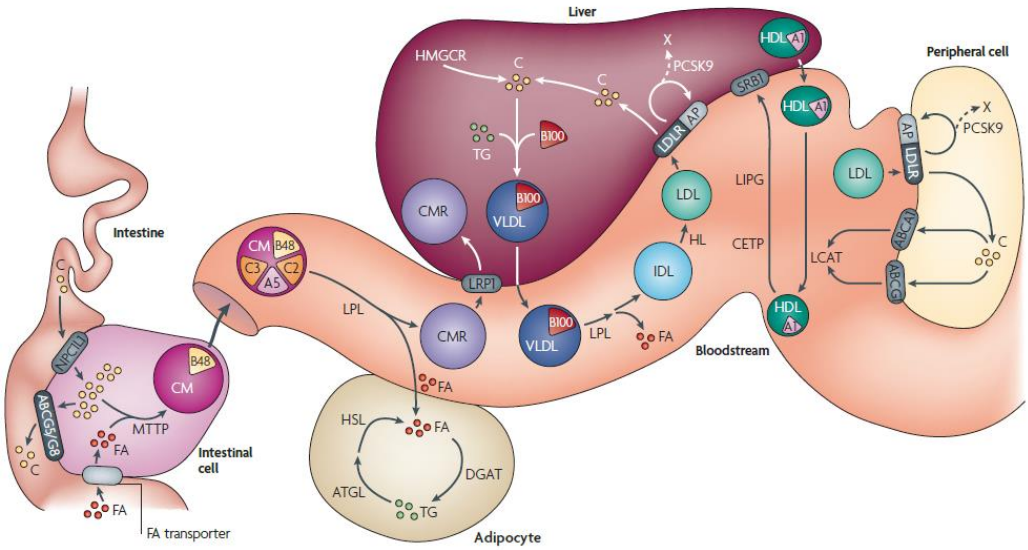


Figure 1.2 – Overview of the main steps and actors in the lipoprotein metabolism. Adapted from Hegele et al 2009 (Hegele 2009).

During the TG metabolism, the fatty acid transporter allows the entrance of dietary hydrolyzed fats in the enterocytes (Hegele 2009). Then, the microsomal TG-transfer protein (MTTP) assembles the reconstituted TGs with cholesterol ester and ApoB48 to create CMs, containing also ApoA5, ApoC2 and ApoC3, that are secreted the lymphatic system. CMs pass through the vena cava and circulate in the organism until the interaction with the lipoprotein lipase (LPL), anchored to the endothelium, causes the hydrolysis of triglycerides in free FAs and monoacylglycerol, and CMs are transformed in CM remnants (CMRs). Free FAs are able to enter in the peripheral cells and in the adipocytes where they are reassembled to form TGs, through acylCoA:diacylglycerol acyltransferase (DGAT), which in turn can be hydrolyzed by the adipose TG lipase (ATGL) and hormone sensitive lipase (HSL). Instead, CMRs entered in the liver though the hepatic LDL receptor or by LDLR-related protein-1 (LRP1), if LDLR is absent. In the hepatocytes, the VLDL are produced assembling TG, cholesterol and ApoB100 and secreted in the bloodstream. Also in this case, LPL hydrolyzes TGs in the VLDL realizing FAs and VLDL remnants or IDL, in turn transformed in LDL after the hydrolyzation by the hepatic lipase (HL). For what concerns the LDL-C metabolism, the Niemann-Pick C1-like 1 (NPC1L1) transporter allow the transport of sterols from the intestine to the enterocytes and part of them can be re-secreted in the intestinal lumen by the heterodimeric ATP-binding cassette transporter G5/G8 (ABCG5/G8). In the intestinal cell, CMs are formed through the package of cholesterol and TG, as described above. In the liver, cholesterol can be recycled or synthesized *de novo*, and VLDL are secreted. Through the previous described steps, VLDL are converted to LDL that can be endocytosed by peripheral cells and hepatocytes through the complex LDLR and LDLR adaptor protein (LDLRAP1), and the activity of LDLR is modulated by proprotein convertase subtilisin/kexin type 9 (PCSK9) (see below, paragraph 1.2.1).

Finally, in the HDL metabolism the interaction of HDL-ApoA1 and ATP-binding cassette A1 (ABCA1)/ABCG1 transporters on non-hepatic cells promotes the reverse cholesterol transport. Through the activity of lecithincholesterol acyltransferase (LCAT), cholesterol in the peripheral cells is esterified and incorporated in HDLs that, after being remodeled by cholesterol ester transfer protein (CETP) and endothelial lipase (LIPG), access in the hepatocytes via another receptor called scavenger receptor class B type I (SRB1).

1.1.1.2 The role of lipids and lipoproteins in the pathophysiology of atherosclerosis

All lipoproteins containing ApoB and with a diameter lower than 70 nm have the potential ability to cross the endothelial barriers. This could be more frequent in pathological condition characterized by endothelial dysfunction, because of the interaction between lipoprotein and extracellular structure, for example proteoglycans (Tabas 2007) . The lipoproteins accumulation in the arterial wall triggers a process leading to lipid deposit, initiation of an atheroma and a non-resolving low-grade inflammation (Boren 2016, Pirillo 2018). The continuous exposure to chronic excess of ApoB-containing lipoproteins interferes with the arterial relaxation and alters the normal process of catabolism. Thus, these atherogenic particles are recognized by scavenger receptors of macrophages localized on arterial wall instead of being internalized with the physiological receptor-mediated endocytosis. Consequently, internalized lipids in the macrophages tend to oxidize and generate damaging intermediates that stimulate the production of cytokines and chemotaxis of inflammatory intermediates (Lusis 2000, Rader 2008). The altered macrophages convert to foam cells, creating atherogenic plaques that could evolve to occlusive plaques. The increment of atherosclerosis could lead to plaque disrupt with the formation of thrombosis that obstructs the blood flow, resulting in atherosclerotic cardiovascular disease (ASCVD) (i.e. coronary heart disease [CHD] or stroke) or death in the most severe cases (Hegele 2009, Mach 2020). This evidence provides the rational to promote a lipid lowering treatment in order to reduce the ApoB-containing lipoproteins both in primary and secondary prevention (Ference 2018).

- *LDL-C and risk of atherosclerosis*

The plasmatic levels of LDL-C are an estimation of the circulating-LDL concentration and are a measurement of cholesterol mass carried by LDL particles. The log-linear relationship between the absolute changes in plasmatic LDL-C and risk of ASCVD has been strongly demonstrated by randomized clinical trials (RCT), epidemiological and Mendelian randomization studies (Cholesterol Treatment Trialists 2010, Emerging Risk Factors 2012, Ference 2012, Willer 2013, Holmes 2015, Nikpay 2015, Silverman 2016, Ference 2018). Consequently, it has been confirmed the causal association between LDL-C and risk of ASCVD, and that the reduction in LDL-C concentration proportionally reflects the reduction of risk of ASCVD (Baigent 2005). Thus, Mendelian randomization studies are a valid strategy to introduce a randomization in an observational study, to evaluate if a detected association

exposure-outcome is likely to be causal. They have shown that variants (present in our genome since birth and therefore able to mimic a long-life exposure not reproducible with RCTs) in more than 50 genes associated with lower LDL-C are also associated with a lower risk of CHD. This evidence supports that LDL particles possess both a causal and a cumulative effect of ASCVD risk, that is consequently influenced by the absolute magnitude and by the duration of the exposure to LDL-C (**Figure 1.3**) (Ference 2017).

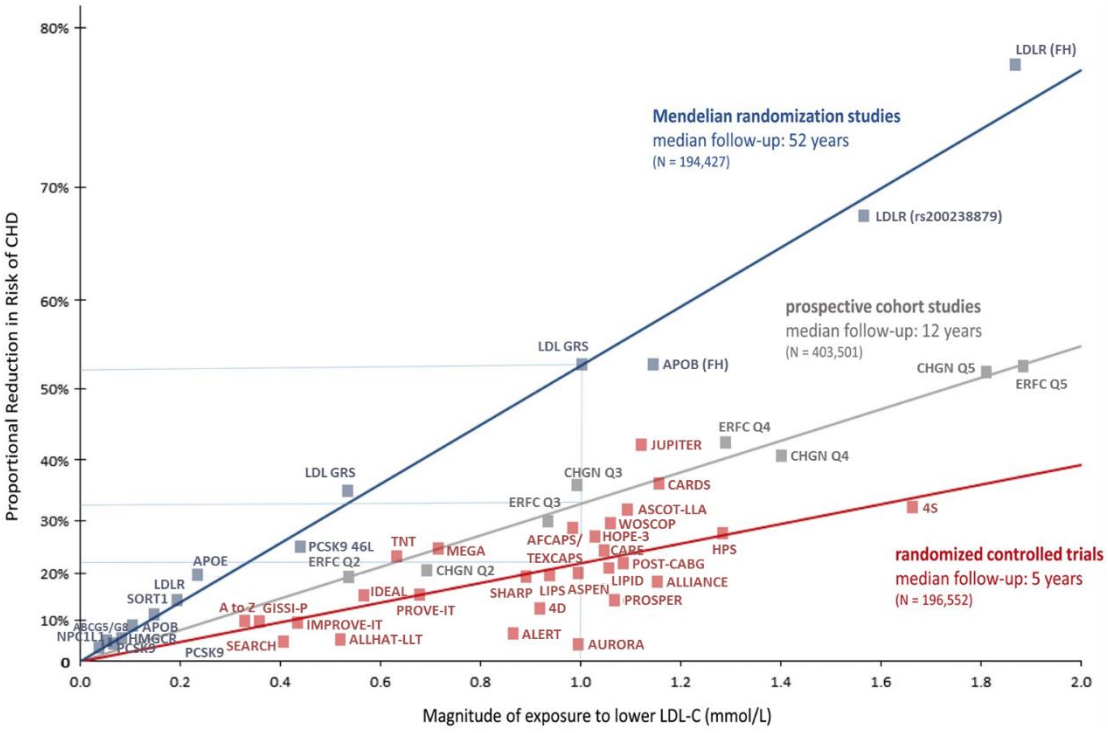


Figure 1.3 – Log-linear association per unit change in LDL-C and risk of CHD as emerged in the meta-analyses of Mendelian randomization studies, prospective cohort studies and RCTs. Adapted from Ference et al. 2017 (Ference 2017).

- *Triglyceride-rich lipoprotein and risk of atherosclerosis*

The majority of circulating TGs is carried by TG-rich VLDL particles and their remnants. Consequently, the plasmatic concentration of TGs corresponds to the proportion of circulating ApoB-containing TG-rich lipoproteins. High concentrations of plasma TGs are associated with an increment in the ASCVD risk; however, this association disappears after the adjustment for non-HDL-C that is considered an estimation of the overall concentration in ApoB-containing lipoproteins (Emerging Risk Factors 2012, Silverman 2016). In a similar

way, the effect of fibrates in the reduction of TGs suggests that the observed reduction in CV events is comparable to one observed with LDL-C-lowering therapy (Silverman 2016). Also, this observation leads to conclude that the effect of plasmatic concentration of TGs on CV event is mediated by modification in the TG-rich lipoproteins concentration estimated by non-HDL-C. Therefore, recent results of Mendelian randomization studies suggest that the causal effect of TG-rich lipoproteins and their remnants on ASCVD risk is due to the concentration of circulating ApoB-containing particles instead of the content of TG itself (Triglyceride Coronary Disease Genetics 2010, Ference 2019, Mach 2020).

- *HDL-C and risk of atherosclerosis*

The evidence from observational epidemiology showed an inverse association between plasma HDL-C and risk of ASCVD, while results from Mendelian randomization studies seem not to confirm it (Frikke-Schmidt 2008, Emerging Risk Factors 2012, Voight 2012). However, the design of Mendelian randomization studies makes difficult to estimate the effect of HDL-C on the ASCVD risk because most genetic variants associated with HDL-C are also associated with opposite changes in TGs and/or LDL-C. Moreover, RCTs did not show that a therapeutic increment in the plasma concentrations of HDL-C leads to a reduction in CV events as reported by the results of dal-OUTCOMES, ACCELERATE and REVEAL trials (Schwartz 2012, Hps Timi Reveal Collaborative Group 2017, Lincoff 2017).

- *Lp(a) and risk of atherosclerosis*

Also lipoprotein(a) presents a component of ApoB and can potentially across the endothelial barrier with atherosclerotic effects, increasing the risk of ASCVD.

The association between elevated concentration of Lp(a) and increased ASCVD risk seems to be weaker compared to the one observed for LDL-C (Emerging Risk Factors 2009, Nordestgaard 2010). However, evidence from Mendelian randomization studies strongly demonstrated a causal association between long-life exposure to high levels of Lp(a) and increased risk of ASCVD (Clarke 2009, Kamstrup 2009).

On the contrary, RCTs for therapies reducing Lp(a) by 20-30% did not show a reduction in the ASCVD risk higher than the one expected by the reduction in ApoB-containing lipoproteins even if recent results for PCSK9 inhibitors suggested a possible role for Lp(a) lowering in the reduction of CV risk (O'Donoghue 2019).

After these conflicting results, data from a recent Mendelian randomization study showed that the causal effect of Lp(a) on the ASCVD risk is proportional to the absolute modification in the plasma levels of Lp(a). This study underlined also that subjects with extremely high concentrations of Lp(a) (higher than 180 mg/dL) could present an increased lifetime risk of ASCVD comparable to the one observed in HeFH (Burgess 2018).

1.2 Familial hypercholesterolemia

Among the genetic dyslipidemias, familial hypercholesterolemia is the most common form being also one of the most common inherited disease (**Figure 1.4**) (Wiegman 2015, Pirillo 2021).

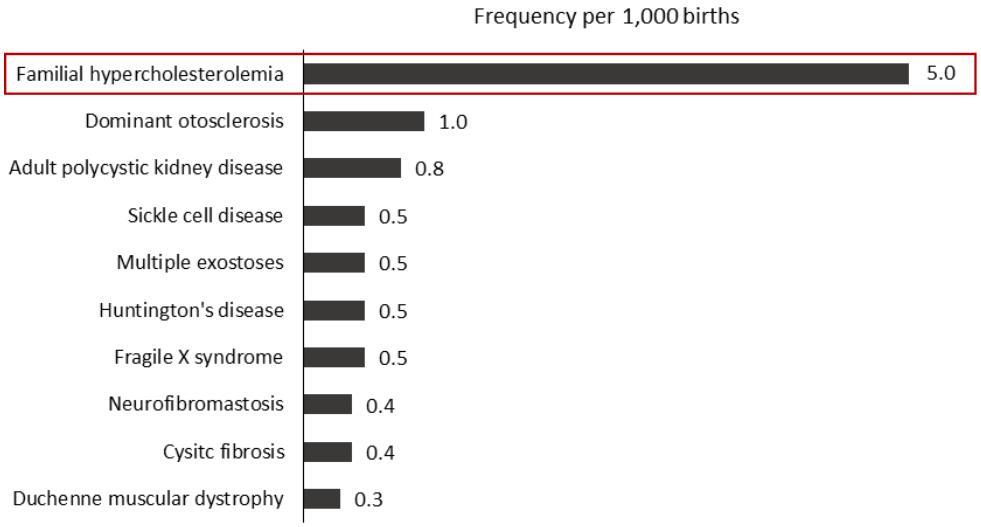


Figure 1.4 – Frequency per 1,000 births of common genetic disorders – Adapted from Wiegman A et al, 2015 (Wiegman 2015).

Familial hypercholesterolemia (FH) is a common disease of lipid metabolism, characterized by lifelong high levels of LDL-c because of genetic defects in the genes involved in the pathway of LDL receptor (LDLR). The elevated concentration of LDL-c accelerates the atherogenic process with a consequent higher risk to develop premature coronary heart disease (CHD) (**Figure 1.5**).

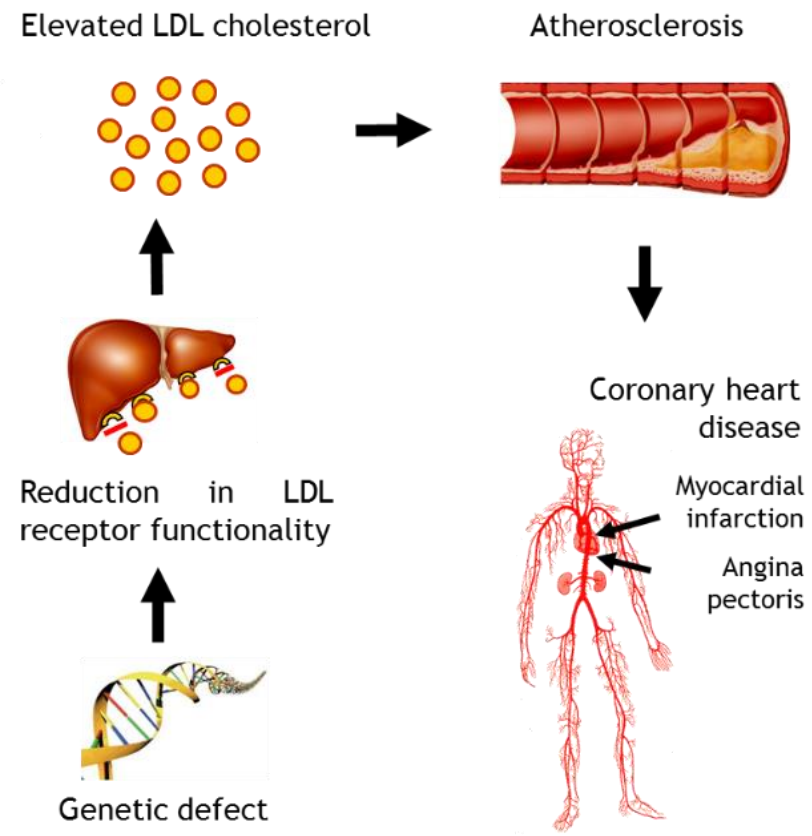


Figure 1.5 – General features of familial hypercholesterolemia – Adapted from Nordestgaard BG et al, 2013 (Nordestgaard 2013).

Although the early identification and treatment are crucial to prevent cardiovascular events and achieve a normal life expectancy, FH still remains underdiagnosed and undertreated in the general population (Nordestgaard 2013). If untreated, heterozygous FH can present levels of total cholesterol (TC) between 310-580 mg/dL and develop premature CHD (before 55 years for men and before 60 years for women) while higher levels can be reached by homozygous FH with the risk to develop a CHD very early in life, dying before 20 years (**Figure 1.6**) (Goldstein 1995).

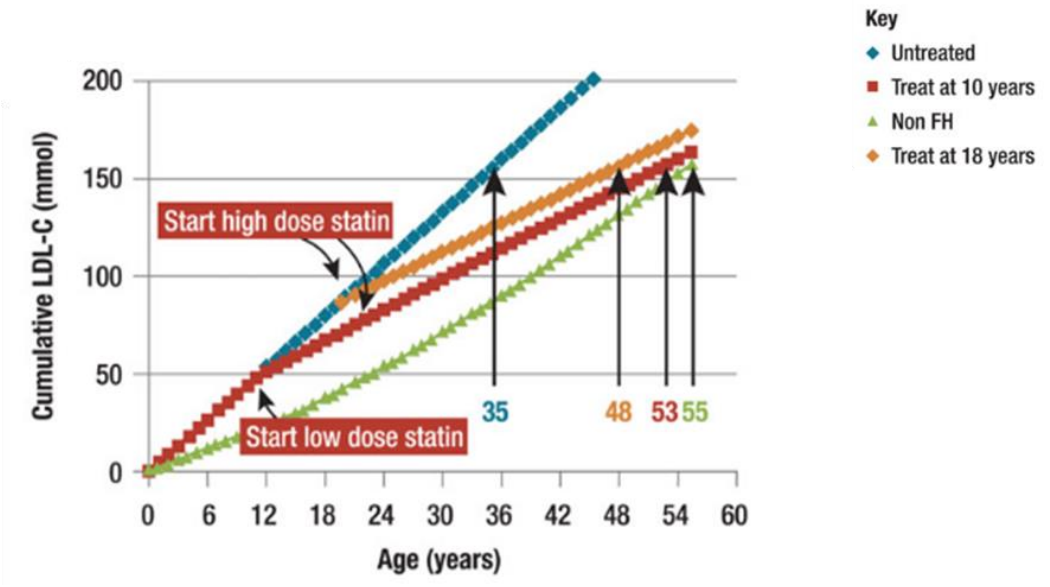


Figure 1.6 – Impact of statin therapy in LDL-c burden in FH subjects. The cumulative LDL-c burden (160 mmol) reached by a non-FH subjects at the age of 55 years is reached at the age of 35 years in an untreated FH, at the age of 48 years if the treatment was started at the age of 18 years and further delayed (53 years) in those treated from 10 years old. – Adapted from Nordestgaard BG et al, 2013 (Nordestgaard 2013).

1.2.1 Pathophysiology and genetic basis

Familial hypercholesterolemia is mainly due to mutations in genes codifying for proteins involved in the LDLR metabolic pathway, causing a reduction in the LDL cellular uptake and an excess deposition of cholesterol in tissues.

In physiological condition, the LDL receptor, a glycoprotein localized on the cellular surface, specifically binds the LDL particles in the extracellular fluids through ApoB, the surface protein of LDL particles. The complex LDLR-LDL particles via clathrin-coated vesicles is then transported into endosome, through the interaction with the LDL receptor adaptor protein 1 (LDLRAP1) (**Figure 1.7**) (Soutar 2007). Once that the complex is internalized, the acid cellular conditions cause the dissociation of the ligand-receptor complex and LDLR is recycled back to the cell surface while the LDL particle is degraded in the lysosomal compartment. On the contrary, the LDLR intracellular recycling can be blocked by the complex of LDLR with the proprotein convertase substilisin/kexin type 9 (PCSK9) to reduce the number of LDLR on the surface by post-translational mechanism that is not yet fully clarified.

The accumulation of intracellular free cholesterol inactivates the sterol regulatory element binding protein (SREBP - a transcription for genes involved in the synthesis of cholesterol and LDLR) while induces the production of another protein Inducible Degrader of LDL-Receptor (IDOL) that binds the LDLR inducing the LDLR lysosomal degradation.

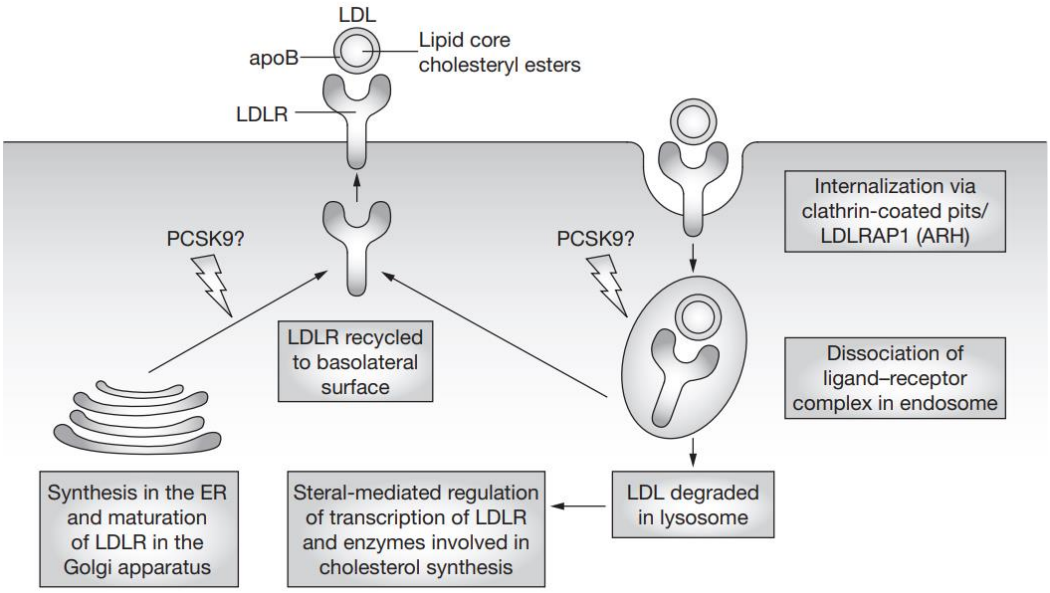


Figure 1.7 – The pathway of LDLR for LDL particles uptake and degradation. Adapted from Soutar et al., 2007 (Soutar 2007).

Consequently, mutations in genes coding for proteins involved in the described pathways are responsible for familial hypercholesterolemia.

Based on the several actors involved in the LDLR pathway, the main biological modifications that could happen in the FH subjects include a decrease in the number of LDLR, a reduction in the clearance of LDL-C from plasma due to a reduction of the activity of LDLR mainly in the hepatocytes, an alteration in ApoB structure that hampers the binding between LDL-C and LDLR and/or overespression or hyperactivity of PCSK9, reducing the number of LDLR on the cell surface due to an acceleration in the internalization and degradation of LDLR.

As described in previous paragraphs, elevated concentrations of cholesterol are present since birth in FH patients, causing early atherosclerotic lesions. The attenuate clearance of plasma LDL-C concentrations, because of absent or altered LDLR, increases the concentration of circulating LDL that could penetrate and accumulate in the artery wall, be oxidized and start the inflammatory response (Stocker 2004, van Wijk 2014). These conditions lead to vascular

damages, creation of atherosclerotic plaque and potential development of CV events also in the first decades of life (**Figure 1.8, panel A**). However, a prompt detection of FH and treatment could slow down the atherosclerotic process, reducing the lifelong burden of elevated LDL-C concentrations and preventing CV events (**Figure 1.8, panel B**) (Wiegman 2015).

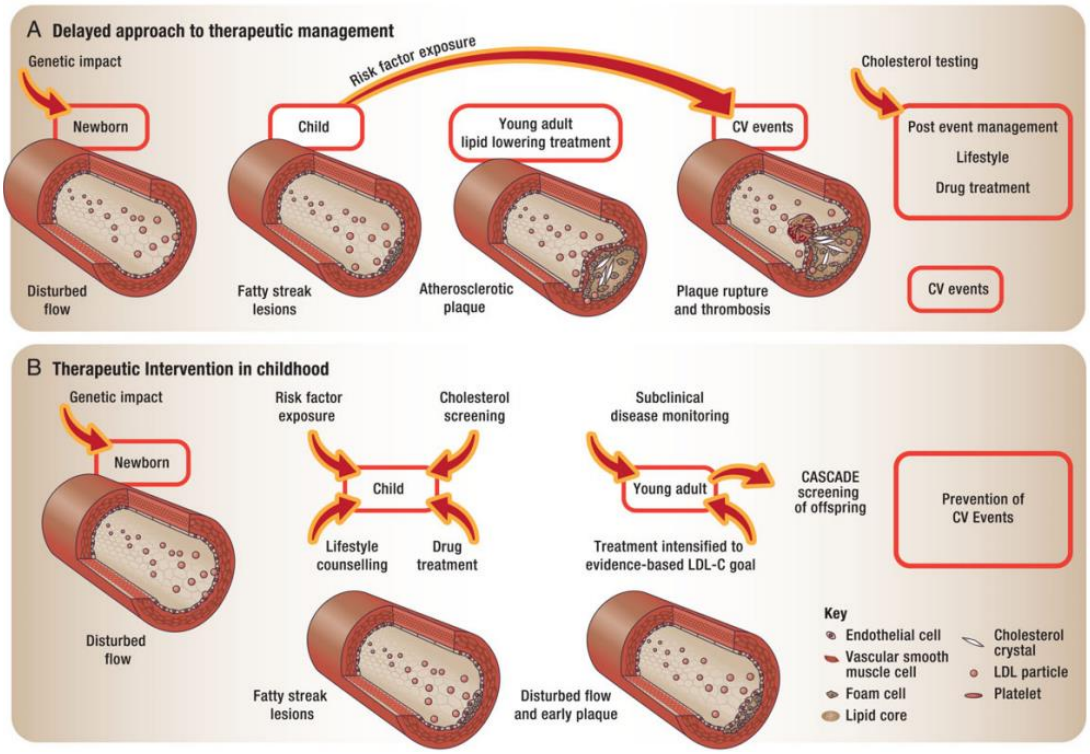


Figure 1.8 – Process of early atherosclerotic disease in FH (panel A), showing the potential benefits of an early detection and therapy in childhood. Adapted from Wiegman et al 2015 (Wiegman 2015).

1.2.2 Prevalence

It is historically assumed a prevalence of 1:500 for heterozygous FH and 1:1,000,000 for homozygous FH in the general population. However, these estimates remained unreliable because related to a limited number of countries (22 on 200 countries/territories), leading to an under-estimation of this disease (Nordestgaard 2013).

In the last decade, the creation of nationwide FH registries and the huge effort to improve the knowledge of FH worldwide confirmed that FH is more common than what previously

supposed, although there is still a lack of information in most countries (EAS Familial Hypercholesterolaemia Studies Collaboration 2018, Gazzotti 2020).

Nowadays, the HeFH is more frequent reaching one subjects on 250/200 in the general population, about 2-fold higher than previously thought, even if the worldwide prevalence still remains debated. Based on this estimate, the total number of HeFH in Europe could reach 4.5 million subjects and 35 million worldwide (**Figure 1.9**). About the 20-25% of them are represented by subjects under 18 years, this means that worldwide a baby with FH is born every minute (Wiegman 2015).

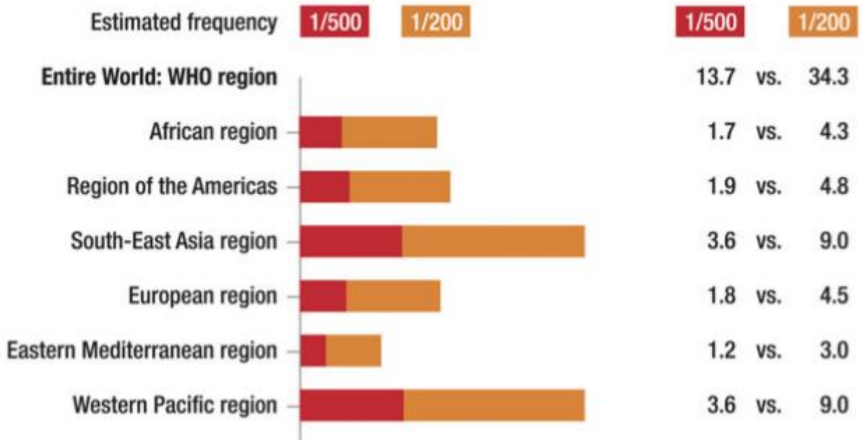


Figure 1.9 – Worldwide prevalence of FH based on estimated frequencies of 1/500 and the more recent 1/200 in the general population. Adapted from Nordestgaard et al 2013 (Nordestgaard 2013).

Based on the HeFH frequencies, also the HoFH could affect a higher number of individuals, from 1/160,000 to 1/300,000 (Hegele 2020). Higher estimates can be observed in some subpopulation with founder effect or with an elevated rate of consanguinity as Afrikaners in Sud Africa, French-Canadians in Quebec, in Lebanon or in the Hokuriku region of Japan (Leitersdorf 1989, Moorjani 1989, Fahed 2011, Mabuchi 2011).

In the last years, two meta-analyses provided a comprehensive assessment and a more reliable estimates of the prevalence of FH among general population or specific sub-groups (subjects with atherosclerotic cardiovascular disease [ASCVD], with ischemic heart disease [IHD] or premature IHD, and with severe hypercholesteroleamia), including a total of more than 60 studies and 7.3 million of individuals in Hu et al (Hu 2020), and more than 100 studies and 11 million of individuals in Beheshti et al (Beheshti 2020).

The estimation of FH in general population were comparable: 1/311 with no significant differences among the available world region, and 1/313, respectively. In the analysed sub-cohorts, the prevalence of FH was strongly elevated: 18-fold higher in subjctes with ASCVD compared to general population (1/17) in the first study and 10-fold (1/31), 20-fold (1/15) and 23-fold (1/14) higher among individuals with IHD premature IHD and severe hypercholesterolemia, respectively in the other study (**Figure 1.10**).

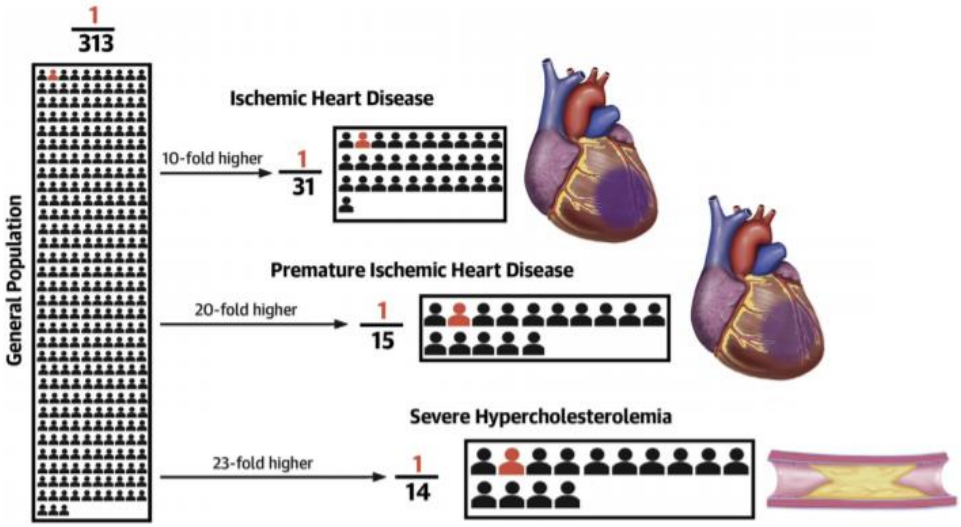


Figure 1.10 – Estimated prevalence of FH in general population compared to individuals with IHD, premature IHD and with severe hypercholesterolemia (LDL-c>190 mg/dL. Adapted from Beheshti et al 2020 (Beheshti 2020).

Although data about prevalence is still missing for the majority of countries, these prevalences confirmed that FH is the most common genetic disease in the general population, suggesting the importance of investigating cost-effectiveness screening strategies in the health policies programs in order to promote an early identification and treatment.

1.2.3 Diagnosis

Historically, FH was diagnosed from a clinical point of view, detecting the most severe cases with a most severe phenotype characterized by elevated levels of LDL-c, family and personal history of premature CHD and/or presence of typical signs of FH. Later, with the better understanding of the causative genetic role, also the molecular diagnosis was implemented in order to detect causative variants in known genes. These progresses allow to figure out a

proportion between 10-40% of individuals in whom the clinical and genetic diagnosis did not coincide because of the presence of a clinical phenotype without the identification of any known detectable mutations and *viceversa* (**Figure 1.11**) (Nordestgaard 2013).

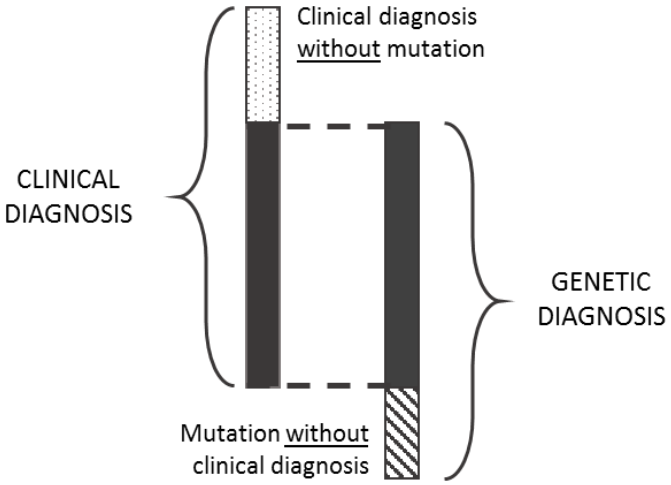


Figure 1.11 – Overlap of clinical and molecular diagnosis di FH. Adapted from Nordestgaard et al 2013 (Nordestgaard 2013).

A diagnosis of FH entails a high lifetime risk of CVD and the need to start an intensive lipid lowering therapy and screening to identify asymptomatic affected relatives. Although FH is a genetic condition and adequate therapies are available, it is not typically detected in infancy or early childhood, but more frequent in adolescence or young adults, as the longer time of exposure to very high levels of LDL-C results in detrimental effects on affected subjects (Defesche 2017).

Nowadays, FH still remains underdiagnosed and undertreated in the general population (Nordestgaard 2013), even if this gap in care is being strongly addressed by physicians and scientists at global levels, as showed by the increasing numbers of publications about FH in the last five years (EAS Familial Hypercholesterolaemia Studies Collaboration 2018, Watts 2020).

Based on the international guidelines for the management of FH, a suspected of FH should be supposed in individuals with:

- severely high levels of LDL-c (>190 mg/dL in adults and >150 in children, if HeFH suspected) or in their first-degree family
- premature CHD (before 55 years for men, before 60 years for women) or in their family members

- tendon xanthomas or in their family members
- sudden premature cardiac death in a family member (Mach 2020).

However, before confirming the diagnosis of FH and starting the cascade screening, is crucial to exclude possible secondary causes of hypercholesterolemia as hypothyroidism, nephrotic syndrome, liver disease, diet, and specific drugs (Catapano 2016).

1.2.3.1 Clinical diagnosis

The clinical diagnosis is still the starting point for identifying FH patients in daily clinical practice. The FH phenotype is mainly correlated to the exposure to elevated levels of LDL-C since birth. The clinical factors that contribute to a clinical diagnosis are a weighted combination of physical signs, personal or family history of hypercholesterolemia, early onset of ASCVD and LDL-C concentration. The clinical manifestations of FH are usually more severe in homozygous compared to heterozygous FH.

The physical signs include tendon xanthomas, a deposition of yellowish cholesterol-rich material, mainly at the level of Achilles, subpatellar and hand extensor tendons, xanthelasma, a deposit of cholesterol underneath the skin, and arcus cornealis before 45 years old, a lipid deposition in the cornea (**Figure 1.12**). The time of onset is variable and xanthomas could already appear in the first decade of life in the more severe cases (Defesche 2017).

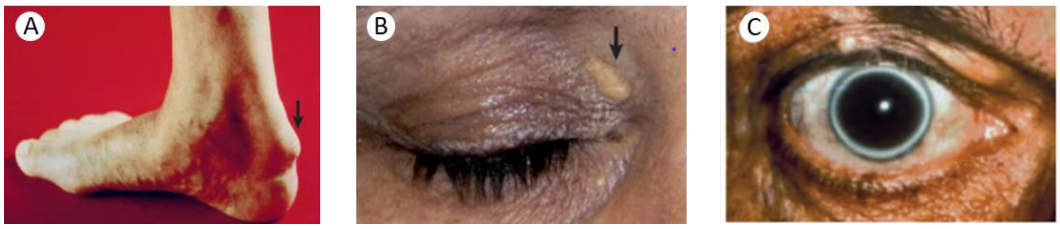


Figure 1.12 – Examples of typical signs of familial hypercholesterolemia due to deposits of cholesterol: Achilles tendon xanthoma (A), xanthelasma (B), arcus cornealis (C). Adapted from Defesche et al 2017 (Defesche 2017).

In addition, the long-life exposure to high levels of cholesterol is responsible for the increment of the risk of developing premature coronary heart disease. In the most severe conditions, rare cases of angina pectoris, myocardial infarction and death were reported in

HoFH in the first years of life, even if cardiovascular events develop in the adolescence for untreated HoFH individuals and later in life for HeFH (Kolansky 2008, Macchiaiolo 2012).
Moreover, there is a great variability in the LDL-C concentration, a suspected HeFH can be supposed starting from LDL-c > 190 mg/dL while HoFH with untreated levels of LDL-c higher than 500 mg/dL.
Different groups had suggested scoring criteria for the identification of FH, with some differences in the parameters values; the mainly used criteria (**Figure 1.13**) are the Simon Broome criteria (Simon Broome Register Group 1991) the MEDPED (Make Early Diagnosis to Prevent Early Deaths) (WHO Human Genetics Programme 1998) and the Dutch Lipid Clinic Network (DLCN) score (Defesche 2004).

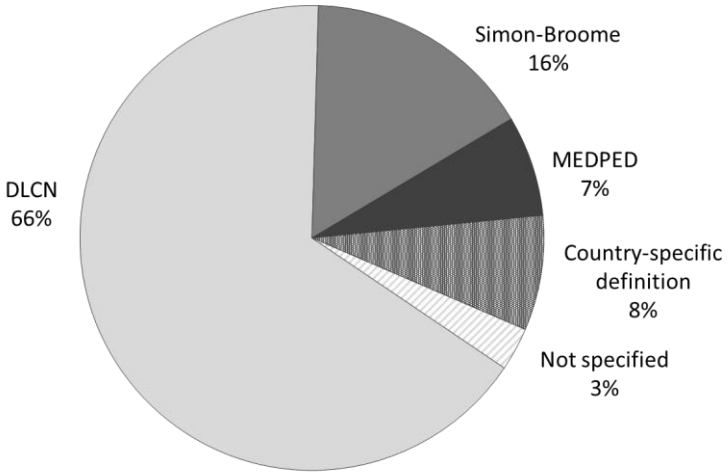


Figure 1.13 – Available diagnostic tools for the clinical diagnosis of FH

- **THE SIMON BROOME CRITERIA**

The Simon Broome diagnostic criteria consider the TC and LDL-c concentrations, providing different cut-off for adults (TC 290 mg/dL, LDL-C 190 mg/dL) and children under 16 years (TC 260 mg/dL, LDL-C 155 mg/dL), the presence of tendon xanthoma in the patient or in his/her 1st and 2nd degree relatives, the family history of premature CHD in 1st (before age of 60 years) and in 2nd (before age of 50 years) degree relatives, the family history of hypercholesterolemia both in 1st and 2nd degree relatives, and the genetic results. Based on the copresence of some of these factors, a definite or a possible diagnosis of FH can be performed, as reported in the **Table 1.4** (Simon Broome Register Group 1991).

Table 1.4 – The Simon Broome Criteria

Criteria	Description
A	**Total cholesterol** >260 mg/dL (6.7 mmol/L), if <16 years old >290 mg/dL (7.5 mmol/L), if >16 years old OR **LDL-cholesterol** >155 mg/dL (4.0 mmol/L), if <16 years old >190 mg/dL (4.9 mmol/L), if >16 years old
B	Tendon xanthomas in patient or in 1st degree relative (parent, sibling, child) or in 2nd degree relative (grandparent, uncle, aunt)
C	DNA-based evidence of an LDLR mutation or familial defective apo B-100 or a PCSK9 gain-of-function mutation
D	Family history of myocardial infarction: younger than 50 years of age in a 2nd degree relative or younger than 60 in a 1st degree relative
E	Family history of raised cholesterol greater than 7.5mmol/L in adult 1st or 2nd degree relative or greater than 6.7mmol/L in child or sibling aged younger than 16 years
FH diagnosis **DEFINITE**: Criteria A and B or C **POSSIBLE**: Criteria A and D or A and E	

- **THE US MEDPED CRITERIA**

The US MEDPED Criteria is based on age-specific and relatives-specific criteria only for total cholesterol levels and FH is diagnosed only if the TC value exceed the cut-off reported in the **Table 1.5**.

Table 1.5 – The US MEDPED Criteria

Age (years)	1st degree relative with FH (TC*)	2nd degree relative with FH (TC*)	3rd degree relative with FH (TC*)	General population (TC*)
<20	221	228	340	271
20-29	240	252	259	290
30-39	271	279	290	341
≥40	290	302	310	360

*TC cut-off expressed in mg/dL

The TC cut-off in the general population is higher compared to the one used for patients with suspected FH with parents of 1st, 2nd, 3rd degree already affected by FH. Although this criterion can easily be applied, it presents strong limitations because it does not consider other typical features of FH. Moreover, it has not specific cut-off for the paediatric subjects that are all included in the range < 20 years.

- **THE DUTCH LIPID CLINIC NETWORK (DLCN) SCORE**

The DLCN score can be considered an implementation of the Simon Broome criteria, introducing a scoring system that associates specific points to each voice. It is structured in five sections (**Table 1.6**) (Defesche 2004):

  - family history only on 1st degree family members, evaluating the presence of premature coronary artery disease (CAD), hypercholesterolemia, tendon xanthoma/arcus cornealis, and children younger than 18 years old with hypercholesterolemia (LDL-c above the 95th percentile for age and sex;
  - personal clinical history, evaluating the development of a premature CAD (men <55 years, women < 60 years) and /or premature cerebral or peripheral vascular disease;
  - physical examination, evaluating the presence of tendon xanthoma and/or arcus cornealis prior to age 45 years;
  - LDL-c levels, stratified by LDL-c classes with different points: <155 mg/dL: 0 points, 155-190 mg/dL: 1 point, 191-250 mg/dL: 3 points, 251-325 mg/dL: 5 points and >325 mg/dL: 8 points;
  - genetic results, considering the presence of a mutation in the known causative genes even if the DLCN score is mainly used to identify suspected FH before performing the molecular test.

