PhD in Pharmacological biomolecular sciences, experimental and clinical

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SD BIO/14

GENETIC DYSLIPIDAEMIAS IN THE ITALIAN AND RUSSIAN POPULATIONS: FROM THE CLINIC TO THE BENCH

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Abstract

Genetic dyslipidemias are a heterogeneous group of disorders, and familial hypercholesterolemia (FH) is the most common one, that are characterized by abnormal levels of circulating lipoproteins and leading to premature cardiovascular diseases (CVD).

Despite the need for an accurate and timely diagnosis due to high cardiovascular risk of those patients

- there is still no well-managed system for a diagnosis and treatment of patients with severe dyslipidemias,
- there are still some gaps between clinical and genetic diagnosis scores that need to be improved to increase their reliability at the population level.

To overcome the above mentioned challenges, national registries on lipid disorders have been created as a tool to improve a screening programs and to make a progress towards formulating a joint consensus on methods to improve accuracy of its diagnosis and cardiovascular risk stratification.

The Italian and Russian Genetic Network (a background for the current PhD project) has been initiated with the primary focus on familial hypercholesterolemia as a genetically determined lipid disorder with the highest prevalence worldwide. The key goal of the current PhD thesis was to provide a phenotype – genotype characterization of the two populations aiming to use these data for searching of new approaches for improvement of an accuracy of FH diagnostics and cardiovascular risk stratification to promote in a future personalized approach in a disease management. The additional advantage of the current Network is an availability of a two-population

validation of potential new markers for FH detection to will allow for an improvement of current algorithms.

Thus, we studied two cohorts from north cities of Italy and Russia (Milan and Saint Petersburg), a phenotype – genotype characterization for both cohorts was performed as well as concreate gaps between clinical and genetic diagnosis were described. The ultrasound measurement of Achilles tendon thickness was considered as a potential marker to fill in the gap between clinical and genetic FH diagnosis that brings an additional value to identification of FH subjects with higher LDL-C burden that may affect the level of the aggressiveness for the therapeutic strategies. Furthermore, proteomic data analysis demonstrated that a set of immune-inflammatory proteins associated with increased CVD risk, significantly characterize the clinically determined FH phenotype. Crossing the clinical phenotype with genetic analysis allowed to identify genetically positive FH individuals that, in addition to a higher LDL-C burden, were also characterized by a peculiar set of immune-inflammatory proteins as compared to genetically negative ones. By pairing genetically positive and negative FH patients for LDL-C levels, a number of significantly different proteins was indicated, that let to suggest that the prognostic value of these proteins should be longitudinally addressed.

In conclusion, the Italian and Russian Genetic Network contributed to reinforcing of knowledge about FH in both countries within the access to performing and interpretation of genetic results and analysis of approaches for an increase FH detection and risk stratification accuracy to driving improvement in standards of care for FH patients globally.

Riassunto

Le dislipidemie genetiche sono un gruppo eterogeneo di disturbi caratterizzati da livelli anomali di lipoproteine circolanti che portano a malattie cardiovascolari premature. Tra queste l'ipercolesterolemia familiare (IF) è la più comune.

Nonostante la necessità di una diagnosi accurata e tempestiva a causa dell'alto rischio cardiovascolare di questi pazienti
- non esiste ancora un sistema ben gestito per la diagnosi e il trattamento dei pazienti con dislipidemie gravi
- ci sono ancora alcune lacune tra i punteggi di diagnosi clinici e genetici che devono essere migliorate per aumentare l'affidabilità di questi strumenti di diangosi a livello di popolazione.

Per superare questi limiti, sono stati istituiti registri nazionali per i disturbi lipidici come strumenti per migliorare i programmi di screening e per progredire verso la formulazione di un consenso comune sui metodi da applicare in pratica clinica per migliorare l'accuratezza della diagnosi e la stratificazione del rischio cardiovascolare dei pazienti.

L'*Italian and Russian Genetic Network* è stato avviato ponendo inizialmente attenzione sull'ipercolesterolemia familiare, dato che quest'ultima è un disordine lipidico geneticamente determinato con la più alta prevalenza nel mondo. L'obiettivo chiave dell'attuale tesi di dottorato è stato quello di fornire una caratterizzazione fenotipica e genotipica delle due popolazioni (italiana e russa) al fine di utilizzare questi dati per la ricerca di nuovi approcci per migliorare l'accuratezza della diagnosi di IF e la stratificazione del rischio cardiovascolare al fine di promuovere in futuro un

approccio personalizzato per la gestione di questa malattia nella pratica clinica. L'ulteriore vantaggio della rete è la possibilità di convalidare in queste due popolazioni nuovi potenziali marcatori per l'identificazione di soggetti con IF che permetterà un miglioramento degli algoritmi attuali di diagnosi.

Nello studio oggetto di questa tesi, sono state valutate due coorti delle città di Milano (Lombardia, Italia) e San Pietroburgo (Russia). E' stata eseguita una caratterizzazione fenotipica e genotipica per entrambe le coorti e sono state descritte le lacune esistenti relative alla diagnosi clinica e genetica di IF. La misurazione ecografica dello spessore del tendine d'Achille è stata considerata come un potenziale marcatore per colmare il divario tra le due tipologie di diagnosi. Le evidenze ottunute hanno validato il valore aggiunto che ha questo marcatore nell'identificare soggetti con IF con esposizione prolungata ad alti livelli di colesterolo LDL che può influenzare il livello di aggressività delle strategie terapeutiche. E' stata inolte condotta un'analisi di proteomica che ha evidenziato un insieme di proteine immuno-infiammatorie, associate ad un aumento del rischio di malattie cardiovascolari, che caratterizzano significativamente il fenotipo di IF clinicamente determinato. Incrociando il fenotipo clinico e l'analisi genetica con i dati di proteomica, è stato possibile identificare gli individui geneticamente positivi che, oltre a un maggiore *burden* di colesterolo LDL, erano anche caratterizzati da un particolare insieme di proteine immunitarie-infiammatorie che li distingueva dai soggetti geneticamente negativi. Successivamente, appaiando pazienti con IF geneticamente positivi e negativi per i livelli di colesterolo LDL, è stato individuato un numero di proteine diverso da quello precedente. Ciò ha evidenziato che il valore prognostico di queste proteine dovrebbe essere valutato longitudinalmente.

In conclusione, l'*Italian and Russian Genetic Network* ha contribuito ad approfondire le conoscenze su questa patologia, attraverso l'accesso

all'esecuzione e all'interpretazione dei risultati genetici e grazie all'identificazione di nuovi approcci per aumentare l'accuratezza di rilevamento e stratificazione del rischio cardiovascolare di soggeti con IF al fine di determinare un miglioramento degli standard di cura per i pazienti con IF a livello globale.

Chapter I

BACKGROUND

I. Introduction

Atherosclerotic cardiovascular disease (ASCVD) and its clinical manifestations are the leading cause of morbidity and mortality throughout the world. Multiple exposures have been reported to be associated with an increased risk of cardiovascular events [*Yusuf 2004*]. The most extensively studied of these exposures by far is low-density lipoprotein cholesterol (LDL-C). Multiple lines of evidence have established that cholesterol-rich LDL-C and other apolipoprotein B (apoB)-containing lipoproteins, including very low-density lipoproteins cholesterol (VLDL-C) and their remnants, intermediate density lipoproteins cholesterol (IDL-C), and lipoprotein(a) [Lp(a)], are directly implicated in the development of ASCVD [*Goldstein 2015*]. While the focus is on LDL-C, this does not diminish the importance of the role of other apoB-containing lipoproteins on the development of ASCVD nor does it exclude potential atherogenic actions of the individual components of the lipidome and proteome of LDL beyond cholesterol and apoB. Recent meta-analyses of genetic studies, prospective epidemiologic studies, Mendelian randomization studies, and randomized clinical trials, that include over 200 studies involving over 2 million participants with over 20 million person-years of follow-up and more than 150 000 cardiovascular events, provide remarkably consistent and unequivocal evidence that LDL-C causes ASCVD [*Ference 2017*].