A score is associated to each section and the final sum allow to obtain a definite (score >8 points), probable (score between 6-8 points), possible (score between 3-5 points) or unlikely (score <3 points) diagnosis of FH. This criterion is the one mainly used in Italy and suggested by the ESC/EAS European guidelines for the management of dyslipidaemia.

Table 1.6 – The DLCN score criteria

Feature	Points
FAMILY HISTORY	
1st degree relative with known premature coronary and/or vascular disease (males <55 years, females <60 years) *OR* 1st degree relative with known LDL-C above the 95th percentile for age and sex	1
1st degree relative with tendinous xanthomata and/or arcus cornealis *OR* Children aged less than 18 years with LDL-C above the 95th percentile for age and sex	2
CLINICAL HISTORY	
Premature coronary artery disease (males <55 y, females <60 y)	2
Premature cerebral or peripheral vascular disease (males <55 y, females <60 y)	1
PHYSICAL EXAMINATION	
Tendinous xanthoma	6
Arcus cornealis prior to age 45 years	4
LDL-C CLASSES	
>325 mg/dL	8
251-325 mg/dL	5
191-250 mg/dL	3
155-190 mg/dL	1
<155 mg/dL	0
DNA ANALYSIS	
Functional mutation in LDLR, APOB or PCSK9 genes	8
Diagnosis	**Score**
Definite	>8
Probable	6-8
Possible	3-5
Unlikely	<3

Although all these criteria are useful tools for clinicians in the daily clinical practice, all of them presented some limitations:

- they have been created and validated long ago,
- they do not consider the paediatric population (with the exception of the Simon Broome Criteria which however uses only 16 years old as cut-off),
- they are not adapted by country or by age,
- they require crucial information that sometimes are difficult to be found, and the impact of missing data is not considered.

1.2.3.1.1 The role of Achilles tendon xanthoma in the clinical diagnosis

The Achilles tendon xanthoma is a typical sign of FH and is considered by both Simon Broome and DLCN score criteria, contributing with several points to the diagnosis of FH (Beeharry 2006, Santos 2016). Although the presence of xanthoma is associated with higher prevalence and severity of ASCVD, most FH subjects do not present xanthoma at physical examination (Junyent 2005, Oosterveer 2009, Mangili 2017) . Moreover, the detection of xanthoma at the physical exam has a poor sensitivity and is largely influenced by the clinician judgement (Tsouli 2005). To overcome this gap, some groups has evaluated the usefulness of Achilles tendon ultrasonography to identify the Achilles tendon lesions associated with genetic defects, concluding that the ultrasonography is a safe, reproducible and more sensitive method for the identification of tendon xanthoma (Kutkiene 2019, Scott 2019, Paantjens 2020). Nowadays, the search of xanthomas through the ultrasound-based method is employed in clinical practice and is a valid tool for the identification of focal lesions and modification in the tendon structure and echotexture, and for the detection of tendon thickness (Kutkiene 2019) .

1.2.3.2 Genetic diagnosis

Due to the genetic etiology of this disease, the clinical diagnosis can be verified by the search of causative mutation in the known genes. However, published studies showed that causative variants were identified only in the 60-80% of individuals with a definite or probable HeFH, suggesting that a relevant proportion of subjects with a clinical phenotype of FH can present a polygenic cause or causative variants in still unknown genes (Mach 2020) . Moreover, the role of the molecular testing is still debated: s.ome experts promoted the DNA sequencing in all suspected FH (Nordestgaard 2013), while others suggest that the molecular test should be not mandatory and that the diagnosis and treatment should be driven by the levels of LDL-C (Santos 2016). However, it is crucial to underline that the CVD risk is strongly higher in subjects with pathogenic variants; consequently, it could be considered that the genetic test should be done if affordable and available. The presence of FH mutation leads to a more elevated cumulative exposure to high levels of LDL-C in the life time with a consequent higher risk of CAD within any stratum of LDL-C compared to hypercholesterolemic subjects without genetic predisposition (**Figure 1.14**). In this latter

group, a single elevation in LDL-C could reflect a time-limited exposure to high cholesterol levels (Khera 2016).

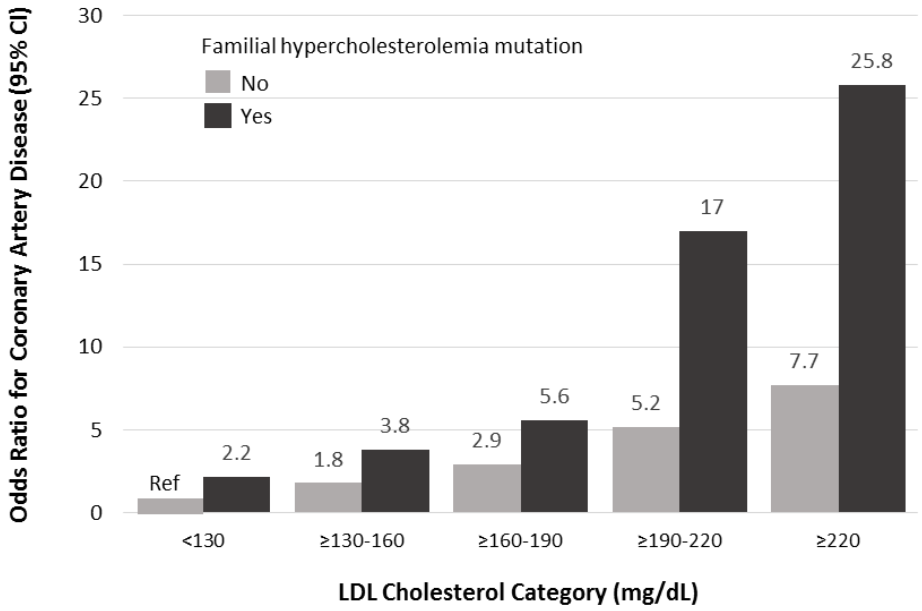


Figure 1.14 – Risk of CAD among LDL-C and FH mutation status categories. Adapted from Khera et al 2016 (Khera 2016).

Moreover, the identification of causative mutation in the index case will be useful for the cascade screening and the research of the same causative variants in the family members. At the contrary, the presence of only a polygenic cause of FH hampers the cascade screening because of the loss of the autosomal dominant inheritance pattern. Nowadays, the targeted next-generation sequencing of the main genes is the most commonly used methods for the molecular diagnosis. These improvements in the genetic tests have been accompanied by new challenges for an accurate interpretation of sequences to confirm their involvement in the disease. To overcome the problem of the novel genetic variant classification/interpretation, the American College Medical Genetics and Genomics (ACMG) published guidelines for Mendelian disorders (Richards 2015). These recommendations describe a process to classify the genetic variant based on population databases (useful for obtaining variants frequency in large populations), computational (*in silico*) predictive programs, functional and segregation data into five classes: (I) pathogenic, (II) likely pathogenic, (III) uncertain clinical significance (VUS), (IV) likely bening, (V) bening. The presence of pathogenic/likely pathogenic variants determines a positive diagnosis of FH,

while variants with uncertain clinical signifance can provide only an inconclusive diagnosis because evidences are still conflicting (criteria for bening and pathogenic are contradictory) and require further studies. Presently, there are five genes involved in the LDL-C metabolism and responsible for causing a FH phenotype: *LDLR, APOB, PCSK9, APOE* and *LDLRAP1*.

1.2.3.2.1 Mutations in *LDLR*

Familial hypercholesterolemia is mainly due to mutation in the *LDLR* gene (chromosomal location 19p13.2), accounting for more than 90% of cases (Defesche 2017, Berberich 2019). Most of detected mutations consisted of missense, nonsense or splicing substitutions (60.1%), small deletions and insertions (22.7%) and large rearrangements (17.2%) and changes mainly happened in the largest exon of *LDLR* (exon 4) (Gabcova-Balaziova 2015). Based on the effect on the protein formation, Hobbs et al (Hobbs 1990) divided the LDLR mutation into five groups:

- First class: null receptor. The LDLR is not synthetized due to point mutations that cause a premature termination in protein coding region, extensive deletions, nonsense and frameshift mutations or mutations in promoter region, blocking the transcription.
- Second class: slow or absent processing of the precursors. Part of these mutations leads to the inability of receptor precursors to pass through the membrane, the endoplasmic reticulum, and/or the Golgi apparatus to reach the cell surface. Other mutations allow the LDLR transportation in the endoplasmatic reticulum but fail the transport to the cell membrane. They affected the ligand-binding domain and the epidermal growth factor precursor-like domain.
- Third class: defective ligand-binding. These mutations are located in the ligand-binding domain and in the epidermal growth factor precursor-like domain too, but the receptors reach the hepatocyte membrane without being able to bind ApoB.
- Fourth class: internalization defective. These mutations are mainly large deletions located in the cytoplasmic or transmembrane domain and do not allow the internalization of the complex LDLR-LDL particle into the clathrin-coated vesicles.
- Fifth class: recycling defective. These mutations are located only on the EGF precursor-like domain. They cause a truncated receptor that is able to bind and internalize the ligand but fails in its release in the endosomes. Consequently, the altered receptor is degraded without recycling it on hepatocyte surface.

The number of LDLR gene variants associated with FH strongly increased in the last decade, with the modern genetic techniques allowing to identify more than 2600 variants (ClinVar database, https://clinvarminer.genetics.utah.edu) compared to the 300 known at the end of nineties (Hobbs 1992, Day 1997).

When a new variant is identified, functional studies are necessary to establish its causality or not in the disease. Two main historical approaches are used to perform the functional characterization of *LDLR* variants and evaluate the residual receptor activity. The first approach involves the use of cultured fibroblast of homozygous FH or the transfection of cells with plasmid carrying the homozygous variants (Goldstein 1974, Goldstein 1983). The second one requires the isolation of T-lymphocytes directly extracted from patient's blood and measures the residual activity directly in the patient with mutant or normal LDLR (Romano 2011).

1.2.3.2.1.1 LDLR residual activity

Causative variants can have a different impact on LDLR functionality that translates the genetic defects in a more or less severe phenotype.

The first observations were obtained through functionality studies on American (Sprecher 1985), French Canadian (Moorjani 1993) and Italian FH homozygotes in the '80s and '90s, proposing the idea that the more receptor activity was compromised, the higher the cholesterol levels were, and the faster the atherosclerosis progression was (Bertolini 1999). Moreover, the impact of mutation on LDLR activity is also linked to the presence of the defect as homozygous or heterozygous, resulting in the number of affected alleles.

Based on the LDLR residual activity measured in cultured skin fibroblasts, the *LDLR* causative variants can be divided in receptor-negative (or null) mutations, when the residual activity is lower than 5%, and in receptor-defective mutation, when the receptor maintains from 5 to 30% of its normal activity (Bertolini 2013).

1.2.3.2.2 Mutations in *APOB*

The prevalence of mutations in *APOB* gene (chromosomal location 2p24.1) is lower compared to *LDLR* and varies from 2% to 5% of all FH cases. Apolipoprotein B is a non-replaceable apolipoprotein of LDL and variants in its gene affected the ability of LDL to bind the LDLR with a consequent increment of LDL-c concentration in plasma (Gabcova-Balaziova

2015) . Nowadays, about 30 causative variants (downloaded from the Human Gene Mutation Database (http:// www.hgmd.cf.ac.uk/ac/index.php)) were detected in the APOB gene while the number of variants with uncertain clinical significance is increasing but needed structural and bioinformation studies to confirm their involvement in the disease. These mutations were identified in some families with segregation of FH phenotype but without any detectable *LDLR* mutations and are more common in central Europe than other geographical area.

The first one was identified in 1987 and consists of a nucleotide substitution c.10580G>A, resulting in amino acid change from arginine to glutamine in position 3527 (p.Arg3527Gln) (Innerarity 1987). Another mutation was detected also in the same codon but with different amino acid change c.10579C>T p.(Arg3527Trp). Generally, causative variants in *APOB* result in a less severe phenotype compared to the ones in *LDLR* (Defesche 2017) .

1.2.3.2.3 Mutations in *PCSK9*

Based on protein structure, mutations on *PSCK9* gene (chromosomal location 1p32.3) are divided in loss-of-function (LOF) and gain-of-function (GOF).

The first ones decrease the functionality of PCSK9, losing its capability in mediating the LDLR degradation and leading to a continuous LDL-c catabolism. Consequently, they are associated with a reduction of LDL-c and decrease the cardiovascular risk.

At the contrary, the GOF mutations are associated to FH and lead to an increase of LDL-c because the altered PSCK9 constantly promotes the degradation of LDLR, losing its ability to efficiently remove LDL-c from circulation.

Less than 30 GOF mutations are known and the first one was identified in a French family with a dominant segregation of FH phenotype but without mutation in LDLR or APOB gene. However, their prevalence is low and mutations in *PCSK9* account for about the 1% of all FH (Abifadel 2003).

In fact, only few variants were detected in families with FH phenotype (p.Ser127Arg, p.Asp129Gly, p.Arg215His, p.Phe216Leu, p.Arg218Ser, p.Asp374Tyr, p.Asp374His) and in the last decade the list was increased. Abifadel et al identified other two new mutations c.323T>G p.(Leu 108Arg), for whom *in vitro* studies confirmed the gain-of-function impact, and c.103G>T p.(Asp35Tyr) responsible of an enhancement of PCSK9 intracellular activity (Abifadel 2012).

1.2.3.2.4 Mutations in *APOE*

Just few mutations in *APOE* gene (chromosomal location 19p13.2) are associated with hypercholesterolemia and affected the physiological function of apolipoprotein E involved in the lipoprotein clearance from plasma. The confirmed one is c.500_502del p.Leu167del, a deletion of three base-pair at position 167 that causes a deletion of a leucine in the LDLR binding region of apoE, and determined a dominant FH phenotype. Its prevalence is low and just rare case were identified in the published FH cohorts (Awan 2013, Marduel 2013, Cenarro 2016, Rashidi 2017).

1.2.3.2.5 Mutations in *LDLRAP1*

Mutations in *LDLRAP1* (chromosomal location 1p36.11) are responsible of autosomal recessive hypercholesterolemia (ARH), a hypercholesterolemic condition with a phenotype comparable to FH.

The first cases were identified between 1970s and 1980s in children with a phenotype at the interface between heterozygous to homozygous *LDLR* patients but with normocholesterolemic parents. Based on these observations, it was hypothesized the rare possibility of a recessive pattern of inheritance initially called pseudo-homozygous type II hyperlipidemia (Khachadurian 1973, Morganroth 1974, Harada-Shiba 1992).

In Italy, the first cases were identified in 1979 in Sardinia with a more elevated probability of consanguinity. The familial segregation allows to identify sudden death and hypercholesterolemia in the previous six generations. In the following years, the number of detected cases increased and were detected also in other countries (Fellin 2015).

The genetic defect was fully characterized at the beginning of 2000s and six different mutations were detected; this kind of hypercholesterolemia was defined as ARH (Garcia 2001). Sardinian subjects with ARH use to present lower levels of LDL-c compared to Italian HoFH but with a wide range (from 372 mg/dL to 766 mg/dL), similar prevalence of planar, tuberous or tendon xanthomas since childhood but lower percentage of subjects with coronary artery disease than HoFH with receptor negative mutations, closer to the one detected in HoFH with a receptor defective mutation (Fellin 2003, Pisciotta 2006, Bertolini 2013).

1.2.3.2.6 Homozygous FH and genotype-phenotype association

The presence of a heterozygous or homozygous condition reflects in the different phenotypes but sometimes the less severe homozygous forms are at the interface with heterozygotes. Consequently, HoFH is difficult to be confirmed without the genetic result. Most of the HoFH cases are caused by a homozygous mutation in LDLR gene but also biallelic mutations were identified in APOB and PCSK9 genes. Based on the underlying mutations, it is possible to distinguish three different conditions (**Figure 1.15**):

- true homozygotes: carriers of the same causative variants in both alleles of the same gene;
- compound heterozygous: carriers of different mutations in each allele of the same gene;
- double heterozygous: mutations in two different genes (*LDLR* and *APOB* or *LDLR* and *PCSK9*), affecting the LDL receptor pathway (Cuchel 2014).

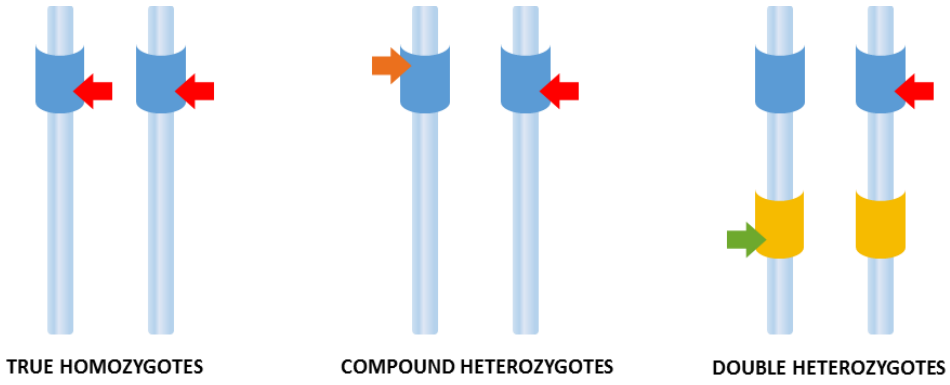


Figure 1.15 – Genetic diversity of HoFH. Adapted from Al-Ashwal A et al. (Al-Ashwal 2015).

The different genetic causes reflect in a great variability of the phenotype, leading to a progressive increment in LDL-C levels (**Figure 1.16**). Overall, the mean LDL-C concentrations by genotype gradually increase as follows: HeFH < double heterozygous (LDLR-APOB or LDLR-PCSK9 gain-of-function mutations) < homozygous APOB or PCSK9 gain-of-function mutation < homozygous LDLRAP1 or LDLR-defective mutations < compound heterozygote with a *LDLR*-defective *and a LDLR*-negative mutation < homozygous LDLR-negative mutations (Cuchel 2014).

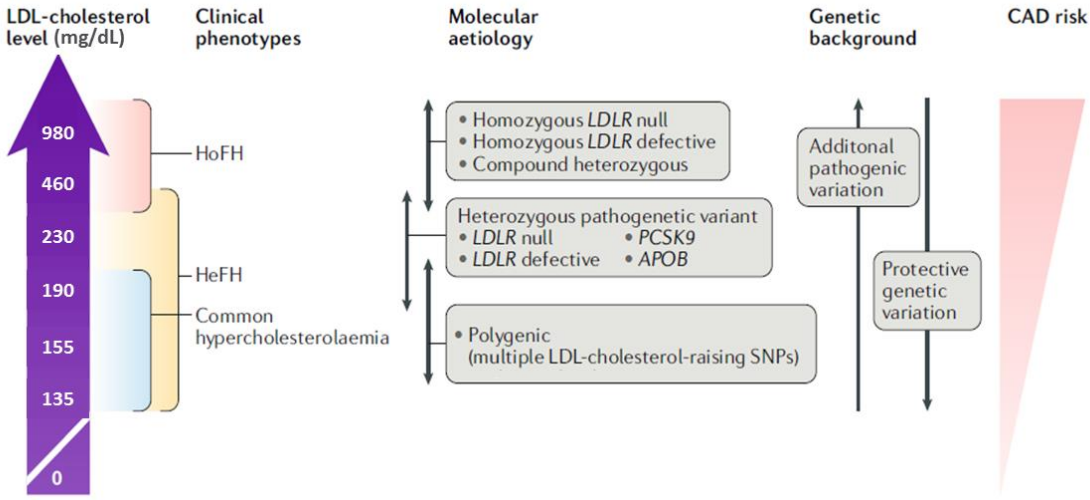


Figure 1.16 – Phenotypic and genetic spectrum of FH. Adapted from Watts et al 2020 (Watts 2020).

In addition, there are also other factors responsible of phenotype variability, as the polygenic contribution of raising LDL-C single nucleotide polymorphisms (SNPs).

1.2.3.2.7 Polygenic hypercholesterolemia

About 20-40% of subjects with a clinical FH phenotype do not present mutations in any tested genes. The failure in the detection of causative variants raises the question of the existence of still unknown causative genes and strongly supports the hypothesis of a polygenic contribution in the FH phenotype. To address the hypothesis that the FH phenotype can be determined also by an accumulation of common small-effect LDL-c-raising alleles, polygenic risk scores were created. In 2010, the Global Lipid Genetic Consortium published a meta-analysis of genome-wide association studies (GWAS) that identified several loci associated with common variants raising the LDL-c value (Teslovich 2010, Willer 2013). Based on them, Talmud et al. proposed a polygenic risk score (PRS) including 12 common LDL-c raising single nucleotide polymorphisms (SNPs) in 11 genes (*PCSK9*, *CELSR2*, *APOB*, *ABCG8*, *SLC22A1*, *HFE*, *MYLIP*, *ST4GAL4*, *NYNRIN*, *LDLR* and 2 in *APOE*) (**Table 1.7**) (Talmud 2013).

Table 1.7 – 12-SNPs included in the polygenic risk score. Adapted from Talmud et al.

SNP	Chromosome number	Gene	Minor Allele	Common Allele	GLGC weight for score calculation
rs2479409	1	PCSK9	G*	A	0.052
rs629301	1	CELSR2	G	T*	0.15
rs1367117	2	APOB	A*	G	0.10
rs4299376	2	ABCG8	G*	T	0.071
rs1564348	6	SLC22A1	C	T*	0.014
rs1800562	6	HFE	A	G*	0.057
rs3757354	6	MYLIP	T	C*	0.037
rs11220462	11	ST3GAL4	A*	G	0.050
rs8017377	14	NYNRIN	A*	G	0.029
rs6511720	19	LDLR	T	G*	0.18
rs429358	19	APOE†	C	T	··
rs7412	19	APOE†	T	C	··
ε2ε2	19	APOE	··	··	−0.9
ε2ε3	19	APOE	··	··	−0.4
ε2ε4	19	APOE	··	··	−0.2
ε3ε3	19	APOE	··	··	0
ε3ε4	19	APOE	··	··	0.1
ε4ε4	19	APOE	··	··	0.2

*Risk alleles (LDL-C-raising). †APOE weights were based on haplotypic effects taken from Bennet et al. 2007 (Bennet 2007).

In their paper, Talmud et al. showed that carriers of LDL-c raising SNPs could reach LDL-c levels comparable to the ones with a causative mutation in known genes. Thus, the polygenic risk score was significantly higher in the group of clinical FH subjects with no monogenic mutations compared to healthy controls (mean score 1.00±0.21 vs 0.90±0.23; $p=4{\cdot}5\times10^{-16}$) (**Figure 1.18, panel A**). These data support the possibility of a polygenic aetiology of FH, where the cumulative effect of common LDL-c raising SNPs leads to increase LDL-c levels at the range observed in patients with monogenic FH (Talmud 2013, Futema 2018). Moreover, a polygenic contribution was observed also in mutation positive subjects, that presented a mean PRS value at the interface between controls and FH/M- (controls: 0.90±0.23 vs FH/M+: 0.95±0.20 vs FH/M-: 1.00±0.21) (**Figure 1.17, panel B and C**). Consequently, the presence of an additional polygenic component also in the mutation positive FH could be an explanation of part of the phenotypic variability among carriers of the same mutation. In the same paper, Talmud et al. used a Belgian FH cohort to validate those results with comparable inclusion criteria to the original one.

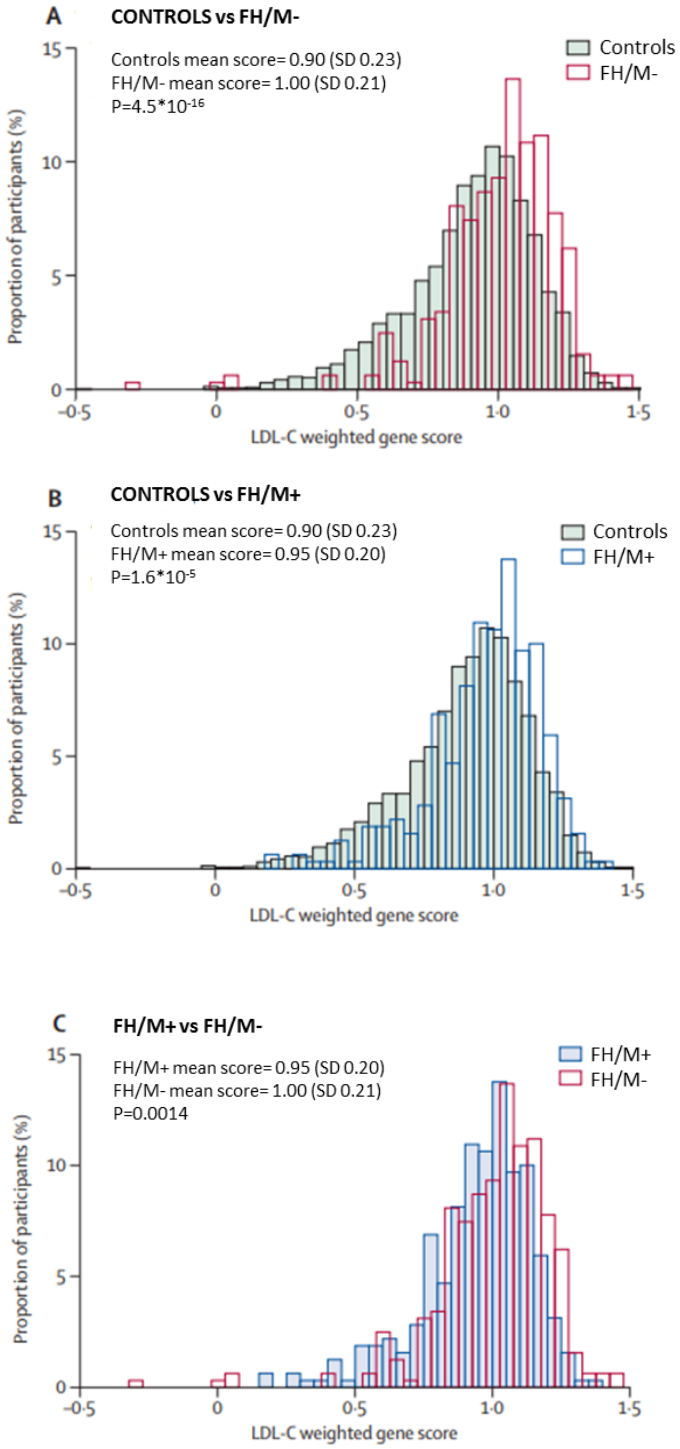


Figure 1.17 - Distribution of weighted LDL-C polygenic risk score in controls vs FH/M- (panel A), controls vs FH/M+ (panel B) and FH/M+ vs FH/M- (panel C). Adapted from Talmud et al 2013 (Talmud 2013).

Later, the 12 SNPs PRS has been refined in order to improve the selection of SNPs. The increment to 33 SNPs PRS did not show an improvement in discriminating between mutation negative FH and healthy controls while the removal of SNPs with lower effect allow to obtain a weighted 6 SNPs PRS (rs629301-*CELSR2*, rs1367117-*APOB*, rs6544713-*ABCG5/8*, rs6511720-*LDLR*, rs429358-*APOE*, rs7412-*APOE*), with the same performance of the 12 SNPs PRS (**Figure 1.18**) and lower genotyping costs (Futema 2015).

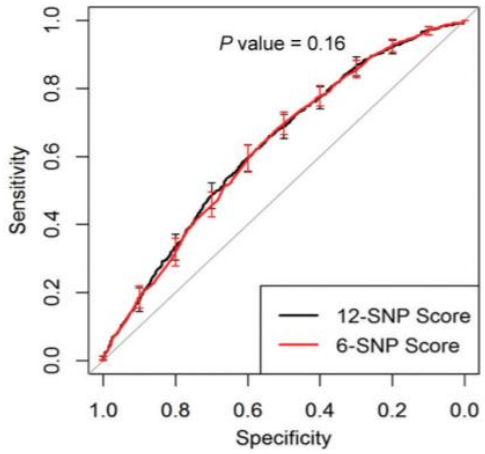


Figure 1.18 – ROC curve analysis of the discrimination between healthy controls and mutation negative FH subjects by using the 12-SNPs vs 6-SNPs LDL-c score. Adapted from Futema et al 2015 (Futema 2015).

The LDL-c SNP score was tested in 7 independent cohorts from 6 different countries (The Netherlands, Greece, Canada, Italy, Poland, Israel) proving similar results and confirming a significant higher mean PRS for all FH/M- compared to controls from general population. Also in this case, the mean PRS was higher in mutation-positive FH compared to controls, confirming an additional polygenic contribution on the phenotype determined by the large effect of a single mutation. In the following years, the 6 SNPs PRS was validated also in other cohort as in the Portuguese population (Mariano 2020).

Despite the growing attention to polygenic risk scores in the last decade, their clinical potential is still debated and it is still necessary to establish whether and to what extent a polygenic involvement should be investigated in the clinical practice (Cupido 2021).

1.2.4 Screening

Primary dyslipidemias, including FH, are associated to a high risk of cardiovascular events (Khera 2016, Mach 2020). Consequently, the prompt identification of affected subjects is crucial to start an adequate treatment and prevent premature events. However, how to promptly identify FH individuals among the general population still remains really debated. Three approaches for screening have been proposed: cascade, opportunistic and universal screening.

1.2.4.1 Cascade screening

Because of the co-dominant inheritance pattern of FH, the cascade screening revealed to be a valid approach for the identification of new FH individuals once that the index case has been identified. However, it must necessarily start from the identification of a proband, for whom other strategies are necessary (see above) (Nordestgaard 2013).

After the identification of index cases, the process of cascade screening starts with 1st degree family member including parents, sibling and children. If an affected parent is identified, the screening is extended to as many relatives as possible from that parent's side (**Figure 1.19**). Children need to be screened too, because the dietary recommendations and treatment are indicated also in childhood. To maximize cost-effectiveness, it is necessary that the cascade screening is systemic, central coordinated in a specialized and carried out evaluating a combination of lipid profile and/or the genetic test centres. The national program in the Netherlands allow to identify more than 28,000 FH individuals (almost 23,000 through the cascade screening) with more than 500 different causative variants. However, despite this approach, about 30% of estimated cases remained not disgnosed because no index cases were identified (Huijgen 2010, Kwiterovich 2012). Even in Norway, the screening program identified more than 5600 of 15,000/20,000 estimated FH, confirming that also strategies for the identification of index cases need to be implemented (Wiegman 2015).

However, there are potential barriers that are related to the family structure and dynamics, the potential geographic dispersion of some family members, the health care literacy mainly in countries with a private health care system and privacy concerns (Knowles 2017).

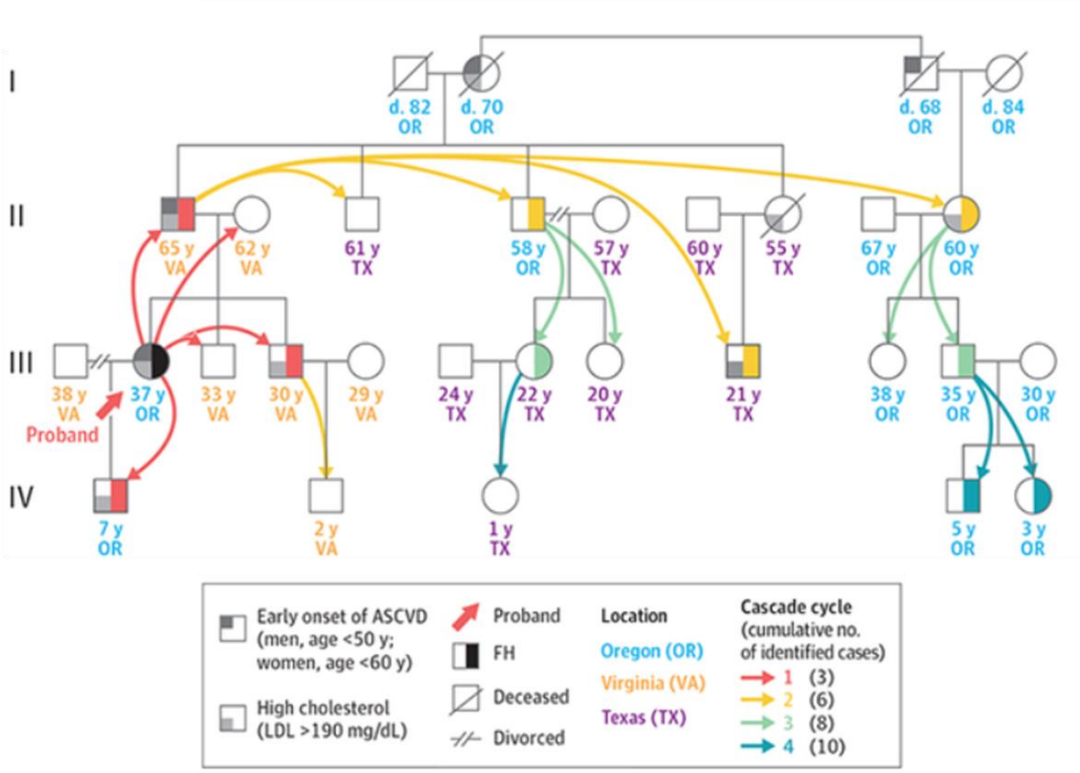


Figure 1.19 – Example of the cascade screening after the identification of index case. Adapted from Knowles et al 2017 (Knowles 2017)

Cascade screening can reduce the age at diagnosis, as reported in a study conducted in Japan where the mean age of cascade-screened FH patients was significantly lower compared to the age of index cases (39 years vs 57 years, p<0.0001) (Tada 2021). Consequently, also the lipid-lowering therapy is started before in life, resulting in a reduction of premature events and in a better prognosis (Knowles 2017). Kerr et al. estimated that in a 30 years-perspective, 23 deaths and 139 adverse cardiovascular diseases will be avoided per 1000 relatives tested (Kerr 2017).

Despite the potential benefits, cascade screening was not yet common practice in many countries. To overcome these issues, in the last years specific programs have been started, as happened in South Africa (Raal 2020) where despite the high prevalence of FH (about 1/80 because of the founder effect) no systemic programs were available until the end of 2016 or in Belgium (Descamps 2021).

1.2.4.2 Opportunistic screening

Opportunistic screening is the approach to identify index cases affected by familial hypercholesterolemia through analyses or clinical evaluations for other reasons. Some strategies have been proposed but remain still debated.

A potential contest for opportunistic screening could be represented by the community laboratories that perform an enormous quantity of cholesterol testing, usually requested by general practitioners, selecting LDL-c cut-off for the detection of suspected FH through the available diagnostic algorithms. However, a close interaction with doctors is crucial to exclude other causes of dyslipidemia and to account for therapy and family history. In addition, a considerable overlap in LDL-c between FH cases and normal population hampers the identification of a unique LDL-c threshold. Consequently, some FH subjects with lower LDL-c levels could be excluded by the selection and only detected via cascade screening (Bell 2012). Another setting could be represented biobanks (Alver 2019), blood donors (Jackson 2019) or by the primary care itself, promoting and increasing the sensibility of general practitioners in the detection of FH.

Based on the evidence in literature, the higher prevalence of FH among subjects with atherosclerotic cardiovascular diseases and premature events could drive the identification of specific cohorts where index case can be detected although this option fails in the primary prevention of CVD events (Beheshti 2020).

Finally, another option for selective screening is the measurement of cholesterol in children with a positive family history of hypercholesterolemia or premature cardiovascular disease in first- or second- degree family members (Kavey 2003). In Austria, a prevention-study was conducted with the submission of questionnaires in the schools with simple question about presence of high cholesterol, typical sign of FH and/or premature heart attack/stroke in the family members. Only families of children with at least one “yes” to the questions were contacted and the screening was carried out (Kreissl 2019).

1.2.4.3 Universal screening

Universal screening is an approach suggested to identify the highest number of FH cases as soon in life. Nowadays the universal child-parent screening has been implemented only in Slovenia at the moment of immunizations visit in pre-school children (Kusters 2012).

This program started in 1995 but its implementation was slow; the measurement of cholesterol was introduced as mandatory exam during the 5 years-old programmed visit at primary care paediatrician allow to reach about 20,000/year children within the last years (Klancar 2015). Moreover, starting from 2011, the genetic testing was introduced in addition to the lipid profile monitoring, implementing the universal screening program in two steps:

1) universal screening based on lipid measurement at the age of 5 years at primary care paediatricians
2) genetic FH screening in children referred to the tertiary care level in accordance with the guidelines and cascade screening for all family members.

Some preliminary retrospective results of this approach showed that among 2012-2016, 280 children were identified as suspected FH and in almost half of them the FH diagnosis was genetically confirmed (Groselj 2018).

Although universal screening of children and the child-parent (reverse) cascade screening could be an effective method to identify index cases, it presented limitations related to its feasibility in countries with different health-care systems and its less cost-effectiveness compared to the cascade screening (Jackson 2019).

Moreover, also the optimal age for performing it needs to be established although most guidelines suggested before puberty to avoid the hormonal influence and the start a dietary and pharmacological interventions as soon as possible. In US, universal screening is recommended between 9-11 years, because the selective screening based on family history is not completely efficient in the identification of FH children because the young age of children/adolescents is frequently associated also to a young age of parents that could still present a negative family history of premature events (Ritchie 2010, Expert Panel on Integrated Guidelines for Cardiovascular 2011).

1.2.5 Lipid lowering therapies

Based on the current European guidelines, FH individuals are at high or very high cardiovascular risk. All subjects and their affected relatives should start an intensive educational program for improving lifestyle habits (including i.e. interventions on smoke, diet, physical activity) in addition to a pharmacological therapy. Starting these approaches as soon as possible in life is crucial to reduce the risk to develop premature cardiovascular disease (Nordestgaard 2013).

A lipid lowering treatment (LLT) should be started immediately after the diagnosis in adulthood and is strongly suggested to be initiated at age of 6-10 years and even sooner in case of HoFH. The LDL-C goals vary based on the CVD risk: for FH at very-high risk of ASCVD because a prior ASCVD or presence of at least a major risk factor, a ≥50% reduction of LDL-C from baseline and LDL-C <55 mg/dL is recommended. For adults at high risk (with no ASCVD or one major risk factor), the LDL-C goals are a reduction of LDL-C ≥50% from baseline and an LDL-C <70mg/dL.

For children/adolescents, ESC/EAS Guidelines suggests to start with a statin therapy at the lower doses and to increase the dosage to reach LDL-C goals: LDL-C<135 mg/dL for children older than 10 years and a ≥50% reduction of LDL-C for younger ages (Mach 2020).

In case of HoFh children, an intensive lipid-lowering therapy shuld be considered and the maximal tolerated pharmacological therapy should be maintained, considering LDL apheresis as an additional therapeutic option (Wiegman 2015, Reiner 2018).

The pharmacological therapy of FH to reduce LDL-C includes statins as drugs of first choice because the huge amount of their evidence in reducing major CV events in clinical trials and observational studies in adults (Marks 2003, Baigent 2005, Versmissen 2008).

However, also the highest doses of statins could be not enough to reach the LDL-C goal, and other lipid lowering agents should be coadministrated as ezetimibe (an inhibitor of cholesterol absorption thorugh the block of the intestinal NPCL1), bile acid sequestrants (resins able to bind bile acids blocking their enterohepatic recirculation), PCSK9 inihibitors (monoclonal antibodies preventing the attack, internalization and degradation of LDLR by PCSK9) or newer agents as lomitapide (inhibitor of the protein MTTP necessary for the synthesis of CMs and VLDL in the intestine and livel) or inclisiran (a double-stranded small interfering RNA agent, suppressing the translation of PCKS9 in the liver loweing the levels of circulating PCSK9 and LDL-C) (**Figure 1.20**).

Although the number of RCTs in children is lower in FH children compared to adults due to their young age and ethical reasons, most of the available studies confirmed the short-term efficacy, safety and tolerability of the main lipid-lowering drugs (statins, ezetimibe, bile acid sequestrants, and PCSK9 inhibitors). Furthermore, long-term results are mainly provided by open-lable and observational studies (Collins 2016, Gazzotti 2021).

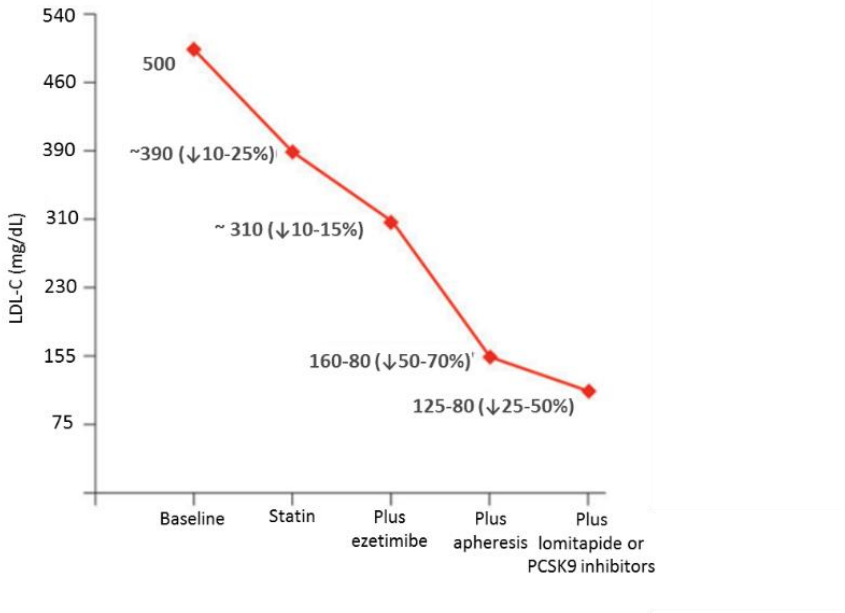


Figure 1.20 – Cumulative effect of lipid lowering drugs on LDL-C. Adapted from Schuff-Werner et al 2012 (Schuff-Werner 2012)

- *Statins*

Statins are inhibitors of HMGCoA reductase, the rate-limiting step of cholesterol biosynthesis. Their action is based on the reduction of intracellular cholesterol that stimulates an increased expression of LDLR on the surface of hepatocytes. Thus, the uptake of LDL from bloodstream increases and the plasma concentration of LDL and other ApoB-containing lipoprotients decreases. The risk/benefit ration for statin treatment have clearly established by large RCTs (Collins 2003, Colhoun 2004, Raal 2011, Catapano 2016). By decreasing the LDL-C levels, statins reduce CV morbidity and mortality as well as the necessity of coronary artery interventions in HeFH and HoFH (Collins 2003, Colhoun 2004, Raal 2011, Catapano 2016).

The decrement of LDL-C is dose-dependent and differs based on the statin. On average, a moderate-intensity statin treatement reduced LDL-C by 30 to 50% while a decrease higher than 50% is expected for high-intensity regimen. The effect of statins has been analysed in several meta-analyses (Baigent 2005, Mills 2008, Cholesterol Treatment Trialists 2010, Cholesterol Treatment Trialists 2012, Cholesterol Treatment Trialists 2015). For example, the Cholesterol Treatment Trialists (CTT) meta-analysis, including more than 170,000 individual participants of 26 RCTs, for each 1mmol/L reduction in LDL-C, major vascular events, major coronary events, CAD death, total stroke and total mortality decreased over 5 years of about

22%, 23%, 20%, 17% and 10%, respectively (Cholesterol Treatment Trialists 2010). Similar results were also obtained by the Cochrane review in 2013 (Taylor 2013).

The absolute reduction in LDL-C with statins is similar and even more pronounced in FH individuals compared to the general population, due to the higher baseline level of LDL-C (Defesche 2017). Similarly, observational studies confirmed that statins reduce CHD in FH and the survival without CHD is comparable to the one in the general population for FH individuals treated before the onset of CHD (**Figure 1.21**) (Versmissen 2008, Perez de Isla 2019).

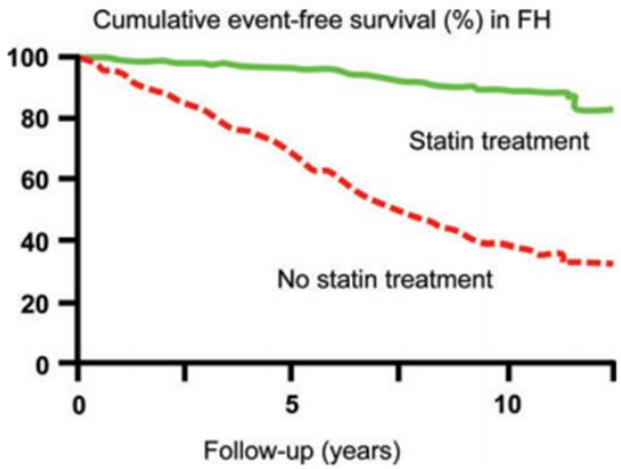


Figure 1.21 – Kaplan-Meier curve estimated of cumulative CHD-free survival in subjects with FH according to statin therapy. Adapted from Versmissen et al 2008 (Versmissen 2008).

In FH subjects, statin are generally safe and well tolerated, even if the higher dosages could lead to more frequent adverse events. The most frequent side effect is rapresented by muscle syntoms, although their prevalence is still debated (Stroes 2015, Casula 2021). In addition, other adverse events are alanine aminotransferase (ALT) elevation and increased risk of new-oneset diabetes; other effects still need to be clarified, as increased risk of neurocognitive effects, renal dysfuction, haemorrhagic stroke, and cataract (Mach 2018).

The management of statin intolerance in FH is similar to the one in other groups with high CV risk and requires statin rechallenge and use of other lipid lowering drugs because LDL-C reduction needs to be a priority in FH.

- *Non-statin drugs*

Most of FH subjects do not reach the LDL-C goals with statins alone, and a combination therapy is necessary. The second-line drug mainly used is ezetimibe, an inhibitor of cholesterol absorbation with few side effects and high compliance, rarely used alone in FH individuals. Its addition to statin therapy, it produced a further reduction of 21-27% in LDL-C compared to placebo in hypercholesterolemic patients with or without CHD. Starting a LLT directly with the association statin-ezetimine showed a 15% greater decrement in LDL-C compared the the same statin and dosage alone (Morrone 2012). The reduction in LDL-C with the addition of ezetimibe is also associated to a further reduction in CV events (Rossebo 2008, Sharp Collaborative 2010, Baigent 2011, Cannon 2015, Tsujita 2015).

In FH subjects with also high levels of triglycerides, statins can be combined with fibrates, especially fenofibrate that can also lower LDL-C. Its action on LDL-C led it to be considered as alternative in case of statin intolerance. Other therapeutic options for intolerant FH are acid-binding resins that can be added to low dose of statin and ezetimibe (Weisweiler 1989, Chapman 2011). The LDL-C reduction can also be implemented with monoclonal antibodies approved in the last years: alirocumab and evolocumab, alone or in combination with statins and/or other lipid lowering drugs. The co-administration with high-intensity statin or the maximum tolerated dose of statin lead to a reduction of 46-73% of LDL-C more than placebo and of 30% more than ezetimibe (Cho 2016). Due to their mechanism of action, mAbs need to be assumed by FH subjects with a LDLR residual activity. The two major studies already finished (FOURIER for evolocumab and ODYSSEY for alirocumab) demonstrated that these mAbs are effective treatment in reducing LDL-C and CV events on top of statin and/or ezetimibe (Sabatine 2017, Schwartz 2018). Furthermore, the two most recent lipid-lowering drugs are lomitapide and inclisiran, that act in a receptor-independent pathway. Lomitapide inhibits the transfer protein MTTP and has been studied in addition to statin therapy with or without apheresis. In Italy, lomitapide is approved only for adults HoFH (Cuchel 2013). Inclisiran is a siRNA, administered twice yearly in addition to the maximal tolerated statin therapy with or without other LDL-C lowering drugs. Pooled analysis of the main trials confirmed that inclisiran is effective, safe and well-tolerated lipid-lowering drug (Wright 2021). Another recently approved drug is the bempedoic acid, the first-in-class lipid-small-molecule inhibitor of ATP citrate lyase. Its co-administration with statin caused a futher reduction of 18% in patients treated with statin and a 28% reduction in statin-intolerant

patients (Banach 2020). The association with ezetimibe caused a reduction of 36.2% in LDL-C levels in statin-intolerants (Ballantyne 2018).

1.3 Other genetic dyslipidemias

In addition to familial hypercholesterolemia, the most frequent primary dyslipidemia, other genetic dyslipidemias have been investigated within this thesis: familial chylomicronaemia syndrome and lysosomial acid lipase deficiency.

1.3.1 Focus on familial chylomicronemia syndrome

Familial chylomicronemia syndrome (FCS) is a very rare autosomal recessive disorder of triglyceride-rich lipoproteins (TRLs). It is characterized by severe hypertriglyceridemia (HTG) with the presence of chylomicrons in fasting condition and fasting triglyceride levels higher than 885 mg/dL, that lead to an increased risk to develop recurrent episodes of potentially lethal pancreatitis (Hegele 2020).

1.3.1.1 Pathophysiology and genetic basis

In physiological conditions after a consumption of a meal containing fat, chylomicrons are secreted by the intestine and eliminated from the circulation within 4-6 hours, resulting undetectable in fasting condition. In this pathological state, this pathway is altered and chylomicrons can not be cleared but accumulated in plasma due to defects in the lipoprotein lipase (LPL), an enzyme on the endothelial surface of adipose and muscle tissue able to hydrolyse TGs into fatty acids and glycerol (Brahm 2015). FCS is mainly caused by biallelic loss-of-function mutations in *LPL* gene (about 90% of cases) or in one of other four genes involved in the lipolysis process (Brahm 2015, Dron 2017, Dron 2019).

The presence of causative variant in *LPL* is responsible in a partial or total deficit in the normal functionality of LPL enzyme with an alteration in the catabolism and removal of triglycerides in VLDL and chylomicrons (Murthy 1996, Jap 2003, Gotoda 2012, Martin-Campos 2014). The second most known and studied form is caused by mutation in the gene of apolipoprotein C2 (*APOC2*) that codified for an activator (apoC2) of LPL (Gotoda 2012, Okubo 2015).

In the last years, rare cases of severe hypertriglyceridemia with autosomal recessive pattern of inheritance were detected in carriers of mutations in other three genes involved in the TRLs catabolism:

- *APOA5* that codified for apolipoprotein A5, a modulator of LPL, able to stabilize the lipoprotein-enzyme complex (Calandra 2006, Nilsson 2011);
- *GPIHBP1* that codified for glycosyl-phosphatidyl-inositol-anchored high-density lipoprotein binding protein 1, a protein that stabilizes the binding of LPL to chylomicrons, creating a molecular platform for lipolysis (Beigneux 2009, Okubo 2015);
- *LMF1* that codified for lipase maturation factor 1, a co-factor involved in the intracellular production of the mature form of LPL (**Table 1.8**) (Peterfy 2012).

Furthermore, mutations in *CREB3L3* gene (encoding for factor cyclic AMP-responsive element-binding protein H) were identified in recent rare cases of autosomal severe hypertriglyceridemia (Lee 2011).

Table 1.8 – Main genes involed in primary monogenic chylomicronemia

Gene	Function	Clinical characteristic	Molecular characteristics	% of causative mutations
LPL	Hydrolysis of TGs and peripheral uptake of FFA	Severe chylomicronemia in infancy or childhood	Severely reduced or absent LPL enzyme activity	95.0%
APOC2	Cofactor of LPL	Severe chylomicronemia in childhood or adolescence	Absent or non-functional apoC2	2.0%
GPIHBP1	Stabilization of chylomicrons-LPL bind	Chylomicronemia in late adulthood	Absent or defective GPI-HBP1	2.0%
APOA5	Enhancer of LPL activity	Chylomicronemia in late adulthood	Absent or defective apoA5	0.6%
LMF1	Chaperone molecule for correct LPL folding/expression	Chylomicronemia in late adulthood	Absent or defective LMF1	0.4%

However, a definite diagnosis of FCS is performed only in 1-2% of subjects affected by hypertriglyceridemia while the multifactorial chylomicronemia syndrome (MCS) is more frequent (Moulin 2018). MCS can be due the polygenic susceptibility, presence of a heterozygous mutation in FCS causing genes, or presence of secondary non-genetic factors

as comorbidity known to increase plasma TG (i.e. uncontrolled diabetes or hypothyroidism), environmental factors (i.e. alcohol abuse, unhealthy diet) or some pharmacological treatment (i.e. estrogens, glucocorticoids, neuroleptic drugs) (Dron 2018). For example, Dron et al. published a study that included 563 subjects with severe hypertriglyceridemia (TGs > 885 mg/dL), finding out that only 6 patients (1.1%) had biallelic (homozygotes or compound heterozygotes) rare variants in causative genes, 87 (14.4%) had heterozygous rare variants and about 32.0% of subjects presented an extreme accumulation of triglyceride-raising common variants, while the remained patients were genetically undefined (Dron 2019).

1.3.1.2 Clinical manifestations and diagnosis

Clinical manifestations of FCS can be detected since infancy and included failure to thrive and gastrointestinal disorders as recurrent abdominal pain and more severe episodes of acute pancreatitis. Other symptoms of FCS are a creamy lipaemic plasma for the excess of TGs, eruptive xanthomas on the trunk and/or extremities as inflammatory response to the deposit of chylomicrons in tissue, *lipemia retinalis* as whitened and/or lipemic retinal vessels, hepatosplenomegaly for the infiltration of macrophages in the liver and spleen as consequence of chylomicron deposit, and possible neurological symptoms such as irritability, memory changes and depression (Brahm 2015).

In clinical practice, the rare phenotype of FCS can be easily confused with the more frequent MCS phenotype, with a delay in the diagnosis of FCS often only after an episode of acute pancreatitis. The molecular test for causative genes is therefore crucial to identify the presence of genetic basis and distinguish FCS from MCS.

However, there are some differences between these two forms of chylomicronemic syndrome, which have been considered by a group of international clinicians and experts at for the creation of a diagnostic score that could be useful for discriminating between FCS and MCS (Moulin 2018). FCS and MCS can be differentiated for TG values which are very high (>885 mg/dL) for several weeks or months in the first case, while they have greater variability and are more responsive to dietary changes or to therapy in subjects with MCS. In addition to the lipid phenotype, some clinical features also support the diagnosis of CSF, rather than the more common MCS: FCS occurs in younger patients, mainly without secondary factors, with the exception of pregnancy and use of contraceptive oral estrogens, while MCS typically occurs in overweight adult patients with metabolic syndrome. In addition, the presence of

acute pancreatitis is more frequent in FCS than in MCS due to the partial response to a low-fat diet and higher plasma TG concentrations (Moulin 2018).

1.3.1.3 Treatment

The therapy of familial chylomicronemia is still challenging and just few drugs are approved for the treatment of patients. Dietary recommendations are crucial and include low-fat diet (ideally <10% of calories from fat), even if the adherence is not optimal for most patients.
The use of fibrates, which increase also LPL activity, result not effective in FCS but an improvement was reported in patients with polygenic chylomicronaemia, for whom also elevated doses of omega-3 fatty acids can be effective due to their action in reducing VLDL level and potentially chylomicron secretion.
However, most FCS patients still have elevated TG concentration and high risk of pancreatits even following diet and medications. Consequently, in the last years, new clinical trials were conducted in order to identify other potential drugs.
Among them, volanesorsen was recently approved. It is an antisense oligonucleotide that targets the mRNA of apolipoprotein C3. ApoC3 is a glycolipoprotein involved in VLDL metabolism, through the promotion of VLDL assembly and secretion by liver and the inhibition of LPL activity, and compromise the removal of TRLs remnants from blood (D'Erasmo 2021). The pre-authorization trials showed that volanesorsen dramatically reduced the levels of TGs in FCS patients (Graham 2013, Gaudet 2014, Gaudet 2015). In the APPROACH study, a 77% reduction in TGs and an 84% reduction in ApoC3 levels was detected in subjects on volanesorsen after 3 months of treatment and were also maintained until 52 weeks (end of the study) (Witztum 2019). However, a critical increase risk of bleeding associated to thrombocytopenia was reported in treated subjects that led U.S. Food and Drug Administration FDA to limit the approval, while EMA approved it for genetically-confirmed FCS subjects at high risk of pancreatitis and not sufficiently responders to diet and TG lowering therapy. To overcome the adverse events of volanesorsen, a new third generation ASO (AKCEA-APOCIII-LRx) is being tested and the results are expected for the second half of 2023 (NCT04568434).

1.3.2 Focus on lysosomal acid lipase deficiency

The lysosomal acid lipase deficiency (LALD) is a rare autosomal recessive disorder related to *LIPA* gene that encodes for lysosomal acid lipase (LAL), a protein responsible for the hydrolysis of cholesterol esters and triglycerides located in the lysosomal compartment after the lipoproteins' endocytosis. There are two forms of this disease, depending on the level of deficiency: Wolman disease (WD), more severe and with an early and fulminant onset, and cholesteryl ester storage disease (CESD), a more attenuated defect with later-onset (Hoeg 1984).

1.3.2.1 Pathophysiology and genetic basis

In physiological conditions, cholesterol ester transported by the LDL-C is internalized by cells, through the interaction of LDL with LDLR. In lysosomes, cholesterol esters are hydrolysed to free cholesterol and fatty acids, by the action of the LAL enzyme. This enzyme also hydrolyses triglycerides into fatty acids and glycerol. The availability of free cholesterol and fatty acids in the cytoplasm regulates the lipid homeostasis. This process is regulated by gene expression, cellular receptors, and activation of transport proteins and the transcription factors SREBPs (Sterol Regulatory Element Binding Proteins) play a crucial role (Jeon 2012). The activation of SREBP-2 depends on the availability of free cholesterol in the cell. When levels of free cholesterol are low, SREBP-2 is activated and stimulates the expression of *LDLR* gene and HMCoA enzyme, while SREBP-2 is inactivated by an excess of free cholesterol in the cell. The free cholesterol obtained by *LAL* action but in excess respect to cellular needs is re-esterified by the enzyme acyltransferase (ACAT). A similar mechanism regulates the synthesis of fatty acids and it is modulated by the action of SREBP-1.

In pathological conditions, the deficiency of LAL activity alters these mechanisms causing a lysosomal accumulation of ester cholesterol and triglycerides and a consequent reduced availability of free cholesterol and fatty acids.

Since the enzyme has ubiquitous expression, excessive accumulation of lipids can be localized in any organ; however, the most affected organs are the liver, the spleen and the hematopoietic system, especially macrophages (Pericleous 2017).

This condition is followed by compensatory mechanisms, which include increased endocytosis of LDL-C and increased endogenous synthesis of cholesterol.

About 100 loss-of-function mutations in *LIPA* gene can cause LALD: homozygous or compound heterozygous mutations cause a complete LALD associated to Wolman disease, while heterozygous mutations in *LIPA* gene reduce LAL activity (residual activity: 4-12%) (Bernstein 2013).

1.3.2.2 Clinical manifestations and diagnosis

The clinical manifestations of LALD differ between and WD and CESD phenotypes. A suspicion of LALD generally could emerge during occasionally routine analysis or for the appearance of signs and symptoms (hepatomegaly, increased transaminases). The diagnosis is confirmed through the evaluation of residual enzymatic activity and the search of causative mutations in the *LIPA* gene.

The typical features of WD are an infantile-onset with malabsorption that cause malnutrition, hepatomegaly due to the deposition of cholesterol ester and triglycerides in hepatic macrophages, and liver disease and adrenal cortical insufficiency due to the calcification of adrenal gland (Hoffman 1993). The accumulation of CE and TGs is also widespread in the other visceral organs and leads to multiple organ failure and consequent death within the first year of life (Jones 2016).

Subjects affected by CESD have a residual LAL activity, which is reflected in a more modest and delayed symptomatology, with onset variable symptoms from infancy to adulthood. Most of the cases reported in literature present a symptoms onset and a consequent diagnosis of the more severe forms of CESD between the first 5-10 years of life, while the milder forms can be diagnosed randomly in adulthood. Patients with CESD have increased transaminases, mixed dyslipidaemia associated with reduced levels of HDL-C. The clinical signs include hepatosplenomegaly, micro vesicular steatosis, malabsorption and accelerated atherosclerosis. The prognosis of CESD patients is better compared to WD ones, and it was not completely clarified whether there is a real decrease of life expectancy for these patients. Death generally occurs for progression of liver disease or ischemic cardiovascular disease (Pericleous 2017).

The rate of progression of fatty liver to fibrosis and cirrhosis in CESD patients appears more rapid than to other chronic liver diseases, as non-alcoholic steatosis (NAFLD) or hepatitis C; in fact, the average interval between the first clinical manifestation of CESD and the onset of fibrosis or cirrhosis was estimated in 3.1 years (Burton 2017). Similarly, several studies

described a media-intimal carotid thickness (index of preclinical atherosclerosis) higher in CESD patients compared to healthy subjects associated for sex and age (Arnaboldi 2020). Splenomegaly and possible alteration in bone marrow due to the LAL deficit can cause anemia, thrombocytopenia and alterations in homeostasis, although they are less frequent than in other lysosomal storage diseases (Gomaraschi 2019). Furthermore, other symptoms include gastrointestinal alterations such as diarrhea, vomiting, abdominal pain, steatorrhea and possible signs of malabsorption (Fasano 2012). Finally, the dyslipidemia can involve deposition of lipids in the renal vessels with impaired renal function.

1.3.2.3 Treatment

For the treatment of the most severe form WD, numerous therapeutic approaches evaluated since its discovery (1956-1961) have been unsuccessful in most cases (Wolman 1961). In 1970s, various strategies were tested (cholestyramine, D-thyroxine, clofibrate, corticosteroids and activity depressant drugs gastrointestinal, associated with a diet enriched in medium fatty acids chain) but the success was variable and unsatisfactory. Unfortunally, improvements were limited to the short term, without influencing the overall survival of new-borns (Meyers 1985). In 1990s, several infants with WD underwent haematopoietic stem cell transplantation, leading to improvements in liver disease in some patients but reporting also numerous cases of therapeutic failure and important adverse effects, even fatal (Krivit 2000).

In CESD patients, the treatment until the end of 2000s was limited and mainly aimed to manage dyslipidaemia and only partially the liver disease. For these subjects, a low-fat diet associated with various lipid-lowering drugs (such as statins, ezetimibe and cholestyramine) was prescribed. Although no RCTs were conducted, this approach showed a variable effect on the control of dyslipidaemia and was unable to influence liver disease (Pericleous 2017, Pisciotta 2017). Furthermore, improvements in lipid profile and liver parameters were described in CESD patients that underwent the liver transplant (mean age 16 years old) due to a detrimental progression of liver disease. However, transplant has no effect on the systemic consequences of enzyme deficiency (Ferry 1991, Martinez Ibanez 1993, Leone 1995, Ambler 2013).

In the last decade, the therapy for LALD has been strongly improved by the development of sebelipase alfa, a human protein recombinant available for the enzyme replacement therapy

of patients suffering from complete and partial LALD (Sheridan 2016). Currently, the efficacy of this recombinant enzyme was evaluated in more than 100 WD or CESD subjects (about half of them in paediatric age) (Balwani 2013, Valayannopoulos 2014, Burton 2015, Rader 2015, Jones 2017, Wilson 2018). Sebelipase alfa has been tested in 9 infants affected by WD and their survival was compared to the course of a historical cohort 21 new-borns with the same characteristics, none of which survived beyond 8 months of life (Jones 2017). Among patients on sebelipase alfa, 6 of them achieved 12 months of life, 5 of which subsequently exceeded 24 months. The most important RCT that evaluated sebelipase alfa was the ARISE study, enrolling 66 children or adults (Burton 2015). At the end of the study, about 31% of treated subjects reached a normal level of alanine aminotransferase level compared to 7% in the placebo group. Moreover, improvements in lipid levels and reduction in hepatic fat content were observed in subjects on active treatment (Valayannopoulos 2014). Further studies are necessary to investigate the impact of atherosclerosis progression and cardiovascular events.

CHAPTER 2

AIMS OF THE PROJECT

Although genetic dyslipidemias are a common risk factor for cardiovascular morbidity and mortality, they remain still widely underestimated, underdiagnosed and undertreated in general population and efforts are necessary to reduce the global burden of diseases and improve awareness and knowledge among specialists, general practitioners and population (Vallejo-Vaz 2015).

In the last years, the Italian Society for the Study of Atherosclerosis (Società Italiana per lo Studio dell'Aterosclerosi, SISA) through its Foundation (Fondazione SISA) has supported the LIPIGEN Project, a network for clinical and molecular diagnosis of dyslipidemias (Averna 2017, Pirillo 2017). Within this framework, the LIPIGEN study was the cornerstone of this PhD thesis.

Taking advantages from the LIPIGEN Network Project, (i) the first objective was to create the first national registry of familial dyslipidemias in Italy. The focus was on the most common form represented by familial hypercholesterolemia, to test and standardize a model of management, data collection, genetic diagnosis and clinicians-researches-patients network, that could be exported to set pathology registries also for other dyslipidemias including the rare forms (Gazzotti 2020). Furthermore, this Italian data contributed to the global initiative EAS-FH Studies Collaboration promoted by European Atherosclerosis Society (EAS) to develop a worldwide registry of FH and generate large-scale robust data (EAS Familial Hypercholesterolaemia Studies Collaboration 2018).

The creation of the LIPIGEN-FH registry was crucial to obtain real-world data and to achieve the (ii) second objective of fully characterizing the clinical and genetic features of the Italian FH cohort, in order to deepen the genetic basis of disease and the genotype-phenotype correlation, to evaluate specific subpopulations (in particular Italian children/adolescents, for whom literature is limited) and to provide a snapshot of baseline treatment.

The baseline characterization developed in the first part of the project was the starting point to overcome critical issues in the detection and diagnosis FH, adapting also the data collection with the development of *ad hoc* sections in the electronic Case Report Form (eCRF). Consequently, the subsequent objectives were (iii) to deepen the discrepancy among clinical and genetic diagnosis investigating the polygenic aetiology of FH and the phenotypic variability among carriers of the same causative mutation, (iv) to improve the detection rate of the DLCN score, identifying the Achilles Tendon xanthoma ultrasonography as a new potential parameter that could be added for adults' evaluation, and (v) to adapt the available

diagnostic tool to subjects under 18 years, tailoring the data collection and taking into account all limits related to their young age.

Finally, the last part of the project focused on the rare dyslipidemias, providing a full characterization of the homozygous FH with both dominant or recessive forms (part of these data merge for the creation of another global registry "HoFH International Clinical Collaborators (HICC)") and describing some case report of subjects affected by familial chylomicronaemia syndrome (for whom the eCRF was developed), and by lysosomal acid lipase deficiency.

CHAPTER 3

THE LIPIGEN STUDY

3.1 The LIPIGEN Network

The LIPIGEN (Lipid TransPort Disorder Italian Genetic Network) Network was created in 2009 by the Italian Atherosclerosis Society (Società Italiana per lo Studio dell'Aterosclerosi - SISA) through its Foundation (Fondazione SISA) in order to promote and facilitate the clinical and genetic diagnosis of familial dyslipidemias. This network involves 51 Italian centres (codified with a unique center code) specialized in the management of patients affected by primary dyslipidemias throughout the national territory, including paediatric clinics and LDL apheresis centres **(Figure 3.1)**.

Figure 3.1 – Map of Italian lipid clinics involved in the LIPIGEN Network

The LIPIGEN Network structure is based on a close interaction between clinical centres, general practitioners, and patient organizations. Main objectives are to create a structured nationwide network for the identification of patients with genetic dyslipidaemias, to facilitate the molecular genetic testing, and to promote research in the field. This initiative also aims at raising awareness and culture of the medical community, patients, and regulatory authorities in our country in the area of genetic dyslipidaemia and encouraging the exchange of information and knowledge according to recommendations from scientific societies. The clinical activity of the centres is complemented by the work of specialized genetic

laboratories in the search for causative mutations for genetic dyslipidaemia in the genes described so far as being associated with these diseases.

Based on these objectives, the LIPIGEN study was started in 2012 focusing on the most common genetic dyslipidemia, familial hypercholesterolemia, to create and consolidate a model that could be implemented also for the other less frequent genetic lipid disorders (**Table 3.1**) (Averna 2017).

Table 3.1 - Estimated prevalence of genetic dyslipidemia in Italy

Dyslipidemia	Expected cases
Familial hypercholesterolemia	230,000
Type III hyperlipidemia	10,000 (?)
Severe hypertriglyceridemia	200 (?)
Familial hypertriglyceridemia	?
Familial hypobetalipoproteinemia	20,000
Familial combined hypolipidemia	?
Abetalipoproteinemia & chylomicron retention disease	50-100
Familial hypoalphalipoproteinemia	?
Familial hyperalphalipoproteinemia	?

3.2 The LIPIGEN FH study

3.2.1 Study Design

The LIPIGEN FH study is an observational, multicenter, retrospective and prospective study aimed to identify FH patients and create the first national FH registry. It was approved by the Ethics Committee of the Coordinating Center (Centro per lo Studio dell'Aterosclerosi IRCCS MultiMedica, Sesto San Giovanni, Milan, Italy) and by the local Ethics Committee of each included centers (**Appendix I, Appendix II**). The study is conducted in accordance with the study protocol and amendments, the ethical principles of the Helsinki Declaration, the standards of the ICH-Good Clinical Practice (ICH-GCP), the data protection laws and other applicable regulations.

To be enrolled, patients of any age and sex should present a clinical diagnosis of FH, according to the specialist's judgment. Clinical diagnosis should be guided by the Dutch Lipid Clinic Network criteria, in a version adapted to the Italian population by LIPIGEN scientific Committee (**Table 3.2**). Adults patients with a DLCN score≥6 and subjects under 18 years, in

which the DLCN is not still validated, are recommended to be genetically tested in order to detect the presence of causative variants.

Table 3.2 - DLCN SCORE (modified for Italy)

Parameters		**Points** (automatically assigned)
Family History		
First degree relative with known premature CHD (men <55, women < 60 years)		Y =1
First degree relative with known LDL-C >190 mg/dL		Y =1
First degree relative with tendon xanthoma and/or corneal arcus at age < 45		Y =2
Children (age<18) with known LDL-C >160 mg/dL		Y =2
Clinical history		
Clinical history of premature CHD		Y =2
Clinical history of premature cerebral or peripheral vascular disease (men <55, women <60 years)		Y =1
Hypercholesterolemia LDL-C >180 mg/dL since age<18		Y=4
Physical Examination		
Tendon xanthoma		Y =6
Arcus cornealis at age <45 years		Y =2
Biochemical Results		
LDL-C mg/dL (if age >=18)	155-190	Y = 1
	191-250	Y = 3
	251-325	Y = 5
	>325	Y = 8
LDL-C (mg/dL) (if age <18)	100-130	Y = 1
	131-159	Y = 3
	160-189	Y = 5
	≥190	Y = 7
TOTAL DLCN SCORE		

3.2.1.1 The LIPIGEN Paediatric Group

In 2018, the Italian lipid clinics involved in the LIPIGEN study constituted the LIPIGEN Paediatric group, including both paediatric and adult centers that, even occasionally, manage FH subjects under 18 years.

The main purpose of this sub-study consists of improving the detection, diagnosis and management of children and adolescents affected by FH, as these age classes present more critical issues compared to adults due to asymptomatic traits and absence of physical signs, except in the more severe cases.

Consequently, the activities of the LIPIGEN paediatric group are aimed to:

- identify and propose changes in the collection, analysis and interpretation of the data entered in the eCRF (See the following paragraph 3.2.2.1);
- promote improvements in the screening, diagnosis and therapy of pediatric FH subjects (See the following paragraph: 3.4.3);
- suggest recommendations to support clinical practice.

At July 2021, the LIPIGEN paediatric group accounted for more than 1600 subjects under 18 years with a clinical and/or genetic diagnosis of FH, followed up by 30 LIPIGEN sites (4 of them specialized in paediatric population).

3.2.1.2 The Italian Study Group on Homozygous FH

Within the LIPIGEN-FH study, data about LIPIGEN HoFH were merged with historical patients and the Italian Study Group of Homozygous FH was created, accounting for 25 LIPIGEN sites. The main aim of the group was to provide a complete molecular characterization of HoFH patients started approximately 30 years ago, including the first cases identified between 1989 and 1999 by the University Centers in Modena and Genova, the ones characterized from 2000 by other Italian Universities in Palermo, Naples and Rome and then the HoFH subjects identified within the LIPIGEN study for a total of 125 subjects with ADH and 66 with ARH (Bertolini 2020). These results were lately merged in the global registry "HoFH International Clinical Collaborators (HICC)".

3.2.2 Data Collection

Due to the observational nature of the study, enrolled subjects undergo only procedures and exams required by physician as routine clinical practice, and all available data are collected in a strictly anonymous way through a dedicated electronic Case Report Form (eCRF): a unique code is assigned to each subject by the system, in order to guarantee the privacy.

For each patient, the eCRF includes 7 sections where anamnestic, biochemical, genetic and follow up data are collected:

1) REGISTRATION: this is the section where the unique code is generated, and only the investigator can access to this part. Here, the birth date is required. Investigator should also indicate here whether the subject is the first case (Index) of FH identified in the family, or a relative of a subject already entered in the eCRF. This data is crucial to create the family tree and better investigate the causative mutations and their segregation within family members (**Figure 3.2**).

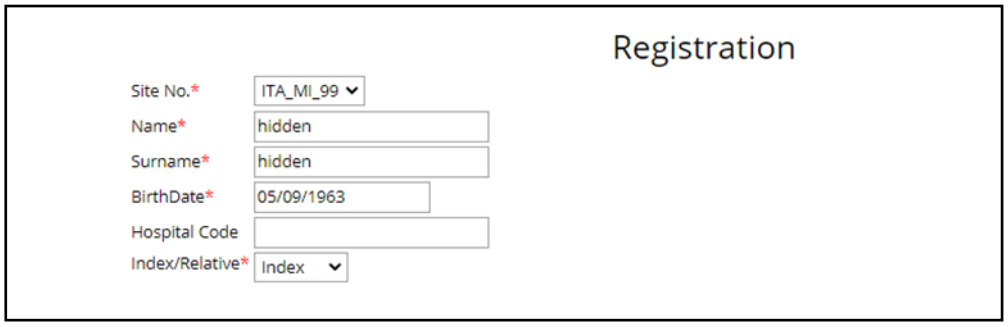


Figure 3.2 - "Registration" section

2) DATA FOR DIAGNOSIS: this section is composed by 9 parts, investigating the baseline characteristics of the subject.
   - *Informed Consent*: the Informed consent form (ICF) is a process by which a subject decides to voluntarily participate in a study and it is documented by means of a written, signed and dated document that needs to be stored at the centre (**Figure 3.3**).

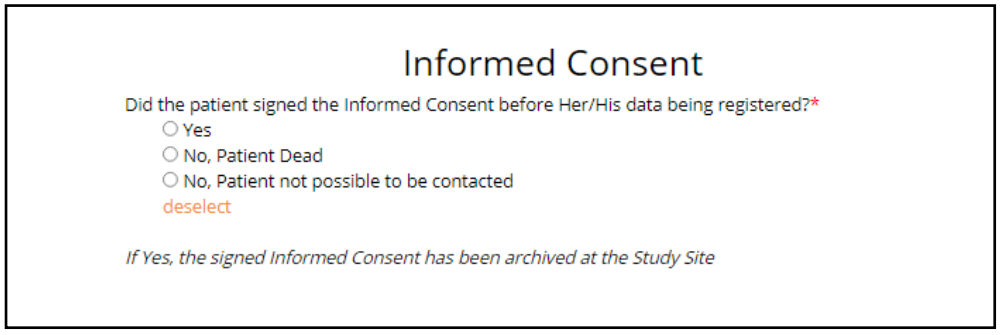


Figure 3.3 – "Informed Consent" section

- *Demography:* this part collects some demographic data about sex, geographic origin and ethnicity (**Figure 3.4**)

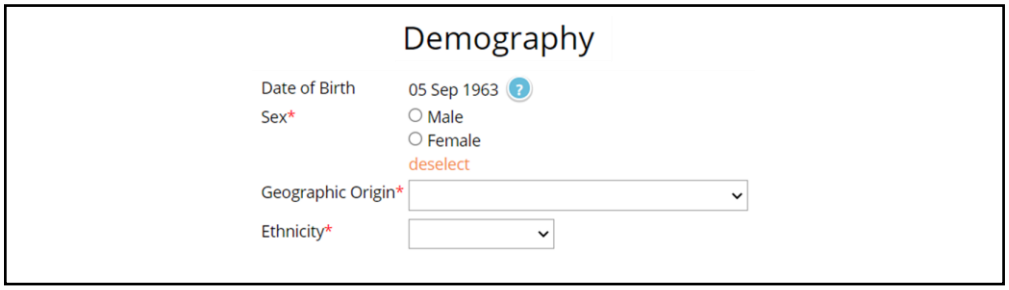


Figure 3.4 – "Demography" section

- *Anamnestic and anthropometric data:* this part collects data about age at FH diagnosis, that could be different from the age at baseline, mainly in adult patients that could present a previous diagnosis of FH without being followed up by a specialized lipid clinic yet. The majority of them only has a clinical diagnosis of FH without the genetic confirmation, that was obtained after the enrolment in the LIPIGEN study. Moreover, height, weight, Body Mass Index (BMI), smoke status and IMT were collected (**Figure 3.5**).

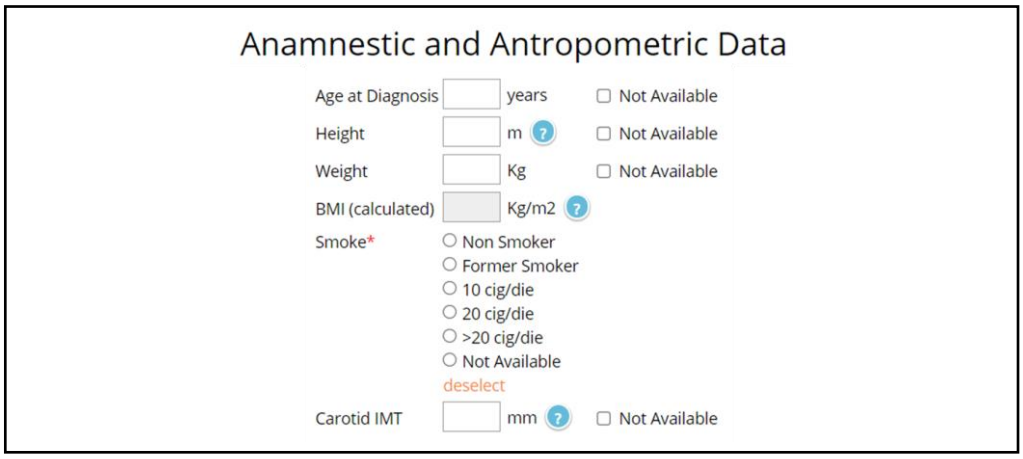


Figure 3.5 – "Anamnestic and anthropometric Data" section

- *Physical Examination (DLCN Score):* this section collects part of the data necessary for the DLCN score evaluation. It takes into account the presence or not of the typical FH signs associated to a long-life exposure to high level of LDL-C: tendon xanthoma and/or arcus cornealis before 45 y (**Figure 3.6**)

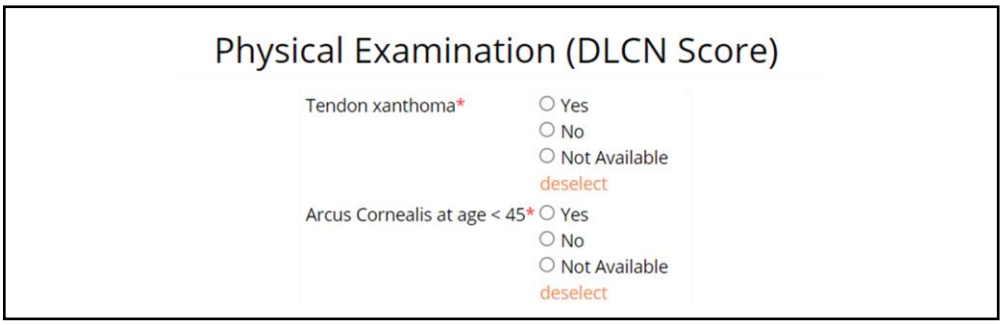


Figure 3.6 – "Physical Examination (DLCN Score)" section

- *Family History (DLCN Score):* this section collects other parameters for the DLCN score as the ones related to the family history: premature CHD, tendon xanthoma and/or arcus cornealis before 45 years, hypercholesterolemia (LDL-C > 190 mg/dL) in first-degree family members and children with LDL-C > 160 mg/dL (**Figure 3.7**).

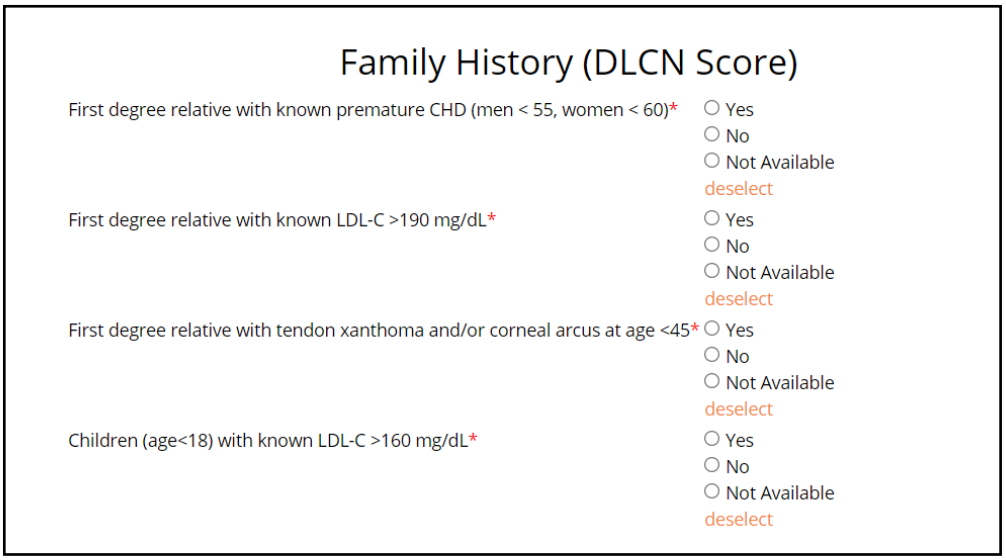


Figure 3.7 – "Family History (DLCN Score)" section

- *Clinical History (DLCN Score):* this is the last part related to the DLCN score and it takes into account the clinical history of the subject as the presence of a premature CHD, premature cerebral or peripheral vascular disease. Moreover, there is an additional parameter implemented for the Italian population related to hypercholesterolemia (LDL-C > 180 mg/dL) from

childhood, to account for the temporal exposure to elevated LDL-C in adults (**Figure 3.8**).

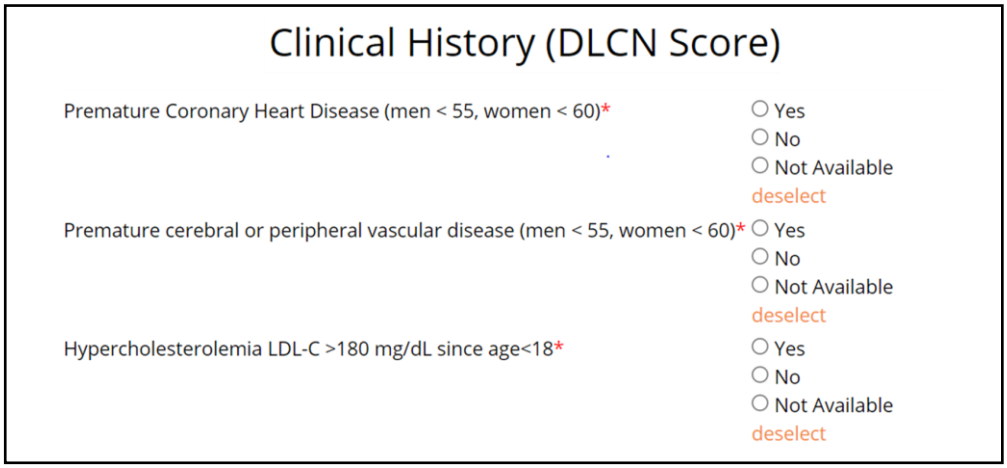


Figure 3.8 – "Clinical History (DLCN Score)" section

- *Biochemistry at baseline:* this part is dedicated to data collection about biochemistry values at baseline, if available: LDL-Cholesterol, total cholesterol, triglycerides, HDL-Cholesterol, lipoprotein(a), glucose and creatinine. The system automatically converts the value from mg/dL to mmol/L and *viceversa.* The year of the "date of sample" is used to calculate the age the baseline for each enrolled subject (**Figure 3.9**).

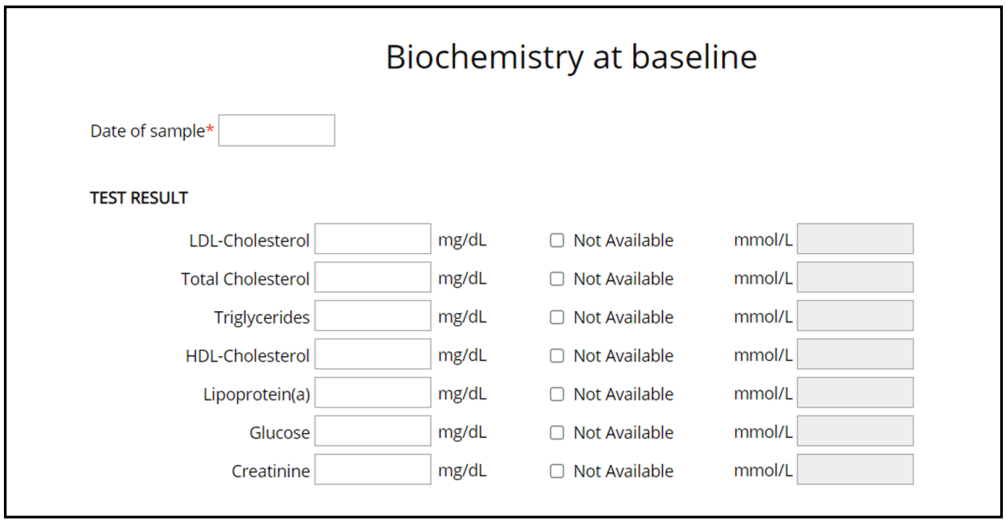


Figure 3.9 – "Biochemistry at baseline" section

- *Lipid Lowering Treatment:* here, investigator should indicate if the subject is on lipid lowering treatment at the moment of the biochemistry exams at baseline, in order to evaluate the lipid profile based on the therapy or not. If the answer to "Is the patient under lipid lowering treatment?" is "Yes", it is required to indicate the lipid lowering drug and the dosage. Moreover, a pre-treatment LDL-C value is necessary for the calculation of DLCN score. If an untreated LDL-C value is available, it could be entered in the "Highest LDL-C level pre-treatment" (**Figure 3.10**). If the value is unknown, the system automatically retro-calculates it using the therapy correction factors (**Appendix III**).

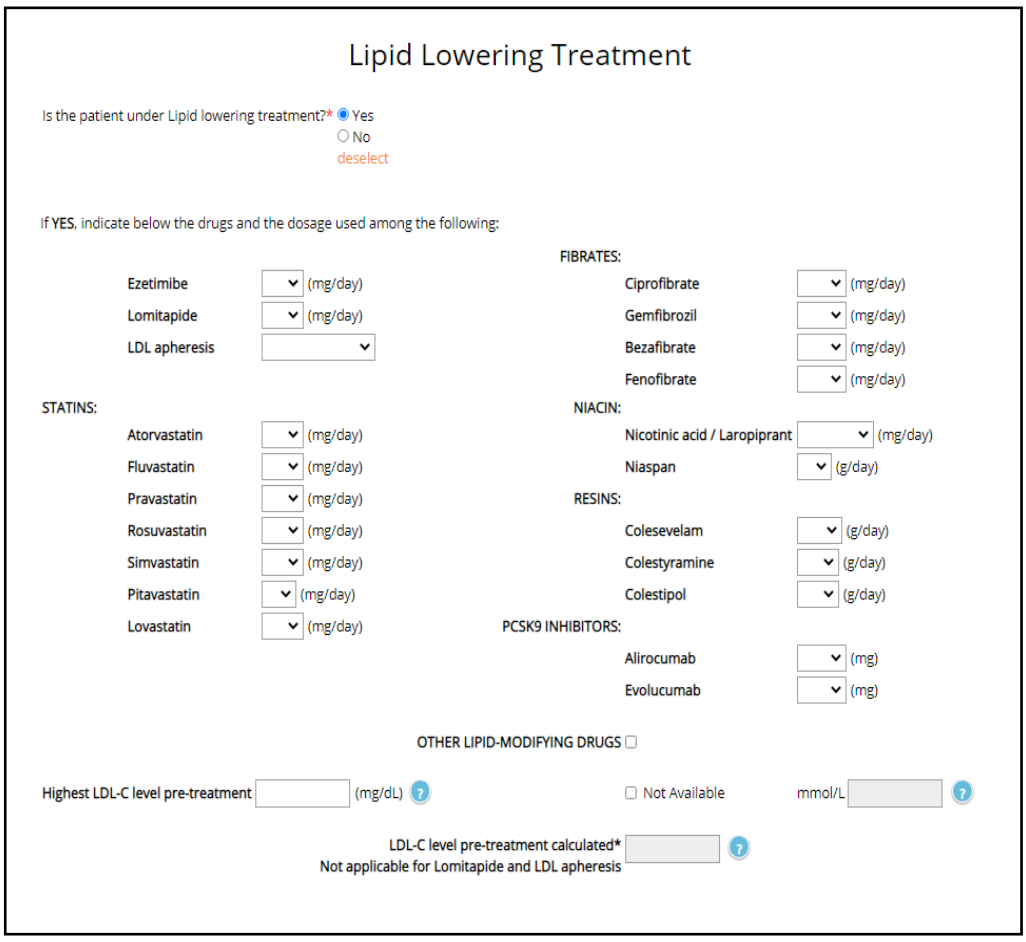


Figure 3.10 – "Biochemistry at baseline" section

- *DLCN Score:* This section summarizes all DLCN score parameters and the final score that allow to classify the clinical diagnosis of FH as unlikely (<3), possible (3-5), probable (6-8) or definite (>8). For all children with a suspicion of FH or adults with a DLCN score ≥6, the genetic test is recommended.