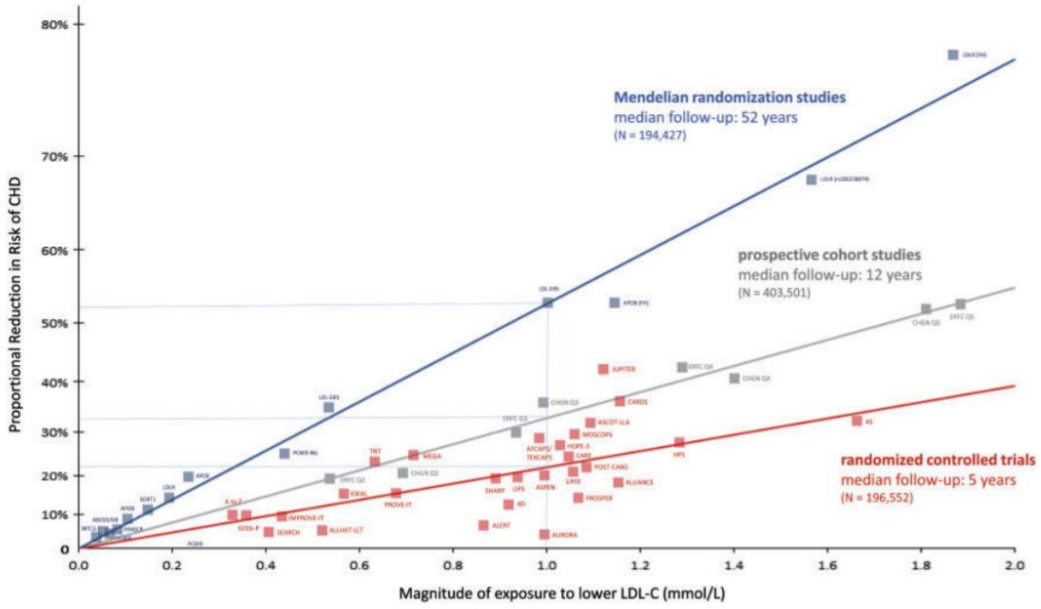
The key events in the initiation of ASCVD are the retention and accumulation of cholesterol-rich apoB-containing lipoproteins within the arterial intima at sites of predilection for plaque formation [*Skålén 2002,*

Williams 2007]. Notably, LDL-C and other apoB-containing lipoproteins < 70 nm in diameter (including VLDL-C, their remnants, IDL-C, and Lp(a)) efficiently enter and exit the arterial intima [*Tabas 2007, Nordestgaard 1988*]. As the concentration of LDL-C increases, the probability of intimal retention of LDL-C leading to the initiation and progressive development of atherosclerotic plaque increases in a dose dependent manner [*Goldstein 2015*].

Although the association between LDL-C and the risk of ASCVD is strong, graded, and reproducible in meta-analyses of prospective cohort studies, these studies are not randomized and are therefore unavoidably vulnerable to confounding, reverse causation, and other forms of bias. Hovewer, Mendelian randomization studies introduced a randomization scheme into an observational study specifically to assess whether an observed association between an exposure and an outcome is likely to be causal [*Lawlor 2008*]. Numerous variants in multiple genes have been reported to be associated with lower LDL-C levels [*Teslovich 2010, Willer 2013*]. Mendelian randomization studies have consistently demonstrated that variants in over 50 genes that are associated with lower LDL-C levels (but not with other potential predictors or intermediates for ASCVD) are also associated with a correspondingly lower risk of CHD [*Nikpay 2015, Ference 2012, Linsel-Nitschke 2008, Holmes 2015*], thus providing powerful evidence that LDL-C is causally associated with the risk of CHD. Indeed, when the effect of each LDL-C variant is plotted against its effect on CHD, there is a continuous, dose-dependent, and log-linear causal association between the magnitude of the absolute change in LDL-C level and the lifetime risk of CHD (Figure 1) [*Ference 2012, Holmes 2015]*.

Figure 1.1. Log-linear association per unit change in low-density lipoprotein cholesterol (LDL-C) and the risk of cardiovascular disease as reported in meta-analyses of Mendelian randomization studies, prospective epidemiologic

cohort studies, and randomized trials.



Adapted from [*Ference 2017*].

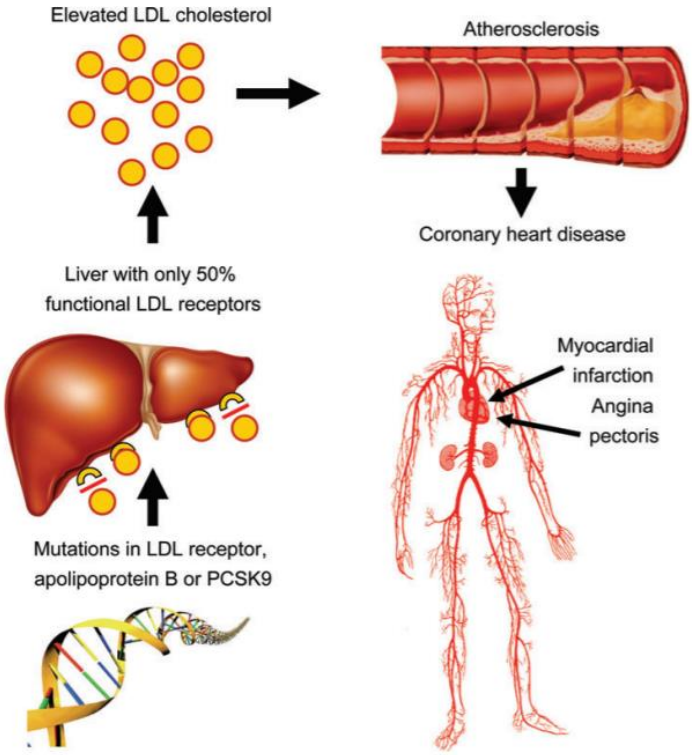
Furthermore, when adjusted for a standard decrement in LDL-C, each of the genetic variants associated with LDL-C has a remarkably similar effect on the risk of CHD per unit lower LDL-C, including variants in the genes that encode the targets of pharmacological agents commonly used to lower LDL-C [i.e. 3-hydroxy3-methyl-glutaryl-coenzyme A reductase (HMGCR), the target of statins; Niemann-Pick C1-like 1 (NPC1L1), the target of ezetimibe; and proprotein convertase subtilisin/kexin type 9 (PCSK9), the target of the monoclonal antibodies alirocumab and evolocumab], with no evidence of any heterogeneity of effect (I 2 = 0%) [*Ference 2012, Holmes 2015, Ference 2015*]. This observation strongly implies that the causal effect of these variants on the risk of CHD is mediated essentially entirely through LDL-C, because it would be implausible that variants in numerous different genes involving multiple distinct biological pathways by which LDL-C is lowered would each have directionally concordant and quantitatively similar pleiotropic effects on the risk of ASCVD. Summarizing, we may talk about the evidence that LDL is causally associated with the risk of ASCVD and that the causal effect of LDL-C

on ASCVD is largely independent of the mechanism by which LDL-C is lowered.

II. Familial hypercholesterolemia as the most frequent model of genetically determined lipid disorders

Among the genetic dyslipidemias, FH is the most common form that result in reduced hepatic capacity to clear atherogenic LDL-C from the circulation, with its consequent accumulation (Figure 1.2.) [*Brown 1986]*.