3) MOLECULAR DIAGNOSIS: this section reports the genetic results if the subject underwent the molecular test. The tested genes include *LDLR*, *APOB*, *PCSK9*, *LDLRAP1*, and *APOE*. Moreover, subjects can be tested for *LIPA* gene in order to identify the presence of rare case of LALD with a phenotype similar to FH. For each detected variant, the gene, the position of the variant (exon or intron), the variation of the amino acid and protein sequence, the genotype (homozygous or heterozygous), the type of mutation (SNV, SNP,CNVDUP, CNVDEL, INDEL) and the pathogenic classification are reported (**Figure 3.11**). Moreover, additional parameters as the LDL-C polygenic risk score (Talmud 2013) in subjects analysed after 2016 are reported.

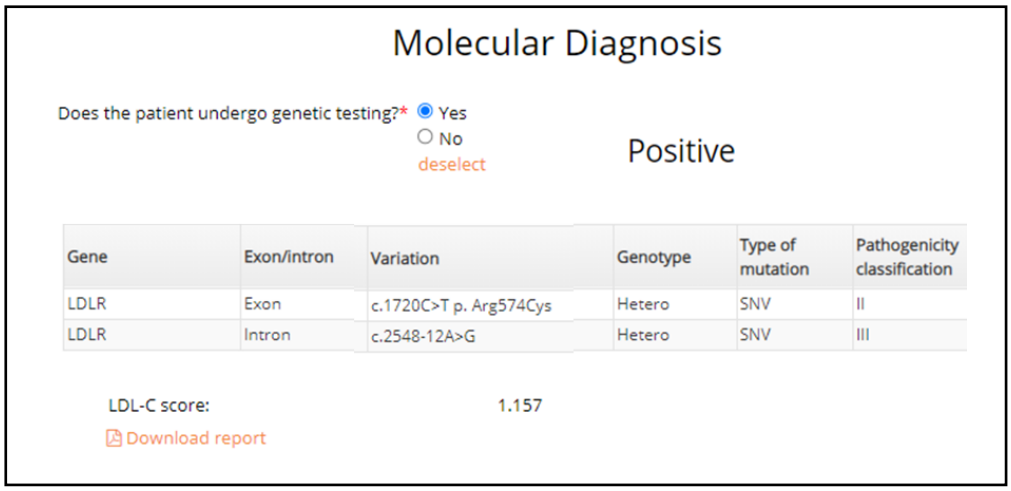


Figure 3.11 – "Molecular Diagnosis" section

The investigator can download the English version of the detailed genetic report, and also a simplified report in Italian that can be handed to the patient.

4) CONCOMITANT DISEASES OTHER THAN FH: in this section, the physician has to report the relevant concomitant diseases other than FH present at diagnosis or occurred after the diagnosis with the onset date (**Figure 3.12**).

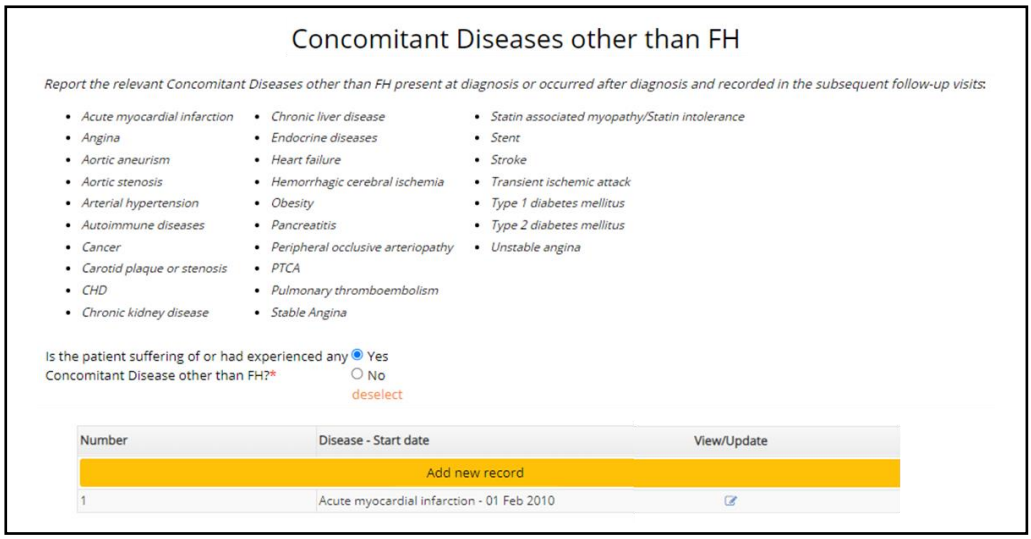


Figure 3.12 – "Concomitant Diseases other than FH" section

5) CONCOMITANT TREATMENTS OTHER THAN LIPID LOWERING: in this section, the physician reports if the patient is taking some concomitant treatments other than the lipid lowering drugs, such as antidepressant, antihypertensive, antithrombotic, hypoglycemic or immunosuppressive drugs (**Figure 3.13**).

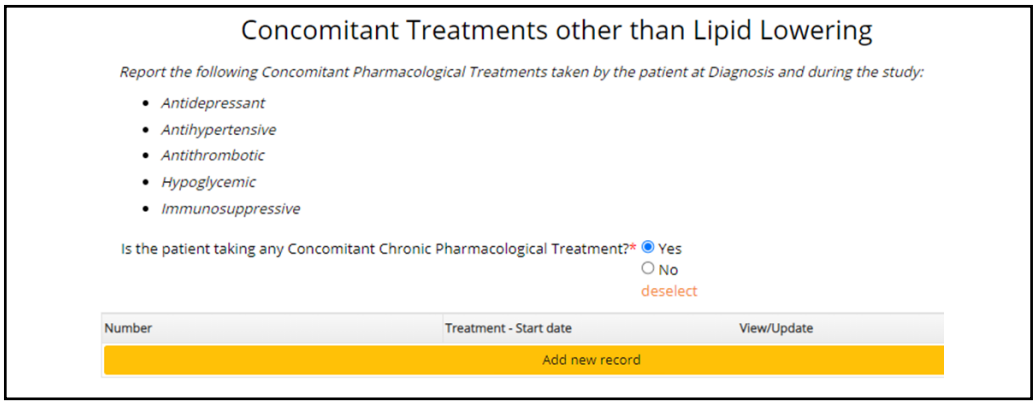


Figure 3.13 – "Concomitant Treatments other than Lipid Lowering" section

6) DEATH: this section reports information about patient's death (**Figure 3.14**).

Figure 3.14 – "Death" section

7) CHANGES IN LIPID LOWERING TREATMENTS AND BIOCHEMISTRY: follow-up sections were recently designed and implemented in the eCRF to record any significant changes in (i) anamnestic/anthropometric data, (ii) lipid lowering therapy and (iii) lipid profile (**Figure 3.15**).

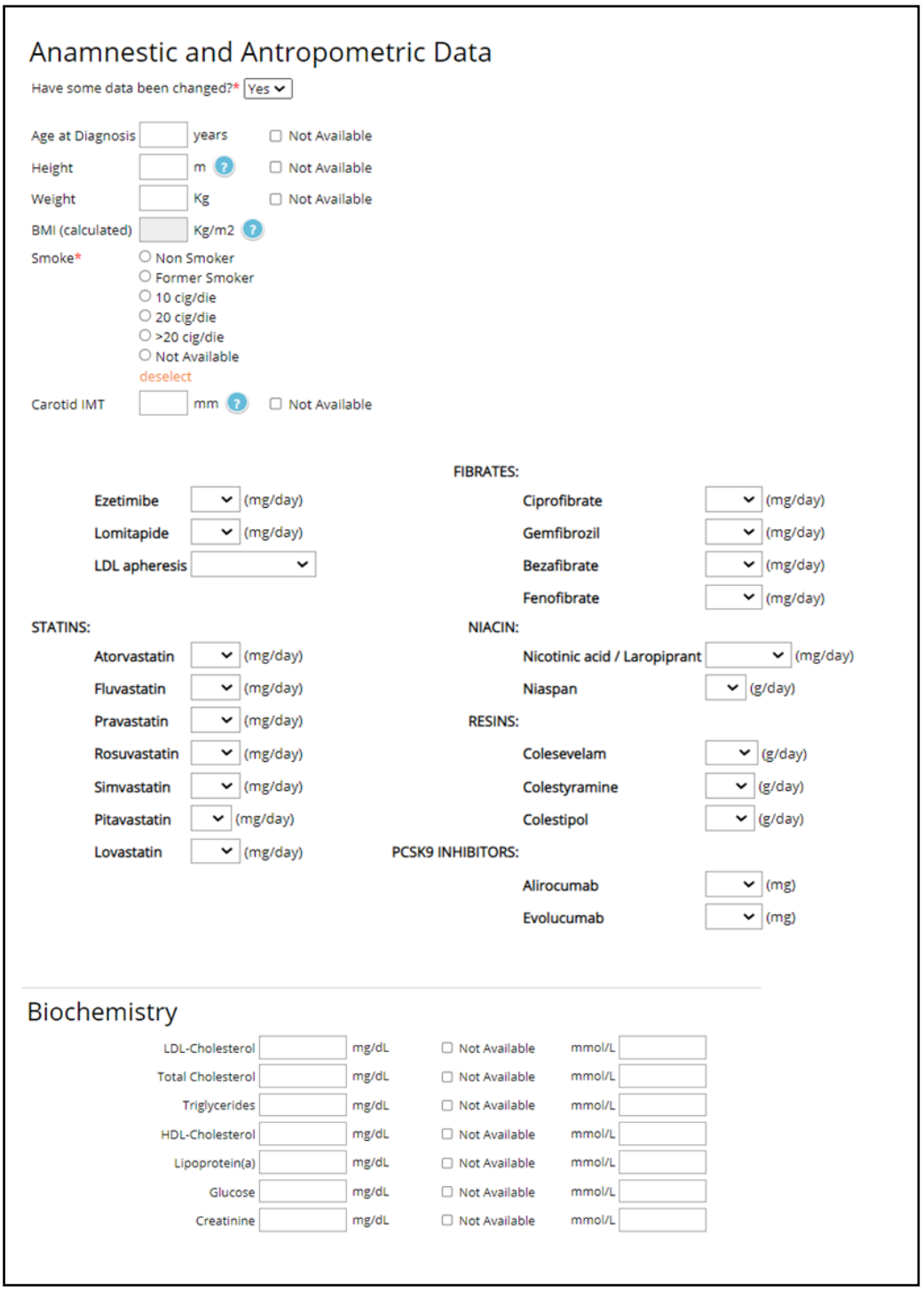


Figure 3.15 – "Changes in Lipid Lowering Treatment and Biochemistry" section

3.2.2.1 Changes and implementation in data collection

Additional parameters were collected for specific sub-studies as data about the Achilles tendon ultrasonography (see paragraph 3.3.2 ACTUS-FH sub-study). In this case, the data collection has been split including not only the tendon xanthoma detectable to physical examination (as collected in the DLCN section) but also the information about the presence of xanthomas detectable with the ultrasonography exam, defined as the presence of a single or multiple focal hypoechoic lesion within the tendon and/or the diffuse loss of tendon fibrillary structure. Moreover, also the evaluation of Achilles tendon thickness was collected, measuring it in three different points bilaterally, moving the ultrasound probe in caudocranial direction, from the calcaneal insertion to the junction with the soleus and the maximum thickness among that measured on the left and that measured on the right Achilles tendon was considered for the analyses.

Furthermore, changes and implementation in data collection were performed within the LIPIGEN Paediatric Group. Compared to adults, the diagnosis of FH in children and adolescents presents critical issues mainly related to their young age, usually associated to a less severe phenotype in the first decades of life, due to a limited long-life exposure to high concentrations of LDL-C. Moreover, there is a lack of diagnostic criteria validated in this sub-population, making the identification more difficult.

Based on this scenario, the LIPIGEN paediatric group suggested new key parameters to be added to the eCRF. Based on the clinical practice and knowledge of LDL-C fluctuations in childhood/adolescence, the LIPIGEN steering committee modified the LDL-C ranges taking into account the young age of these subjects. The cut-off and the points associated were modified as reported below:

- 100-130 mg/dL (point=1)
- 131-159 mg/dL (points=3)
- 160-189 mg/dL (points =5)
- ≥190 mg/dL (points =7).

In addition to these changes, an *ad hoc* section of the LIPIGEN eCRF was implemented to collect further information specific for paediatric FH (**Figure 3.16**). These new data include a more detailed gathering of:

- family members who presented a premature CHD, to extend the data collection to second degree relatives;
- levels of pre-treated LDL-C in both parents, to deepen the family history;

- pubertal development status, to evaluate the impact of puberty on lipid profile by age and sex;
- value of waist circumference, to discriminate an obesity/metabolic syndrome condition that could modify the lipid profile without a real FH condition.

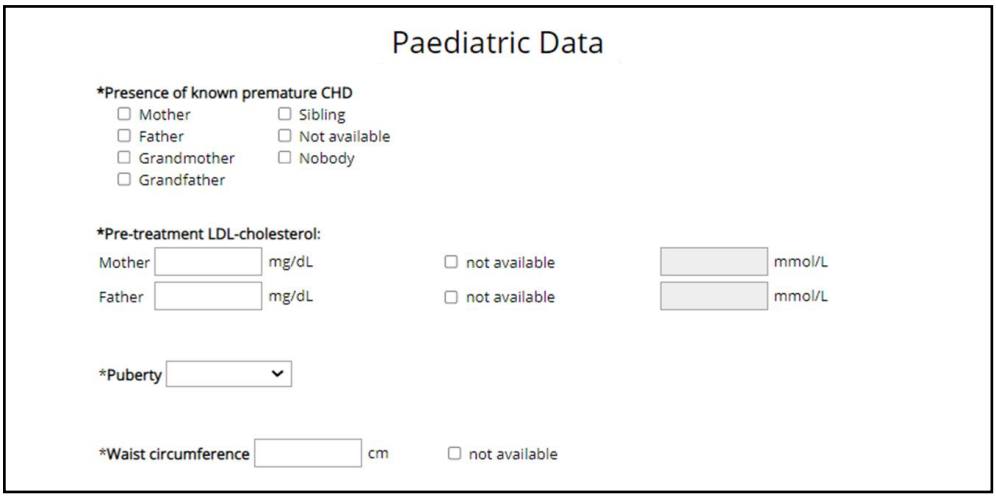


Figure 3.16 – "Paediatric Data" section

3.3 Cohort profile

The eCRF proved to be an effective method for data collection. As required by good clinical practice, all data were controlled and verified with local investigators if inconsistencies were detected during the data cleaning process.

3.3.1 The whole LIPIGEN cohort

At June 2021, the LIPIGEN study accounted for 8730 subjects enrolled by 45 LIPIGEN centers distributed throughout the Italian territory. As reported in **Table 3.3**, the number of enrolled patients largely varies from the highest numbers of historical lipid centers, to the smaller centers that started to manage hypercholesterolemic patients in the last years or have recently obtained the ethics committee approval.

Table 3.3 – Patients entered in eCRF by LIPIGEN lipid centers

Center Code	Frequency (N)	Percentage (%)
ITA-AN-01	108	1.2
ITA-AQ-01	58	0.7
ITA-AT-01	44	0.5
ITA-BA-02	109	1.2
ITA-BG-01	15	0.2
ITA-BO-01	157	1.8
ITA-CA-01	69	0.8
ITA-CH-01	113	1.3
ITA-CT-01	271	3.1
ITA-CZ-01	209	2.4
ITA-FE-01	331	3.8
ITA-FI-01	17	0.2
ITA-FR-01	22	0.3
ITA-GE-01	1409	16.1
ITA-ME-01	135	1.5
ITA-MI-01	391	4.5
ITA-MI-02	496	5.7
ITA-MI-03	63	0.7
ITA-MI-04	23	0.3
ITA-MI-05	346	4.0
ITA-MI-06	141	1.6
ITA-MO-01	324	3.7
ITA-MO-02	20	0.2
ITA-NA-02	516	5.9
ITA-NA-03	55	0.6
ITA-PA-01	800	9.2
ITA-PC-01	42	0.5
ITA-PD-01	284	3.3
ITA-PG-01	86	1.0
ITA-RE-01	7	0.1
ITA-RM-01	922	10.6
ITA-RM-02	107	1.2
ITA-RM-05	360	4.1
ITA-RM-06	76	0.9
ITA-RM-07	27	0.3
ITA-SA-01	33	0.4
ITA-TN-01	43	0.5
ITA-TO-01	41	0.5
ITA-TO-02	168	1.9
ITA-TO-03	13	0.1
ITA-TS-01	26	0.3
ITA-VA-01	39	0.4
ITA-VB-01	20	0.2
ITA-VE-01	9	0.1
ITA-VR-01	185	2.1
TOTAL	**8730**	100.0

Based on available data, the LIPIGEN cohort is composed by 79.9% adults, and the percentages of males and females are comparable (47.2% and 52.8%, respectively). As expected, almost the whole cohort is Caucasian, with also 47 Hispanic/Latino, 41 Black, 28 Asian, and 6 Indian subjects. Enrolled subjects are mainly from Sicily (N=1290), Lazio (N=1146), and Lombardy (N=1068). Moreover, 338 subjects come from Countries other than Italy (**Figure 3.17**). About 85% of patients are index cases (first FH subject identified in a family) while the remaining ones are relatives of LIPIGEN patients already entered in the eCRF. The deepening of this information is important to better identify mutation clusters, providing relevant studies on the different clinical impact of the most frequent mutations and conjecturing some speculation related to the historical aspects of the origin of detected variants (Bertolini 2017).

Figure 3.17 – Geographical origin of LIPIGEN subjects

3.3.2 LIPIGEN Adults

3.3.2.1 Methods

The analyses were conducted on all confirmed LIPIGEN adults entered in eCRF. Parameters collected in the normal clinical practice were analyzed in the study population as age at

diagnosis of FH, anthropometric and demographic data (sex, weight, height, BMI, etc), biochemical profile (triglycerides, total cholesterol, LDL-C, HDL-c, and pretreatment LDL-C), presence of a LLT at baseline and genetic diagnosis, as reported in the paragraph "Data Collection". Interpretation and classification of pathogenicity of the variants were determined following the rules published by the American College of Medical Genetics and Genomic (Richards 2015). Comparisons among the adult cohort were performed stratifying by LLT or genetic results. Continuous variables are expressed as mean ± standard deviation (SD) or median with the interquartile range, whereas categorical variables are presented as cases (N) and percentage rate (%).

3.3.2.2 Demographic and biochemistry data

The LIPIGEN study accounts for a total of 6674 adults, with a mean age at the first visit (baseline) of 47.1±14.6 years (**Figure 3.18**), subjects aged 18-40 years represent the 33.4% of the whole cohort and 37.1% of the patients are 41-60 years old.

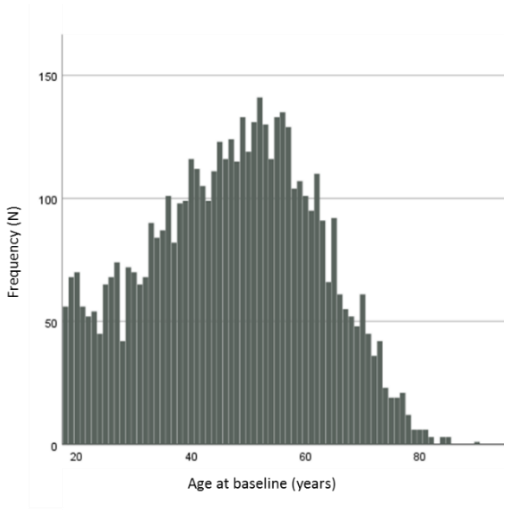


Figure 3.18 – Distribution of age at baseline in the LIPIGEN adult cohort

The lower age at diagnosis (37.3±15.9 years) compared to age at baseline confirms that many patients had a previous clinical diagnosis of FH. However, the majority of them underwent the genetic test once they have been enrolled in the study. Overall, 88.7% of adults were index cases; the percentage of females was slightly higher than males (53.3% vs 46.7%, respectively). The mean height and weight of LIPIGEN adults were 1.67 m and 71.4 kg, respectively and more than half the cohort presented a BMI lower than 25 kg/m^2 (**Table 3.4**).

Table 3.4 – Characteristics of LIPIGEN adult cohort

Characteristic	Value
Males, N (%)	3548 (53.3%)
Height [m], mean±SD	1.67±0.1
Weight [Kg], mean±SD	71.4±14.3
BMI [Kg/m^2], mean±SD	21.2±9.9
BMI classes	
<25, N (%)	3004 (53.1)
25-30, N (%)	1992 (35.2)
>=30 N (%)	661 (11.7)
Age at diagnosis [years], mean±SD	37.3±15.9
Age at baseline [years], mean ± SD	47.1±14.6
Age at baseline classes	
18-30 years old, N (%)	792 (15.7)
31-40 years old, N (%)	890 (17.7)
41-50 years old, N (%)	1157 (22.9)
51-60 years old, N (%)	1227 (24.3)
61-70 years old, N (%)	731 (14.5)
>70 years old, N (%)	245 (4.9)

In both treated and untreated adults, the mean levels of TC, HDL-c, TG and LDL-C were 304.1±88.7 mg/dL, 55.3±15.3 mg/dL, 108.0 [180-153] mg/dL, and 225.6±89.8 mg/dL respectively (**Table 3.5**). The pre-treatment LDL-C value was 267.2±93.7 mg/dL, higher in subjects that already were on LLT (281.0±98.2 mg/dL) than the untreated patients (256.2±88.4 mg/dL). For treated subjects with unknown LDL-C levels (N=1341), it was estimated with the correction factors, although this could lead to an overestimation of LDL-C real value (Casula 2018).

Table 3.5 – Lipid profile of LIPIGEN adult cohort

	All	on LLT	Untreated
Total Cholesterol [mg/dL], mean±SD	304.1±88.7	257.7±85.6	333.9±80.6
Triglycerides [mg/dL], median [IQR]	108.0 [80.0-153.0]	104.5 [77.0-150.0]	114.0 [81.5-160.0]
HDL-C [mg/dL], mean±SD	55.3±15.3	55.5±14.9	56.1±15.9
LDL-C [mg/dL], mean±SD	225.6±89.8	176.7±78.8	256.1±88.1
Pre-treatment LDL-C [mg/dL], mean±SD	267.2±93.7	281.0±98.2	256.2±88.4

3.3.2.3 Genetic results and genotype-phenotype correlation

The genetic results were obtained for 5748 adults, identifying 3980 subjects with at least one causative mutation, while the 10.3% of patients only presented VUS. The 20.5% of adults did not present any mutations in the tested genes (**Figure 3.19**).

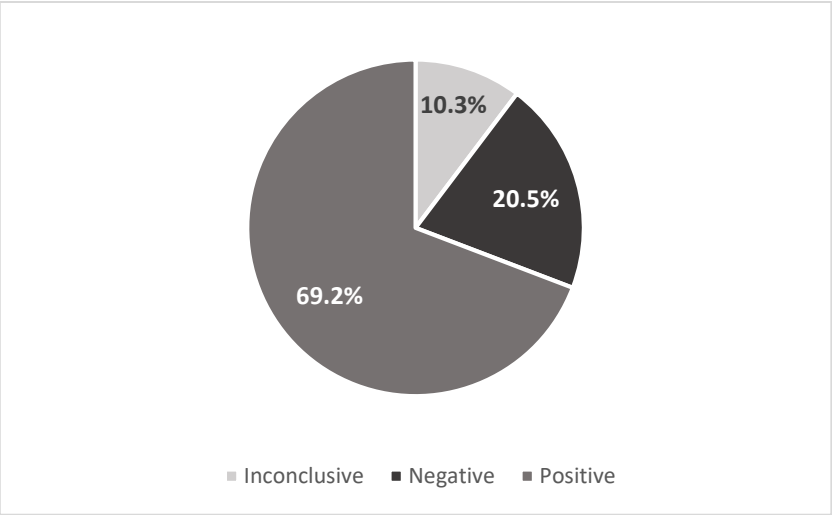


Figure 3.19 – Percentage of adults with a positive (N=3980, 69.2%), negative (N=1176, 20.5%) or inconclusive (N=592, 10.3%) genetic results

Subjects with a positive genetic diagnosis were younger compared to the negative cohort (44.4 years vs 50.8 years) and presented higher levels of pretreatment LDL-C than negative group (296.6±94.9 mg/dL vs 227.0±63.7 mg/dL), while the group with inconclusive diagnosis showed intermediate features. (**Table 3.6**).

Table 3.6 – Lipid profile of LIPIGEN adult cohort

	Positive	Inconclusive	Negative
Age at baseline [years], mean±SD	44.4±15.1	49.5±13.4	50.8±13.2
Total Cholesterol [mg/dL], mean±SD	323.1±89.4	274.5±73.7	268.7±75.2
Triglycerides [mg/dL], median [IQR]	101.0 [75.0-144.0]	115.0 [84.0-166.0]	124.0 [91.0-172.0]
HDL-C [mg/dL], mean±SD	53.3±14.3	57.8±16.1	58.9±16.3
LDL-C [mg/dL], mean±SD	249.2±92.8	190.3±7+68.1	180.5±65.0
Pre-treatment LDL-C [mg/dL], mean±SD	296.6±94.9	229.4±70.5	227.0±63.7
On lipid lowering therapy, %	40.7	40.8	46.1

Among the 3980 subjects with a positive diagnosis of FH, 3862 individuals presented one causative mutation in *LDLR* (N=3774), *APOB* (N=64), *PCSK9* (N=19), or *APOE* genes, while the others had at least two causative variants: 48 *LDLR* homozygotes, 55 compound heterozygotes, 10 double heterozygotes. Three unrelated subjects had three different mutations in *LDLR* gene (**Table 3.7**). Moreover, a diagnosis of autosomal recessive hypercholesterolemia (ARH) was formulated in three unrelated Sicilian subjects. Finally, in 5 subjects with a clinical suspicion of FH, only causative mutations on LIPA gene were detected, suggesting a diagnosis of Lysosomal Acid Lipase Deficiency (See paragraph 3.5.3).

Table 3.7 – Stratification of genetically confirmed FH subjects by causative mutated gene

Causative gene variants	Frequency (N)	Mean untreated LDL-C levels (mg/dL)
LDLR Heterozygous	3774	292±85
APOB Heterozygous	64	245±66
LDLR Compound Heterozygous	55	414±160
LDLR Homozygous	48	507±199
PCSK9 Heterozygous	19	236±71
Double Heterozygous *LDLR - PCSK9*	7	289±82
APOE Heterozygous	4	251±45
LDLR Three mutations	3	371±159
LDLRAP1 Homozygous	3	321±89
Double Heterozygous *LDLR - APOB*	1	/
Double Heterozygous *LDLR - APOE*	1	/
Double Heterozygous *LDLR - LIPA*	1	/

- <u>*LDLR* mutation carriers</u>

More than 400 *LDLR* gene variants were identified in the 3774 *LDLR* heterozygotes subjects, with a mean level of untreated LDL-C of 292±85 mg/dL, higher than *APOB* 245±66 mg/dL or *PCSK9* 236±71 mg/dL heterozygotes but lower than the values that were detected in *LDLR* homozygotes, *LDLR* compound heterozygotes or double heterozygotes (a more in-deph analysis is reported in the paragraph 3.5.1). The three most detected mutation in adults are c.662A>G p.Asp221Gly in 369 subjects, c.1646G>A p.Gly549Asp in 244 subjects and c.1775G>A p.Gly592Glu in 228 subjects. Stratifying the twenty most detected mutations by LDLR residual activitiy, the pre-treatment LDL-C was significantly higher in the carriers of a null mutation compared to carriers of a defective one (302.3±85.6 vs 281.9±79.8 mg/dL, $p<0.001$). The twenty most detected variants are reported in the **Table 3.8.**

Table 3.8 – The twenty most detected causative mutation in *LDLR* gene and their impact on the LDLR residual activity

***LDLR* gene variant**	**Protein change**	**LDLR residual activity**	**N of subjects**
c.662A>G	p.Asp221Gly	Defective	369
c.1646G>A	p.Gly549Asp	Null	244
c.1775G>A	p.Gly592Glu	Defective	228
c.1567G>A	p.Val523Met	Defective	175
c.1415_1418dupACAT	p.Gln474Hisfs	Null	130
c.2054C>T	p.Pro685Leu	Defective	101
c.313+1G>A	p.Leu64_Pro105delinsSer	Defective	93
c.1257C>G	p.Tyr419*	Null	85
c.2312-3C>A	p.Ala771_Ile796del	Defective	84
c.682G>A	p.Glu228Lys	Null	77
c.304C>T	p.Gln102*	Null	68
c.1586+1G>A	p.Thr454_Gly529del, p.Gly529_Phe530ins22	Null	61
c.1135T>C	p.Cys379Arg	Defective	59
c.68-?_1845+?del	p.Val2Glyfs*29	Null	53
c.1735G>T	p.Asp579Tyr	Null	44
c.1846-?_2311+?del	p.Asp616Leufs*17	Null	40
c.1778delG	p.Gly593Alafs*72	Null	35
c.858C>A	p.Ser286Arg	Defective	34
c.352G>T	p.Asp118Tyr	Defective	34
c.367T>C	p.Ser123Pro	Defective	29

- *APOB* Heterozygous carriers

In the APOB heterozygotes, 6 different causative variants were detected. The most common was c.10580G>A p.Arg3527Gln in 55 subjects, followed by another single nucleotide variation that determined the c.10579C>T p.Arg3527Trp in 4 subjects. The other APOB causative variants are reported in the **Table 3.9**.

Table 3.9 – *APOB* gene causative variants

***APOB* gene variant**	**Protein change**	**N of subjects**
c.10580G>A	p.Arg3527Gln	55
c.10579C>T	p.Arg3527Trp	4
c.10136A>G	p.Tyr3379Cys	2
c.13315_13319del	p.Gln4439*	1
c.13480_13482del	p.Gln4494del	1
c.9221A>G	p.Tyr3074Cys	1

- *PCSK9* Heterozygous carriers

As expected, only few PCSK9 heterozygotes were detected and the majority of them were carriers of the gain-of-function variant (GOF) c.60_65dupGCTGCT p.(Leu22_Leu23dup) or c.1399C>G p.(Pro467Ala) (**Table 3.10**).

Table 3.10 – *PCSK9* gene causative variants

***PCSK9* gene variant**	**Protein change**	**N of subjects**
c.60_65dupGCTGCT	p.Leu22_Leu23dup	7
c.1399C>G	p.Pro467Ala	5
c.-331C>A	.	2
c.103G>T	p.Asp35Tyr	2
c.1394C>T	p.Ser465Leu	2
c.381T>A	p.Ser127Arg	1

- *APOE* Heterozygous carriers

The only causative variant in the APOE gene was detected in 3 subjects from Abruzzo (two of them are relatives) and in one Sicilian adult; it consists in the in-frame three base-pair deletion c.500_502del p.Leu167del and the mean pre-treatment LDL-C value was 251.2±44.7 mg/dL.

3.3.2.4 Lipid lowering therapy at baseline

Among the LIPIGEN adults, 2331 subjects were already on a lipid lowering therapy and about 27% of them were in secondary prevention. More than 85% of patients on LLT was treated with a statin, alone (N=1032) or in combination (N=1009) with another lipid lowering drug. The most used statin was rosuvastatin (N=983), followed by atorvastatin (N=614), simvastatin (N=348), pravastatin (N=52), lovastatin (N=27) and fluvastatin (N=20). The combination therapy was mainly characterized by the association statin-ezetimibe (about 97%) with also the addition of PSCK9 inhibitors in 64 patients (**Table 3.11**). Overall, 123 subjects (more than 50% in secondary prevention) were on PSCK9 inhibitors: 56 were treated with evolocumab 140 mg while the others with alirocumab at 75 o 150 mg; one third of subjects in secondary prevention reached a LDL-C level lower than 55 mg/dL and about the 50% a LDL-C level lower than 70 mg/dL. Three subjects were treated with lomitapide at

dosage of 10 mg/die in association with atorvastatin 80 mg/die for one patient in secondary prevention or rosuvastatin 40 mg/die plus ezetimibe 10 mg/die in primary prevention.

Table 3.11 – Type of lipid lowering therapy at the moment of the first visit in one of the LIPIGEN site

One lipid lowering drug	**N of subjects (N,%)**
Statin	1032 (44.3)
Ezetimibe	127 (5.4)
Nutraceuticals	75 (3.2)
PCSK9 inhibitors	25 (1.1)
Fibrates	17 (0.7)
Resins	6 (0.3)
Apheresis	2 (0.1)
Combination therapy	
Statin + ezetimibe	890 (38.2)
Statin + ezetimibe + PSCK9 inhibitors	64 (2.7)
Statin + ezetimibe + other LLT excluded PSCK9 inhibitors	31 (1.3)
Ezetimibe + PSCK9 inhibitors	28 (1.2)
Statin + other LLT excluded ezetimibe	27 (1.2)
Ezetimibe + non statinic drug	7 (0.3)

Overall, the mean pre-treatment LDL-C level was 281.2±97.4 mg/dL reaching a mean value of 159.3±63.1 mg/dL during therapy at the first visit in a LIPIGEN site.

Stratifying the treated subjects by type of lipid lowering therapy (PCSK9 inhibitors alone or in combination [N=99], statin plus ezetimibe [N=844] or only statin [N=975]), higher levels of pretreatment LDL-C were detected in this first group (306±145 mg/dL) compared to the levels of subjects treated with the combination statin-ezetimibe (296±98 mg/dL) or statin alone (274±85 mg/dL) (**Table 3.12, panel A**). As expected, slightly lower mean pretreatment LDL-C levels were reported excluding the subjects with only a retro-calculated LDL-C due to the overestimation of therapy correction factors: 275±91mg/dL [N=49], 281±78 mg/dL [N=308] and 260±66 mg/dL [N=368] (**Table 3.12, panel B**). Overall, the treatment initiation before the first visit in one of the specialized LIPIGEN clinic allow obtaining a percentage reduction in LDL-C concentration of 55.2%, 51.0% and 39.8%, respectively. However, only a limited proportion of subjects has already reached a value in LDL-C lower than 100 mg/dL: 37.7%, 22.6%, and 10.0%, respectively and lower percentage were detected considering a LDL-C <70 mg/dL 32.5%, 5.0% and 1.5%, respectively.

Table 3.12 - Variation in LDL-C levels among subjects already treated at the baseline visit in one of the LIPIGEN site. The panel A included all subjects treated with only statin, statin plus ezetimibe or with PCSK9 alone or in combination; in the panel B, subjects with a retro-calculated pretreatment LDL-C value were excluded.

(A)

	Statin alone (N=975)	**Statin plus ezetimibe** (N=844)	**PCSK9 alone or in combination** (N=99)
Pre-treatment LDL-C [mg/dL], mean±SD	274 ± 85	296 ± 98	306 ± 145
Baseline LDL-C on LLT	165 ± 58	145 ± 59	137 ± 91
Mean LDL-C percentage reduction (%)	39.8	51.0	55.2
Subjects with LDL-C on LLT < 100 mg/dL	10.0	22.6	37.7
Subjects with LDL-C on LLT < 70 mg/dL	1.5	5.0	32.5

(B)

	Statin alone (N=368)	**Statin plus ezetimibe** (N=308)	**PCSK9 alone or in combination** (N=49)
Pre-treatment LDL-C [mg/dL], mean±SD	260 ± 66	281 ± 78	275 ± 91
Baseline LDL-C on LLT	161 ± 68	146 ± 65	127 ± 80
Mean LDL-C percentage reduction (%)	38.1	48.04	53.82
Subjects with LDL-C on LLT < 100 mg/dL	13.4	26.8	39.8
Subjects with LDL-C on LLT < 70 mg/dL	1.7	7.5	33.3

3.3.3 LIPIGEN children and adolescents

3.3.3.1 Methods

The analyses about children and adolescents affected by FH were conducted within the LIPIGEN Paediatric group. To be included, subjects should present a difference between the year of the first biochemistry in the eCRF (age at baseline) and the year of birth date lower than 18, with at least one pre-treatment LDL-C value and data about performing of the genetic testing or not. Based on these criteria, from 1679 subjects under 18 years of the whole cohort, 1520 children/adolescents were selected and analyzed.

As for adult cohort, parameters collected in the normal clinical practice were analyzed taking into account also the ones implemented in the eCRF paediatric section.

Anthropometric, anamnestic and demographic data, biochemical profile (triglycerides, total cholesterol, LDL-C, HDL-C, and pretreatment LDL-C), presence of an LLT at baseline and

genetic diagnosis were analyzed. Age classes at baseline (0-5 years, 6-10 years, 11-13 years, 14-17 years) were applied to analyse the study population taking into account the physical changes during growth. Interpretation and classification of pathogenicity of the variants were determined following the rules published by the American College of Medical Genetics and Genomic (Richards 2015).

Continuous variables are expressed as mean ± standard deviation (SD) or median with the interquartile range, whereas categorical variables are presented as cases (N) and percentage rate (%).

3.3.3.2 Demographic and biochemistry data

As for the entire LIPIGEN cohort, the proportion of males and females were comparable (49.5% and 50.5%, respectively) while the percentage of index cases decreased because a higher number of children were identified after the detection of causative mutation in their parents already enrolled in the LIPIGEN study: two thirds of the paediatric population were FH index cases while the others were identified by cascade screening, with no difference in the age at baseline (9.7±4.0 vs 10.0±4.3 years p=0.158).

The mean (±SD) age at baseline was 9.8 ± 4.1 years, and the study population was composed by 269 (17.7%) subjects within the age class 0-5 years, 574 (37.8%) in the class 6-10 years, 356 (23.4%) of 11-13 years and 321 (21.1%) of 14-17 years (**Table 3.13**).

Table 3.13 – Characteristics of LIPIGEN paediatric cohort

Characteristic	Value
Male, N (%)	753 (49.5)
Index cases, N (%)	1016 (66.8)
Age at baseline [years], mean ± SD	9.8 ± 4.1
Age class 0-5, N (%)	269 (17.7)
Age class 6-10, N (%)	574 (37.8)
Age class 11-13, N (%)	356 (23.4)
Age class 14-17, N (%)	321 (21.1)
LDL Cholesterol [mg/dL], N; mean ± SD	1520; 222.0±98.6
Total Cholesterol [mg/dL], N; mean ± SD	1498; 283.1±97.1
HDL Cholesterol [mg/dL], N; mean ± SD	1488; 53.3±13.6
Triglycerides [mg/dL], N; median [IQR]*	1483; 75.0 [58.0-100.0]

* IQR interquartile range

For what concerns the lipid profile, the values of total cholesterol (mean±SD), HDL-C (mean±SD) and triglycerides (median [IQR]) were 283.1±97.1 mg/dL, 53.3±13.6 mg/dL and 75.0 [58.0-100.0] mg/dL, respectively. The untreated LDL-C levels (mean±SD) were 222.0±98.6 mg/dL and about 90% of individuals in each age group presenting LDL-C> 130 mg/dL.

3.3.3.3 Genetic results and genotype-phenotype correlation

Almost the whole cohort (95.7%) underwent the genetic test. The results showed that about 70% of individuals presented at least one causative mutation while in 119 individuals only VUS were detected, receiving an inconclusive diagnosis of FH.
In the others 308 children/adolescents, no known genetic variants were identified to explain the clinical phenotype (**Figure 3.20**).

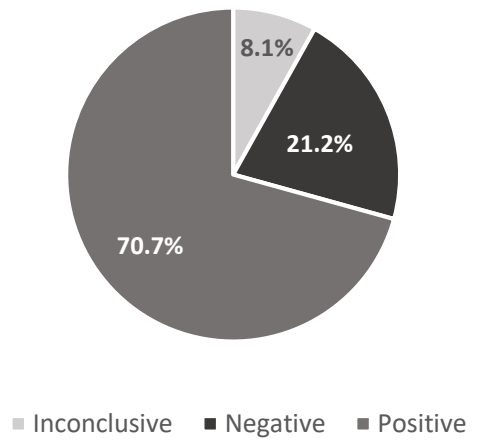


Figure 3.20 – Percentage of paediatric subjects with a positive (N=1027, 70.7%), inconclusive (N=119, 8.1%) or negative (N=308, 21.2%) genetic result

Subjects with a positive genetic diagnosis presented higher level of LDL-C compared to subjects with only VUS or negative diagnosis (248.9 ± 104.0 mg/dL vs 166.3 ± 54.3 mg/dL vs 159.8 ± 48.2 mg/dL, p<.0001). Stratifying the mean LDL-C levels by genetic diagnosis and by age classes, LDL-C levels decrease with increasing age in subjects with positive or negative genetic diagnosis (**Table 3.14**).

Table 3.14 – Mean LDL-C levels stratified by genetic diagnosis and age classes

		Genetic diagnosis			All
		Positive	Inconclusive	Negative	
LDL-C [mg/dL] (mean ± SD)	0-5 y	271.8±125.2	182.7±50.6	174.1±59.6	248.3±118.5
	6-10 y	253.3±114.2	159.0±40.9	158.8±47.0	222.4±106.2
	11-13 y	237.7±88.2	169.1±50.3	156.1±43.5	207.4±83.5
	14-17 y	232.8±72.0	164.0±68.5	157.5±49.5	215.7±75.2

Among subjects with a positive diagnosis of FH (N=1027), 988 individuals presented one causative mutation in *LDLR* (N=973) or *APOB* (N=15) genes, while in 39 subjects two causative mutations were detected (17 LDLR homozygotes, 17 LDLR compound heterozygotes and 5 double heterozygotes) (**Table 3.15**).

Table 3.15 – Stratification of genetically confirmed FH subjects by causative mutated gene

Causative gene variants	Frequency (N)
LDLR Heterozygous	973
LDLR Homozygous	17
LDLR Compound Heterozygous	17
APOB Heterozygous	15
Double Heterozygous LDLR - APOB	4
Double Heterozygous LDLR - PCSK9	1

Stratifying the positive cohort by number of causative variants/alleles (N=1: *LDLR* heterozygous and *APOB* heterozygous, N>1: *LDLR* Homozygous, *LDLR* Compound Heterozygous, Double Heterozygous *LDLR* – *APOB*, Double Heterozygous *LDLR* - *PCSK9*), the difference of LDL-C value among the age classes attenuated, compared (**Table 3.16**) to the difference in the whole cohort (**Table 3.13**). This suggests that the difference in LDL-C is strongly influenced by the type of genetic defect. However, the variation in LDL-C needs to be deepened once all data about puberty will be collected, in order to confirm the influence of hormonal development on the LDL-C concentration.

Table 3.16 – LDL-C levels stratified by number of causative variants (1 of >1) among age classes

	Causative variants			
	1 causative variant		>1 causative variant	
	N of subjects	LDL-C [mg/dL] mean ± SD	N of subjects	LDL-C [mg/dL] mean ± SD
0-5 years	185	246.4 ± 55.1	13	607.5 ± 255.6
6-10 years	361	233.9 ± 59.5	17	665.3 ± 197.6
11-13 years	212	229.0 ± 58.6	4	699.8 ± 161.8
14-17 years	230	229.1 ± 56.3	5	399.5 ± 294.6)
Total	988	234.1 ± 58.0	39	615.3 ± 237.7

- LDLR Homozygous, LDLR Compound Heterozygous, Double Heterozygous

In the 39 LIPIGEN children or adolescents with more than one causative variant, the mean LDL-C value was 615.3 ± 237.7 mg/dL, higher in the homozygous FH (731.7±154.0 mg/dL; min 461 mg/dL max 1029 mg/dL) compared to compound (629.1 ± 216.9 mg/dL; min 284 mg/dL, max 1100 mg/dL) and double heterozygous subjects (222.3 ± 209.0 mg/dL). Twelve distinct causative variants were detected in the homozygous FH (**Table 3.17**), while the different mutations in compound or double heterozygous FH are reported in the **Table 3.18** and **3.19**.

Table 3.17 – Homozygotes for LDLR gene variants

***LDLR* gene variant**	**Protein change**	**N of subjects**
c.1646G>A	p.Gly549Asp	3
c.68-?_1845+?del	p.Val23Glyfs*29	2
c.1567G>A	p.Val523Met	2
c.2054C>T	p.Pro685Leu	2
c.373C>T	p.Gln125*	1
c.671A>G	p.Asp224Gly	1
c.940_940+14del	p.Ser306Aspfs*17	1
c.1056C>G	p.Cys352Trp	1
c.1109A>C	p.Asn370Thr	1
IVS10 c.1586+1G>A	p.Thr454_Gly529del, p.Gly529_Phe530ins22	1
IVS15 c.2311+1G>A	p.[Gln770_Ala771ins30, Lys730fs*17, Thr713_Ala771del]	1
IVS16 c.2390-1G>A	p.Val797Alafs*155	1

Table 3.18 – Compound Heterozygotes carriers of two *LDLR* gene variants

***LDLR* gene 1st variant**	**Protein**	***LDLR* gene 2nd variant**	**Protein**	**N of subjects**
c.1646G>A	p.Gly549Asp	c.81C>G	p.Cys27Trp	3
c.1646G>A	p.Gly549Asp	c.1846-?_2311+?del	p.Asp616Leuf*17	2
c.1775G>A	p.Gly592Glu	c.1135T>C	p.Cys379Arg	2
c.268G>A	p.Asp90Asn	c.666C>A	p.Cys222*	1
c.352G>T	p.Asp118Tyr	c.418G>T	p.Glu140*	1
c.1068T>A + c.1069_1086dup	p.Asp356Glu/Glu357_Asp362dup	c.1846-1894_2140+1498del	p.Asp616Argfs*16	1
c.1118G>A	p.Gly373Asp	c.126C>G,	p.Tyr42*	1
c.1135T>C	p.Cys379Arg	c.1567G>A	p.Val523Met	1
c.1230G>C	p.Arg410Ser	c.1478_1479del	p.Ser493Cysfs*42	1
c.1472C>A	p.Thr491Asn	c.535G>A	p.Glu179Lys	1
c.1775G>A	p.Gly592Glu	c.265T>C	p.Cys89Arg	1
c.1775G>A	p.Gly592Glu	c.407A>T	p.Asp136Val	1
c.1775G>A	p.Gly592Glu	c.694+4_5insT	p.Ala232Glyfs*28	1

Table 3.19 – Double Heterozygotes for *LDLR* and *APOB* (N=4) or *LDLR* and *PCSK9* (N=1) gene variants

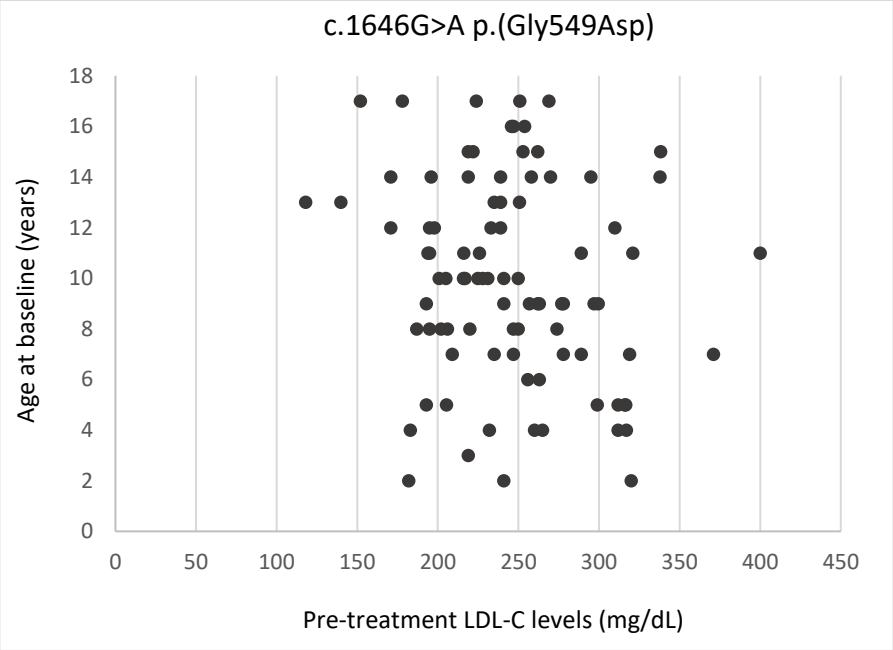
Gene 1st	***LDLR* gene variant**	**Protein**	**Gene 2nd**	***APOB*/*PCSK9* gene variant**	**Protein**
LDLR	c.662A>G	p.Asp221Gly	APOB	c.10580G>A	p.Arg3527Gln
LDLR	c.681C>G	p.Asp227Glu	APOB	c.3400A>G	p.Arg1134Gly
LDLR	c.1135T>C	p.Cys379Arg	APOB	c.13320delT	p.Glu4441Serfs*36
LDLR	c.1257C>G	p.Tyr419*	APOB	c.10672C>T	p.Arg3558Cys
LDLR	c.1694G>T	p.Gly565Val	PCSK9	c.-332C>A	.

- LDLR heterozygotes

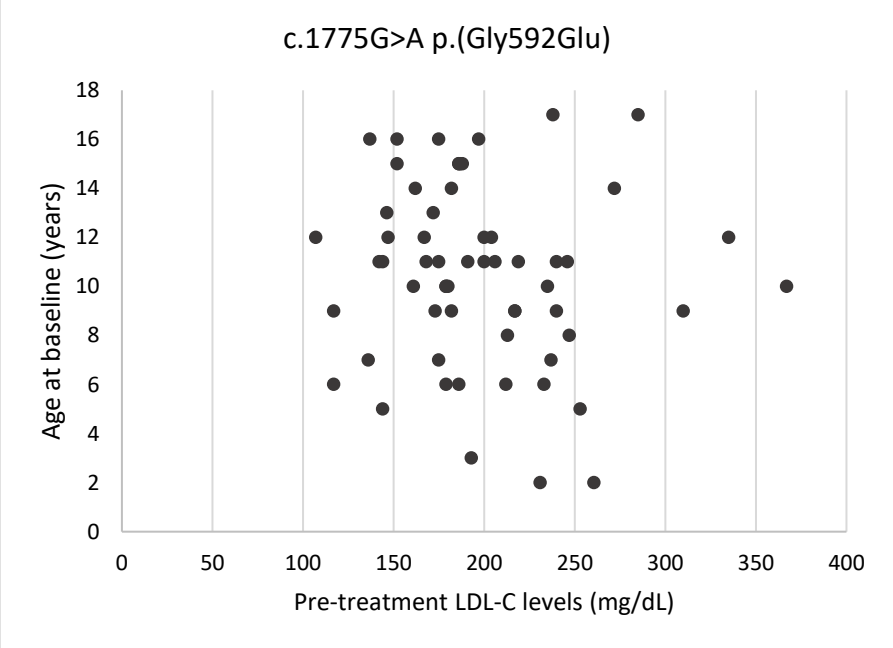
Among the HeFH for *LDLR*, more than 200 causative variants were detected and the stratification by LDLR residual activity showed that children/adolescents with a null mutation (<5% residual activity) (N=464 with 120 different variants, **Appendix IV**) presented significantly higher levels of LDL-C compared to the ones with a defective-receptor mutation (N=509 with 86 different variants, **Appendix V**): 249.5±59.6 mg/dL vs 220.6±52.7 mg/dL, p<0.0001 while no differences in the age at diagnosis were detected (9.9±4.3 vs 9.7±4.1 years, p=0.29).

Among all the *LDLR* heterozygous causative variants, the three mainly reported are c.1646G>A p.(Gly549Asp) (**Figure 3.21, panel A**), c.1775G>A, p.(Gly592Glu) (**Figure 3.21, panel B**) and c.1567G>A p.Val523Met (**Figure 3.21, panel C**).

(A) Pre-treament LDL-C levels in carriers of c.1646G>A p.(Gly549Asp)



(B) Pre-treament LDL-C levels in carriers of c.1775G>A p.(Gly592Glu)



(C) Pre-treament LDL-C levels in carriers of c.1567G>A p.(Val523Met)

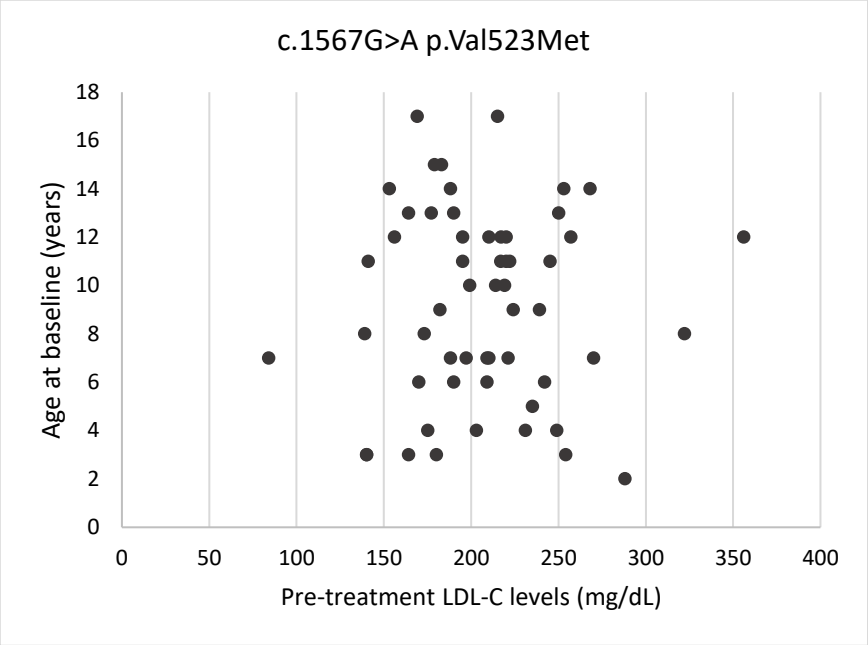


Figure 3.21 – Distribution of pre-treatment LDL-C levels by age among the carriers of the same causative variant. The panel **(A)** shows data related to carriers of c.1646G>A p.(Gly549Asp), the panel **(B)** of c.1775G>A p.(Gly592Glu) and the panel **(C)** of c.1567G>A p.(Val523Met).

Within each of these variants, the mean baseline LDL-C levels were 245.0±50.4 mg/dL (min 118 mg/dL, max 400 mg/dL), 198.0±51.1 mg/dL (min 107 mg/dL, max: 367 mg/dL), 207.1±46.6 mg/dL (min: 84 mg/dL, max: 356 mg/dL), respectively. The higher levels of LDL-C in the carrier of the first mutation could be justified by its impact on LDLR residual activity (null-mutation) compared to the other two receptor-defective mutations; nevertheless, a great variability in the LDL-C values was observed also within the same mutation.

- <u>APOB heterozygotes</u>

Four different APOB variants were detected in 15 subjects (**Table 3.20**). The most reported is c.10580G>A p.Arg3527Gln and determined an amino acid change in the protein. *APOB* heterozygotes presented a mean untreated LDL-C value lower than *LDLR* heterozygotes (191.0±40.6 mg/dL vs 234.1±57.9 mg/dL, respectively).

Table 3.20 – Variants in *APOB* gene

Gene variant	Protein	N of subjets
c.10580G>A	p.Arg3527Gln	10
c.10579C>T	p.Arg3527Trp	2
c.10136A>G	p.Tyr3379Cys	2
c.10672C>T	p.Arg3558Cys	1

3.3.3.4 Lipid lowering therapy at baseline

At the moment of the first visit at the LIPIGEN center, only 73 subjects were already on LLT with a mean age at baseline of 10.8 years (91.8% were 6 or more years old). This means that the treatment was probably started by the general practitioners or by other physicians. The pretreatment LDL-C level of these subjects was 273.8±88.9 mg/dL, with the value on LLT reduced to 199.9±54.4 mg/dL. Thirty-five subjects were on statin (N=28) or statin in combination with ezetimibe (N=7). The statin mainly prescribed was atorvastatin (N=17) at dosage of 10 mg/die or 20 mg/die, followed by pravastatin (N=8), rosuvastatin (N=4), lovastatin (N=3) and simvastatin (N=3). The non-statin treatment mainly prescribed was cholestyramine (from 4 g/die to 16 g/die), a bile acid resin/sequestrant, while 7 patients were only on ezetimibe and one child was treated with fenofibrate (**Table 3.21**). The 17.8% of subjects under 18 years was treated with non-pharmacological agents, as nutraceuticals.

Table 3.21 – Type and dosage of lipid-lowering drugs for subjects already treated at the first LIPIGEN visit

Drug and dosage		N of subjects
Atorvastatin	10 mg/die	4
	20 mg/die	13 (3 plus ezetimibe)
Lovastatin	10 mg/die	3
Pravastatin	10 mg/die	5
	20 mg/die	2
	40 mg/die	1
Rosuvastatin	5 mg/die	
	20 mg/die	4 (1 plus ezetimibe)
Simvastatin	10 mg/die	2 (1 plus ezetimibe)
	20 mg/die	1
Ezetimibe	10 mg/die	7
Fenofibrate	145 mg/die	1
Cholestyramine	4 g/die	8
	8 g/die	3
	16 g/die	3

For all other subjects that were not on therapy at the first visit, data about treatment initiation and type of drugs were available through the new eCRF section "Follow up" (**Figure 3.15**).

3.3.3.5 Treatment initiation

Since the activation of the follow up section in the eCRF, data about treatment initiation were collected for 207 subjects, with therapy started within about 2.5 years after the baseline visit. Only diet was prescribed to 81 subjects. More than half of the 207 subjects started a statin therapy mainly with atorvastatin (mainly dosage of 10 mg/die) and pravastatin (mainly dosage of 20 mg/die), and in 6 of them the association with ezetimibe was prescribed. Colestyramine was prescribed to 48 subjects, and 12 subjects started ezetimibe in monotherapy. In addition, about the 15% took a nutraceutical (**Table 3.22**).

Table 3.22 – Type of lipid lowering agents among subjects starting a therapy after the baseline visit

Lipid lowering agent	N of subjects
Statin	118
Atorvastatin	47
Fluvastatin	1
Lovastatin	1
Pravastatin	42
Rosuvastatin	19
Simvastatin	6
Ezetimibe	12
Cholestyramine	48
Nutraceuticals	31

Taking into account the subjects already treated at baseline and the ones who started it after the first visit, data about therapy were available for 280 subjects. Overall, 151 subjects were on statin and the others were on non-statin therapy (ezetimibe, resins or nutraceuticals). Patients on non-statin treatment were younger and presented lower level of LDL-C at baseline compared to the ones on statins (age at baseline: 8.3±3.8 vs 10.8±4.0 years, LDL-C at baseline: 234.2±66.1 vs 256.8±88.6 mg/dL). During treatment, the LDL-C level respectively decreased of 15.9% and 29.6% on average, reaching 196.9±63.2 mg/dL and 180.8±66.1 mg/dL (**Table 3.23**).

Table 3.23 – Characteristics of subjects on non-statin or on statin therapy.

	Non-Statin	Statin
Age at baseline (mean ± SD)	8.3±3.8	10.8±4.0
Pre-treatment LDL-C [mg/dL]; (mean ± SD)	234.2±66.1	256.8±88.6
On treatment LDL-C [mg/dL] (mean ± SD)	196.9±63.2	180.8±66.1
Average LDL-C reduction (%)	-15.9%	-29.6%

3.4 How to implement the FH diagnosis: The LIPIGEN Sub-Studies

The results described in the previous paragraphs provided a full characterization of the baseline LIPIGEN cohort composed both by adults and children, and were the starting point to plan further analyses focused on the improvement of FH detection and diagnosis.

The data collection and evaluation with LIPIGEN register allowed to highlight some critical issues in the diagnosis of FH (previous paragraph 1.2.3). Three of them were mainly deepened within the LIPIGEN study.

The first one is related to the discrepancy in the clinical and genetic diagnosis: a relevant proportion of subjects (about 20%) with clinical features of FH phenotype does not present any mutations in the tested genes, leading to hypothesize a polygenic etiology of the disease, or the presence of mutations in still unknown genes. Based on this point, the polygenic risk score sub-study was conducted.

The second scenario takes into account the importance of identifying sooner in life subjects with FH through clinical parameters, especially when the genetic test is not or poorly available due to its costs. Based on it, the LIPIGEN data was used to identify new parameters and to evaluate their role in improving the detection rate of the available diagnostic algorithms that still fail to identify part of FH subjects.

The last scenario was focused on the paediatric cohort, in order to deal with the previously described limits related to the young age of subjects and the lack with validated diagnostic tools. The additional paediatric parameters were analyzed and some improvements for the detected and diagnosis were proposed.

3.4.1 Polygenic risk score sub-study

The main aim of this LIPIGEN sub-study was to describe the distribution of the 12 SNPs LDL-C raising alleles polygenic risk score, comparing it in subjects with (FH/M+) or without (FH/M-) a causative mutation in one of the FH candidate genes, and evaluating the correlation of the score with LDL-C levels.

3.4.1.1 Methods

The analysis was conducted on LIPIGEN adults with a clinical diagnosis of FH that underwent the genetic test in the centralized laboratory to identify the presence of genetic variants in the candidate genes (*LDLR*, *APOB*, *PCSK9*, *APOE*, *LDLRAP1*) and evaluate the 12 LDL-C raising SNPs included in the polygenic risk score (LDLc-score), previously described by Talmud et al (Talmud 2013) (see paragraph 1.2.3.2.7).

The selected cohort was divided in two groups based on the genetic results: mutation-positive FH patients (FH/M+), in which at least one FH causative mutation was detected, and mutation-negative FH patients (FH/M-), with no mutations (neither VUS) in any of the tested genes.

Continuous variables are expressed as mean ± standard deviation (SD) or median with the interquartile range, while categorial variables are presented as cases (N) and percentage rate (%). When the distributions did not fail the assumptions of normality, the Student t test was applied to compare mean LDLc-score values between FH/M+ and FH/M- groups. A ROC analysis was also performed to determine if the LDLc-score could be considered a valid tool in clinical practice to distinguish between individuals with a causative mutation and mutation-negative subjects.

Sensitivity and specificity are presented as the measures to assess the effectiveness of the polygenic score, which indicates the ability of LDLc-score to discriminate FH/M+ from FH/M- subjects.

The identification of the optimal cut-off point was obtained using the Youden index method and the proposed value was the point maximizing the Youden function that consists of the difference between true positive rate and false positive rate over all possible cut-point values. Correlations between LDLc-score and LDL-C levels in FH/M+ and FH/M- subjects were assessed using a Pearson correlation coefficient.

All analyses were performed using Statistical Analysis System software, version 9.4 (Statistical Analysis System Institute, Inc, Cary, NC). Statistical significance was set at the 0.05 level for every analysis performed.

3.4.1.2 Results

The analysis was carried out on 1519 clinically diagnosis FH subjects with a positive or negative genetic test.

3.4.1.2.1 Cohort Description and Comparison

A pathogenic/likely pathogenic mutation was identified in 875 patients [FH/M+] (females 54.6%, mean age 42.5±15.0 years) while the other 644 patients were classified as mutation negative [FH/M-] (females 54.2%, mean age 49.7±13.5 years). Although all FH/M- patients had a clinical phenotype compatible with a diagnosis of FH, the mean levels of total cholesterol (272.7±72.6 vs 313.1±86.3 mg/dL, p-value <.0001) and pre-treatment LDL-C (217.1±55.5 vs 270.5±68.6 mg/dL, p-value <.0001) were lower than FH/M+ while the levels of HDL-Cholesterol (59.9 ±17.1 vs 56.1±15.1 mg/dL, p-value <.0001) and triglycerides (median, 126 [89-177] vs 98 [71-137] mg/dL, p-value <.0001) were higher compared to the FH/M+ group (**Table 3.24**).

Table 3.24 – Demographic and biochemical profile in FH/M+ and FH/M- individuals

	FH/M+ N=875	**FH/M- N=644**	**p-value**
Females; N (%)	478; 54.6	348; 54.2	0.83
Age at baseline, [years]; mean±SD	42.5 (15.0)	49.7 (13.5)	<.0001
Total Cholesterol [mg/dL], mean±SD	313.1 (86.3)	272.7 (72.6)	<.0001
Triglycerides [mg/dL], median [IQR]	98 [71-137]	126 [89-177]	<.0001
HDL Cholesterol [mg/dL], mean±SD	56.1 (15.1)	59.9 (17.1)	<.0001
Lp(a) [mg/dL], N; median [IQR]	172; 19.1 [8.6-37]	124; 40.0 [8.4-98]	0.003
Glucose [mg/dL], mean±SD	89.3 (18.4)	94.8 (23.8)	0.0002
Pre-treatment LDL Cholesterol [mg/dL], mean±SD	270.5 (68.6)	217.1 (55.5)	<.0001

Comparing the FH/M+ and the FH/M- groups, no statistically significant differences for the clinical history of premature CHD and of premature cerebral or peripheral vascular disease were detected. While the prevalence of tendon xanthoma and arcus cornealis before the age of 45 years were significantly higher in the FH/M+ group (17.5% vs 4.2% and 13.7% vs 10.6%, respectively). No significant differences in lipid lowering therapies were observed (**Table 3.25**).

Table 3.25 – DLCN parameters and clinical characteristics of FH/M+ and FH/M- adults

	FH/M+ N=875	FH/M- N=644	p-value
First-degree relative with premature CHD, and/or with LDL-C >95th percentile; N (%)	799; 91.3	503; 78.1	<.0001
First-degree relative with tendon xanthoma and/or arcus cornealis, and/or children <18 years with LDL-C >95th percentile; N (%)	278; 31.8	97; 15.1	<.0001
Clinical history of premature CHD; N (%)	76; 8.7	50; 7.8	0.52
Clinical history of premature cerebral or peripheral vascular disease; N (%)	35; 4.0	27; 4.2	0.85
Tendon xanthoma	153; 17.5	27; 4.2	<.0001
Arcus cornealis prior to age 45 years; N (%)	120; 13.7	68; 10.6	0.07
Pre-treatment LDL-C value; N (%)			
155-190 mg/dL	52; 5.9	93; 14.4	<.0001
191-250 mg/dL	308; 35.2	335; 52.0	
251-325 mg/dL	322; 36.8	124; 19.3	
>325 mg/dL	171; 19.5	20; 3.1	
Lipid lowering therapy; N (%)	268; 30.6	198; 30.8	0.96

3.4.1.2.2 Correlation of LDLc-score and LDL-C levels

The FH/M- subjects presented a higher mean value of the LDLc-score compared to the FH/M+ ones (1.00±0.18 vs 0.94±0.20; p-value for the difference between means <.0001) and its distribution by genetic diagnosis is shown in **Figure 3.22**.

The higher levels of LDLc-score in FH/M- compared to FH/M+ were maintained also stratifying the cohort by pre-treatment LDL-C classes, with a significant difference in the LDL-C range between 150-350 mg/dL (**Figure 3.23)**

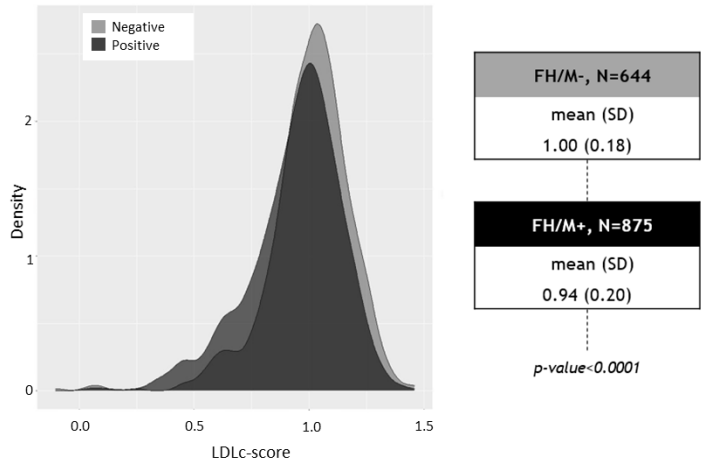


Figure 3.22 - Distribution of the LDLc-score in FH/M- and FH/M+ patients

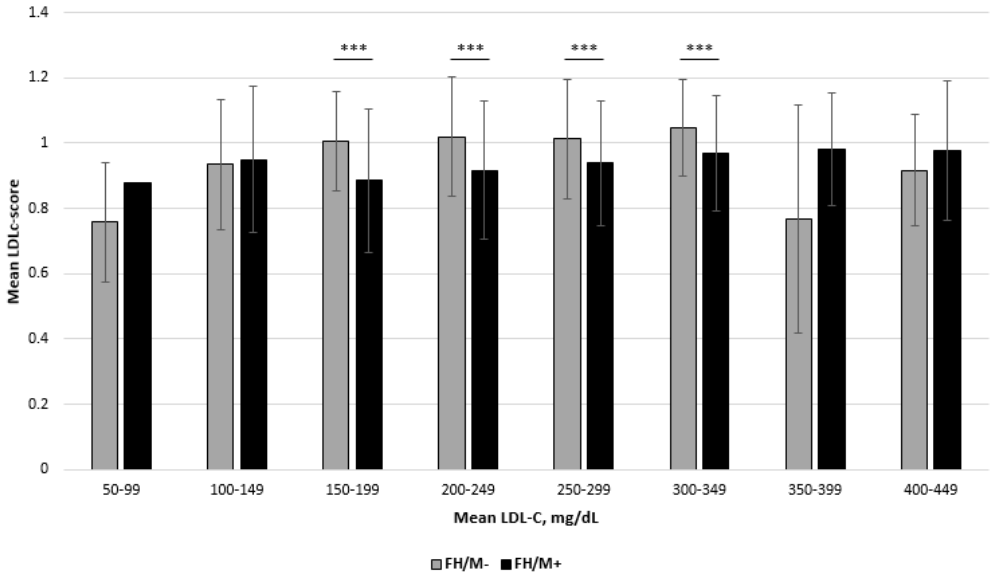
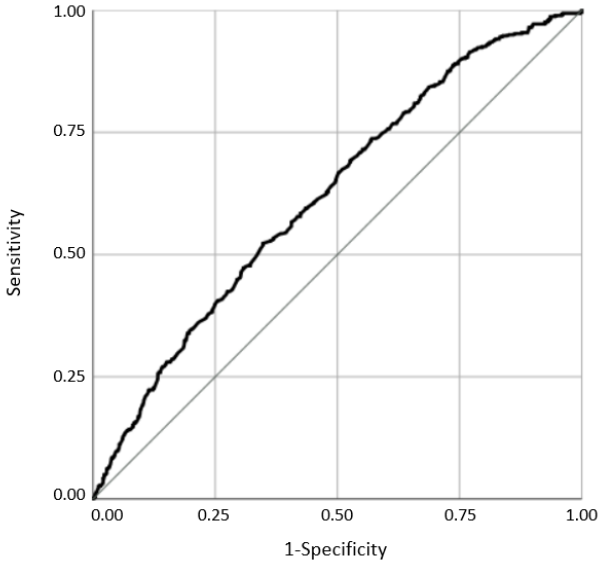


Figure 3.23 - Mean values of LDLc-score by LDL-C classes in FH/M- and FH/M+ subjects

However, when a ROC analysis was performed (**Figure 3.24**), the area under the curve predicting polygenic hypercholesterolemia was 0.59 (95% CI, 0.56-0.62), with sensitivity and specificity being 77% and 36% at 0.905 as a cut-off value.

Based on the ROC analysis result, the value of 0.905 was identified as a decision threshold point (cut-off) to define the probability of having hypercholesterolemia with a polygenic aetiology. Based on it, the value of 77% indicated the proportion of subjects who are correctly diagnosed as having a polygenic aetiology while 36% the proportion of subjects who are correctly diagnosed as FH/M+.



	FH/M-	FH/M+	total
≤0.905	148	315	463
	31.97	68.03	
	22.98	36.00	
>0.905	496	560	1056
	46.97	53.03	
	77.02	64.00	
Total	644	875	1519

Figure 3.24 – ROC curve for LDLc-score for the diagnosis of a polygenic aetiology and classification of adults with (FH/M+) and without (FH/M-) according to a 0.905 cut off in LDLc-score

In the last part of the study, the correlation between LDL-C score and pretreatment LDL-C values in FH/M- or FH/M+ was investigated, finding out not only a positive trend in the first group (FH/M-, R=0.12, p-value=0.002; **Figure 3.25**) but an unexpected stronger impact on FH/M+ (R=0.16, p-value<.0001; **Figure 3.26**), as demonstrated by the Pearson correlation coefficient.

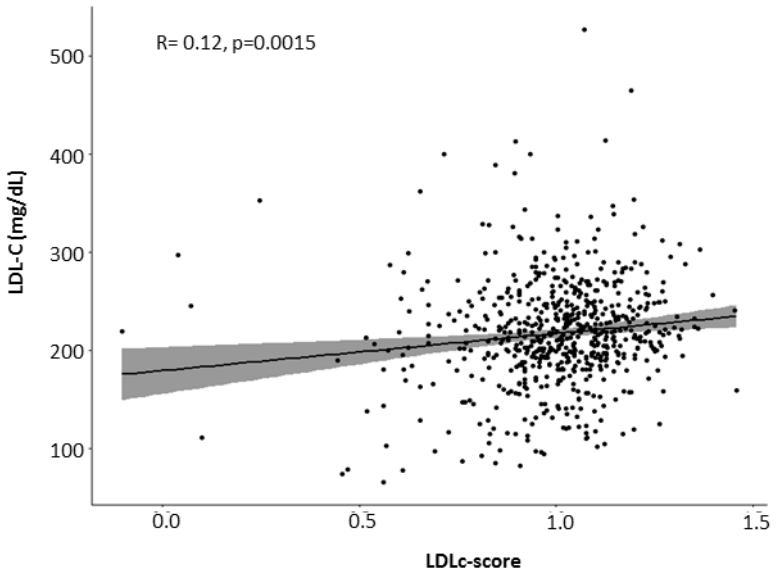


Figure 3.25 – Correlation between LDL-C levels and LDLc-score in FH/M- subjects

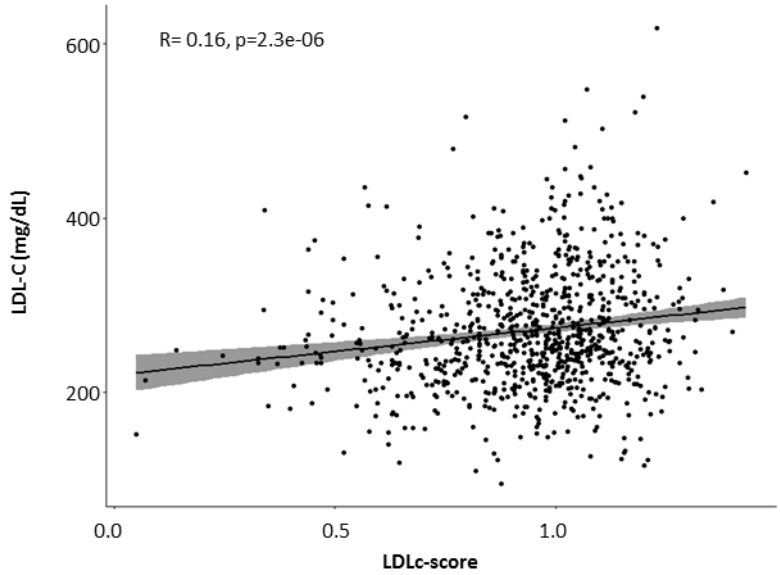


Figure 3.26 – Correlation between LDL-C levels and LDLc-score in FH/M+ subjects

The correlation in FH/M+ was also confirmed in a sub-group of subjects with the same causative mutation, choosing the carriers of the most detected causative variant affecting *LDLR* (c.662A>G, p.Asp221Gly [N=72]; R =0.26) (**Figure 3.27**).

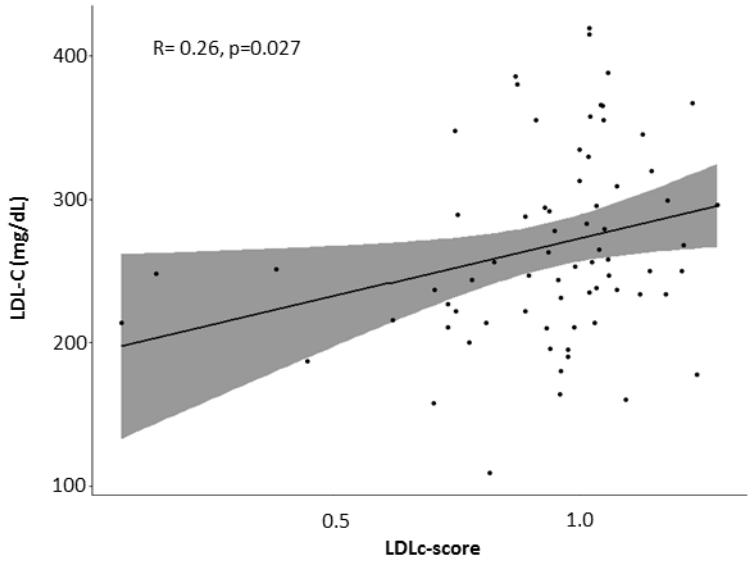


Figure 3.27 – Correlation between LDL-C levels and LDLc-score in FH/M+ subjects carrying the mutation c.662A>G (p.Asp221Gly)

3.4.2 ACTUS-FH sub-study

The ACTUS-FH "AChilles Tendon UltraSonography in Familial Hypercholesterolemia" project involved 5 LIPIGEN centres, and aimed to evaluate the diagnostic role of Achilles tendon ultrasonography in the diagnosis of familial hypercholesterolemia.

3.4.2.1 Methods

This sub-study was initially started within the LIPIGEN Network with the main aim of standardizing the use of the Achilles tendon ultrasonography for the clinical evaluation of FH subjects. Next, the LIPIGEN data collection was implemented for 5 LIPIGEN centres with data derived by the Achilles tendon ultrasonography, a share integrated database was created for research purpose and analyses with all data were planned. All patients included in the ACTUS-FH project were adults with a clinical diagnosis of FH who performed also the genetic test to identify the presence or not of mutation in the causative genes. The data collection included, if available:

- Demographic and clinical data: gender, age, BMI, smoking status, carotid intima-media thickness [IMT], past medical history. Moreover, the clinical data used for the evaluation of the DLCN score included: presence of FH clinical signs (xanthoma and corneal arcus), personal history of premature cardiovascular or cerebrovascular events and family history of hypercholesterolemia or premature cardiovascular diseases. Moreover, the acquisition of tendon xanthoma ultrasonography data and the measurements of xanthoma thickness were standardized. Subjects with previous Achilles tendon lesions or inflammatory/degenerative tendinopathy were excluded.

- Biochemical data: lipid profile with the values of total-cholesterol, LDL-Cholesterol, HDL-Cholesterol and triglycerides associated with the information about any current lipid-lowering treatment and the start date. Moreover, a pre-treatment LDL-C value was necessary; if not available, it was estimated with correction factors based of the type and dosage of the drugs.

- Genetic results: presence of causative mutation in the promoter, coding DNA sequences, exon-intron boundaries regions of *LDLR*, *APOB*, *PCSK9*, *APOE*, and *LDLRAP1* genes. The pathogenicity of each variant was evaluated based on the

criteria of the American College of Medical Genetics and Genomic (Richards 2015) and subjects with at least one pathogenic or likely pathogenic variants were classified as mutation positive (FH/M+) while subjects with no mutation (neither VUS) as mutation negative (FH/M-). Finally, subjects with only variants with uncertain clinical significance were classified with an inconclusive diagnosis and excluded for the analysis.

Continuous variables were expressed as mean±SD or median with the interquartile range, whereas categorical variables were expressed as cases (N) and percentage rate (%).
Continuous variables normally distributed were compared using the Student's t-test while the ones without normal distribution using the Mann–Whitney U-test. The chi-square and Fisher's exact tests were used for categorical variables. Differences between the subgroups of subjects according to the presence/absence of clinically apparent or ultrasound-detected ATX were assessed with the Analysis of variance (ANOVA) and chi-square test. Pearson and correlation coefficients were used to evaluate correlations between Achilles tendon thickness and several covariates. Receiver operating characteristic (ROC) curves were performed to investigate the diagnostic performance of maximum tendon thickness for identifying FH/M+ subjects as compared to FH/M-. The areas under the ROC (AUROCs) curves with 95% CI were recorded and the optimal cut-offs were identified using the Youden index. All tests were 2-sided and P values less than 0.05 were considered statistically significant. Statistical analysis was performed by using both SAS Software version 9.3 (SAS, NC) and R Software version 3.6.2.

3.4.2.2 Results

The analyses were carried on 769 adults with a clinical diagnosis of FH, followed by the 5 LIPIGEN clinics involved in the ACTUS-FH sub-study: Milan (N=187), Modena (N=179), Ferrara (N=174), Padua (N=140) and Catania (N=89).

3.4.2.2.1 Cohort description and comparison

The tendon xanthoma was identified in the 9.8% of the cohort at physical examination (CA-ATX; N=75) while the number increased to 255 subjects considering also the Achilles xanthoma undetectable at clinical exam but detected at ultrasonography (US-ATX).

Patients with only US-ATX were younger (50.2±12.7 vs 53.5±13.2 years), with lower pre-treatment LDL-C (300.5±78.5 vs 347.6±85.3 mg/dL) and lower prevalence of premature cardiovascular events (13.3% vs 24.0%) than CA-ATX but older (50.2±12.7 vs 45.5±15.3 years), with significantly higher levels of pre-treatment LDL-C (300.5±78.5 vs 254.4±63.4 mg/dL) and higher prevalence of premature CV events (13.3% vs 7.8%) than those with no xanthoma (No-ATX; N=514) at all (**Table 3.26**). An increasing gradient in the other covariates (BMI, glucose and diabetes prevalence) was detected from No-ATX to CA-ATX subjects.

Table 3.26 – Comparison of demographic and biochemical parameters among subjects with clinically detected xanthoma (CA-ATX), ultrasonography xanthoma (US-ATX) or absence (No-ATX).

	CA-ATX	**US-ATX**	**No-ATX**	***p value***			
	N 75	**N 180**	**N 514**	***overall***	**CA-ATX vs US-ATX**	**US-ATX vs No-ATX**	**CA-ATX vs No-ATX**
Age [years], mean±SD	53.5±13.2	50.2±12.7	45.5±15.3	*<.0001*	*0.06*	*<.0001*	*<.0001*
Men, N (%)	32 (42.7)	83 (46.1)	266 (51.8)	*0.20*	*0.61*	*0.19*	*0.14*
BMI [kg/m^2], mean±SD	27.0±4.7	26.4±4.6	25.2±3.9	*<.0001*	*0.28*	*0.002*	*0.001*
Glucose [mg/dL], mean±SD	97.2±16.5	92.8±13.1	89.8±11.3	*<.0001*	*0.09*	*0.02*	*0.002*
Diabetes, N (%)	8 (10.7)	7 (3.9)	12 (2.3)	*0.001*	*0.04*	*0.0002*	*0.0002*
Total Cholesterol [mg/dL], mean±SD	247.5±94.8	230.7±75.7	253.7±79.1	*0.005*	*0.18*	*0.001*	*0.59*
LDL-C [mg/dL], mean±SD	170.7±86.9	153.2±68.6	172.9±73.4	*0.009*	*0.09*	*0.002*	*0.84*
Triglycerides [mg/dL], median (IQR)	96 (72-133)	82 (63-110)	100 (72-139)	*0.0005*	*0.02*	*0.0001*	*0.79*
HDL-C [mg/dL], mean±SD	54.4±12.7	58.2±14.4	58.5±14.7	*0.07*	*0.05*	*0.80*	*0.02*

	CA-ATX	US-ATX	No-ATX	*p value*			
	N 75	N 180	N 514	*overall*	CA-ATX vs US-ATX	US-ATX vs No-ATX	CA-ATX vs No-ATX
Pre-treatment LDL-C [mg/dL], mean±SD	347.6±85.3	300.5±78.5	254.4±63.4	*<.0001*	*<.0001*	*<.0001*	*<.0001*
Statin treatment (at the time of ultrasound evaluation), N (%)	61 (81.3)	149 (82.8)	298 (58.0)	*<.0001*	*0.78*	*<.0001*	*0.0001*
Cardiovascular event, N (%)	22 (29.3)	30 (16.7)	49 (9.5)	*<.0001*	*0.02*	*<.0001*	*<.0001*
Cerebrovascular event, N (%)	2 (2.7)	7 (3.9)	8 (1.6)	*0.18*	*0.63*	*0.49*	*0.49*
Early cerebrovascular event, N (%)	0 (0.0)	6 (3.3)	7 (1.4)	*0.10*	*0.11*	*0.31*	*0.31*
Carotid IMT [mm], mean±SD	0.80±0.21	0.80±0.21	0.74±0.30	*0.009*	*0.83*	*0.13*	*0.13*
Subclinical carotid atherosclerosis, N (%)	42 (60.9)	84 (58.3)	142 (35.2)	*<.0001*	*0.72*	*<.0001*	*<.0001*
Positive at genetic test, N (%)	72 (96.0)	170 (94.4)	336 (65.4)	*<.0001*	*0.61*	*<.0001*	*<.0001*
DLCN score, N (%) Unlikely (0-2) Possible (3-5) Probable (6-8) Definite (>8)	 0 (0) 0 (0) 0 (0) 75 (100)	 3 (1.7) 27 (15.0) 56 (31.1) 94 (52.2)	 35 (6.8) 229 (44.5) 169 (32.9) 81 (15.8)	*<.0001*	*<.0001*	*<.0001*	*<.0001*
Achilles tendon thickness [mm], mean±SD	10.3±4.2	7.0±1.8	5.4±1.0	*<.0001*	*<.0001*	*<.0001*	*<.0001*

3.4.2.2.2 The diagnostic implication of US-ATX

Considering the clinical characteristics, family history and the lipid profile, the DLCN score was evaluated: all subjects with clinical tendon xanthoma presented a score higher than 8 while a definite diagnosis of FH was identified in about half of US-ATX subjects and in the about 16% of patients without any detection of xanthoma. Once that the DLCN parameter related to the CA-ATX was substituted with US-ATX, the overall percentage of ACTUS-FH subjects with a definite DLCN score diagnosis increased from 32.5% to 43.2% and the percentage of individuals above the cut-off of 5 points from 61.8% to 65.7% (**Figure 3.28**).

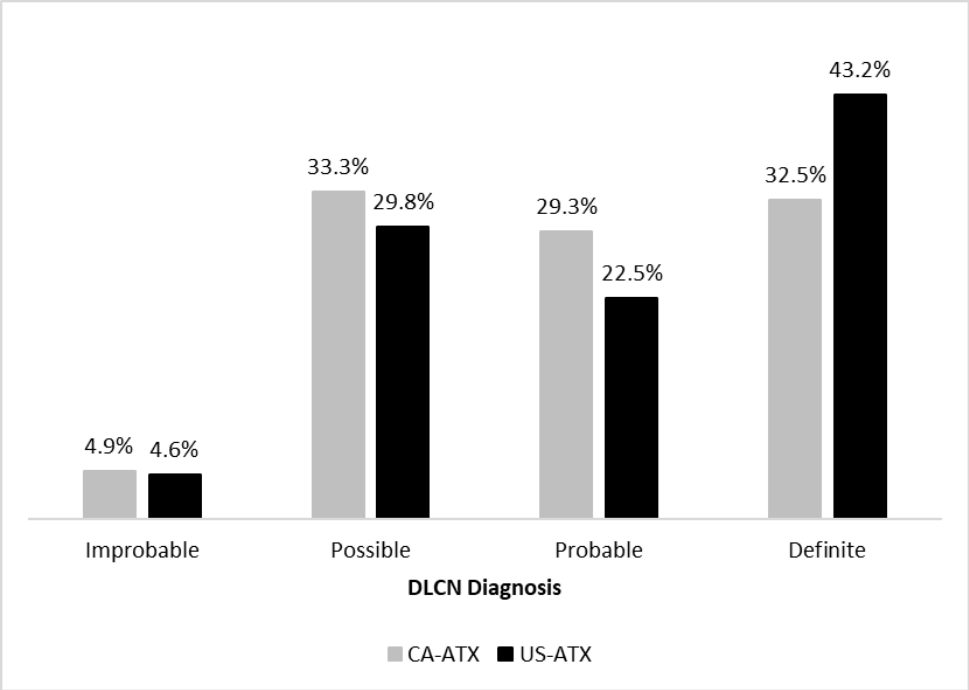


Figure 3.28 - Reclassification of DLCN categories using the US-ATX instead of CA-ATX

Stratifing the cohort by genetic results, the presence of causative variants (FH/M+) was detected in 578 while the others had a negative genetic diagnosis (FH/M-) but a clinical diagnosis of FH. The FH/M+ subjects were younger (45.7±15.3 vs 52.4±12.0, p<.0001) and presented higher pre-treatment LDL-C levels (289.5±78.9 vs 228.2±39.9, p<.0001) compared to FH/M-. Moreover, the FH/M+ showed a higher prevalence of clinical apparent (12.5% vs 1.6%, p<.0001) and only ultrasound detected xanthomas than FH/M- (29.4% vs 5.2%, p<.0001). Using the US-ATX instead of CA-ATX, the number of subjects with tendon xanthoma and genetic FH increased from 72 to 242 (**Table 3.27**).