Figure 1.2. Pathophysiology of heterozygous FH

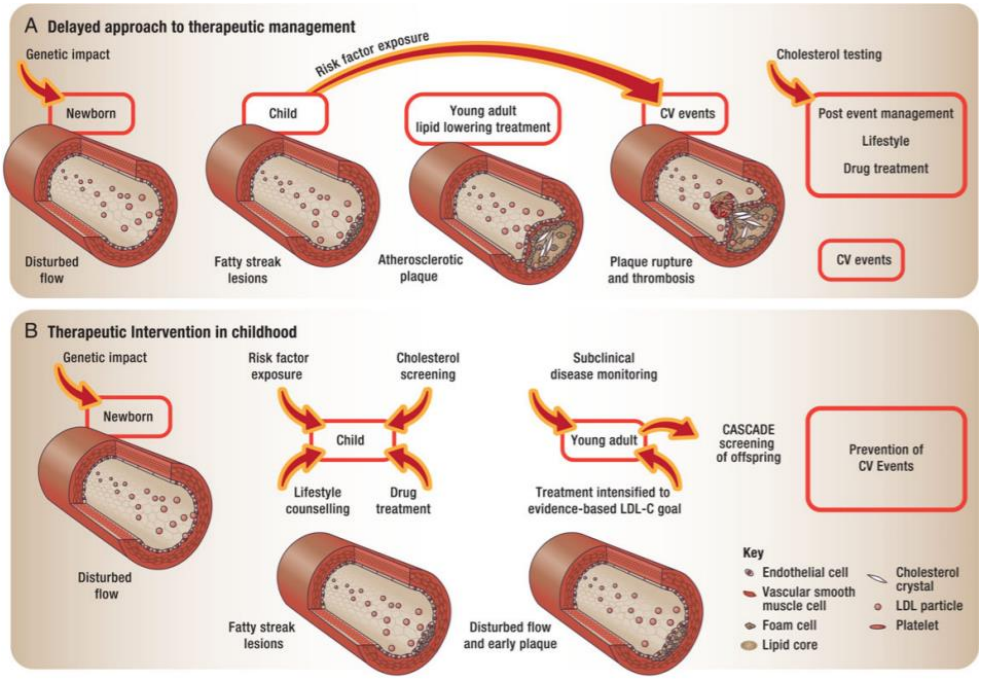


Adapted from [*Nordestgaard 2013*]

An exposure to the arterial wall by elevated LDL-C levels accelerates cholesterol deposition and vascular inflammation, developing atherosclerosis,

especially in the coronary arteries and aorta, thus leading to premature CHD [*Brown 1978, Buja 1979*]. There are two forms of FH: heterozygous and homozygous. Heterozygous FH(HeFH) is common, present in 1 per 200–250 of the general population [*Sjouke 2015, Nordestgaard 2013, Watts 2014*]. That means, there are potentially as many as 4.5 million individuals in Europe with HeFH and probably 35 million worldwide, of whom 20–25% are children and adolescents. FH in its homozygous form (HoFH) is a rare disease with an estimated prevalence of 1 per 160 000–300 000 in European populations [*Sjouke 2015, Cuchel 2014*]. Individuals with HoFH are at extremely high risk and, if untreated, many will manifest coronary or other cardiovascular disease in a very young age. Figure 1.3 demonstrates the potential of early recognition of FH, combined with treatment from a young age, to substantially delay atherosclerosis progression.

Figure 1.3. Development of early atherosclerotic vascular disease in familial hypercholesterolaemia showing the potential impact of early recognition and treatment on evolution of the condition.



[*Wiegman 2015*]

Clinical diagnosis

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. Probands within the primary screening should be identified according to the following criteria:

- plasma total cholesterol ≥8 mmol/L (≥310 mg/dL) in an adult or adult family member(s) (or >95th percentile by age and gender for country),
- premature CHD in the subject or family member(s),
- tendon xanthomas in the subject or family member(s),
- sudden premature cardiac death in a family member.

Diagnosis of FH adults in European countries relies on five criteria: family history, clinical history of premature CHD, physical examination for xanthomas and corneal arcus, very high LDL cholesterol on repeated measurements, and/ or a causative mutation detected by molecular genetics (Table 1.1) [*Defesche 2004*]. A "definite FH" diagnosis can be made if the subject scores > 8 points. A "probable FH" diagnosis can be made if the subject scores 6 to 8 points. A "possible FH" diagnosis can be made if the subject scores 3 to 5 points. An "unlikely FH" diagnosis can be made if the subject scores 0 to 2 points. There is an indication to use the diagnostic algorithm: per group only one score, the highest applicable, can be chosen.

Table 1.1. Dutch Lipid Clinic Network criteria for diagnosis of heterozygous familial hypercholesterolaemia in adults.

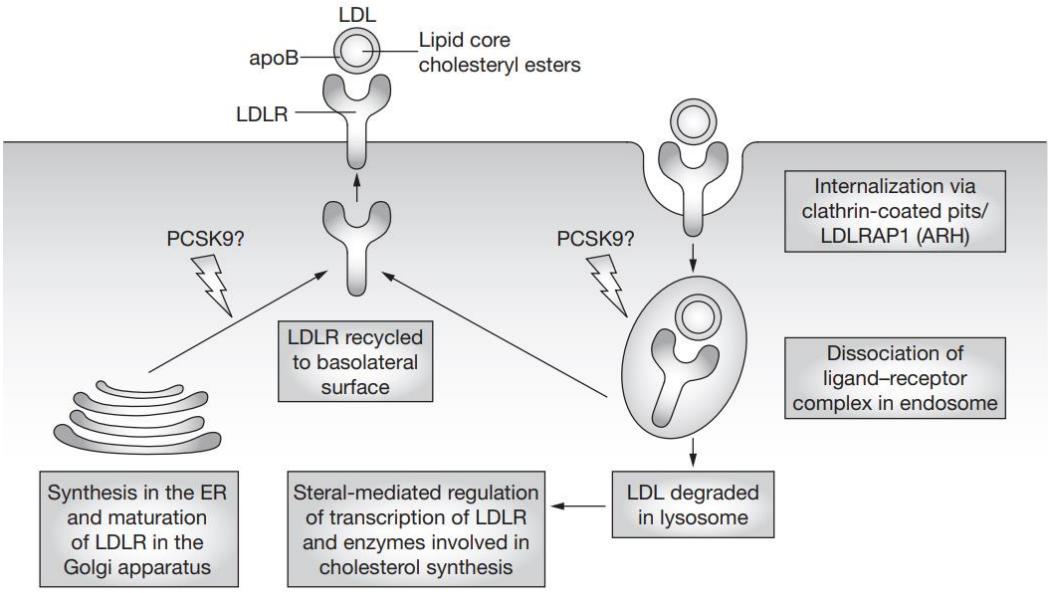
Family history		
First degree relative with known premature coronary and/or vascular disease (men aged <55 years, women aged <60 years) OR First degree relative with known LDL-cholesterol above the 95th percentile for age and gender		**1**
First degree relative with tendinous xanthomata and/or arcus cornealis OR Children aged <18 years with LDL-cholesterol above the 95th percentile for age and gender		**2**
Clinical history		
Patients with premature coronary artery disease (men aged <55 years, women aged <60 years)		**2**
Patients with premature cerebral or peripheral vascular disease (men aged <55 years, women aged <60 years)		**1**
Physical examination		
Tendinous xanthomata		**6**
Arcus cornealis before 45 years of age		**4**
Investigation		
LDL-cholesterol (mmol/L)	LDL-C ≥8.5	**8**
NB. This is the **untreated** LDL-cholesterol concentration. See supporting documentation for method of calculation.	LDL-C 6.5–8.4	**5**
	LDL-C 5.0–6.4	**3**
	LDL-C 4.0–4.9	**1**

Diagnosis	Total
Definite FH	**>8**
Probable FH	**6-8**
Possible FH	**3-5**
Unlikely FH	**<3**

Genetic diagnosis

FH 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 1.4) [*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 1.4. The pathway of LDLR for LDL particles uptake and degradation.