Table 3.27 - Classification by (A) CA-ATX and (B) US-ATX among FH/M- and FH/M+ subjects

(A)

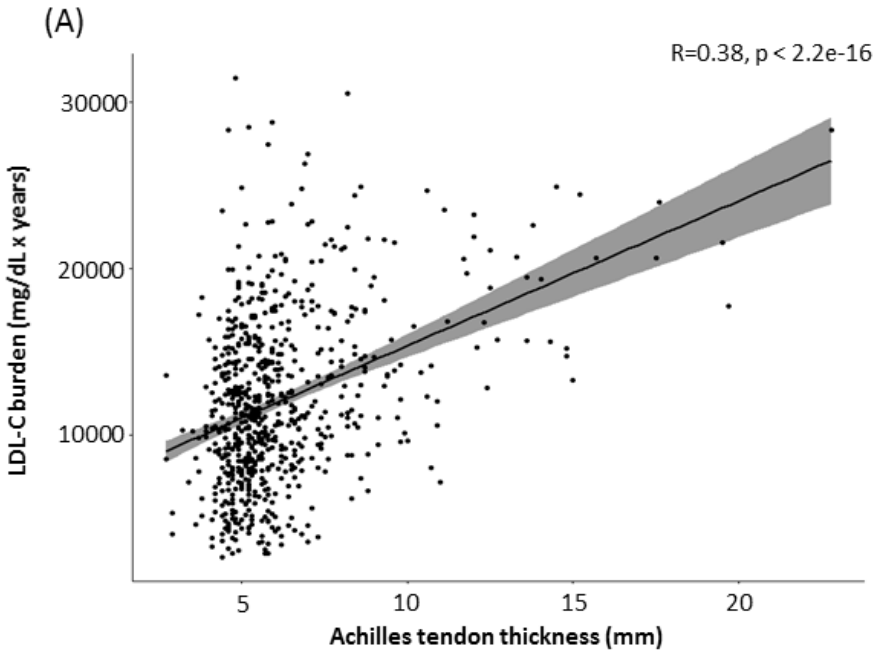
	FH/M-	FH/M+	Total
No	188 27.1% 98.4%	506 72.9% 87.5%	694
Yes	3 4.0% 1.6%	72 96.0% 12.5%	75
Total	191	578	769

(B)

	FH/M-	FH/M+	Total
No	178 34.6% 93.2%	336 65.4% 58.1%	514
Yes	13 5.1% 6.8%	242 94.9% 41.9%	255
Total	191	578	769

For what concerns the thickness of Achilles tendon, the maximum thickness showed an increasing gradient from No-ATX with the lowest thickness (5.4±1.0 mm), to only US-ATX (7.0±1.8 mm) and to the highest value in the CA-ATX (10.3±4.2 mm).

Analysing the maximum Achilles tendon thickness and the LDL-C burden, a significant positive correlation was identified (R=0.38, p<.0001), but only confirmed in FH/M+ (0.41, p<.0001) (**Figure 3.29**).



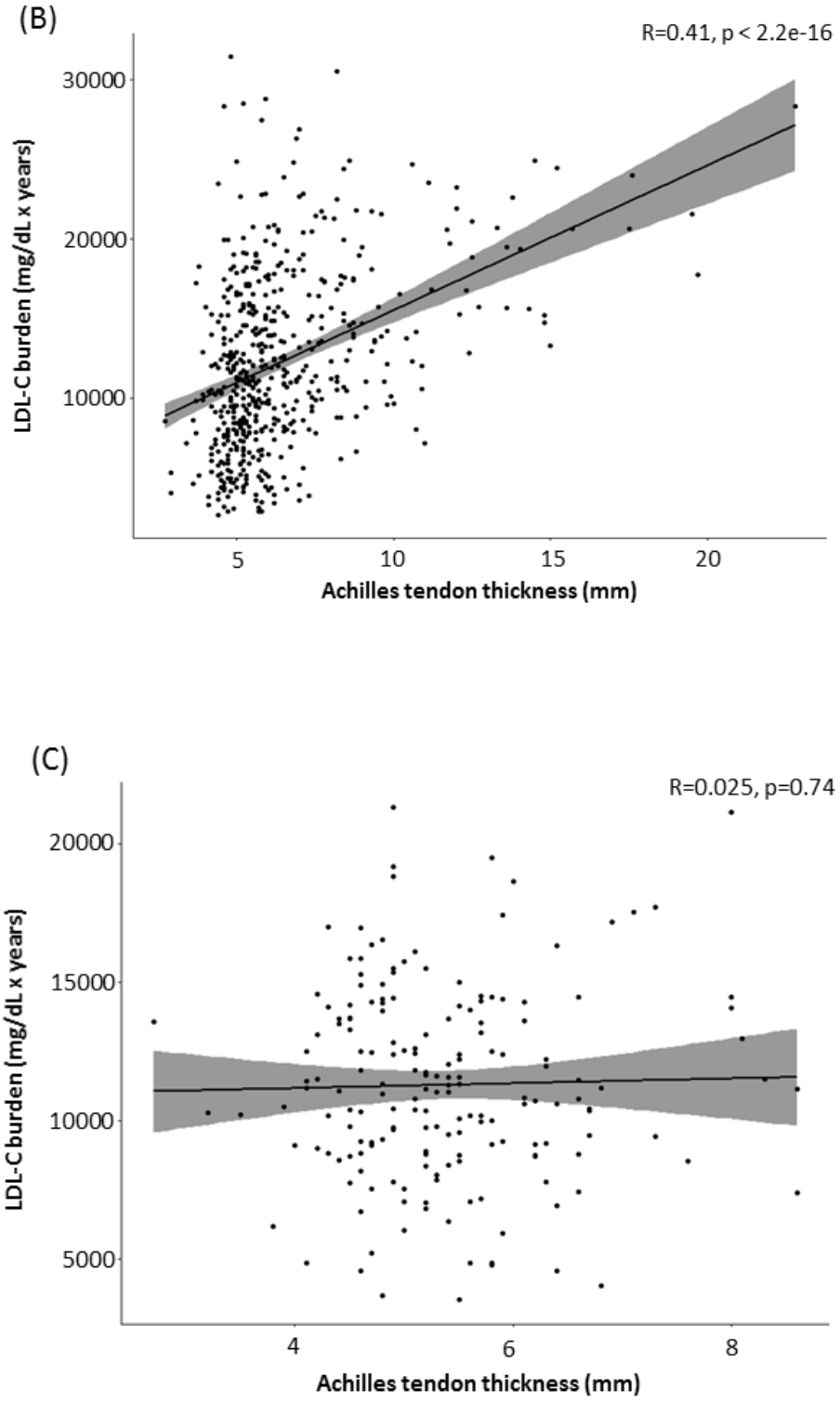


Figure 3.29 – Correlation between the maximum Achilles tendon thickness and LDL-C burden in the all ACTUS-FH subjects (A), in FH/M+ (B) and in FH/M- (C).

An increasing trend of maximum Achilles tendon thickness was also found taking into account the DLCN score classes: from 5.43 mm in the class with DLCN score < 6, to 5.76 mm in the class with 6-8 points and 7.60 mm in the class with DLCN score ≥9. Finally, the ROC analysis was performed to evaluate the role of maximum Achilles tendon thickness in the identification of subjects with a definite diagnosis of FH.

The value of AUROC was 0.73 with a sensitivity and specificity of 67% and 71%, respectively, at a cut-off value of 6.5 mm, failing also in the estimation of a threshold value of maximum Achilles tendon thickness able to identify FH/M+ with a high sensitivity (**Figure 3.30, A**). Actually, by using the cut-off value of 6.5 mm, the proportion of FH subjects who were correctly diagnosed as having a monogenic aetiology was 34% (**Figure 3.30, B**).

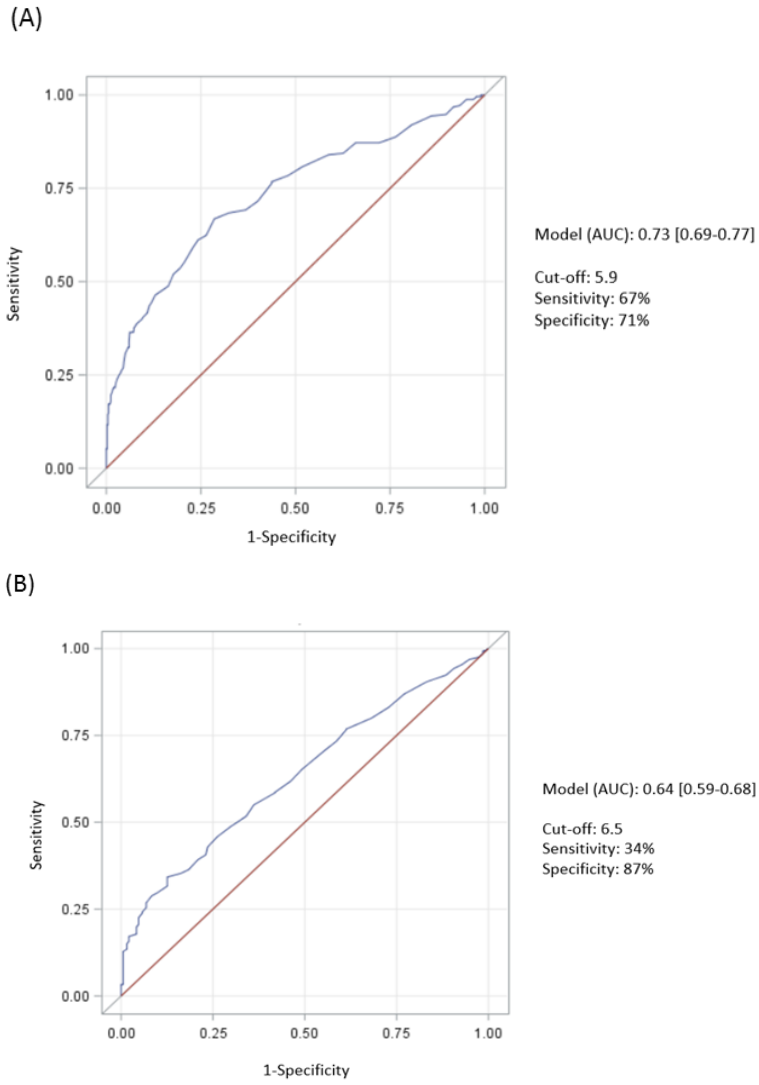


Figure 3.30 – ROC analyses of maximum Achilles tendon thickness for the clinical diagnosis (DLCN score class: definite, A) and for the genetic diagnosis (FH/M+, B) of FH

3.4.3 DLCN criteria in paediatric FH subjects

The main aims of this part were to overcome the limits in the diagnosis of FH in children and adolescents because of their young age and propose some improvements in refining the diagnostic tools.

3.4.3.1 Methods

First, specific LDL-C cut-offs for children/adolescents were identified based on the percentile distribution of LDL-C classes among adults with a positive genetic diagnosis of FH, including only subjects with a value of LDL-C pre-treatment available and not retro-calculated. Then, the DLCN criteria were compared in genetically-confirmed FH adult (N=607) and paediatric (N=659) patients with no missing DLCN data or at most one missing parameter in the family history section of DLCN score.

Finally, the additional collected data (available for 293 children/adolescents) about premature CHD in second-degree family members were applied to evaluate improvements in the detection rate of modified DLCN score in subjects under 18 years. Statistical analysis was performed by using SAS Software version 9.3 (SAS, NC) and IBM SPSS Statistics version 27.

3.4.3.2 Results

3.4.3.2.1 Identification of specific LDL-C cut-off in subjects under 18 years

A total of 875 genetically confirmed adults and 988 subjects under 18 years were analyzed in order to identify LDL-C cut-offs specific for the Italian children/adolescents that could substitute the ones used in the DLCN score for adult population.

In adult cohort, the cut-off of 155 mg/dL corresponded to the 2.7 LDL-C percentiles, 191 mg/dL to 8.6 percentiles, 251 mg/dL to 44.1 percentiles and 326 mg/dL to 80.8 percentiles. Shifting these percentiles to the LDL-C distribution in children/adolescents, the new identified cut-offs were 140, 165, 220 and 275 mg/dL, respectively (**Table 3.28**).

Table 3.28 – New identified LDL-C cut-off for children/adolescents based on the adult LDL-C classed of DLCN score

DLCN points	Cut-off for adults	Cut-off for subjects under 18 years
0	<155	<140
1	155-190	140-164
3	191-250	165-219
5	251-325	220-274
8	326+	275+

3.4.3.2.2 Comparison and implementation of DLCN parameters among adults and children/adolescents

According to the original DLCN score, probable/definite FH (score≥6) was found in 59.1% of adults, but only in 28.1% of children/adolescents (**Figure 3.31**). In this latter group, the percentage increased to 50.7% using the new identified LDL-C classes reported in **Table 3.28**.

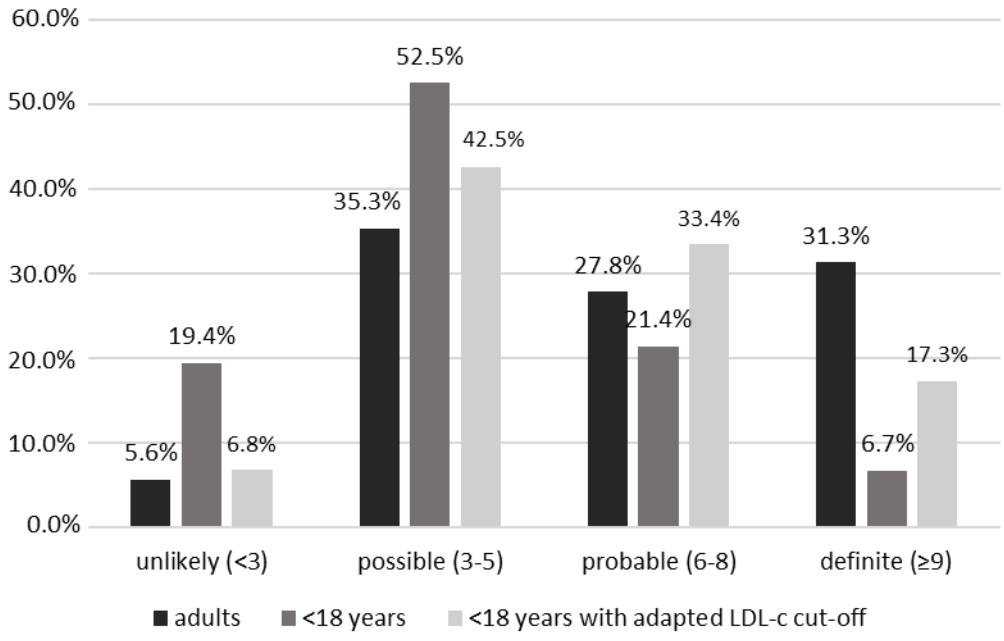


Figure 3.31 – Classification of adults and subjects under 18 years based on the official DLCN score and with the new identified LDL-C classes adapted for children/adolescents.

The prevalence of the DLCN score parameters was compared between 607 adults and 659 children/adolescents with all available characteristics or only one missing in the family history.

The lower prevalence of the typical features of FH in children/adolescents compared to adults was confirmed: tendon xanthoma was identified in the 12.9% of adults vs only in the 2.1% of children/adolescents, a similar difference was also detected for the arcus cornealis 11.9% vs 0.8% (**Table 3.29)**. No children presented a clinical history of premature CHD or cerebral/peripheral vascular disease, identified in the 7.7% and 3.8% of adults, respectively. For the family history, the presence of first-degree relatives with tendon xanthoma and/or corneal arcus at age < 45 was the most missing parameter in the selected cohort (24.9% in adults and 9.3% in children/adolescents). On valid data, its prevalence was comparable among adults and children (20.6% and 18.7%, respectively) as the presence of hypercholesterolemia in first degree family members (93.6% vs 93.1%, respectively). At the contrary, a premature CHD in first-degree relatives was reported in the 39.2% of adults while only in the 21.0% of subjects under 18 years.

Table 3.29 – Prevalence of DLCN parameters in adults vs children/adolescents

	Adults	**Subjects < 18 y**
Physical examination		
Tendon xanthoma	12.9%	2.1%
Arcus cornealis at age < 45	11.9%	0.8%
Clinical hystory		
Clinical history of premature CHD	7.7%	0.0%
Clinical history of premature cerebral or peripheral vascular disease	3.8%	0.0%
Family history		
1st degree relative with known premature CHD	39.2%	21.0%
1st degree relative with known LDL-C >190 mg/dL	93.6%	93.1%
1st degree relative with tendon xanthoma and/or corneal arcus at age < 45	20.6%	18.7%

Furthermore, the deepening of the parameter related to the presence of a premature event in family members for children/adolescents was possible because of the “Paediatric Data” section in the eCRF. While a premature CV events in parents was reported in 61 of 293 subjects, the percentage increased from 21.0% to 56.7% extending the evaluation also in second degree family members (**Table 3.30**).

Table 3.30 – Frequency of subjects with the presence of premature CV event(s) in 1st and/or 2nd degree family members

Family member(s)	Frequency (N)
One parent (father or mother)	45
One grandparent	106
One parent and one grandparent	11
Two grandparents	2
Both parents and two grandparents	1
Father and sibling	1
Nobody	127

However, the substitution of the voice of the DLCN "1st degree relative with known premature CHD" with "1st and 2nd degree relative with known premature CHD" (maintaining the point associated to this parameter as 1) did not lead to an improvement in the classification of children/adolescents as probable or definite. However, a shift from the lower class to the highest points was detected, suggesting the need not only to modify the parameters collected through the score but also the points associated to each voice.

3.5 Cases of rare dyslipidemias

3.5.1 Subjects affected by homozygous FH

The full characterization of all Italian homozygous FH (included the LIPIGEN subjects) was performed in collaboration with the Italian study group on Homozygous FH. Of 125 subjects (114 of whom apparently unrelated) with autosomal dominant hypercholesterolemia, 60 were homozygous FH, 58 compound heterozygous FH and 7 double heterozygous (4 for *LDLR* and *APOB*, and 3 for *LDLR* and *PCSK9*) (**Figure 3.32**) (Bertolini 2020).

The age at molecular testing ranged from 1 to 73 years with a mean age of 28.4±19.6 years, similar among HoFH (27.8±19.5 years) and CHE (26.9±19.4 years) but higher in DHE (43.2±17.6). About the 95% of subjects were carriers of two pathogenic or likely pathogenic variants in LDLR.

Figure 3.32 - HoFH Distribution of 125 ADH patients based on the individual patient's regional origin (HO: true homozygotes, CHE: compound heterozygotes, DHE: double heterozygotes)

In increasing gradient in the severity of phenotype was identified from DHE to HO, with CHE that presented intermediate features in terms of lipid profile and prevalence of ASCVD. A value of LDL-C higher than 500 mg/dL was observed in about the 70.0% of HO and 45.0% of CHE (**Table 3.31**).

Table 3.31 – Main characteristics of homozygous (HoFH), compound heterozygous (CHE) and double heterozygous (DHE) ADH subjects

	HO	**CHE**	**P-value**	**DHE**
TC [mg/dL], mean±SD	666.3±170.9	584.3± 148.5	0.006	471± 87.8
LDL-C [mg/dL], mean±SD	606.7± 173.2	519.7± 152.7	0.005	392.9± 90.5
HDL-C [mg/dL], mean±SD	35.9± 13.1	42.2± 11.2	0.006	54.1± 15.9
TG [mg/dL], mean±SD	116.0± 42.5	104.5± 88.6	ns	130.2± 74.4
Tendon xanthomas, N (%)	53/60 88.3%	47/58 81.0%	ns	71.4%
Arcus cornealis, N (%)	34/60 56.7%	24/58 41.4%	ns	14.2%
ASCVD, N (%)	40/60 66.6%	25/58 43.1%	0.009	2/7 (28.5%)
Age at first ASCVD event (yrs), mean±SD	38.4±16.0	43.0±15.7	ns	37.5±3.5

The stratification of HoFH and CHE subjects by LDLR residual activities allowed to divide the cohort in two groups: R-NEG/NEG and R-DEF/DEF, including in this latter class also carriers of R-DEF/NEG mutation because of their similarity to R-DEF/DEF subjects. Individuals with R-NEG/NEG genotype were significantly younger than R-DEF/DEF at the time of molecular diagnosis and presented a more severe phenotype in terms of LDL-C levels, presence of cutaneous xanthomas, prevalence of ASCVD and age at the first ASCVD event (**Table 3.32**).

Table 3.32 – Comparison between subjects with R-NEG/NEG and R-DEF/DEF genotypes

	R-NEG	**R-DEF**	**P-value**
HO-ADH/CHE-ADH	18/7	42/51	<0.03
Age at molecular diagnosis (yrs), mean±SD	16.5±16.7	30.4±19.1	0.001
TC [mg/dL], mean±SD	831.0±176.7	570.8±109.4	0.0001
LDL-C [mg/dL], mean±SD	776.1±176.7	506.9±111.8	0.0001
HDL-C [mg/dL], mean±SD	29.8±8.5	41.8±12.4	0.0001
TG [mg/dL], mean±SD	127.5±39.0	105.4±42.5	0.03
Tendon xanthomas, %	88.0%	83.9%	Ns
Arcus cornealis, %	36.0%	52.7%	Ns
ASCVD, %	72.0%	50.5%	0.07
Age at first ASCVD event (yrs), mean±SD	22.4±10.9	46.3±12.4	0.0001

3.5.2 Subjects affected by familial chylomicronaemia syndrome

The Familial Chylomicronemia syndrome was identified in two carriers of homozygous mutations in *LPL* gene.

- *Case report - case 1*

The first case is a 58 years old woman with the missense mutation c.984G>T p.Met328Ile on exon 6, actually treated with volanesorsen. The suspicion of a genetic disease affecting the triglycerides started at the age of 22 with levels of TG between 4000 and 5000 mg/dL and total cholesterol of more than 500 mg/dL.

The treatment was started at the beginning of 1990s with nicotinic acid at dosage of 250 mg (3 capsules/die) that lowered the TG levels to 990 mg/dL (and TC to 195 mg/dL) but was not enough to prevent an episode of pancreatitis at the age of 30. After that, the treatment with

gemfibrozil and the association between eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was prescribed with a fluctuation of TG levels in the following years without reaching satisfactory results.

In 2014, the patient started to be treated in one of the LIPIGEN center and the therapy was incremented with medium chain fatty acids oil and hypolipidic diet. However, the patient continued not to optimally respond to therapy and TG concentrations were higher than 2000 mg/dL. Based on these values and after monitoring the baseline platelets, the treatment with volanesorsen at the dosage of 285 mg/week has been started in 2020. The baseline levels of TG, TC and platelets were 2393 mg/dL, 312 mg/dL and 245,000/uL, respectively and after 2 weeks of therapy triglycerides significantly decreased to 740 mg/dL with a decrement in the count of platelets at 177,000/uL, a value that is still tolerated.

The check at 4 weeks after the treatment initiation showed a further decrement in TGs. The patient continues to be followed up by the LIPIGEN sites following the constant monitoring check required by the regulatory authorities.

- *Case report - case 2*

The second patient with available data is a 47 years old male with the homozygous mutation in *LPL* gene with more severe clinical conditions compared to the case report 1 with seven episodes of acute pancreatitis, one of them as hemorrhagic.

Pretreatment baseline parameters were not available. Initial therapies included nicotinic acid in addition to ezetimibe (10 mg/die), simvastatin (20 mg/die), medium chain fatty acids oil and a hypolipidic diet (with a lipid composition <7%), with TG levels higher than 3700 mg/dL. Just adding lomitapide TGs level decreased reaching a value < 1000 mg/dL.

However, the lomitapide dose titration from 5 mg to 40 mg (as off-label use) caused a 3ULN increment of transaminase (ALT and AST) and the dosage was decreased to 20 mg/die. Afterwards, the instrumental exams (hepatic RMN and FibroSCA) detected a progression of steatosis and an onset of steatohepatitis, suggesting the non-alcoholic fatty liver disease (NAFLD).

Thus, lomitapide was interrupted for safety reasons and TG showed great variability reaching more than 7900 mg/dL on the association therapy with fenofibrate 145 mg, EPA and DHA 2g/die and medium chain fatty acids oil. Consequently, the patient has to add the plasma exchange weekly but the request for prescription of volanesorsen was made to achieve a better control of triglyceridemia during this summer.

3.5.3 Subjects affected by lysosomal acid lipase deficiency

Among the LIPIGEN study, five unrelated subjects did not presented FH causative variants but were carriers of the LIPA mutation: c.894G>A p.Gln298=, leading to lysosomal acid lipase deficiency. Three of them were carriers of the heterozygous form while other two individuals presented a homozygous form which, in any case, has been found to be compatible with life and the genetic confirmation was obtained later in life when the clinical phenotype suggested the presence of a genetic dyslipidemia.

- *Case Report*

For one LIPIGEN subject, detailed data were available and are related to a woman of 77 years with a clinical phenotype of familial hypercholesterolemia even if the genetic testing identified a homozygous mutation in the *LIPA* gene, leading to the diagnosis of a deficit in the lysosomal acid lipase. The patient presented a family history of cardiovascular diseases and is affected by hypertension, TSA and aortic arteriopathy.

She was sent to a LIPIGEN center because the lipid-lowering therapy did not allow to reach optimal goals. Baseline therapy was not available but previous intolerances to statin were detected. In details, previous therapies with simvastatin 20 mg/die and then ezetimibe/simvastatin 10/10 mg/die and cholestyramine needed to be implemented but the change to rosuvastatin at the dosage of 30 mg/die caused the development of cramps.

Because of statin intolerance, alirocumab (150 mg every 2 weeks) was prescribed but the patient did not respond (LDL-C 292 mg/dL) to the therapy and was referred to the LIPIGEN clinic where re-started the actual treatment with statin at lower dosage (rosuvastatin 20 mg/dL) in association with ezetimibe (10 mg/die) achieving a concentration of 89 mg/dL in LDL-C.

The other homozygote presented a severe hypercholesterolemia with pretreatment LDL-C of 344 mg/dL closed to the value detected also in two LIPA heterozygous subjects (381 and 312 mg/dL). For the last LIPIGEN carriers of heterozygous LIPA mutation, pretreatment biochemistry was not available but the therapy with simvastatin 40 mg/die associated with ezetimibe 10 mg/dL leads to a value of 82 mg/dL in LDL-C concentration.

CHAPTER 4

DISCUSSION

The LIPIGEN study is the first example of a national register of genetic dyslipidemias in Italy, involving more than 50 lipid clinics throughout the territory. Although the local distribution of LIPIGEN sites varies in terms of number of centers in each region, almost the entire territory is covered.

The choice of familial hypercholesterolemia as the first genetic dyslipidemia to be addressed by the register is related to the fact that is one of the most common genetic disease, translating in a higher number of affected subjects managed in several different contest.

In this way, it was possible to deal with different Italian realities, each with its own protocols, regulations and management methods in clinical practice.

The presence of a pathology register is important at national as well as at international level, as strongly underlined by the "global call to arms" of European Atherosclerosis Society FH Studies Collaboration (FHSC) (Vallejo-Vaz 2015), a worldwide initiative in which also Italy is involved with the LIPIGEN study, aimed at dealing with the FH burden at global level.

Only the integration of large-scale real-world data could strongly cover all aspects involved in FH, trying to fill the gaps in the knowledge and estimate the impact of FH in general population, improving the awareness of FH among clinicians, general practitioners-population and ensuring the most adequate and updated program of cares (Kindt 2017, Gazzotti 2020).

These benefits are well reported by the experiences of some registries established from the longest time as the SpAnish Familial HypErcHolEsterolaemiA CohoRt Study [SAFEHEART] in Spain (Mata 2011, de Isla 2017), the CAscade SCreening for Awareness and DEtection of Familial Hypercholesterolemia) registry [CASCADE FH] in the United States (Duell 2019), or the more recent Canadian FH registry in the British Columbia of Canada (Brunham 2018).

Pathology registers also suggest several research questions, as the deepening of the genetic basis of disease, the correlation genotype-phenotype, the local adaptation of diagnostic algorithms, the identification and analysis of specific subgroups, the real-world evidence of pharmacological approaches and gaps in the therapy, and the identification of factors modifying the cardiovascular risk. Some of them were analyzed within this PhD thesis related to the Italian scenario.

4.1 General cohort descriptions

At June 2021, LIPIGEN study enrolled more than 8700 subjects with a diagnosis of HeFH or HoFH, representing one of the wider cohorts in literature. The inclusion criteria for the LIPIGEN study included both a clinical or genetic diagnosis of FH, as in CASCADE FH, in the national French FH registry (Beliard 2016), or in the national HELLAS FH registry in Greece (Rizos 2017), while the Spanish SAFEHEART registry required the presence of a genetic diagnosis for index cases and for their relatives >15years. Moreover, the SAFEHEART included also a control group of relatives >15 years without a genetic diagnosis of FH, group that is not considered in the LIPIGEN study where relatives analysed with cascade screening were entered in eCRF only in presence of a clinical or genetic diagnosis of FH. Other registries, as the Canadian FH registry, are mainly focused on the clinical diagnosis because genetic testing is not available in the most of the country (Brunham 2018).

In LIPIGEN, the enrollment took place at the specialized lipid clinics involved in the SISA LIPIGEN Network, similarly as the majority of other registers, while the CASCADE FH planned to use a hybrid enrolment, including also a self-enrolment through an online screening mechanism accessible to general public, and the identification of possible cases using the electronic health records (EHRs) (O'Brien 2014).

- *Clinical and genetic characteristics*

Through the LIPIGEN data, a full clinical and molecular characterization of Italian subjects was performed. For LIPIGEN adults, the age at enrollment was 47 years old, similar to one of the Spanish cohort (46 years old), but younger compare to CASCADE-FH Registry (57 years old). The clinical diagnosis of FH was performed using the DLCN score, the algorithm mainly used at global levels (EAS Familial Hypercholesterolaemia Studies Collaboration 2018), though (as for all the algorithms supporting the clinical diagnosis) it is essential that all the required data should be available in order to avoid the possibility of misclassification (Casula 2018).

Regardless of the underlying genetic defect, the median level of pretreatment LDL-C (266 [215-329] mg/dL) and on-treatment LDL-C (152 [115-195] mg/dL) were slightly higher compared to values of CASCADE cohort (239 [211–294] mg/dL and 134 (100–183] mg/dL, respectively) and of German population (239 [192 343] mg/dL and 151 [107-204] mg/dL, respectively). Of note, the LIPIGEN study include both HoFH and HeFH and do not require the genetic confirmation in addition to the clinical judgment. An in-depth focus was conducted

on subjects that underwent the genetic testing, identifying higher LDL-C levels in the positive cohort compared to negative cohort, and intermediate values for subjects with only VUS. These data reflect the comparison between positive and negative FH subjects of other programs, as the one conducted in Korea (KFH) (LDL-C = 246 mg/dL vs 210 mg/dL) (Kim 2021), Norway (LDL-C = 313 mg/dL vs 217 mg/dL), Poland (TC = 406 mg/dL vs 368 mg/dL) (Sharifi 2016), and Brazil (LDL-C = 254 mg/dL vs 114 mg/dL) (de Paiva Silvino 2020).

As other FH cohorts, mutations in *LDLR* gene represented the more frequent cause of FH and were detected in about 98% of Italian subjects with a positive diagnosis of HeFH, followed by heterozygous mutations in *APOB* (N=64, 1.6%) and *PCSK9* (N=19, 0.6%). Although *LDLR* mutations are the most detected variants (80-90%), a slightly higher prevalence of *APOB* causative variants were detected in other small cohorts: in the CASCADE-FH sub-cohort (APOB 5.8%), in the Korean KFH registry (5.8%) (Kim 2021), in the Norwegian (5.4%), in Slovak (6%) (Gabcova 2017), in the Polish (8%) (Sharifi 2016) and in the Icelandic cohorts (4.5%) (Bjornsson 2021), being c.10580G.A, p.Arg3527Gln and c.10579C>T, p.Arg3527Trp the most detected causative variants in *APOB,* as in Italy. *PCSK9* mutations were not detected, or presented a prevalence lower than 1%.

The most reported mutations and the number of *LDLR* mutations varied based on the countries, and the analysis of each national cohort frequently allow the determination of new mutations not identified before (Pirillo 2017). We identified more than 400 pathogenic or likely pathogenic mutations, while the number in other cohorts also depends by the size of the selected cohort. Among LIPIGEN adults, the most detected mutations were c.662A>G p.Asp221Gly (as in Poland), 1646G>A p.Gly549Asp (as in Greece) and p.Gly592Glu (most common variants in other countries of Central Europe as Slovakia, Czech Republic and Poland) (Gabcova 2017). As in Italy, some countries present several mutations with comparable prevalence, probably due to the presence of ancestry that were carriers of different genotype. For example, in Norway the main detected mutation were c.313+1G>A, p.Cys231Gly and p.Pro685Leu; in Spain were c.1342C>T, p.(Gln488*) and c.97C>T p.Gln33* (Bourbon 2017).

At the contrary, just few mutations accounted for the most of cases in other countries because of the geographical isolation or social behaviors. For example, in Icelander cohort, c.694+2T>C is the most common single causative *LDLR* variant, known to be the founder mutation, while other variants became part of the Icendic genotype only during the last

century or have been detected in families of foreign origin (Bjornsson 2021). Another example is a small cohort from the Southeastern region of Brazil, where the most reported mutation was p.Asp224Asn, a pathogenic variant reported as one of the most detected in Portugal, probably because of historical features for the immigration of Portuguese in that part of Brazil in the 18th century and the high rate of consanguinity (de Paiva Silvino 2020).

Because of the elevated number of causative variants in *LDLR*, the genotype-phenotype correlation could be deepened taking into account their impact on biological function through the LDLR residual activity. In our LIPIGEN study, we identified the 20 mostly detected mutations and stratified the carriers by LDLR residual activity (as null mutation carries and defective mutation carries), confirming higher levels in LDL-C in carriers of a null mutation compared to defective ones (302.3±85.6 mg/dL vs 281.9±79.8 mg/dL, p<0.001). The more severe phenotype was also confirmed in other European cohort as the SAFEHERART registry, where carriers of null variants (N=911) had higher levels of LDL-C and higher prevalence of tendon xanthoma compared to defective carriers (N=1259) (LDL-C: 264 mg/dL vs 254 mg/dL, p<0.001; prevalence of tendon xanthoma: 16.6% vs 10.7%, p<0.0001) (Bourbon 2017).

- *Lipid-lowering therapy status and achievements of LDL-c goals*

Another aspect preliminary investigated in the LIPIGEN adults is related to the lipid lowering therapy. At entry, only the 35% of subjects (cohort mean age 47 yeas) were treated, a lower percentage compared to other countries, for example in Spain more than 80% was already on LLT at the inclusion on SAFEHEART (Mata 2011). Furthermore, in LIPIGEN, most of treated patients did not achieve the goal recommended by guidelines, and a level of LDL-C< 100 mg/dL was obtained by the 15.4% of treated cohort (with an increasing gradient from patients on only statin, patients treated with statin plus ezetimibe and on PCSK9 alone or in combination), decreasing to 4.5% when the goal of 70 mg/dL was considered. This undertreated condition was reported also in the Spanish cohort, where only the 3.4% reached a LDL-C value below 100 mg/dL. For what concerns the Italian scenario, this data showed a condition of under-treatment started later in life but mainly referred to the clinical practice of general practitioners, the physicians who managed patients before referring them to the specialist centers.

The involvement of lipid clinics, as in LIPIGEN study, could potentially lead to an improvement in LLT and goals achievement, managing also the most innovative drugs and implementing

the association therapy. However, the impact of the specialized work in modifying and setting an efficient high-intensity therapy would be evaluated only in future when the follow up data will be available. To do that, within this thesis, the *ad hoc* section was designed and implemented in the LIPIGEN eCRF "Changes in Lipid Lowering Treatments and Biochemistry". The compilation of follow up information will be crucial also to obtain a real-world snapshot of the Italian cohort in terms of achievement of LDL-C goals, type of prescribed therapy and incidence of CV events and death, trying to fill the treatment gap in care.

In fact, longitudinal data from SAFE HEART showed a further decrement of 16% in LDL-C levels after a 5 year follow up compared to baseline parameters, increasing the number of subjects on maximal LLT (71.8%) and the number who reached LDL-C goals based on ASCVD status although this percentage still remain too low (about 10%) (Perez de Isla 2016). A slightly higher percentage was detected in a longitudinal analysis of CASCADE FH registry, showing that, during a 20-months follow up, 48% of treated subjects achieved LDL-C< 100 mg/dL and 22% the goal of <70 mg/dL (Duell 2019). Similarly, low rates in the achievement of LDL-C goal were reported in German CaReHigh registry (Schmidt 2018) (the LDL-C goals of previous ESC/EAS Guidelines 2016 (Catapano 2016) were reached only by the 18% and 15% of patients with and without CVD, respectively) and in the PLANET registry of Czech Republic and Slovakia, where about the 15% of HeFH achieved the LDL-C goals, increasing to 17.3% in patients treated with a high intensity lipid lowering therapy (about the 55% of the cohort) (Vrablik 2018). Furthermore, the latest cross-sectional analysis of EAS-FHSC showed that among more than 42,000 HeFH adults enrolled in the EAS-FHSC registry only the 2.7% reached the LDL-C goal of 70 mg/dL, mainly reached by subjects treated with a combination therapy including also anti-PCSK9 mAbs (EAS Familial Hypercholesterolaemia Studies Collaboration 2021).

The obtainment of these large-scale real-world data was possible only because of the presence of national registries, included LIPIGEN, with standardized, harmonized and merged data into a unique global registry. The data aggregation from different local centers into registers and from national registries to a global one represent also a source of information for the determination of factor leading to a late identification, late referral and treatment failure and allow to evaluate the current practice, patient management and the identification of gaps in care.

4.2 Specific sub-cohort description and analyses

The elevated number of FH patients in the LIPIGEN study also allow identifying specific subgroups, as the ones reported in this PhD thesis, based on genetic defects or on age classes. The presence of a unique LIPIGEN dataset (extracted from the eCRF) revealed to be useful for this selection, allowing to verify all data at the same time without singularly contacting each lipid center.

- Homozygous cohort

The first identified sub-cohort was represented by homozygous FH, including also double heterozygotes and compound heterozygotes. Recently detected LIPIGEN HoFH patients were merged to the historical subjects, within the Italian Study Group of Homozygous FH, providing a full characterization of available HoFH data and taking advantage of years of experience of clinicians and geneticists involved in the project (Bertolini 2020).

A similar increasing gradient in LDL-C levels from DHE (393 mg/dL), CHE (520 mg/dL) and HO (607 mg/dL) was previously reported also in the Spanish cohort (397 mg/dL in CHE and 635 mg/dL in HoFH) but only the 55% of subjects presented LDL-C higher than 500 mg/dL, the historical cut-off for a clinical diagnosis of HoFH. This evidence confirms what reported also in other cohorts where LDL-C concentrations higher than 500 mg/dL were detected only in about 50% of Netherlands (Sjouke 2015), Spanish (Sanchez-Hernandez 2016) and Norwegian (Leren 2021) cohorts, and reinforces the decision to decrease the LDL-C cut-off to 400 mg/dL. The wide range of LDL-C in our homozygous cohort showed a great variability (from 309 mg/dL to 1084 mg/dL in HO, and from 276 mg/dL to 1100 mg/dL in CHE) in plasma concentration that could be explained the heterogeneity in *LDLR* gene variants. Consequently, the stratification by LDLR residual activities confirmed a more severe phenotype in R-NEG subjects compared to R-DEF with significantly higher level of LDL-C (774 mg/dL vs 503 mg/dL, p=0.0001) and a younger age at the first ASCVD event (22 years vs 46 years, p=0.0001). Similar differences were identified also in the Spanish cohort, confirming higher levels of LDL-C in R-NEG compared to R-DEF (788 vs 488 mg/dL) as also a higher prevalence of ASCVD at younger age (prevalence of ASCVD: 50% vs 44%, mean age at the first event: 23 vs 39 years old) (Sanchez-Hernandez 2016). Similar pattern were also reported in the Chinese (Sun 2018), Greek (Mollaki 2014) and German (Grenkowitz 2016) cohorts.

- Paediatric cohort

The other deeply analyzed cohort is the one of subjects under 18 years, facilitated by the creation of the LIPIGEN paediatric group. This initiative is similar to other sub-studies created in several countries as the *UK National Paediatric Familial Hypercholesterolaemia Register* in the United Kingdom (Ramaswami 2017), the *Czech MedPed registry (Vrablik 2017),* or the *Greek Paediatric FH Register* (Mollaki 2013). The LIPIGEN paediatric cohort with more than 1500 subjects is one of the largest cohorts of FH children and adolescents. Comparing the main features of Italian subjects with children and adolescents of other countries, the age at diagnosis (10 years old in LIPIGEN) does not differs from other registries/databases. As reported by Ramaswami et al. (Ramaswami 2020), the median age at diagnosis of paediatric cohorts of different European countries (Norway, UK, The Netherlands, Czech Republic, Austria, Portugal) involved in the International paediatric FH register was 10 years, ranging from 7 to 10 years. Moreover, a mean age at diagnosis of 9 years old was also detected in the US cohort within the CASCADE Registry (de Ferranti 2021). Few exceptions were reported in Slovenia (mean age at diagnosis 6 years), within a universal screening program in pre-school children (EAS Familial Hypercholesterolaemia Studies Collaboration 2018, Groselj 2018), and in Greece, with a median age at diagnosis of 3 years because of the presence of a systematic screening program for the detection of cholesterol in all children around 3 years. If concentrations exceeded the 97th percentile for age and sex, children were referred to a lipid clinic (Mollaki 2016).

The mean level of untreated LDL-C (222 mg/dL) in LIPIGEN children/adolescent was similar to the ones detected in other paediatric cohort with a variation from 188 to 240 mg/dL in Ramaswami et al. and 238 mg/dL in CASCADE FH registry (de Ferranti 2021).

Although it is known that children could reach a physiological reduction until the 10% in LDL-C concentrations during the pubertal development, the stratification of our cohort by age-classes and genetic diagnosis (Table 3.14) identified higher levels of LDL-C in the class 0-5 years, with a decreasing trend only in the positive group. The same trend was identified also in positive subjects after their stratification by number of causative mutations (1 causative variant including all heterozygotes, more than 1 causative variant including all homozygotes, compound heterozygotes and double heterozygotes). Among carriers of more than one variant, the higher mean LDL-C levels were detected in the first three age classes while a decrement of about 300 mg/dL was reported among subjects with 14-17 years old (Table

3.16); these differences could be related to an earlier diagnosis in the first decade of life of the more severe cases while patients with a milder phenotype would be identified later. Moreover, the difference could be influenced by the low number of subjects in the considered age classes but this is an unmodifiable parameter related to the lowest prevalence of rare diseases. As for adult cohort, the decreasing trend in mean LDL-C levels was confirmed across HoFH, CHE and DHE children and adolescents.

Among positive subjects, the most common cause of FH was the presence of the least one mutation in *LDLR,* with a similar prevalence to other European paediatric cohort (Futema 2021), while the prevalence of *APOB* causative variants was 1.5%. The majority of *APOB* mutation carriers presented the variant p.Arg3527Gln, as in other cohorts, although their prevalence is variable, from 0% in Greece to 39% in Czech Republic. As in the other European cohort, no mutations in *PCSK9* were identified, with the exception of Portugal (1%) and Norway (2%).

The genotype-phenotype correlation was further investigated through the stratification of *LDLR* heterozygotes by LDLR residual activity and confirmed the more severe phenotype in R-NEG compared to R-DEF as detected in adults. Furthermore, the LDL-C concentrations were investigated within the carriers of the same causative variants, choosing the most frequent mutations. Carriers of the C.1646 G>A p.Gly549Asp presented a mean untreated LDL-C of 245 mg/dL varying from 118 to 400 mg/dL, while carriers of 1775G>A p.Gly592Glu had LDL-C of 198 mg/dL with a minimum of 107 mg/dL to a maximum 367 mg/dL. The difference in mean LDL-C levels could be justified by the impact of LDLR residual activity: p.Glu549Asp is a R-NEG variant while p.Gly592Glu a R-DEF variant, nevertheless the range from the lowest to the highest LDL-C value was similar, highlighting the great variability in LDL-C also within carriers of the same causative variants. Moreover, c.1646G>A p.Gly549Asp was the most detected one in the Greek paediatric cohort, with similar LDL-C (256 mg/dL) compared to LIPIGEN children. The variant p.Gly592Glu was the most identified also in Czech Republic, with comparable mean LDL-C levels (199 mg/dL) to the LIPIGEN paediatric carriers (Futema 2021). Interestingly, the presence of the same mutation in Italian and Greek cohort could be explained by historical reasons that date back to the VIII-IV century B.C. during the century of Greek colonization of the southern part of mainland Italy and Sicily, regions where this mutation cluster has also previously described (Bertolini 2017).

This baseline characterization of children and adolescents affected by FH was performed collecting data through the parameters entered in the eCRF primarily designed for adults. Consequently, after researches in literature and the comparison with the pediatric lipidology specialists, some changes in the data collection were suggested in order to deepen specific points related to the young age of children and adolescents. The structure of the new section was planned to include only closed text fields in order to avoid any ambiguity during the data entry. After the creation of a draft of eCRF, the project was implemented with the help of informatics that finalize the new section. The rationale of the new "Paediatric data section" was to integrate the typical features for a diagnosis of FH in adults that could reveal to be inefficient in children. In fact, their young age and temporal limited exposure to high levels of LDL-C could lead to the lack of typical sign of FH (usually developed after decades, with the exception of the most severe cases (Maliachova 2018, McGowan 2019, Ramaswami 2020, Peterson 2021)). Moreover, their young age is frequently associated to the young age of parents that have not still developed a relevant cholesterol burden, suggesting the opportunity to consider not only the first-degree family member but also the second-degree ones.

Based on these considerations, a comparison between the FH phenotype of adults respect of children was carried out, in order to identify the typical parameters collected in the clinical practice. Considering that the eCRF was initially set for adults and the absence of algorithms considering the numerous changes in children and adolescents in the first decades of life, the prevalence of DLCN score features was compared between the two cohorts. Although the DLCN score was not validated in subjects under 18 years, it was the only tool applicable to both cohorts based on the available information.

In the first part, specific LDL-C cut-offs were identified for our paediatric population in order to reflect the same percentile distribution in adults. The increment in the proportion of children with a probable or definite diagnosis of FH (from 28.1% to 50.7%) demonstrated that the new identified cut-offs are more sensitive for the paediatric subjects but are not enough to identify the majority of genetically confirmed FH subjects. However, the percentage of subjects with a DLCN score higher or equal to 6 did not differ too much from the percentage in adults (59.1%), leading us to deepen also the other features of DLCN. The choice of selecting only positive subjects with no missing or only one missing parameters in the family history was based on the rationale to reduce the relevant impact of missing data on DLCN

performance (Casula 2018). The low prevalence of parameters included in the physical examination and clinical history confirmed the major limit of this algorithm. In fact, based on the official DLCN score, most of the points that lead to the final score derived from these parameters were absent in the majority of subjects due to a limited cumulative exposure to high levels of LDL-C. The absence of typical FH signs was confirmed also in the Portuguese FH cohort where no one of the 295 children (mean age 10 years old) presented any CHD or tendon xanthoma (Medeiros 2014). This comparison showed also another main difference in the prevalence of 1st degree relative with known premature CHD, present only in the 21% of children compared to 39% of adults. To deepen it, data collected within the "Paediatric Data section" allow to extend the family history information to 2nd degree family members showing an increment of the prevalence of premature CHD in 1st and 2nd degree relative to more than 56%. This trend was also observed in the Dutch registry where a history of CVD was reported in 20% of FH parents (mean age 41 years old) and in 49% of grandparents (mean age 51 years) (Galema-Boers 2015). However, the integration of this feature in the DLCN score did not allow to increase the proportion of children or adolescents with a probable or a definite diagnosis of FH. This observation suggested a potential importance in the family history but pointed out also a crucial issue related to the scoring system. The lower prevalence of typical FH features in children should lead to an adaptation of the DLCN score not only in term of evaluated features but also in term of points associated to each voice. However, the most critic limitation in this analysis is the absent of a control cohort that will be crucial for the future evaluation of the specificity of an adapted diagnosis algorithm that would include new identified parameters as well as a new modulation of scoring points.

At baseline, only few paediatric patients were already on lipid lowering therapy, despite guidelines and regulatory agencies approved statin from 6-8 years. The majority of evidences for children and adolescents derived from observational studies and clinical practice because of limitations of RCTs in children due to ethics reason. However, all available results confirmed the efficacy and safety of statin in the short-term, without any significant adverse effect on pubertal development, and further studies for the most innovative molecules are still on going.

Finally, to integrate the baseline characteristics with longitudinal data, the follow up section in eCRF was implemented in subjects under 18 years as in adults. Considering the huge number of enrolled individuals, the follow up information, collected starting from the last

year, will be crucial for the future development to investigate the treatment initiation and the use of lipid lowering drugs in children and adolescents, still undertreated.

At June 2021, follow up data were available only for 207 subjects, and showed that a lipid lowering treatment started within about 3.5 years after the first visit at LIPIGEN site. This preliminary information showed that patients with a non-satin therapy were younger compared to patients on statin. The mean age of therapy initiation was in line with other cohort as 13.6 years in The Netherlands (Galema-Boers 2015) and 12.5 years in Norway (Bogsrud 2018). The extension in data collection of follow-up information within the LIPIGEN registry will be also crucial to collect long-term based evidence of treatment with LLT in children and adolescents and the response to treatment, in terms of real-life effectiveness and safety. As reported by Ramaswami et al. (Ramaswami 2020), the statin therapy was able to significantly reduce the LDL-C in several European cohorts by an average of 46%, varying from 28% in Austria and 44% in Czech Republic. The achievement of LDL-C < 3.5 mmol was obtained in 55% of children over 10 years.

As future perspective, the finalization of a specific questionnaire for the physician of lipid clinic will be crucial to obtain a snapshot of the Italian management and treatment of FH children, and to underline the similitudes and differences in the clinical approach, taking into account that the LIPIGEN paediatric group includes both paediatric and adult centers that occasionally treated also subjects under 18 years. All these considerations could reveal to be a useful starting point for writing a position paper and/or recommendations for improving and standardizing the management of paediatric FH in the Italian scenario. A multidisciplinary framework integrated across primary care, paediatric specialists, and adult services aiming at optimal models of detection and care will ultimately change the natural history of this common and life-threatening disease.

4.3 Implementation of FH diagnosis

Furthermore, the implementation of DLCN score was investigated also in the adults, that are often diagnosed later in life (Nordestgaard 2013). Although this algorithm is the one mainly used for the identification of FH, its performance is not optimal (Casula 2018) and several modifications were proposed to improve the detection rate (Haralambos 2015). Among the DLCN parameters, the presence of tendon xanthoma strongly contributes to the final score for a clinical diagnosis of FH (Junyent 2005). Nevertheless, the detection of tendon xanthoma

at physical examination has poor sensitivity and is strongly influenced by the clinician judgement and most of FH does not present it (Tsouli 2005). However, the use of the Achilles tendon ultrasonography revealed to be a safe, reproducible and economic instrumental tool for filling the gap between physical examination and xanthoma detection (Tsouli 2005, Scott 2019, Paantjens 2020). Based on these considerations, the results of the ACTUS-FH analysis demonstrated that the ultrasonography was able to identify patients with a tendon xanthoma undetectable with the physical examination, increasing the number of subjects classified as definite FH by the DLCN score and those with the genetic confirmation. These results reflect the findings previously reported in monocentric experiences. In the study conducted by Descamps et al. (Descamps 2001), the use of ultrasonography was able to increase the number of patients with tendon xanthoma from 30%, percentage of subjects with tendon xanthoma at physical examination, to 75% of subjects with genetically-confirmed FH. The same trend was also reported in another study where the identification of tendon xanthoma increased from 43% to 68% in FH carriers of causative variant and from 22% to 46% in mutation-negative patients (Junyent 2005).

The stratification of our study cohort by genetic results (FH/M+ and FH/M-) showed a significantly higher prevalence of xanthoma in FH/M+ compared to FH/M-, suggesting the potential use of tendon ultrasonography as tool for the identification of FH individuals with a more severe phenotype. However, further studies with longitudinal data will be crucial to investigate the potential prognostic role of Achilles tendon ultrasonography. In the second part of the study, the diagnostic performance of the Achilles tendon thickness was investigated, showing an increasing trend from subjects without xanthoma, to subjects with only ultrasonography xanthoma to the ones with clinical detected xanthoma, regardless the genetic diagnosis. However, it was not possible to calculate a specific cut-off to discriminate FH/M+ with high sensitivity. In fact, the cut-off obtained by the ROC analysis (6.5 mm) was able to identify only the 32% of monogenic FH, suggesting caution in the interpretation of the results related to Achilles tendon thickness for clinical purpose. Although the cross-sectional nature of this study does not allow to investigate if the presence of xanthoma could guide the therapeutic choice, a positive correlation between Achilles tendon thickness, LDL-C burden, and marker of pre-clinical atherosclerosis was found in FH/M+ subjects. Association between ultrasonography, LDL-C (Kutkiene 2019), IMT (Kiortsis 2006, Jarauta 2009, Michikura 2017, Genkel 2020) and/or subclinical atherosclerosis (Jarauta 2009,

Michikura 2017) were detected in previous studies but only follow up data will be crucial to better investigate a possible prognostic role of Achilles tendon ultrasonography in the FH subjects.

The study presented some limitations related to the multicenter nature that could lead to a heterogeneity in the detection and measurement of Achilles tendon because there is not a univocal use in the ultrasonography but could depend also by the operator. However, all centers decided to standardize the activity with a share protocol to minimize the variability. Moreover, it was not possible to evaluate *a priori* the effect of additional metabolic risk factors as BMI even if a correlation between BMI, xanthoma and Achilles tendon thickness was identified and was probably related to the higher load on the tendon (Scott 2015).

Another criticality emerged from the baseline characterization of LIPIGEN cohort was the absence of a causative mutation in any of conventionally tested genes in about the 20% of subjects. Even higher percentages were detected in other FH cohorts, with variations depending on the molecular diagnostic testing and the clinical criteria (Benn 2012, Wang 2016). These observations led to hypothesize the presence of a "polygenic" cause (Teslovich 2010, Talmud 2013), where the sum of the effect of single LDL-C raising SNPs could explain both the LDL-C increment in subjects without monogenic variants and the variable phenotype observed in positive carries of the same causative mutation (Mariano 2020). Based on it, the analysis of polygenic risk score investigated the polygenic score proposed by Talmud et al. in our FH Italian adult cohort (Talmud 2013). The LIPIGEN FH study cohort was wider than the original study population of Talmud et al. (FH/M+: 875 vs FH/M-:644 and vs FH/M+:319 vs F/M-: 321) but the LDL-C levels (271 mg/dL in FH/M+ vs 217 mg/dL in FH/M- in LIPIGEN cohort and 272 mg/dL in FH/M+ vs 227 mg/dL in FH/M-in Talmud et al. cohort) and LDLc-score mean values (0.94 in FH/M+ vs 1.00 in FH/M- in LIPIGEN cohort, and 0.95 in FH/M+ vs 1.00 FH/M- and in Talmud et al. cohort) were comparable. These results confirmed the application of the LDLc-score not only in the Belgian (Descamps 2003) validation cohort (LDLc-score; 0.92 in FH/M+ vs 0.99 in FH/M-) used in the original paper but also in other European population as our Italian cohort (Martin 2019).

Although an Italian healthy population was not available within this project, the increasing trend in LDL-C concentration from the first LDLc-score decile to the last one (from 145 mg/dL to 190 mg/dL), detected in the Talmud et al. healthy group of the UK Whitehall II study (Martin 2019), was confirmed in the LIPIGEN cohort both in FH/M+ (from 182 mg/dL to 230

mg/dL) and in FH/M- (from 258 mg/dL to 281 mg/dL). Moreover, the Pearson correlation coefficients showed an even relevant impact of LDLc-score in FH/M+ cohort, supporting the presence of an additional influence of polygenic etiology on monogenic FH subjects, also confirmed by the correlation of LDLc-score and LDL-C concentration among carriers of the same causative variant. This suggested the evidence in literature supporting the coexistence of monogenic and polygenic etiology in FH, more as interacting instead of exclusive entities (Cupido 2021). This is also corroborated by the higher mean LDL-C levels in FH/M+ compared to FH/M- within the same LDLc-score deciles.

Furthermore, the modest difference in polygenic score among FH/M- and FH/M+, reported also in other cohorts, leading to hypothesize that the increment of LDL-C in FH/M- subjects was not driven only by the presence of polymorphisms but also by the influence of other additional factors as diet or lifestyle habits (Rieck 2020). In addition, the comparison of LDL-C values among LIPIGEH FH/M- and healthy controls of Talmud et al. showed about 30% higher LDL-C concentration in our negative cohort compared to healthy subjects, despite the polygenic score values were comparable. Consequently, it is possible to speculate of the concomitant presence of genetic and environmental factors that are able to modulate to FH phenotype in absence of a known causative mutation. This multifactorial nature (genetic-epigenetic-environmental factors) has already been proposed for CV disease (Sing 2003) and other disorders as diabetes or cancer (Tremblay 2019, Mbemi 2020), in which the exposure to environmental factors can modulate the genetic predisposition and *viceversa* (Ottman 1996, Yang 1997). However, further studies are necessary to completely clarify it (Flowers 2012).

Beyond the research purpose, the question about the diagnostic and prognostic utility of polygenic score in the management of dyslipidemic subjects still remain debated because evidence is still limited. The aim of early identification of FH subjects is finalized to reduce the cumulative exposure to high levels in LDL-C to reduce CV risk. However, the characterization of LDL-C trend from childhood to adulthood among polygenic subjects has not be clarified yet and the possible contribution of environmental factors in the increase of LDL-C could appear also later in life. This suggested the need to deepen also the possible interaction between gene and environment in order to clarify if the effect of polygenic risk score could be influenced by the environmental risk factors (Lewis 2020). As demonstrated by previous studies, the LDLc-scores are independently associated with the CVD risk that

increased in a dose-dependent manner with the increment of LDL-C polygenic scores (Trinder 2020). The inclusion of means of LDLc-scores in the CV risk models could lead to a clinical application of these scores for improving diagnosis and long-term prognosis (Lewis 2020, D'Erasmo 2021). However, it is necessary to consider that the polygenic hypercholesterolemia does not provide a dichotomous diagnosis of FH but a continuous gradient that increases CVD risk in a dose dependent manner, indicates the probability of a possible increment of LDL-C that is not so certain as in monogenic subjects and does not allow the genetic screening because is not inherited with autosomal dominant pattern (Umans-Eckenhausen 2003, Knowles 2017).

4.4 Rare dyslipidemias

Finally, the last part of this thesis is focused on the rare genetic dyslipidemias, including both HoFH but also other two familial dyslipidemias through the descriptions of cases reports as first steps to extend the data collection within the LIPIGEN study. For what concerns the familial chylomicronemia syndrome, the eCRF was designed during the last years with the help of physicians and informatics and will be available in the next months. Moreover, also some cases of LALD were identified through the analysis of *LIPA* gene within the FH genetic panel due to the similarities of phenotype with FH subjects. However, a systematic data collection needs to be improved as in the other genetic dyslipidemias to better investigate the impact of causative mutation on the phenotype. The extension of the LIPIGEN study to other monogenic dyslipidemias will allow to improve the detection of affected subjects in Italy, promoting the use of shared protocols and validating the diagnostic suspicion with the genetic testing. The analysis of the collected data will allow to estimate the prevalence of rare forms of genetic dyslipidemia and the identification of clusters and/or subpopulations at higher risk. These results could lead to the identification of priorities for future health interventions and for studies concerning the etiopathogenesis and the identification of factors that can influence the prognosis of specific rare forms.

4.5 Conclusions

To conclude, this thesis provided a full clinical and genetic characterization of both adults and children/adolescents affected by familial hypercholesterolemia in Italy. From these baseline features, critical issues were identified and developed in the second part of the project in

order to implement the detection and diagnosis of FH. In details, this thesis deals with the discrepancies between clinical and genetic diagnosis, deepening the polygenic aetiology of FH in the Italian cohort, confirming the role of SNPs in the modulation of LDL-C concentration even in FH monogenic subjects and supporting the application of PRS in the refinement of diagnosis and in the prediction of future cardiovascular risk. Moreover, this thesis evaluates the inclusion of the Achilles tendon ultrasonography in the diagnostic algorithm, identifying the ultrasound detected xanthoma as a valuable marker for clinical practice and as support for physician in the identification of FH subjects with higher LDL-C burden, who require to be earlier and more aggressively treated. Finally, it deals with some of the main obstacles related to the young age of FH children and adolescence, identifying the differences respect adults, the importance to establish *ad hoc* criteria for their identification, with the final aim of improving and standardizing the management of FH in the paediatric population.

Thus, the development of the LIPIGEN study contributed to improving the knowledge of genetic dyslipidemia in Italy (increasing also the number of lipid clinics that decide to join the project), facilitate the access to the execution and interpretation of genetic results, promote the process of cascade screening in the family members and contribute to international collaboration to face the burden of FH at global level. The extension of the LIPIGEN registry also to other genetic dyslipidemias will allow the pathology registers to grow, representing a valid tool to increase the number of subjects included in national databases, promoting awareness in among physician, general population, and supporting patient organizations. Moreover, efforts in the standardization of national registers will facilitate the comparison of results and the creation of large multinational databases merging local experiences, and contributing to the evidence-based enhancement of uncommon diseases at global level.

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APPENDICES

Appendix I – LIPIGEN study protocol

LIPIGEN

(LI**pid **T**rans**P**ort **D**isorders **I**talian **GE**netic **N**etwork**)

Registro delle Dislipidemie Familiari in Italia

Protocollo di studio

Versione 2.0 del 23 Aprile 2015

FONDAZIONE S.I.S.A.

Per la promozione della ricerca sulle malattie da arteriosclerosi

1. INFORMAZIONI GENERALI

PROTOCOLLO DI STUDIO	
Titolo: LIPIGEN (*LIpid TransPort Disorders Italian GEnetic Network*) Registro delle Dislipidemie Familiari in Italia	**Versione n.**: 2.0 **Data Versione:** 23 Aprile 2015
Codice studio: LIPIGEN	**Fase studio:** Studio Osservazionale
Sponsor: FONDAZIONE SISA Via Balzaretti, 7 20133 Milano	

1 INFORMAZIONI AMMINISTRATIVE

Sponsor	FONDAZIONE SISA Via Balzaretti, 7 - 20133 Milano Tel.: 02 49637591 Fax: 02 49633384 E-mail: fondazione@sisa.it Prof. Alberico Luigi CATAPANO *Direttore*
Sperimentatore Coordinatore	Dr. Fabio PELLEGATTA, MD *Centro per lo studio dell'Aterosclerosi* *IRCCS Multimedica, Sesto San Giovanni (MI)*
Comitato Scientifico	Prof. Marcello ARCA *Università di Roma "La Sapienza"* *Azienda Policlinico Umberto I* Prof. Maurizio AVERNA *Università degli Studi di Palermo* *A.O.U. Policlinico "P. Giaccone" – Palermo* Prof. Stefano BERTOLINI *Università degli Studi di Genova* *Ospedale San Martino di Genova* Prof. Sebastiano CALANDRA *Università degli Studi di Modena e Reggio Emilia Policlinico di Modena* Prof. Alberico Luigi CATAPANO *Università degli Studi di Milano* *Ospedale Bassini – Cinisello Balsamo (MI)* Prof.ssa Patrizia TARUGI *Università degli Studi di Modena e Reggio Emilia Policlinico di Modena*

LISTA DELLE ABBREVIAZIONI E DEGLI ACRONIMI

ADH	Ipercolesterolemia autosomica dominante
Apo	Apolipoproteina
CAD	Malattia Coronarica (Coronary Artery Disease)
Carotid IMT	Carotid Intima-media thickness (spessore medio intimale carotideo)
CE	Colesterolo Esterificato
CETP	Cholesterol Ester Transfer Protein
CRF	Case Report Form (Scheda Raccolta Dati)
FCHL	Iperlipidemia familiare combinata
FED	Fish Eye Disease
FH	Ipercolesterolemia Familiare
FHBL	Ipobetalipoproteinemia familiare
FLD	Familial LCAT Deficiency
ICH-GCP	Good Clinical Practice
IDL	Lipoproteine a densità intermedia
LCAT	Lecitin-Cholesterol acyl transferase
LMF	Lipase Maturation Factor - Fattore di Maturazione delle Lipasi
LPL	Lipoprotein Lipasi
NAFLD	Steatosi Epatica non alcolica
TG	Trigliceridi

2 introduzione e razionale

2.1 Dislipidemie oggetto dello studio

2.1.1 Ipo ed Ipercolesterolemie Genetiche

I livelli plasmatici di LDL-C sono sotto controllo genetico e ambientale. Ad oggi è stato studiato il ruolo di alcuni geni e tra questi i geni per LDL-R, Apo B; inoltre i polimorfismi dell'ApoE spiegano circa il 3- 6% della varianza dei livelli di LDL-C.

La ipobetalipoproteinemia familiare (FHBL), un disordine autosomico co-dominante con una frequenza di 1:500-1:1000 **(in Italia il numero di soggetti con FHBL è stimato nell'ordine delle 20.000 unità)**. La maggior parte dei soggetti FHBL sono eterozigoti; essi hanno livelli plasmatici di colesterolo inferiori al 5° percentile (< 128 mg/dl nella popolazione siciliana). La maggior parte di essi sono asintomatici o presentano una modesta sintomatologia. L'FHBL può derivare da difetti legati al gene apoB o indipendenti da esso. Le FHBL meglio caratterizzate sono quelle dovute a mutazioni del gene apoB. Con una sola eccezione, tutte le mutazioni del gene apoB riportate fino ad ora determinano l'inserimento di codoni di stop prematuri nell'mRNA, con formazione di forme troncate di apoB di dimensioni variabili da apoB-2 ad apoB-89 (cioè dal 2% all'89% delle dimensioni dell'apoB-100). Le apoB troncate possono essere visibili o non visibili nel plasma a seconda della loro lunghezza. ApoB troncate con dimensioni inferiori al 29% dell'apoB-100 (inferiori ad apoB-29) non sono visibili nel plasma in quanto non secrete in forma di lipoproteine. Ad oggi sono stati descritti solo pochi casi di FHBL con apoB troncate inferiori ad apoB-29. Recentemente è stata descritta una mutazione missenso del gene apoB (R463W) responsabile di FHBL. Essa è associata ad una ridotta secrezione di VLDL dovuta ad una prolungata ritenzione dell'apoB mutata nel reticolo endoplasmatico, probabilmente per un aumentato legame con MTP. Ci sono evidenze che in alcune grandi famiglie, l'FHBL non cosegrega con il gene apoB. In alcune di queste è stata osservata una cosegregazione con un locus sul cromosoma 3p21.

Recentemente è stato osservato che alcuni individui con un fenotipo FHBL-simile, sono portatori di mutazioni nonsenso del gene PCSK9. Questo gene codifica per un enzima proteolitico che sembra degradare il recettore LDL (LDL-R) negli epatociti. La perdita di funzione di questo enzima si traduce in un

aumento del numero di LDL-R a cui consegue un aumentato uptake di LDL dal plasma.

Un numero ancora indeterminato di soggetti con un fenotipo caratterizzato da ridotti livelli di LDL-C e HDL-C (ipolipidemia combinata) sono portatori di mutazioni a carico del gene ANGPTL3.

Un numero indeterminato di FHBL eterozigoti, con mutazioni che si traducono in forme troncate di apoB, presentano NAFLD (steatosi epatica non-alcolica). Queste mutazioni determinano diverse alterazioni funzionali, quali:

a) ridotta capacità delle apoB troncate di trasportare TG;
b) ridotta o assente secrezione di alcune forme troncate di apoB;
c) ridotta secrezione dell'apoB-100 normale.

L' entità di accumulo di TG sembra essere in relazione alla dimensione delle apoB troncate. In base alla nostra esperienza soggetti portatori di apoB troncate inferiori ad apoB-40 sviluppano sempre NAFLD di grado severo. In presenza di apoB troncate più lunghe di apoB-40 la capacità di esportare TG dal fegato non è ridotta al punto da indurre NAFLD conclamata, a meno che non siano presenti fattori steatogenici addizionali (consumo di alcool, obesità, infezione da HCV ecc.). Schonfeld e coll. hanno osservato che il contenuto epatico di TG nei soggetti FHBL portatori di forme troncate di apoB di varia lunghezza (da apoB-4 ad apoB-89) era 5 volte superiore rispetto ai controlli. Questa differenza non era influenzata da altri fattori steatogenici noti. Anche nell'infanzia la FHBL è associata a NAFLD particolarmente nei casi di individui portatori di apoB troncate corte non visibili. Purtroppo non sono disponibili studi prospettici di follow-up di soggetti FHBL genotipizzati riguardanti la progressione della NAFLD ad epatopatie più severe. Soggetti FHBL sembrano predisposti alla colelitiasi, condizione peraltro frequente in soggetti con NAFLD dovuta ad altre cause.

L'ipercolesterolemia autosomica dominante (ADH) è una delle malattie monogeniche più frequenti ed è caratterizzata clinicamente da elevati livelli plasmatici di LDL-colesterolo, xantomatosi tendinea, arco corneale e coronaropatia precoce su base aterosclerotica. I geni classicamente associati alla ADH sono il gene del recettore delle LDL (Ipercolesterolemia Familiare o FH) e il gene dell'Apo B (Familial Detective ApoB o FDB). Recentemente sono state descritte due mutazione del gene PCSK9 responsabile di ADH (FH3).

La frequenza della FH in eterozigosi è stimata in circa 1/500 nella popolazione generale, e fa di questa malattia una delle sindromi monogeniche più comuni. La forma omozigote, la cui espressione fenotipica ha caratteristiche di estrema gravità, è molto rara, con una frequenza stimata di 1/1 milione. I soggetti affetti da FH in eterozigosi presentano livelli plasmatici di colesterolo totale e di LDL-colesterolo compresi tra 300 e 500 mg/dl. Negli omozigoti i livelli sono sensibilmente più alti, con concentrazioni comprese tra 600 mg/dl e 1200 mg/dl. Nei soggetti con FH, il colesterolo in eccesso veicolato dalle LDL si deposita preferenzialmente in alcuni tessuti quali cute, tendini e arterie, dando origine a lesioni caratteristiche. La gravità di queste lesioni, soprattutto quelle a carico della parete arteriosa, è direttamente correlata sia ai livelli plasmatici di LDL-colesterolo che al tempo di esposizione ad elevati livelli di LDL-colesterolo. In eterozigosi la FH comporta livelli plasmatici di LDL-colesterolo elevati fin dalla nascita, anche se i soggetti affetti rimangono solitamente asintomatici per decadi fino allo sviluppo di sintomi correlati soprattutto alla coronaropatia su base aterosclerotica.

I soggetti eterozigoti per FH presentano anche una maggiore probabilità di sviluppare cardiopatia ischemica ed infarto miocardico acuto prima dei 60 anni: l'85% rispetto ad un 15% circa dei soggetti non FH. Gli omozigoti mostrano invece caratteristiche cliniche molto più uniformi: xantelasmi e xantomatosi sono presenti nei primi anni di vita e le manifestazioni cliniche della malattia coronarica su base aterosclerotica sono evidenti già nella prima infanzia e non sono rari i casi di decesso per infarto miocardico acuto prima dei 20 anni.

Dal punto di vista clinico è possibile distinguere i soggetti affetti da Ipercolesterolemia Familiare in: a) Ipercolesterolemia Familiare definita e b) Ipercolesterolemia Familiare probabile. La forma definita è caratterizzata da livelli plasmatici di colesterolo totale (CT) > 290 mg/dl negli adulti o > 260 mg/dl nei bambini sotto i 16 anni, oppure livelli di LDL-colesterolo > di 190 o 155 mg/dl rispettivamente negli adulti o nei bambini, associata alla presenza di xantomi tendinei nella famiglia. La forma probabile è caratterizzata da livelli di colesterolo come i precedenti ma è anche associata ad una storia familiare di infarto miocardico in assenza di xantomatosi tendinea nella famiglia.

Gli elevati livelli di LDL colesterolo riscontrati in soggetti FH sono dovuti ad una mancata o ridotta rimozione delle LDL dal circolo sanguigno, il che può essere dovuto ad un deficit di sintesi o ad una funzione alterata del recettore delle LDL causati da mutazioni del gene codificante il recettore delle LDL. Le mutazioni

nel gene per il recettore delle LDL note ad oggi sono più di 700 (www.ucl.ac.uk/fh).

Identificare una mutazione in un soggetto con Ipercolesterolemia Familiare significa poter fare una diagnosi definitiva (che potrebbe essere essenziale in vista di una probabile terapia genica) e permettere che in una famiglia siano identificati tutti i componenti affetti (anche in età pediatrica), in modo tale da poterli sottoporre a terapie ipocolesteromizzanti adeguate in tempi precoci rispetto all'insorgere dei sintomi. L'identificazione di un numero significativo di soggetti affetti dalla stessa mutazione sarebbe, inoltre, di enorme aiuto per lo studio delle relazioni tra genotipo e fenotipo e soprattutto per poter correlare specifiche mutazioni con le risposte alle terapie ipolipemizzanti.

In alcuni paesi come il Sud Africa, la Finlandia e il Quebec sono presenti solo poche mutazioni responsabili di FH in queste popolazioni (effetto capostipite) e ciò permette di fare diagnosi molecolare con maggiore facilità perché permette l'utilizzo di test veloci e poco costosi per l'identificazione della mutazione causale della malattia. In Italia, particolarmente in Sicilia, il quadro è invece molto eterogeneo rispecchiando il dato storico dell'incontro di molteplici etnie. Nel nostro paese sono state descritte più di 89 differenti mutazioni che includono sia grossi riarrangiamenti che mutazioni puntiformi. Anche in Sicilia, comunque, alcune mutazioni sembrano avere una maggiore frequenza rispetto ad altre.

2.1.2 Ipertrigliceridemie e le forme miste

2.1.2.1 Ipertrigliceridemie

Si definisce ipertrigliceridemia l'aumento dei livelli di trigliceridi (TG) nel plasma, che è dovuto alla presenza in eccesso di VLDL e/o di chilomicroni.

La classificazione fenotipica classica proposta da Frederikson comprende la iperlipoproteinemia di tipo I caratterizzata dalla presenza in circolo di chilomicroni a digiuno, la iperlipoproteinemia di fenotipo IV con livelli di VLDL elevati e le forme caratterizzate dalla contemporanea presenza nel plasma a digiuno di elevate quantità di VLDL e di chilomicroni (iperlipoproteinemia di fenotipo V).
L'Ipertrigliceridemia moderata è una forma comune di dislipidemia ed è caratterizzata da livelli di trigliceridi sierici generalmente compresi tra 200-500 mg/dl e comunque inferiori ai 1000 mg/dl; queste forme di ipertrigliceridemia

sono spesso secondarie associate a patologie di base (Diabete, malattie renali, obesità, abuso di bevande alcoliche) che concorrono all'aumento del rischio cardiovascolare.

Se l'ipertrigliceridemia sia associata direttamente all'aumentato rischio di CAD o sia un marker di altre anomalie lipoproteiche è ancora una questione controversa: sicuramente la ipertrigliceridemia correla fortemente con la presenza di LDL piccole e dense e con la riduzione delle HDL-2 (entrambi i fattori sono stati associati ad un maggiore rischio di CAD).

Le forme severe di Ipertrigliceridemia sono invece caratterizzate da livelli sierici di TG > a 1000 mg/dl ed aumentato rischio di pancreatite.

2.1.2.2 Presentazione clinica dell'ipertrigliceridemia ereditaria ed epidemiologia

In soggetti con ipertrigliceridemia, la presenza di elevazione dei trigliceridi nei familiari di primo e secondo grado è sospetto per una forma ereditaria. Indicazione basata usl consenso

E' questa una forma genetica con trasmissione autosomica dominante caratterizzata da un aumento dei livelli di TG (200-500 mg/dl) nel probando e nei familiari di I e II grado. L'Ipertrigliceridemia Familiare si manifesta in genere in età adulta e si associa a bassi livelli di HDL-C mentre le concentrazioni di LDL-C sono normali. Non è del tutto chiara la prevalenza di questa forma di iperTG, anche perché obesità, ridotta tolleranza al glucosio, iperinsulinemia e iperuricemia sono frequentemente associati alla ipertrigliceridemia familiare.

Le migliori stime indicano la prevalenza delle forme primitive attorno allo 0,2-0,3%. Livello B

In certe fasi della malattia (ad esempio dopo abusi alimentari) i livelli di TG possono essere molto più elevati e superare i 1000 mg/dl. Non vi è probabilmente un difetto genetico unico: nella maggioranza dei casi l'alterazione non è nota, ma difetti in eterozigosi del gene della lipoproteinlipasi possono determinare questo fenotipo.

Inoltre questa forma è talora mascherata da altre patologie con una elevata aggregazione familiare (ad es. la sindrome metabolica) e che comportano indipendentemente un elevazione della trigliceridemia.

2.1.2.3 Altre forme di ipertrigliceridemia familiare

Il riscontro di livelli di trigliceridi superiori a 1000mg/dl in pazienti con familiarità deve portare a sospettare la chilomicronemia familiare. Indicazione basata sul consenso

La chilomicronemia familiare è un raro disordine genetico del metabolismo lipidico trasmessa con modalità autosomica recessiva ed è caratterizzata da una massiva ipertrigliceridemia con presenza di chilomicroni a digiuno.

La frequenza stimata di questa di questa rara forma di ipertrigliceridemia severa è di circa 1:1.000.000.

Le due forme familiari di chilomicronemia maggiormente conosciute e studiate sono causate da mutazione del:

a) gene della Lipoprotein lipasi (LPL - OMIM 238600) con conseguente deficit della normale funzione dell'enzima LPL e

b) gene dell'Apolipoproteina C-II (ApoCII - OMIM 608083) con conseguente produzione di una apoCII alterata e conseguente alterazione della funzione di attivatore della LPL.

La LPL è l'enzima deputato al catabolismo ed alla rimozione dei trigliceridi e delle lipoproteine ricche in trigliceridi (VLDL e chilomicroni).

Recentemente sono stati descritti casi di ipertrigliceridemia severa a trasmissione autosomica recessiva causate da mutazioni della:

a) apolipoproteina A-5 (ApoA-V - OMIM 606368) con conseguente deficit di questa proteina che svolge una funzione di attivatore della LPL e

b) del gene LMF1 (Lipase Maturation Factor - Fattore di Maturazione delle Lipasi) che interferiscono con il normale processo di maturazione dell'enzima LPL. Ad oggi è stato descritto un solo caso di mutazione;

c) c) del gene GPIHBP1 (Glycosylphosphatidylinositol-anchored HDL binding protein 1) che interferiscono con l'ancoraggio della LPL alle cellule endoteliali. Da ricordare che la chilomicronemia può essere secondaria a paraproteinemia e sono state descritte forme transitori di deficit transitori di LPL: nella prima forma le paraproteine legandosi direttamente alla lipasi oppure alla apoproteina CII impediscono l'attivazione della lipasi mentre le seconde sono probabilmente legate ad inibizioni enzimatiche su base auto-immune.

La malattia, come detto precedentemente, è caratterizzata da livelli molto elevati di TG (> 1000 mg/dl ma talora anche > 7-8000 mg/dl); i livelli di HDL-C e di LDL-C sono bassi.

La chilomicronemia comporta un elevato rischio di pancreatite acuta. Livello A.

È una patologia grave per l'elevato rischio di pancreatite acuta che comporta. Viene solitamente diagnosticata in età pediatrica per la comparsa di dolore addominale ricorrente.

Altri sintomi tipici sono la xantomatosi eruttiva, la lipemia retinalis ed un epato-splenomegalia, mentre coesistono non di rado alterazioni della concentrazione o anche cefalea, legati all'iperviscosità ematica dovuta alla chilomicronemia. Livello A

La xantomatosi cutanea si manifesta in forme molto diverse e può interessare qualsiasi distretto della superficie cutanea. La xantomatosi eruttiva compare nei pazienti con grave ipertrigliceridemia indipendentemente dall'eziologia del disturbo metabolico. Sedi preferite degli xantomi eruttivi sono le superficie estensorie degli arti, la regione glutea ed il dorso. Queste lesioni regrediscono abbastanza rapidamente durante il trattamento dell'ipertrigliceridemia.

La lipemia retinalis è una complicanza causata dall'eccessivo accumulo di lipoproteine ricche in trigliceridi, che diluiscono i globuli rossi del sangue e fanno assumere ai piccoli vasi retinici un aspetto lattescente. Inoltre, se le lipoproteine trasudano dal letto capillare formano sulla retina degli essudati lipidici che, quando interessano la macula, possono compromettere l'acuità visiva del paziente.

La diagnosi si basa sulla valutazione dell'aspetto del plasma posto per 24 ore a 4° (con il tipico aspetto caratterizzato dall'orletto cremoso su un infranatante limpido, anche se non sempre è possibile visualizzarlo), sull'analisi dell'apoCII attraverso il dosaggio e/o di metodiche elettroforetiche in grado di discriminarne alterazioni strutturali, sulla determinazione della attività enzimatica della lipoproteinlipasi in campioni post-eparinici di plasma da parte di laboratori specializzati oppure, più semplicemente, sulla documentazione delle mutazioni nei geni che codificano per la LPL, l'apoCII o l'apo AV, LMF1 e GPIHBP1.

La terapia delle chilomicronemie è esclusivamente dietetica, con la drastica riduzione dei grassi che danno luogo alla formazione dei chilomicroni.

In sostituzione dei grassi usuali si possono utilizzare acidi grassi a catena mediocorta (Medium Chain Triglycerides, olio MCT) che vengono assorbiti dall'intestino legandosi all'albumina, evitando così la sintesi intestinale dei chilomicroni.

La disbetalipoproteinemia è una patologia legata all'accumulo di lipoproteine a densità intermedia (IDL) e di remnants, entrambe altamente aterogene.

In pazienti con aumento rilevante della CT e della trigliceridemia (TG) (entrambi >300 mg/dL) va sospettata una disbetalipoproteinemia. Indicazione basata sul consenso

Nel passato la disbetalipoproteinemia (dislipidemia tipo III nella classificazione di Frederickson) si diagnosticava attraverso l'osservazione al lipidogramma di una "larga banda beta", la quale però, nel tempo, si è visto essere un carattere molto aspecifico. Il difetto patogenetico è costituito dalla presenza di un'isoforma dell'apoproteina E (ApoE) (indispensabile per la rimozione delle IDL) dotata di minore affinità per il recettore specifico (ApoE2, oppure una forma mutata dell'ApoE).

La prevalenza della disbetaliproteinemia è stimata tra 0,2 e 1 caso su mille. Livello A

Un aspetto peculiare è rappresentato dal fatto che la presenza dell'omozigosi per l'ApoE2 è la condizione necessaria, ma non sufficiente per lo sviluppo della malattia. Infatti, sebbene l'omozigosi per l'ApoE2 abbia una frequenza di circa 0,6% della popolazione generale, la frequenza della disbetalipoproteinemia è molto inferiore, circa 0,02–0,1%. Ciò suggerisce che altri fattori concorrono a favorire la comparsa della dislipidemia in associazione alla presenza dell'omozigosi per l'ApoE2. Queste condizioni sono tutte quelle in grado di aumentare la produzione di lipoproteine ricche in trigliceridi (come il diabete, l'obesità, l'ipotiroidismo), poiché tale aumento non può essere compensato dall'incapacità dell'ApoE disfunzionale a smaltire l'eccesso di lipoproteine in circolo.

Il paziente con disbetalipoproteinemia presenta spesso arco corneale lipidico, xantelasmi, xantomi tuberosi e tubero-eruttivi e xantomi striati palmari. In questa malattia è alta l'incidenza di vasculopatie sia coronariche che periferiche. Gli xantomi striati palmari, strie giallastre localizzate tipicamente nelle pieghe interdigitali o sulla superficie palmare delle mani sono segni clinici patognomonici della malattia.

La diagnosi può essere fatta esclusivamente attraverso la documentazione dell'omozigosi per il genotipo ApoE2 o dell'eventuale mutazione del gene dell'ApoE in omozigosi.

2.1.2.4 Iperlipidemia Familiare Combinata

L'iperlipidemia familiare combinata (FCHL) è una dislipidemia la cui causa genetica è ancora sconosciuta, anche se si ritiene che essa abbia una trasmissione autosomica dominante a penetranza incompleta.

La FCHL è una causa molto comune di dislipidemia nella popolazione generale, con una frequenza di 3–5/1.000. Livello A

I soggetti con FCHL hanno spesso una storia familiare di cardiopatia ischemica prematura. Livello A

I risultati di numerosi studi hanno suggerito che la FCHL è causata dall'aumentata produzione epatica di ApoB e di VLDL, precursore metabolico delle LDL. La cascata metabolica che porta alla produzione di LDL dalle VLDL è regolata dall'attività della LPL che subisce svariate influenze da fattori ambientali (fumo, inattività fisica, sovrappeso).

Per tale motivo il fenotipo può essere molto variabile e caratterizzato dall'aumento isolato di LDL (fenotipo IIA di Fredrickson), dall'aumento isolato di VLDL (e quindi dei TG - fenotipo IV di Fredrickson) o da entrambe le alterazioni (fenotipo IIB di Fredrickson). Si ritiene che ciò sia, almeno in parte, legato alle condizioni metaboliche co-esistenti. È importante sottolineare che la variabilità dell'espressione fenotipica della FCHL nello stesso individuo in vari tempi o nei familiari, rappresenta l'elemento più caratteristico di questa patologia.

La diagnosi si basa sul riscontro di valori di CT ≥240 e/o TG tra 200 e 500 mg/dL nel paziente e in uno o più familiari. Livello A

Può esserci una variabilità intraindividuale e intrafamiliare del fenotipo dislipidemico; il rilievo di valori elevati di ApoB è stato recentemente proposto come un fondamentale indicatore di FCHL.

Il gene (o i geni) responsabile della FCHL non è noto.

2.1.3 Ipo Iper Alfalipoproteinemie

Il ruolo delle Lipoproteine ad alta densita'-HDL-sul rischio cardiovascolare e' stato evidenziato sin dai pioneristici primi studi epidemiologici osservazionali, Framingham Heart Study, ed e' stato ripetutamente confermato dagli studi piu' recenti-PROCAM study- Gli studi di intervento- Coronary Heart Project, Helsinki Heart Study e piu' recentemente il VA-Hit, hanno confermato le prime osservazioni. Possiamo oggi ritenere che un aumento del 1% del colesterolo HDL determina una riduzione del rischio cardiovascolare del 2-3%.

Nonostante queste certezze, i meccanismi fisiopatologici attraverso cui si sviluppa la protezione vascolare da parte delle HDL, non sono completamente chiariti; inoltre spesso bassi livelli di colesterolo HDL si associano ad altre anomalie metaboliche aterogene, quali elevati livelli di trigliceridi e di lipoproteine remnants, ad obesità, insulino-resistenza, sindrome metabolica e diabete. Lo studio di patologie genetiche caratterizzate da elevati o bassi livelli di colesterolo HDL ha consentito di approfondire la conoscenza dei complessi rapporti tra HDL e patologia vascolare.

2.1.3.1 Ipoalfalipoproteinemie Familiari

Appartengono a questo gruppo una serie di malattie genetiche causate da un difetto molecolare di un singolo gene, a trasmissione recessiva o co-dominante, che si associano ad un fenotipo biochimico caratterizzato da bassi livelli di Colesterolo HDL e talvolta ad un fenotipo clinico caratterizzato da un aumentato rischio di eventi cardiovascolari. Fra queste prenderemo in considerazione le patologie caratterizzate dall'alterazione di alcuni geni: Lecitin-Cholesterol acyl transferase o LCAT, ATP binding cassette transporter A1 o ABCA1, mutazioni del gene dell'apolipoproteina AI.

2.1.3.2 Deficit di LCAT

L'enzima LCAT realizza l'esterificazione del colesterolo libero a colesterolo estere, utilizzando un gruppo acilico (acido grasso) prelevato da un fosfolipide noto come lecitina. (figura 1).

L'assenza di tale enzima quindi realizza un blocco maturativo a carico delle lipoproteine HDL, da cui i ridotti livelli circolanti, ed una relativa abbondanza di

colesterolo libero, che normalmente non rappresenta più di un terzo del colesterolo totale.