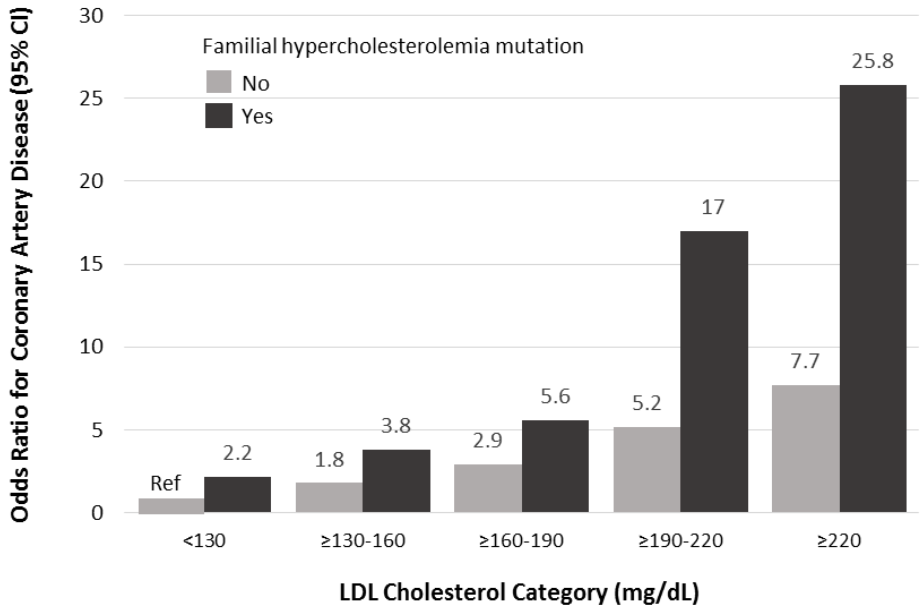
Adapted from [*Soutar 2007*]

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 overexpression or hyperactivity of PCSK9, reducing the number of LDLR on the cell surface due to an acceleration in the internalization and degradation of LDLR.

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: some experts promoted the DNA sequencing in all suspected FH, 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. 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 1.5). In this latter group, a single elevation in LDL-C could reflect a time-limited exposure to high cholesterol levels [*Khera 2016*].

Figure 1.5. Risk of CAD among LDL-C and FH mutation status categories.



Adapted from [*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: pathogenic, likely pathogenic, uncertain clinical significance (VUS), likely benign, benign. 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*.

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*].

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*].

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*].

Other mutations

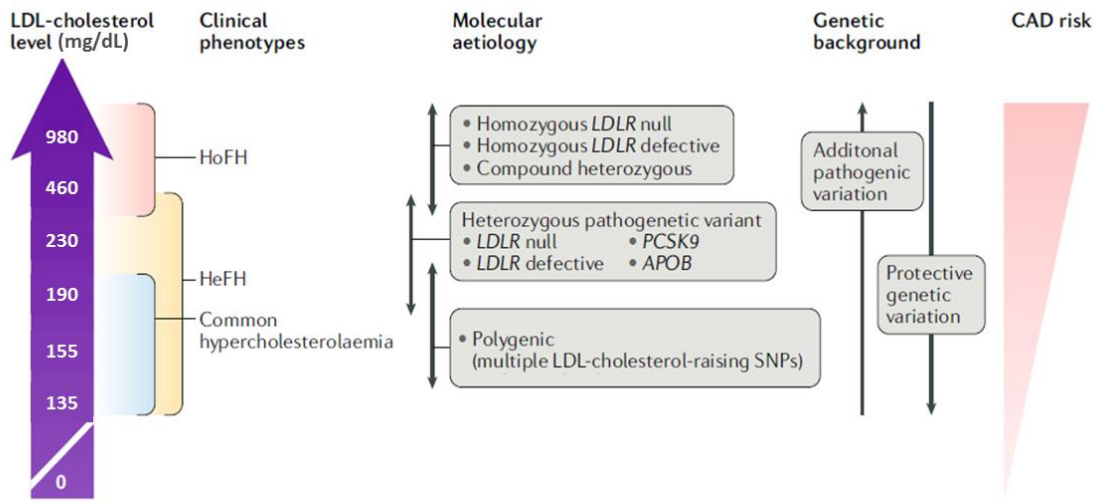
Just few mutations in *APOE* gene (chromosomal location 19p13.2) are associated with hypercholesterolemia and affected the physiological function of apolipoprotein E involved in the lipoprotein clearance from plasma. The confirmed one is c.500_502del p.Leu167del, a deletion of three base-pair at position 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 [*Cenarro 2016, Rashidi 2017*].

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 [*Morganroth 1974, Harada-Shiba 1992*]. 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*].

The different genetic causes reflect in a great variability of the phenotype, leading to a progressive increment in LDL-C levels (Figure 1.6). 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*]. In addition, there are also other factors responsible of phenotype variability, as the polygenic contribution of raising LDL-C single nucleotide polymorphisms (SNPs).

Figure 1.6. Phenotypic and genetic spectrum of FH.



Adapted from [*Watts 2020*]

FH management protocols

Cholesterol-lowering treatment should be initiated as soon as possible after a diagnosis has been made. Based on the current guidelines to improve risk assessment, the use of imaging techniques to detect asymptomatic atherosclerosis is recommended [*ESC/EAS guidelines for the management of dyslipidaemias: Lipid modification to reduce cardiovascular risk 2020*]. Treatment should be initiated with high-intensity statin therapy, in most cases in combination with ezetimibe. In FH patients at very-high risk of ASCVD due to a prior history of ASCVD or another major risk factor, LDL-C goals are a ≥50% reduction of LDL-C from baseline and an LDL-C < 1.4 mmoL/L (< 55 mg/dL). In the absence of ASCVD or another major risk factor, patients with FH are categorized as high-risk, and LDL-C goals are a ≥50% reduction of LDL-C from baseline and an LDL-C < 1.8 mmoL/L (< 70 mg/dL).

Table 1.2. Recommendations for the detection and treatment of patients with heterozygous familial hypercholesterolemia [*ESC/EAS guidelines for the*

management of dyslipidaemias: Lipid modification to reduce cardiovascular risk 2020].

Recommendations	Class[a]	Level[b]
It is recommended that a diagnosis of FH is considered in patients with CHD aged <55 years for men and <60 years for women, in people with relatives with premature fatal or non-fatal CVD, in people with relatives who have tendon xanthomas, in people with severely elevated LDL-C [in adults >5 mmol/L (>190 mg/dL), in children >4 mmol/L (>150 mg/dL)], and in first-degree relatives of FH patients.	I	C
It is recommended that FH should be diagnosed using clinical criteria and confirmed, when possible, via DNA analysis.	I	C
Once the index case is diagnosed, family cascade screening is recommended.	I	C
It is recommended that FH patients with ASCVD or who have another major risk factor are treated as very-high-risk, and that those with no prior ASCVD or other risk factors are treated as high-risk.	I	C
For FH patients with ASCVD who are at very-high risk, treatment to achieve a ≥50% reduction from baseline and an LDL-C <1.4 mmol/L (<55 mg/dL) is recommended. If goals cannot be achieved, a drug combination is recommended.	I	C

In primary prevention, for individuals with FH at very-high risk, an LDL-C reduction of ≥50% from baseline and an LDL-C goal of <1.4 mmol/L (<55 mg/dL) should be considered.	IIa	C
Treatment with a PCSK9 inhibitor is recommended in very-high-risk FH patients if the treatment goal is not achieved on maximal tolerated statin plus ezetimibe.	I	C
In children, testing for FH is recommended from the age of 5 years, or earlier if HoFH is suspected.	I	C
Children with FH should be educated to adopt a proper diet and treated with a statin from 8–10 years of age. Goals for treatment should be LDL-C <3.5 mmol/L (<135 mg/dL) at >10 years of age.	IIa	C

The European Atherosclerosis Society FH initiative

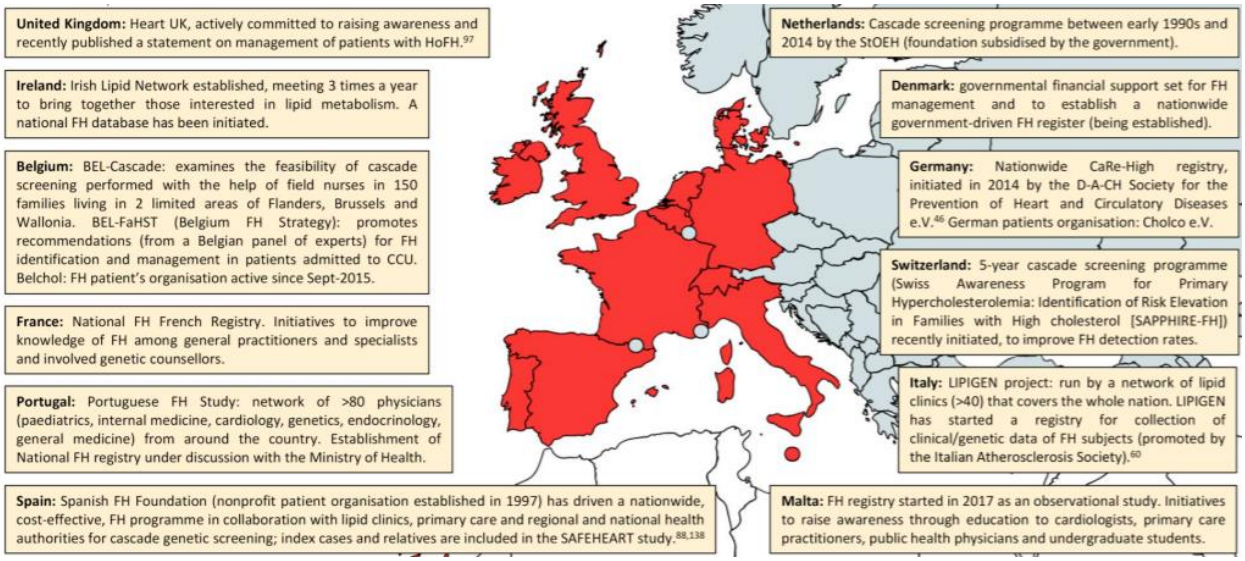
The European Atherosclerosis Society FH Studies Collaboration (EAS FHSC) was initiated with an aim to develop a worldwide, cross-regional registry of FH patients and promote a network of investigators interested in FH (www.eas-society.org/fhsc). Investigators from more than 70 countries were involved, with > 10,000 cases already included in the registry (Figure 1.8 A and B). The results of the survey to provide an overview of FH status (prevalence/ management/initiatives) in different countries involved in the EAS FHSC demonstrated low rates of FH detection across all regions. National registries and education programmes to improve FH awareness/knowledge are a recognised priority, but funding is often lacking. There are only a few national official government programmes for FH. Under-treatment is an issue as FH therapy is not universally reimbursed. PCSK9-inhibitors are available only in ~2/3 countries [*EAS Familial Hypercholesterolaemia Studies Collaboration*

2018]. It should be also mentioned, that in many countries, diagnosis primarily relies on the Dutch Lipid Clinics Network criteria and a genetic testing for clinically suspicious FH patients routinely is not provided.

Italian registry - The LIPIGEN

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 specialized in the management of patients affected by primary dyslipidemias throughout the national territory, including pediatric clinics and LDL-C apheresis centres. The LIPIGEN Network structure was based on a close interaction between clinical centres, general practitioners, and patient organizations. Main objectives were 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 aimed 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 was 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.

Figure 1.7 A. FH-related initiatives in countries involved in the EAS FHSC network in the WHO region of Europe – Italy as a participant.

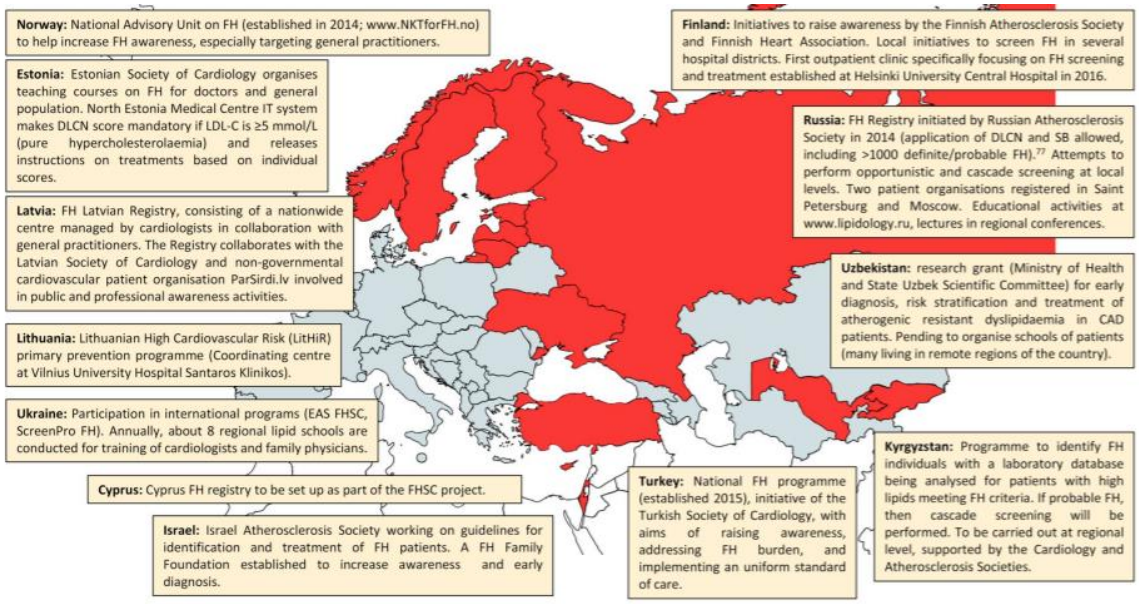


[EAS Familial Hypercholesterolaemia Studies Collaboration 2018]