Il deficit di LCAT e' responsabile di diverse forme cliniche; il deficit classico definito "Familial LCAT Deficiency" o FLD, e il deficit parziale di LCAT, definito "Fish Eye Disease" o FED.

Il FLD venne identificato nel 1967 in una famiglia norvegese; questo difetto è caratterizzato da ridotta o nulla attività LCAT e ridotta presenza di colesterolo estere circolante. In tale patologia sono presenti bassi livelli di colesterolo HDL ed anomalie a carico delle lipoproteine LDL e VLDL. Clinicamente il quadro e' caratterizzato dalla presenza di opacità corneali, da anemia con proteinuria e dall'evoluzione verso l'insufficienza renale. Il FED venne identificato originariamente in una famiglia svedese, ed e' caratterizzato da minori alterazioni cliniche e biochimiche rispetto al FLD, infatti, presenta una attività esterificante il colesterolo plasmatico apparentemente normale, livelli parzialmente ridotti di LCAT circolante, bassi valori di colesterolo HDL con elevati valori di trigliceridi, anomalie delle VLDL e LDL, ma soprattutto assenza di segni clinici tipici del FLD, se si eccettua la presenza di opacità corneali, evidenti con il progredire dell'età, che giustificano il nome della malattia: "fish eye disease" o malattia dell' occhio di pesce. La spiegazione di questa apparente normalità dei livelli di colesterolo esterificato (CE) circolante, si ottenne analizzando le singole frazioni lipoproteiche. A discapito di una normale presenza di CE nella VLDL e LDL, i livelli di CE nelle HDL erano molto bassi. Questo fece supporre l'esistenza di una attività alpha LCAT esterificante le HDL (lipoproteine alpha) carente nella FED, ed una attività beta LCAT, esterificante VLDL e LDL (lipoproteine beta). In realtà il gene LCAT è unico nell'uomo, e queste due attività sono rappresentate da un unico enzima. Ad oggi sono note circa 40 mutazioni dl gene LCAT, e non esiste la possibilità di predire se il fenotipo sarà FLD o FED partendo dalla sede di mutazione. Un punto ancora discusso è dato dalla mancanza di associazione del deficit LCAT, caratterizzato da livelli molto bassi di HDL, con la malattia coronarica. Questo paradosso ha determinato la necessita di rivedere quello che sappiamo del trasporto inverso del colesterolo, con la nascita di nuove ipotesi. Una di queste ridimensiona il ruolo delle HDL piccole come accettrici di colesterolo libero cellulare e sede della sua esterificazione e considera le VLDL ed LDL come primo accettore su cui si svolge prevalentemente il lavoro del LCAT. In atto questo rappresenta un problema aperto.

2.1.3.3 Deficit di ABCA1

Il gene ABCA1 e il suo ruolo nel trasporto inverso del colesterolo venne identificato in soggetti che esprimono la malattia di Tangier. Tale malattia prende il nome dall' isola omonima, dove sono stati identificati primi casi. Il quadro clinico è caratterizzato prevalentemente dall'accumulo di colesterolo in diverse sedi anatomiche. Sono caratteristiche le grandi tonsille arancioni, la epato-splenomegalia, le alterazioni timiche e linfonodali, e l'accumulo in corrispondenza della parete intestinale. Le alterazioni neurologiche caratterizzano una neuropatia definita "syringomyelia-like sindrome" che presenta alterazioni della sensibilità termico-dolorifica, ipostenia agli arti, opacità corneali con possibile evoluzione verso la cecità.

Fra le caratteristiche biochimiche la più rilevante è la assenza di colesterolo HDL circolante o, in una forma definita come "familial HDL deficiency" o FHD, livelli inferiori al 5° percentile della normale distribuzione plasmatica.

Nel 1999 venne identificato il gene ABCA1 come responsabile della malattia e si è valutato il possibile ruolo di ABCA1 nel determinare i livelli di HDL colesterolo circolante. Analizzando i soggetti eterozigoti nelle famiglie di portatori di casi di Tangier si è infatti visto come questi soggetti presentano livelli più bassi di colesterolo HDL, livelli piu' alti di trigliceridi ed una frequenza circa tre volte più elevata di malattia coronarica. ABCA1 fa parte della famiglia degli ATP binding cassette transporter, una serie di proteine che regolano flussi trans-membranari di svariate molecole con particolare azione sugli steroli. ABCA1 realizza il primo passo della formazione delle HDL, cioè la lipidazione della apolipoproteina AI circolante da parte del colesterolo che viene rilasciato dalle membrane cellulari, processo che viene definito "efflusso di colesterolo". Questa prima attività viene svolta con consumo di energia, sotto forma di ATP, da ABCA1. Una volta che il processo è avviato, la lipidazione continua in modo piu' rapido tramite altri sistemi di flusso passivo di colesterolo, senza dispendio energetico. I dati sperimentali derivano prevalentemente da modelli animali, e non sempre sono stati consistenti. Infatti, sebbene la sovra-espressione di ABCA1 in topi sottoposti a dieta aterogena abbia ridotto l'entità della aterosclerosi aortica, quando in topi ABCA1 deficienti sono stati inseriti macrofagi esprimenti ABCA1 tramite trapianto di midollo osseo, questi non hanno mostrato modifiche di livelli di colesterolo HDL o apolipoproteina AI circolanti rispetto ai macrofagi privi di ABCA1, suggerendo che il ruolo di ABCA1

nei tessuti periferici sia marginale per il trasporto inverso del colesterolo rispetto al ruolo di ABCA1 epatico.

2.1.3.4 Mutazioni di apolipoproteina AI responsabili di Ipoalfalipoproteinemia

L' apoliproteina AI rappresenta la componente proteica principale delle lipoproteine HDL. Essa oltre ad avere funzione strutturale presenta numerose altre funzioni di interazione con enzimi chiave del metabolismo lipidico corrispondenti a domini proteici specifici che all'interno della lipoproteina AI riconoscono determinate funzioni. Ad esempio riconosciamo un dominio di legame per i lipidi con funzione strutturale. Mutazioni aminoacidiche in alcuni punti chiave di questo dominio (Leu222, Phe225, Phe229) o di un area ricca di leucina (Leu211, Leu214, Leu218-219) rendono impossibile l'assemblaggio di una HDL normalmente strutturata. In una rivisitazione della letteratura del 2002 sono state classificate 46 mutazioni del gene dell'apolipoproteina AI. Circa la meta' di esse determina bassi livelli di colesterolo HDL, e possono essere fenotipicamente racchiuse in due classi: le mutazioni che determinano ridotta attivazione della LCAT, e quelle associate alla deposizione amiloide. Le mutazioni della prima classe sono concentrate nella parte centrale del gene, che codifica per sequenze ripetute "repeats" 5, 6, e 7, approssimativamente nella regione compresa tra gli aminoacidi 121 e 187.

In questo caso la apolipoproteina AI non attivando la LCAT impedisce l'accumulo di esteri di colesterolo all'interno delle HDL e ne arresta la "maturazione" verso le HDL ricche in colesterolo estere (HDL2).

Fra queste vengono considerate diverse mutazioni identificate con l'area di appartenenza geografica; Apo AI Seattle, (del 146-160) Mallorca (del 165-175) rappresentano due grosse delezioni del repeat 6 mentre altre mutazioni puntiformi sono associate a bassi livelli di HDL (Fin,Zavalla etc.). L'analisi dei pedigree di questi soggetti affetti da mutazioni dell'apo AI non ha mostrato univocamente l'associazione con la malattia coronarica, solo approssimativamente in un terzo dei casi è stato accertato un eccesso di malattia rispetto ai consanguinei non affetti. Nell' altra classe di mutazioni, caratterizzata da accumulo di amiloide, è stato dimostrato che la proteina che forma amiloide e' quasi sempre la transtiretina o prealbumina, vista la sua particolare conformazione proteica di tipo beta-elica. Queste mutazioni sono caratterizzate da un quadro clinico di nefropatia e cardiomiopatia amiloidotica,

come nei primi casi identificati di apo AI Iowa, nella quale l'accumulo di amiloide non era rappresentato da transtiretina ma un frammento proteolitico di apo AI. In altre sette mutazioni identificate il quadro clinico era sovrapponibile, con livelli ridotti di apoAI circolanti dovuta alla instabilita' della proteina che veniva eliminata dal circolo plasmatico rapidamente e in ambiente extravascolare degradata con formazione di amiloide. Una mutazione con caratteristiche particolari prende il nome di apo AI Milano; tale mutazione presenta una sostituzione della arginina 173 con una cisteina. La presenza di una cisteina consente la formazione di un dimero apo AI (M) – apo AI (M) che e' caratterizzato da una maggiore efficacia nella funzione di rimozione del colesterolo periferico, anche a causa del prolungato tempo di residenza in circolo di tale dimero.

A dimostrazione di questo, i soggetti con apo AI Milano presentano una minore incidenza di malattia coronarica sebbene i livelli di HDL circolanti siano estremamente ridotti. La somministrazione terapeutica di questa proteina ha dimostrato in studi su animali una azione anti-aterosclerotica effettiva. Gli studi clinici, dei quali sono attesi i risultati, chiariranno il potenziale ruolo terapeutico per l'uomo. Recentemente è stata ipotizzata l'esistenza di un dominio proteico che interagisce con il trasportatore ABCA1. Una mutazione in questo dominio renderebbe impossibile la lipidazione iniziale della apolipoproteina AI che rende possibile la formazione delle così dette HDL "nascenti". Alcune mutazioni spontanee, ed alcune mutagenesi in vitro hanno mostrato la presenza di alcuni domini all'interno delle eliche 5-7 della apoAI che interagiscono con l'enzima LCAT.

2.1.4 Iperalfalipoproteinemie familiari

Si definisce iperalfalipoproteinemia un aumento delle HDL plasmatiche con valori superiori al 90° percentile rispetto alla popolazione di appartenenza. Sebbene il postulato che lega i bassi livelli di colesterolo HDL all'aumentato rischio cardiovascolare sia ormai ampiamente acclarato, lo stesso non può dirsi per i livelli molto alti di colesterolo HDL, e cioè che questi siano associati a riduzione del rischio di malattia coronarica.L'osservazione nacque in Giappone nel decennio scorso. Infatti gli studi epidemiologici mostravano che nella curva di associazione fra HDL colesterolo e malattia coronaria i soggetti con livelli molto alti di HDL colesterolo presentavano una ripresa del rischio coronarico. Questo fece sospettare che tali livelli fossero determinati da condizioni di alterato metabolismo delle HDL che potessero produrre elevati livelli di

lipoproteine, però disfunzionanti. Tale approccio ha permesso di comprendere l'esistenza di veri quadri patologici monogenici associati ad alti livelli di HDL, in particolare il Deficit di Cholesterol Ester Transfer Protein o CETP o della lipasi epatica o "hepatic lipase" (HL), ma sono stati anche identificati quadri a trasmissione familiare caratterizzati da aumentata sintesi di apolipoproteina AI o dalla presenza di una apolipoproteina CIII mutata.

2.1.4.1 Il Deficit di CETP "Cholesterol Ester Transfer Protein"

Rappresenta probabilmente la causa monogenica più comune di Iperalfalipoproteinemia (HALP). Questo deficit è stato riscontrato con relativa frequenza in popolazioni dell'estremo oriente, ma successivamente altre osservazioni sono state riscontrate in pz caucasici. La CETP è una proteina coinvolta nel trasporto inverso del colesterolo. Secondo l'ipotesi classica, il colesterolo che dalla "periferia", ossia dalle membrane cellulari dell'intero organismo, viene captato dalle HDL nascenti, e poi esterificato in situ dalla LCAT sotto forma di colesterolo estere, viene poi trasferito nuovamente dalla CETP sulle lipoproteine contenenti apolipoproteina B, cioè VLDL e LDL. Lo scopo è quello di veicolare il colesterolo al fegato tramite il sistema del LDL recettore, e di "scaricare" le HDL dall' eccesso di colesterolo, in modo da renderle nuovamente in grado di accogliere colesterolo. Nel deficit di CETP questo meccanismo non è funzionale, così che le HDL non sono i grado di smaltire gli esteri di colesterolo, si infarciscono di colesterolo, aumentano le loro dimensioni e vengono catabolizzate più lentamente. Entrambi i meccanismi spiegano come i livelli plasmatici di colesterolo HDL aumentino. Tale aumento di colesterolo HDL è però protettivo nei confronti dell'aterosclerosi. I dati provenienti dalla letteratura hanno mostrato che su modelli animali, aumentati livelli di CETP, riducendo i livelli circolanti di HDL colesterolo, determinavano un aumento dell'aterosclerosi indotta sperimentalmente, la soppressione del gene CETP sembra migliorare il quadro vascolare. Tuttavia il quadro nell' uomo sembra eterogeneo; esistono infatti famiglie con deficit CETP gravate da alta incidenza di ictus cerebri ed altre che presentano segni di malattia cardiovascolare. Nell' unico caso in cui il deficit di CETP è quasi endemico, nella città giapponese di Omagari, i soggetti che raggiungevano gli 80 anni di età avevano meno deficit di CETP rispetto ai soggetti giovani, e inoltre presentavano alterazioni vascolari all' Eco Doppler più ingenti rispetto ai soggetti con CETP normale. La possibilità che la soppressione della CETP ed il conseguente aumento dei livelli di HDL possano ridurre l'incidenza di malattia

coronarica in soggetti con ipoalfalipoproteinemia hanno fatto considerare la CETP come un target farmacologico. Uno studio pilota condotto con un inibitore specifico della CETP, il torcetrapid, ha mostrato un aumento selettivo del colesterolo HDL dal 40 al 100% in relazione alla dose somministrata. Tuttavia, studi prospettici sono attesi per dimostrare che l'interruzione del trasporto inverso del colesterolo tramite CETP comporti un reale beneficio in termini di riduzione della malattia coronarica.

2.1.4.2 Deficit di HL "hepatic lipase"

La lipasi epatica è un enzima che idrolizza fosfolipidi e trigliceridi delle lipoproteine circolanti. Rimane ancorata alla superficie dei sinusoidi epatici e ne viene separata dall'infusione di eparina. Il suo ruolo è quello di permettere il "denudamento" delle lipoproteine, in particolare HDL, catabolizzando colesterolo libero ed esterificato, trigliceridi e fosfolipidi delle HDL senza rimuoverle dal circolo plasmatico, consentendo quindi alle lipoproteine di rinnovare la loro funzione di accettori di colesterolo. Interviene anche nella eliminazione delle lipoproteine residue o "remnants" presenti in fase postprandiale. Basse attività di HL sono legate a livelli più alti di HDL colesterolo, suggerendo che ridurre l'attività HL è anti-aterogenico.

In realtà i soggetti con deficit di HL presentano evidenti segni di aterosclerosi quando si ritrovano in un contesto aterogeno, ad esempio in soggetti con elevato LDL colesterolo. Infatti nei casi di deficit di HL si ritrovano alti livelli di trigliceridi nelle HDL che sembrano determinare un deficit funzionale, che come nel caso del deficit di CETP, potrebbe giustificare come alti livelli di HDL possano non essere protettivi nei confronti della malattia cardiovascolare.

3 OBIETTIVO DELLO STUDIO

L'obiettivo del progetto é la creazione di un Registro Nazionale delle Dislipidemie Familiari. L'analisi dei dati raccolti permetterà di effettuare la stima della prevalenza, dell'incidenza delle forme rare di Dislipidemie Genetiche e l'identificazione di eventuali "clusters" e/o sottopopolazioni a rischio; da questi risultati si potranno derivare priorità sia per intraprendere interventi sanitari mirati sia per indirizzare studi riguardanti la eziopatogenesi e la identificazione di fattori che possono condizionare la prognosi di specifiche forme rare.

4 POPOLAZIONE IN STUDIO

In questo studio saranno coinvolti circa 10.000 pazienti di qualunque sesso ed età, affetti da una dislipidemia familiare, seguiti presso circa 40 centri italiani attrezzati per la diagnosi ed il trattamento dei disordini del metabolismo lipidico. I pazienti coinvolti saranno in grado di comprendere le procedure dello studio ed accettare volontariamente di partecipare alla ricerca fornendo il consenso informato scritto.

Per i soggetti minori verrà richiesto ai genitori o al legale rappresentante di esprimere il consenso alla partecipazione del minore allo studio.

In accordo a quanto previsto dal Garante per la Protezione dei dati personali con "*Autorizzazione n. 9/2014 – Autorizzazione generale al trattamento dei dati personali effettuato per scopi di ricerca scientifica – 11 dicembre 2014*", saranno inoltre inclusi i dati di soggetti da parte di cui non è possibile ottenere il consenso scritto in quanto deceduti o non più rintracciabili, fermo restando l'obbligo e l'impegno del medico ricercatore a mettere in atto ogni ragionevole strategia per contattare i pazienti non rintracciabili ed ottenerne il consenso

5 DISEGNO, MetODOLOGIA E ConduZIONE DELLO STUDIO

5.1 Disegno di studio

Studio osservazionale, multicentrico, in parte retrospettivo ed in parte prospettico, volto all'individuazione ed alla registrazione di pazienti affetti dalle forme di dislipidemia familiare sopra descritti.

I pazienti partecipanti allo studio non verranno sottoposti ad alcuna procedura che esuli dalla normale pratica clinica quotidiana; allo stesso modo, le variabili cliniche che verranno raccolte per lo studio sono quelle che vengono comunemente raccolte dal Medico nella pratica clinica quotidiana.

5.2 Durata studio

Lo studio in oggetto si propone essere un registro di popolazione in cui raccogliere dati per un periodo di tempo di almeno 15 anni.

La data prevista di inizio studio, ovverosia la data di inizio raccolta dati, è stata stimata il 01/10/2011, mentre per quanto riguarda la data di fine studio, trattandosi di un registro nazionale, è auspicabile che la raccolta dati possa

continuare nel tempo, prevedendo, comunque, come data di termine, il 30/09/2026.

Per ogni singolo soggetto la partecipazione allo studio durerà dalla data di firma del consenso informato fino alla data di conclusione dello studio, a meno che il paziente non decida di uscire prematuramente dallo studio.

Per i soggetti deceduti o non rintracciabili, i dati disponibili verranno raccolti retrospettivamente.

5.3 Metodologia

Lo studio è a carattere osservazionale, non interventistico.

I pazienti saranno trattati secondo quanto previsto dalla pratica clinica in accordo al giudizio del Medico. Rientra, in particolare, nella pratica clinica corrente di pazienti affetti da dislipidemie, l'anamnesi della storia familiare e personale di malattia cardiovascolare e di dislipidemia, l'analisi biochimica del profilo lipidico e l'effettuazione di un test genetico a scopo diagnostico, come previsto dai prontuari regionali.

Il medico sperimentatore dovrà verificare ed identificare presso il proprio centro gli eventuali soggetti eleggibili da poter includere nello studio.

Un soggetto sarà considerato arruolato nello studio solo dopo che il medico sperimentatore avrà ottenuto il consenso informato scritto da parte del soggetto stesso o, nel caso di soggetti minorenni o non in grado di dare personalmente il consenso, dei suoi genitori/rappresentante legale, mentre, per pazienti deceduti o non più rintracciabili, la data di inclusione nello studio corrisponderà alla data della raccolta delle informazioni basali in CRF da parte del medico sperimentatore.

5.3.1 Informazioni da raccogliere

Per ogni soggetto arruolato nello studio, lo sperimentatore dovrà registrare in CRF, per tutta la durata dello studio, le informazioni disponibili, qui di seguito riportate, sia retrospettivamente che prospetticamente, in particolare:

5.3.1.1 <u>Dati basali alla diagnosi di dislipidemia</u>

- Disponibilità del Consenso informato scritto

- Dati demografici: data di nascita, sesso, origine geografica e appartenenza etnica
- Anamnesi e dati antropometrici: età alla diagnosi di dislipidemia, altezza, peso, fumo, IMT carotideo (spessore medio intimale carotideo)
- Esame obiettivo
- Anamnesi familiare
- Storia Clinica
- Patologie concomitanti
- Terapie concomitanti generali e specifiche per dislipidemie
- Esami biochimici
- Diagnosi molecolare

5.3.1.2 Dati visite di controllo

- Data visita di controllo
- Dati antropometrici: altezza, peso, fumo
- Terapie concomitanti generali e specifiche per dislipidemie
- Esami biochimici
- Eventi avversi (solo quelli rilevanti per lo studio)

5.3.2 Interruzione prematura dello studio da parte del soggetto

Un soggetto arruolato nello studio potrà uscire in qualsiasi momento lo desideri e per qualunque ragione.

5.4 Gestione dei dati

Lo Sperimentatore di ogni centro o il personale da lui designato dovrà riportare le informazioni richieste dal protocollo di studio sulla specifica Scheda Raccolta Dati (CRF) messa a disposizione da parte dello Sponsor.

I dati raccolti nella CRF saranno in forma rigorosamente anonima ed il soggetto verrà unicamente identificato con un codice specifico.

Tutti i dati verranno elaborati ed analizzati dal comitato scientifico Lipigen.

Tutti i dati raccolti saranno archiviati elettronicamente in maniera rigorosamente anonima, in base alla Linee guida per i trattamenti di dati

personali nell'ambito delle sperimentazioni cliniche di medicinali - 24 luglio 2008 G.U. n. 190 del 14 agosto 2008.

Lo sperimentatore garantirà la protezione dell'accesso ai dati.

6 Pubblicazione dei dati

Lo sponsor non pone vincoli alla pubblicazione dei dati.

7 Accesso diretto ai Dati ed ai documenti originali

A meno che non sia richiesto dalla legge, solo il medico sperimentatore od il suo staff, coinvolti nello studio, i Comitati Etici e gli Ispettori rappresentanti di organismi governativi possono accedere direttamente agli archivi per collegare i dati dello studio ai singoli pazienti.

In casi particolari, come ad esempio durante un'ispezione da parte di agenzie regolatorie, le informazioni legate di ogni singolo paziente e riportate in forma anonima in CRF potranno essere collegate ai suoi dati clinici mediante la chiave conservata dallo sperimentatore.

8 aspetti etici

Lo studio dovrà essere condotto in accordo al protocollo, ai principi contenuti nella Dichiarazione di Helsinki (ultima revisione, ottobre 2008), alle norme di Buona Pratica Clinica (ICH GCP) alle leggi di protezione dei dati e alle altre regolamentazioni applicabili.

Appendix II – List of the LIPIGEN sites

Center code	Coordinator center	City
MI-01	Centro per lo Studio dell'Aterosclerosi IRCCS Multimedica Via Milanese 300 - 20099	Sesto San Giovanni (MI)

Center codeo	Center	City
AN-01	Clinica di Medicina Interna e Geriatria Centro di riferimento regionale ipertensione arteriosa e malattie cardiovascolari INRCA Ospedale "Sestilli" e Azienda Ospedaliero-Universitaria Ospedali Riuniti di Torrette di Ancona Via Conca, 71 - 60126	Ancona
AQ-01	Centro Ipertensione Arteriosa e Prevenzione Cardiovascolare UOC Medicina Interna e Nefrologia Via Saragat, località Campo di Pile - 67100	L'Aquila
AT-01	SOC Diabetologia e Malattie metaboliche c/o Asti Azienda Sanitaria Locale di Asti - ASL AT Corso Dante Alighieri, 202 - 14100	Asti
BA-01	U.O. Endocrinologia Ambulatori di Diabetologia e Malattie Metaboliche A.O. Universitaria Policlinico Consorziale Università degli Studi di Bari "Aldo Moro" Piazza Giulio Cesare, 11 - 70124	Bari
BA-02	U.O. di Medicina Interna "Frugoni" e Centro di Assistenza e Ricerca Malattie Rare A.O. Universitaria Policlinico Consorziale Università degli Studi di Bari "Aldo Moro" Piazza Giulio Cesare, 11 - 70124	Bari
BG-01	U.O.C. Malattie Endocrine e Centro regionale per il Diabete (Diabetologia) Ospedale "Treviglio-Caravaggio" di Treviglio P.le Ospedale, 1 - 24047	Treviglio (BG)
BO-01	U.O. di Medicina Interna, Centro aterosclerosi Ambulatorio dislipidemie Ospedale Policlinico S. Orsola-Malpighi Via Albertoni, 15 – 40138	Bologna
CA-01	Centro per le Malattie Dismetaboliche e l'arteriosclerosi, Associazione ME.DI.CO Onlus Viale Trento, 27/A – 09123	Cagliari

Center codeo	Center	City
CH-01	Centro di alta specializzazione per la prevenzione dell'arteriosclerosi, Centro di eccellenza ESH per l'ipertensione arteriosa, centro di riferimento regionale per le Dislipemie, Ospedale Policlino S.S.Annunziata. Via dei Vestini - 66013	Chieti
CT-01	U.O. Medicina Interna Ospedale "Garibaldi Nesima" Via Palermo, 636 - 95122	Catania
CZ-01	A.O.U. Mater Domini , UOC di Nutrizione Clinica, ambulatorio Dislipidemie P.O. Via Tommaso Campanella, 115 - 88100	Catanzaro
FE-01	U. O Medicina Interna Universitaria Centro per lo Studio delle dislipidemie e dell'aterosclerosi Azienda Ospedaliero-Universitaria di Ferrara, Polo di Cona Via A.Moro, 8 - 44124	Ferrara
FI-01	Ambulatorio Malattie Aterotrombotiche, AOUC Azienda Ospedaliero-Universitaria Careggi Largo Brambilla, 3 - 50134	Firenze
FR-01	UOSD 'Prevenzione cardiovascolare', Dipartimento di Scienze Mediche, Azienda Sanitaria Locale Via A. Fabi - Palazzina Q - 03100	Frosinone
GE-01	U.O. Clinica di Medicina Interna 1 Ambulatorio dislipidemie IRCCS - A.O.U. San Martino - IST Largo Rosanna Benzi, 10 -16132	Genova
ME-01	Dipartimento di Medicina Interna e Terapia Medica Centro per la diagnosi e cura della dislipidemia e prevenzione dell'aterosclerosi, A.O. Universitaria Policlinico "G.Martino" Via Consolare Valeria, 1 - 98100	Messina
MI-02	Centro Universitario Dislipidemie "E. Grossi Paoletti" A.O. Ospedale Niguarda Ca' Granda Piazza Ospedale Maggiore, 3 - 20162	Milano
MI-03	Ambulatorio Dislipidemie Centro per lo studio e la prevenzione dell'Arteriosclerosi Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano Via Francesco Sforza, 35 - 20122	Milano
MI-04	Dip. Medicina Interna, Centro Prevenzione e Cura dell'aterosclerosi, A.O. "Guido Salvini" Viale Forlanini, 121 - 20024	Garbagnate Milanese (MI)

Center codeo	Center	City
MI-05	U.O. Clinica Pediatrica, Servizio clinico dislipidemie per lo studio e la prevenzione dell'aterosclerosi in età pediatrica Ospedale San Paolo Via di Rudinì, 8 - 20142	Milano
MI-06	U.O. Ambulatorio Prevenzione Aterosclerosi IRCCS Cardiologico Monzino Via Parea, 4 - 20138	Milano
MI-07	IRCCS Istituto Auxologico Italiano Dipartimento Cardio-Neuro-Metabolico Piazzale Brescia, 20 - 20149	Milano
MO-01	U.O. Medicina ad indirizzo metabolico-nutrizionistico Centro dislipidemie e centro di riferimento regionale per le malattie metaboliche rare Nuovo Ospedale S. Agostino Estense (NOCSAE) Via Giardini, 1355 - 41126	Modena
MO-02	U.O. Clinica Pediatrica Policlinico di Modena Via del Pozzo, 71 - 41124	Modena
NA-01	U.O. Medicina Interna 5 Centro per le malattie da arteriosclerosi AORN Cardarelli Via A. Cardarelli, 9 - 80131	Napoli
NA-02	Centro Coordinamento regionale per le iperlipidemie AOU Policlinico Federico II Via Sergio Pansini, 5 - 80131	Napoli
NA-03	U.O.C Cardiologia Clinica a direzione Universitaria con UTIC, AORN di Caserta "Sant'Anna e San Sebastiano" Via Ferdinando Palasciano - 81100	Caserta
PA-01	UO Medicina Interna e Malattie Metaboliche Centro di riferimento regionale per la prevenzione, diagnosi e cura delle malattie rare del metabolismo e delle dislipidemie genetiche A.O.U. Policlinico "P. Giaccone" Via del Vespro, 141 - 90127	Palermo
PC-01	Centro Dislipidemie in Età evolutiva U.O. Pediatria e Neonatologia Ospedale G. da Saliceto Via Taverna, 49 - 29121	Piacenza
PD-01	U. O. Clinica Medica 1 Centro Dislipidemie e Aterosclerosi A.O. di Padova Via Giustiniani, 2 - 35128	Padova

Center codeo	Center	City
PG-01	U.O. Medicina Interna Angiologia Malattie da Arteriosclerosi Ambulatorio di malattie del ricambio lipidico Ospedale Santa Maria della Misericordia Piazzale Menghini, 1 - 06156	Perugia
PI-02	U.O. Lipoaferesi Centro Regionale di Riferimento per la diagnosi e cura delle Dislipidemie Ereditarie Fondazione Toscana “G. Monasterio” Via G. Moruzzi, 1 - 56124	Pisa
RE-01	Ambulatorio Dislipidemie Arcispedale S. Maria Nuova - IRCCS Viale Risorgimento, 80 - 42123	Reggio Emilia
RM-01	Dipartimento Medicina Interna e Specialità Mediche "La Sapienza" A.O. policlinico Umberto I Viale del Policlinico, 155 - 00161	Roma
RM-02	Dipartimento Medicina Interna - Centro per l’Aterosclerosi Policlinico Universtario “Tor Vergata” Viale Oxford, 81 – 00133"	Roma
RM-03	U.O. Medicina Interna, Centro Riferimento Diabete Tipo 2, Policlinico Universitario “Tor Vergata” Viale Oxford, 81 - 00133	Roma
RM-05	Dipartimento Medicina Interna e Specialità Mediche "La Sapienza" A.O. policlinico Umberto I Viale del Policlinico, 155 - 00161	Roma
RM-06	Ambulatorio Polispecialistico per le Malattie Rare IRCCS Ospedale Pediatrico Bambino Gesù - GIANICOLO Piazza S. Onofrio 4 - 00165	Roma
RM-07	Endocrinologia e Malattie del Metabolismo Policlinico Gemelli, Largo Agostino Gemelli, 8 - 00168	Roma
SA-01	AOU San Giovanni di Dio e Ruggi d'Aragona Via San Leonardo, 1 - 84131	Salerno
SI-01	U.O Ematologia, Centro coagulopatie ed aterosclerosi, Policlinico Santa Maria alle Scotte V.le Bracci, 16 - 53100	Siena
TN-01	Centro Dislipidemia UO Medicina Interna Ospedale Santa Chiara Largo Medaglie d'oro, 9 - 38122	Trento

Center codeo	Center	City
TR-01	U.O. Medicina Interna Azienda Ospedaliera Santa Maria di Terni P.le Tristano Di Joannuccio	Terni
TO-01	U.O. Dislipidemie e Prevenzione Cardiovascolare Ospedale Regina Margherita P.zza Polonia, 94 – 10126	Torino
TO-02	AOU San Luigi Gonzaga, Regione Gonzole, 10 - 10043	Orbassano (TO)
TO-03	Ospedale Molinette SCDU endocrinologia, diabetologia e metabolismo, dipartimento di scienze mediche, Università di Torino Corso Bramante, 88 - 10126	Torino
TS-01	S.S. Diabetologia e Malattie Metaboliche U.C.O. Clinica Medica Generale Azienda Ospedaliera Universitaria OORR Ospedale Maggiore Piazza dell'Ospitale 2 - 34142	Trieste
VA-01	Ambulatorio ipertensione dislipidemie, U.O. Medicina Generale, ASST Valle Olona, Ospedale di Gallarate Via Pastori, 4 - 21013	Gallarate (VA)
VB-01	Ospedale Castelli ASL VCO - UO SOC Cardiologia Via Fiume, 18 - 28922	Verbania
VE-01	Ambulatorio Dislipidemie UO Medicina Interna Ospedale dell'Angelo di Mestre via Paccagnella 11 - 30174	Zelarino (VE)
VR-01	U.O. Endocrinologia Diabetologia e Malattie del Metabolismo Centro regionale specializzato per la diagnosi e terapia delle dislipidemie e aferesi terapeutica A.O. Universitaria Integrata di Verona P.le Stefani, 1 - 37126	Verona

Appendix III – Lipid lowering therapy correction factors for the retrocalculation of pre-treatment LDL-C

Drug	Dosage (mg/day)	Correction factor
Atorvastatin	10	1.59
	20	1.75
	40	1.96
	80	2.22
Fluvastatin	20	1.27
	40	1.37
	80	1.49
Pravastatin	10	1.25
	20	1.32
	40	1.64
Rosuvastatin	5	1.61
	10	1.75
	20	1.92
	40	2.13
	80	2.38
Simvastatin	5	1.30
	10	1.37
	20	1.47
	40	1.59
	60	1.67
	80	1.72
Pitavastatin	1	1.47
	2	1.56
	4	1.72
Lovastatin	10	1.27
	20	1.35
	40	1.45
	80	1.61
Ezetimibe	10	1.23
Alicuromab	70	1.67
	150	2.22
Evolocumab	140	2.22

Appendix IV – *LDLR* gene variants with a residual activity < 5% (null-mutation) detected in peadiatric subjects

LDLR gene variant	N of subjects
c.1646G>A p.Gly549Asp	90
c.1415_1418dupACAT p.Gln474Hisfs*63	34
c.682G>A p.Glu228Lys	19
c.1735G>T p.Asp579Tyr	18
c.418G>T p.Glu140*	17
c.1478_1479delCT p.Ser493Cysfs*42	17
c.1586+1G>A p.Thr454_Gly529del, p.Gly529_Phe530ins22	15
c.304C>T p.Gln102*	15
c.1694G>T p.Gly565Val	10
c.97C>T p.Gln33*	8
c.126C>A p.Tyr42*	7
c.1104C>A p.Cys368*	6
c.1846-1G>A p.[(Glu615fs*43, Leu570_Thr621del, Glu615fs*16)]	6
c.557delG p.Gly186Valfs*20	6
c.1067delA p.Asp356Valfs*14	5
c.1257C>G p.Tyr419*	5
c.1587-?_1845+?del Exons 11_12del pGly529fs*49	5
c.1846-?_2583+?del Exons 13_18del p.0	5
c.676T>C p.Ser226Pro	5
c.1118G>A p.Gly373Asp	4
c.1778delG p.Gly593Alafs*72	4
c.1846-?_2311+?del Exons 13_15 p.Asp616Leufs*17, Asp616Leufs*132	4
c.2390-1G>A p.Val797Glyfs*153	4
c.648_649insT p.Asp217*	4
c.828C>G p.Cys276Trp	4
c.862G>T p.Glu288*	4
c.1-?_190+?del Exons 1_2del p.0	3
c.1119_1122dupTGGC, p.Tyr375Trpfs*7	3
c.1120_1123dupGGCT p.Tyr375Trpfs*7	3
c.1252G>T p.Glu418Ter	3
c.2079_2083del p.Lys693Asnfs*22	3
c.214delG p.Asp72Thrfs*134	3
c.2215C>T p.Gln739*	3
c.530C>T p.Ser177Leu	3
c.682G>T p.Glu228*	3
c.796G>A p.Asp266Asn	3
c.941-12G>A p.Thr315Profs*59	3
c.(1845+1_1846-1)_(2140+1_2141-1)del	2
c.1068T>A + c.1069_1086dupGAGTGTCAGGATCCCGAC p.Asp356Glu/Glu357_Asp362dup	2

LDLR gene variant	N of subjects
c.1070_1071dupAG p.Cys358Serfs*13	2
c.1162delC p.His388Thrfs*25	2
c.1187-10G>A p.Gly396Aspfs*20	2
c.1187-?_2140+?dup Exons 9_14dup p.Glu693Gly; Ser376_Thr692dup	2
c.129delG p.Lys43Asnfs*163	2
c.12G>A p.Trp4*	2
c.1415_1429delACATCCAGGCCCCCG p.Asp451_Pro455del	2
c.1439_1449del p.Ala480Aspfs*52	2
c.1448G>A p.Trp483*	2
c.1587-1343_1845+1986del Exons 11_12del p.Gly529fs*49	2
c.191-700_940+377dup p.Gly314Val; Ser65_Cys313dup	2
c.191-?_313+?del p.(Leu64_Pro105delinsSer64)Â	2
c.2043C>A p.Cys681*	2
c.233delG - p.Arg78Leufs*128	2
c.2483A>G p.Tyr828Cys	2
c.274C>T p.Gln92*	2
c.27_37delCTGGACCGTCG p.Trp10Leufs*38	2
c.363C>A p.Cys121*	2
c.365_366insC p.Ser123Leufs*7	2
c.366dup p.Ser123Leufs*7	2
c.373C>T p.Gln125*	2
c.465C>A p.Cys155*	2
c.501C>A p.Cys167*	2
c.666C>A p.Cys222*	2
c.2416dupG p.Val806Glyfs*11	2
c.1846-1894_2140+1498del p.Glu594fs*16	1
c.1846-?_2140+?del	1
c.(1186+1_1187-1)_(2140+1_2141-1)dup	1
c.(1845+1_1846-1)_(2311+1_2312-1)dup	1
c.(?_1587-10)_(1845_?)del	1
c.1-5711_940+2447del Exons 1_6del	1
c.1048C>T p.Arg350*	1
c.1070_1073dup p.Cys358T*	1
c.1075C>T p.Gln359*	1
c.1132C>T p.Gln378Ter	1
c.116_117delGCinsAA p.Cys39*	1
c.1176C>A p.Cys392*	1
c.1187del p.Gly396Alafs*17	1
c.1285G>A p.Val429Met	1
c.1358+1G>A p.Ser453Argfs*1	1
c.1365delG p.Gln455Hisfs*52	1
c.1413_1414delAGinsGGACAT p.Gln474Hisfs*63	1
c.1472C>A p.Thr491Asn	1

LDLR gene variant	N of subjects
c.1749_1753delCTCCA p.Ser584Leufs*17	1
c.1846-?_2140+?del Exons 13_14del p.Asp616Argfs*16	1
c.1860G>A p.Trp620*	1
c.1865_1866delAT p.Asp622fs*22	1
c.1886delT p.Phe629Serfs*36	1
c.1891_2311+1062del p.Glu615fs*17, Glu615fs*132, Asp548_Gln770del	1
c.191-?_1586+?del, p.[(Leu64Serfs*19, Leu64Tyr; Ser65_Asp569del, Leu64*)]	1
c.1987+1G>T p.Gly663Valfs*12	1
c.1988-2A>G Gly663Aspfs*30; Gly663_Thr713del	1
c.2000G>A p.Cys667Tyr	1
c.2133C>A p.Cys711*	1
c.2311_2311+15del p.Ala770Serfs*42	1
c.2390-2A>G p.Val797Alafs*155	1
c.2398+2delTAAGinsGGCCCCAT p.Val776Glyfs*61	1
c.2416_2417insG, p.(Val806Glyfs*11)	1
c.265T>C p.Cys89Arg	1
c.316_328del p.Lys107Argfs*95	1
c.320_332del p.Lys107Argfs*95	1
c.328del p.Glu110Lysfs*10	1
c.349delC p.His117Thrfs*89	1
c.369_370delTC p.Arg124Alafs*5	1
c.413C>G p.Ser138*	1
c.431_434dupCGGT p.Leu146Glyfs*35	1
c.590G>A p.Cys197Tyr	1
c.616_617insA p.Ser206Lysfs*12	1
c.641G>A p.Trp214*	1
c.675delA p.Lys225Asnfs*40	1
c.68-?_1186+?dup Exons 2_8dup	1
c.68-?_1845+?del Exons 2_12dup p.(Val23Glyfs*29)	1
c.680_682delACGins14 p.Asp227Alafs*42	1
c.693C>A p.Cys231*	1
c.695-?_940+?del Exons 5_6del p.Ala232_Cys313del	1
c.818-2A>G p.Val273Glufs*31	1
c.81C>A p.Cys27*	1
c.914G>A, p.Trp305*	1
c.940+2delGAGT p.Thr315Leufs*20	1
c.940_940+14del	1
c.943delA p.Thr315Profs*55	1

Appendix V – *LDLR* gene variants with a residual activity 5-30% (defective-mutation) detected in LIPIGEN paediatric subjects

LDLR gene variant	N of subjects
c.1775G>A p.Gly592Glu	61
c.1567G>A p.Val523Met	56
c.662A>G p.Asp221Gly	55
c.2054C>T p.Pro685Leu	37
c.1135T>C p.Cys379Arg	23
c.953G>T p.Cys318Phe	22
c.2312-3C>A p.Ala771_796del	18
c.313+1G>A p[(Leu64Ser, Ser65_Pro105del, Pro105Argfs*13)]	13
c.352G>T p.Asp118Tyr	12
c.1211C>T p.Thr404Ile	9
c.664_681dupTGCAAGGACAAATCTGAC p.Cys222_Asp227dup	9
c.1463T>A p.Ile488Asn	8
c.1698_1704delCACCCTAinsGCCCAAT, p.Ile566_Leu568delinsMetProAsn	8
c.1027G>A p.Gly343Ser	7
c.665G>A p.Cys222Tyr	7
c.761A>C p.Gln254Pro	7
c.81C>G p.Cys27Trp	7
c.1474G>A p.Asp492Asn	6
c.1090T>C p.Cys364Arg	5
c.1195G>A p.Ala399Thr	5
c.1207_1209del p.Phe403del	5
c.1618G>A p.Ala540Thr	5
c.2389G>A p.Val797Met	5
c.671A>G p.Asp224Gly	5
c.681C>G p.Asp227Glu	5
c.922G>A p.Glu308Lys	5
c.1466A>G p.Tyr489Cys	4
c.1871_1873delTCA p.Ile624del	4
c.920A>C p.Asp307Ala	4
c.1109A>C p.Asn370Thr	3
c.1291G>A p.Ala431Thr	3
c.1301C>G p.Thr434Arg	3
c.1414G>T p.Asp472Tyr	3
c.1570G>A p.Val524Met	3
c.1576C>T p.Pro526Ser	3
c.1640T>C p.Leu547Pro	3
c.173A>G p.Glu58Gly	3
c.1897C>T p.Arg633Cys	3
c.1978C>A p.Gln660Lys	3
c.1056C>G p.Cys352Trp	2
c.1246C>T p.Arg416Trp	2
c.1720C>T p. Arg574Cys	2
c.1739C>T p.Ser580Phe	2
c.1879G>A p.Ala627Thr	2

LDLR gene variant	N of subjects
c.2119G>C p.Asp707His	2
c.367T>C p.Ser123Pro	2
c.418G>A p.Glu140Lys	2
c.440C>T p.Thr147Ile	2
c.514G>A p.Asp172Asn	2
c.682_684delGAGinsTGCAAG, p.Glu228delinsCysLys	2
c.826T>C p.Cys276Arg	2
c.858C>A p.Ser286Arg	2
c.859G>T p.Gly287Cys	2
c.920A>G p.Asp307Gly	2
c.1130_1131delinsCT p.Cys377Ser	1
c.1247G>C p.Arg416Pro	1
c.1295T>C p.Leu432Pro	1
c.1468T>G p.Trp490Gly	1
c.1586+5G>A p.[Gly529Phe530ins22; Thr454_Gly529del]	1
c.1681_1683CAGdup p.Gln561dup	1
c.1686G>T p.Trp562Cys	1
c.1730G>C p.Trp577Ser	1
c.1731G>C p.Trp577Cys	1
c.1889G>C, p.Ser630Thr	1
c.1892C>A p.Ala631Asp	1
c.191_313del Exon3del p.Leu64Ser; Ser65_Pro105del	1
c.2132G>A p.Cys711Tyr	1
c.241C>T p.Arg81Cys	1
c.2476C>A p.Pro826Thr	1
c.2479G>A p.Val827Ile	1
c.248T>C p.Ile83Thr	1
c.313+4_313+16delAGTGTGGCCCTGC	1
c.326G>C p.Cys109Ser	1
c.346T>C p.Cys116Arg	1
c.362G>T p.Cys121Phe	1
c.428G>A p.Cys143Tyr	1
c.493T>G p.Trp165Gly	1
c.515A>G p.Asp172Gly	1
c.523G>A p.Asp175Asn	1
c.589T>C p.Cys197Arg	1
c.661_678dup p.Asp221_Ser226dup	1
c.676_684dupTCTGACGAG p.Ser226_Glu228dup	1
c.846C>A, p.Phe282Leu	1
c.898A>G p.Arg300Gly	1
c.910G>A p.Asp304Asn	1
c.974G>A p.Cys325Tyr	1