Russian registry - The RENESSANS

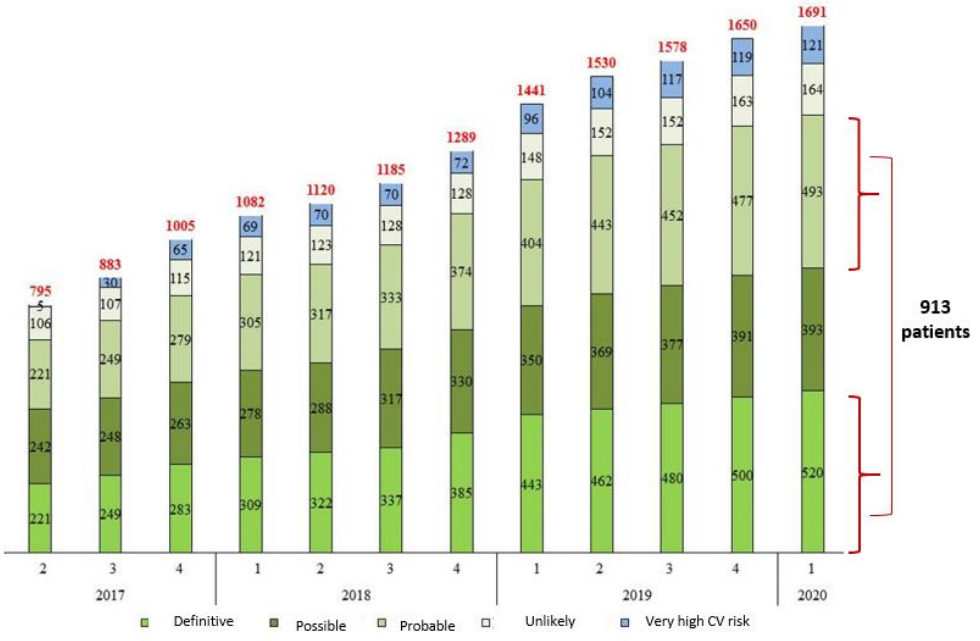
Russian multicenter of patients with FH and very high cardiovascular risk with insufficient effect of hypolipidemic therapy (RENESSANS Registry) was conducted in 2017 [*Yezhov 2019*]. The aim of RENESSANS was maximal inclusion of patients not only with FH, but also those with atherosclerotic cardiovascular diseases (CVD), who did not achieve targeted level of LDL-C using hypolipidemic drug therapy. The RENESSANS Registry was an open, national, observing study that included patients with definite and probable (according to Dutch lipid clinic network and Simon Broome Registry criteria) heterozygous and homozygous FH, as well as patients of very high cardiovascular risk. There were designed two register forms: for patients with FH and for very high cardiovascular risk patients. A very important clinical information was collected. However, the unavailability of a genetic testing was a serious limitation (Figure 1.8).

Figure 1.7 B. FH-related initiatives in countries involved in the EAS FHSC network in the WHO region of Europe – Russia as a participant.



[*EAS Familial Hypercholesterolaemia Studies Collaboration 2018Vallejo-Vaza 2018*].

Figure 1.8. Clinical diagnosis (based on DLCN score data) within the registry RENESSANS.



Up to 2020 year: 913 patients with clinical FH according to DLCN score: 520 – definite diagnosis, 393 – probable diagnosis but no genetic data

Adapted from [*Yezhov 2019*]

To overcome problems of unavailability of a genetic testing and, thus, the number of new FH cases identification in Russia a Genetic Lipid Disorders Network was conducted in Russia in 2020 as a part of a global EAS initiative "Lipid Clinics Network". During 2020-2022 as a part of the current PhD project plan 9 centers entered the Network in order to promote patients from different regions of Russia with a genetic testing under the "umbrella" of Almazov Institute of Molecular Biology and Genetics in Saint Petersburg. Centers – participants are presented in the Figure 1.9.

Figure 1.9. Centers – participants of the Genetic Lipid Disorders Network within the global EAS initiative "Lipid Clinics Network:

- Saint Petersburg (Federal centre level), national coordinator Asiiat Alieva
- Samara, Chief Cardiologist Dmitriy Duplyakov
- Nizhny Novgorod, Chief Cardiologist Elena Timoshenko
- Rostov, Chief Cardiologist Alexey Hripun
- Cheboksary, Chief Cardiologist Natalia Svetlova
- Kazan, Chief Cardiologist Zulfia Kim
- Astrakhan, Chief Cardiologist Maria Kiseleva
- Krasnodar, Chief Cardiologist Elena Kosmacheva
- Ufa, Chief Cardiologist Irina Nikolaeva.

Gaps in FH identification and management

Problematic issues in dyslipidemia management area are related to

- difficulties in the accuracy and timeliness of CV risk stratification in patients, predominantly in the framework of primary prevention
- providing continuity in the management of patients with dyslipidemias between specialized centers and GP-practitioners, and, as a result, failure in achieving LDL-C target values
- patient adherence to lipid-lowering therapy.

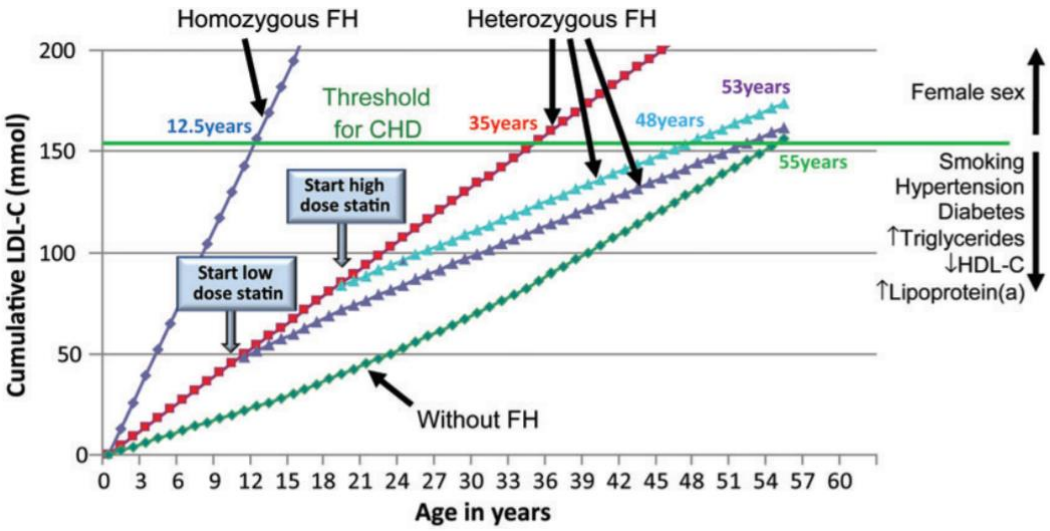
If to consider key gaps in FH identification and management itself, next areas might be considered [*Wiegman 2015*]:

- Evaluation of the potential contributions of invasive and non-invasive imaging for assessment of clinical and incident atherosclerotic vascular disease,
- Efficacy and acceptability of new biologics in refractory FH,
- Long-term safety of current and future cholesterol-lowering treatments, including effects on future fertility,
- Value, cost-effectiveness, and acceptability of universal screening and reverse cascade screening strategies,
- Organization of care between community and specialist settings.

Although last years we got the recognition of the value of Mendelian randomization studies as evidence for the benefit of lifelong low LDL-C and the early identification and treatment are even more 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

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 1.9).

Figure 1.9. The concept of a cumulative LDL cholesterol burden. The cumulative LDL cholesterol burden of a 55-year-old person without FH is typically 160 mmol, a burden sufficient for CHD to develop. For an individual with heterozygous FH, this LDL cholesterol burden is reached by age 35 if untreated, by age 48 if treated since age 18, and by age 53 if treated since age 10. An untreated subject with homozygous FH will reach this level at age 12.5.

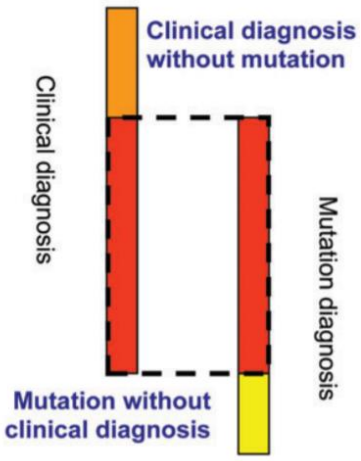


Adapted from [*Nordestgaard 2013*]

Furthermore, from the recent registry data there is an understanding that some 10–40%, depending on referral criteria, of those with a clinical diagnosis do not have a detectable causal mutation; rather, they have a clinical diagnosis of FH, but not a mutation diagnosis (Figure 1.8) [*Civeira 2008, Palacios 2012*]. There may therefore be yet other key genes implicated in this disease; alternatively, these individuals may present a polygenic basis for their LDL-C elevation without contributions from any of the classical FH genes.

Conversely, genetic cascade testing from FH subjects with a detected causative mutation has shown that, while on average, relatives who carry the causative mutation have two-fold higher mean LDL cholesterol levels compared with non-carrier relatives, a significant proportion are below the clinical diagnostic cut-off and thus they have a mutation diagnosis but not a clinical diagnosis of FH [*Palacios 2012, Thorsson 2003, Huijgen 2012, Starr 2008*]. Such individuals may possess other favourable genes and/or a lifestyle that reduces the impact of the mutation, but because of their lifetime LDL-C exposure, they should still be offered appropriate lipid-lowering therapy according to the LDL-C targets given later.

Figure 1.10. Overlap of clinical and mutation diagnosis of heterozygous familial hypercholesterolemia.



Adapted from [*Nordestgaard 2013*]

Thus, a search for new approaches in FH identification and management are highly needed.

III. Approaches to improve FH identification and cardiovascular risk stratification

1. Achilles Tendons ultrasound measurement

The Achilles tendon xanthomas (TXs) are typical signs of FH that is used by DLCN score criteria, contributing with six points to the diagnosis of FH for the proband and with two points to the diagnosis of FH in case of its presence in first degree relatives [*Santos 2016*]. The term originates from the Greek word xantho that means "yellow". TXs are composed of monocyte-derived foam cells resulting from intracellular accumulation of lipids of which the major constituent is cholesterol ester [*Rallidis 2020*]. There are convincing data that TXs are independently associated with the presence and burden of coronary atherosclerosis [*Ferrieres 1995, Civeira 2005, Oosterveer 2009*]. Hovewer, it was shown that the detection of TXs has a poor sensitivity and is largely affected by the clinician judgement [*Tsouli 2005*]. To overcome this gap, the usefulness of Achilles tendon ultrasonography to identify the Achilles tendon lesions was under investigation. Some studies of recent years demonstrated that the ultrasonography is a more reproducible and sensitive method for the TXs identification that allows physicians to identify either focal lesions or diffuse changes in tendon echotexture and to measure calcaneal tendon thickness [*Scott 2019, Kutkiene 2019, Paantjens 2020*]. It also has been shown that not only TXs, but also Achilles tendon thickness (ATT), the early sign of TXs, is a risk factor of CVD in FH individuals [*Sugisawa 2012*]. Next step in a need to identify whether the ATT ultrasound measurement improves the identification of FH patients and brings its impact to the relevance of clinical and genetic FH diagnosis.

2. Proteomics

A substantial proportion of the general population at risk and FH remains unidentified until their first clinical event [*Fernandez-Friera 2017, Nordestgaard 2013*] as the vast majority of single biomarkers suggested for the improvement of a risk assessment are selected based on specific pathophysiological concepts, which do not reflect the true complexity of atherosclerosis. In fact, CV risk is the result of an interplay between comorbidities (chronic inflammatory diseases, metabolic derangements) and exogenous risk factors, propagated by a variety of pathophysiological axes, comprising but not limited to lipids, coagulation, and inflammation [*Hoogeveen 2018*]. Probably the limitations mentioned above could be overcome with proteomics and simultaneous assessment of a large number of plasma proteins may hold a promise to further refine risk assessment [*Lindsey 2015*].

Proteins circulating in blood capture genetically inherited predisposition to diseases and integrate them with the effects of environmental exposures, diet, lifestyle behaviours, and the ageing [*Williams 2019*]. Proteins (directly or indirectly) are the targets of virtually all drugs [*Ferrannini 2020, Williams 2018*]. This ability of proteins to reflect both 'nature' and 'nurture' is essential for understanding and risk-stratifying common, complex diseases, such as atherosclerotic cardiovascular disease, which has a significant non-genetic component. Circulating proteins are an accessible measure of an individual's phenotype. Approximately 19 000 human genes code for 30 000 proteins. About 2200 proteins can enter the blood compartment by directed secretion as they contain the required "signal sequence" [*Lin 2008*]. These proteins, e.g. hormones, cytokines, chemokines, adipokines, and growth factors, are of prime interest as they orchestrate physiological processes in health and pathological processes in diseases. Other proteins enter the bloodstream by 'leakage' during cell damage, with troponin as a familiar

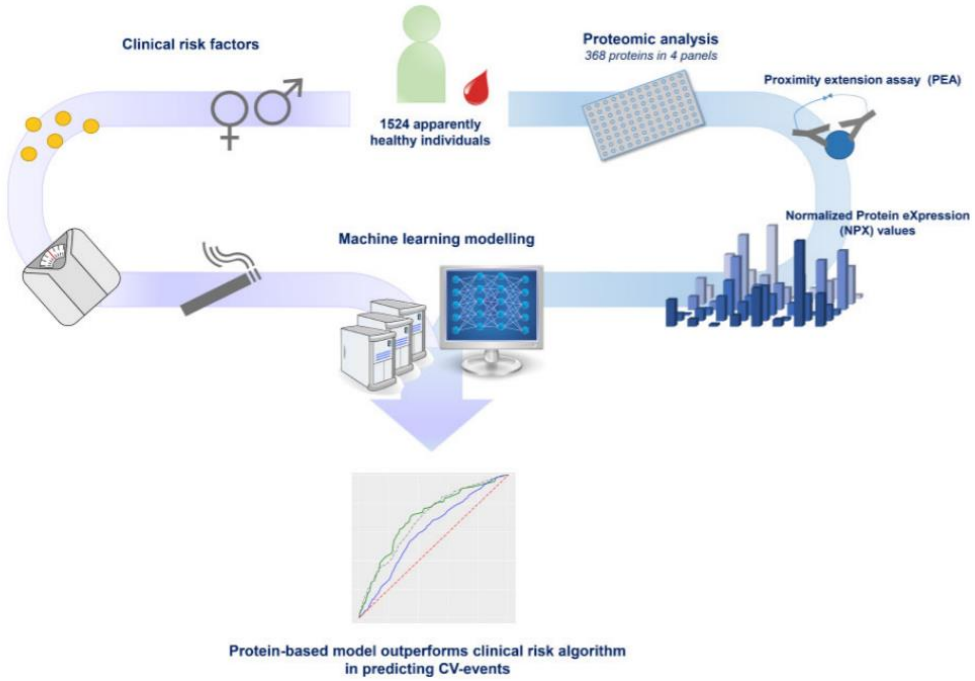
example indicative of myocyte injury. The number of these leakage proteins that can be detected in blood is probably limited only by the sensitivity of the assay. Both secreted and leakage proteins can serve as biomarkers of disease risk and clinical outcomes, and contribute to risk modelling. Until recently, high-throughput proteomic profiling of human plasma had been challenging due to the extraordinary complexity and broad dynamic range of plasma protein concentrations [*Benson 2019*]. Two proteomics platforms have developed affinity reagents that are suitable for assaying large numbers of proteins. Modified aptamers are chemically modified single strands of DNA 40 nucleotides long that bind to proteins with high affinity and specificity. Currently, the modified aptamer platform (SomaScan, SomaLogic Inc, Boulder, CO, USA) can quantify the levels of 5000 unique plasma proteins simultaneously, with excellent reproducibility [*Williams 2019, Benson 2019*]. In another approach, oligonucleotide-labelled antibodies have been incorporated into a novel, commercially available platform (Olink, Uppsala, Sweden) that can be used to quantify levels of over a thousand human proteins. The Olink platform has been available in panels of 92 proteins targeting specific biological domains (e.g. cardiovascular, inflammatory, and metabolic panels). When applied to thousands of participants in clinical investigations, these platforms generate millions of individual protein data points that are integrated with clinical datasets and outcome measures [*Williams 2019]*. Advances in machine learning have been helpful in generating risk models that optimize the discrimination and calibration metrics [*Williams 2019, Ganz 2016*].

Within the study published in 2020 Dr. Hoogeveen and colleagues tested the hypothesis that a protein-based cardiovascular risk model can outperform prediction based on traditional risk factors in the primary prevention setting [*Hoogeveen 2020*]. Focusing on 333 proteins known to be involved in atherogenesis, a cardiovascular risk model was derived in a nested

case–control sample of apparently healthy individuals selected from the EPIC-Norfolk prospective population study, recruited between 1993 and 1997 in Norfolk, UK. A total of 411 individuals who developed acute myocardial infarction, the primary endpoint of the analysis, were compared with 411 individuals who remained free of any clinical cardiovascular disease during a median follow-up of 20 years. The derived protein model was compared with a clinical algorithm similarly optimized to the cohort and comprised of age, sex, body mass index, systolic blood pressure, smoking status, presence of diabetes, the use of antihypertensive medication, total cholesterol levels, and HDL-cholesterol levels. Validation of the protein model was performed in the Progression of the Intima Carotid Lesions (PLIC) observational cohort, enrolled in 1998–2000 in Milan, Italy and followed for a mean of 11 years. Among 702 individuals, 44 had a cardiovascular outcome event. Through machine learning methods, the prediction model for myocardial infarction in the EPIC-Norfolk cohort over the entire 20 years of follow-up was comprised of 50 proteins. It had a receiver operating characteristic curve (ROC) area under the curve (AUC) of 0.754 ± 0.011, a modest but significant improvement over the clinical model AUC of 0.722 ± 0.008. The investigators then applied a statistical technique (Markov chain Monte Carlo) to pinpoint the optimal time horizon for proteomic risk prediction; it was found at 1132 days (3.1 years) using a risk model also comprised of 50 proteins that only partly overlapped with the list of proteins used for the longer 20-year prediction. At this earlier time horizon, the AUC increased to 0.803 ± 0.093 and strongly outperformed the clinical algorithm AUC (0.680 ± 0.223). Improvement in predicting CV events during the entire (median) 20-year follow-up period was significant, albeit modest. In an external independent validation cohort, the predictive value of the protein panel for CV events was confirmed and superior to the clinical risk model (incremental AUC 0.10). Survival analysis showed superiority of the protein model to the clinical risk model at all tested time points ($P < 0.001$).

Collectively, these data showed that a novel proteomic panel offers a significant improvement in CV risk discrimination compared to a clinical risk model based on traditional risk factors (Figure 1.11).

Figure 1.11. Improvement of cardiovascular risk prediction in a primary prevention setting, demonstrating the potential of a proteomics panel to further refine risk assessment.



Adapted from [*Hoogeveen 2020*]

Following the data about the role of proteomics for CV risk stratification in general population a hypothesis about its profits for FH identification might be considered. Hovewer, there are few data demonstrating the impact of protein biomarkers in risk prediction for FH patients. In 2017 Sven Bos and colleagues aimed to check a hypothesis whether proteomics is useful to identify novel protein biomarkers that differentiate genetically confirmed heterozygous patients with FH at high CAD risk from those at low CAD risk [*Bos 2017*]. Sixty genetically confirmed FH patients were recruited and stratified into (1) asymptomatic FH with low atherosclerotic burden (FH,

n 5 20); (2) asymptomatic FH with high atherosclerotic burden; and (3) FH with previously confirmed symptomatic CAD. Six new potential proteins were identified: leucine-rich alpha-2-glycoprotein (LRG1), inter-alpha-trypsin inhibitor heavy chain H3, complement C4-B (C4B), complement C1q subcomponent subunit B (C1QB), monocyte differentiation antigen (CD14), and histidine-rich glycoprotein (HRG). There were significant associations between gender and C4B, C1QB, CD14, and HRG. There were significant associations between smoking and LRG1, CB4, and HRG. All the peptides were significantly associated with advanced CAD stages, independently of age and smoking. However, the absence of the proteins was the strongest marker. The most accurate association with CAD was HRG (area under the receiver operating characteristic curve = 0.922), whereas LRG1, C4B, and C1QB were also associated with CAD (area under the receiver operating characteristic curve > 0.9). For either coronary atherosclerosis or CAD, LRG1, C4B, C1QB, and HRG were relatively well associated. Thus, the study has identified 6 potencial novel protein biomarkers that are associated with more advanced stages of atherosclerotic disease and subsequent coronary events in patients with heterozygous FH. Hovewer, the several limitations of the study included the crosssectional study design and inclusion of a highly selected population with a relatively small sample size (20 patients in each of three groups).

Thus, first of all, the project aimed, by creation of Russian Genetic Lipid Disorders Network and taking advantage of Italian Genetic Lipid Disorders Network data – LIPIGEN, to give a phenotype – genotype characterization of both cohorts, to reveal its particularities, and then, to consider new approaches, such Achilles Tendon xanthoma ultrasonography and proteomics analysis, that might be potencially considered for improvement of FH identification.

Chapter II

STUDY POPULATION

Among 51 Italian centers within the LIPIGEN Network (its structure is described in details in Chapter 1) Milan center was sorted out for the current analysis. It was approved by the Ethics Committee of the Coordinating Center (Centro per lo Studio dell'Aterosclerosi IRCCS MultiMedica, Sesto San Giovanni, Milan, Italy).

Among 9 Russian centers within the Russian Genetic Lipid Disorders Network (its structure is described in details in Chapter 1) Saint Petersburg centre was sorted out for the current analysis. It was approved by the Ethics Committee of the Almazov National Medical Research centre (Protocol № 22082019 dated by August the 12th 2019).

The study was 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 have had to reach 6 or more points according to DLCN score criteria. All the patients enrolled were provided with the genetic testing.

For each patient, the paper and electronic databases included sections where anamnestic, biochemical, genetic and follow up data were collected:

- *Registration data* including *Informed Consent*, that was a process by which a subject decided to voluntarily participate in a study and it was

documented by means of a written, signed and dated document that needs to be stored at the centres

- *Demography*: this part collected some demographic data about sex, geographic origin and ethnicity
- *Anamnestic and anthropometric data:* this part collected 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 had a clinical diagnosis of FH without the genetic confirmation, that was obtained after the enrolment in the current study. Moreover, height, weight, Body Mass Index (BMI), smoke status and IMT were collected
- *Physical Examination (DLCN Score):* this section collected part of the data necessary for the DLCN score evaluation. It took 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 years old
- *Family History (DLCN Score):* this section collected 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
- *Clinical History (DLCN Score):* this section took into account the clinical history of the subject as the presence of a premature CHD, premature cerebral or peripheral vascular disease. Moreover, there was 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

- *Biochemistry at baseline*: this part was 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 converted the value from mg/dL to mmol/L and viceversa. The year of the "date of sample" was used to calculate the age the baseline for each enrolled subject.
- *Lipid Lowering Treatment:* within this section physicians had to indicate if the subject was 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?" was "Yes", it was required to indicate the lipid lowering drug and the dosage. Moreover, a pre-treatment LDL-C value was necessary for the calculation of DLCN score. If an untreated LDL-C value was available, it could be entered in the "Highest LDL-C level pre-treatment". If the value is unknown, the system automatically retro-calculated it using the therapy correction factors (Table 1, Supplementary materials)
- *DLCN Score*: This section summarized 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 was recommended.
- *Molecular diagnosis:* this section reported the genetic results if the subject underwent the molecular test. The tested genes include LDLR, APOB, PCSK9, LDLRAP1, and APOE. Moreover, subjects could 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. Moreover, additional parameters as the LDL-C polygenic risk score [Tal*mud 2013*] in subjects analysed after 2016 are reported. 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*].

- *Concomitant diseases other than FH:* in this section, physicians had to report the relevant concomitant diseases other than FH present at diagnosis or occurred after the diagnosis with the onset date
- *Concomitant therapy other than lipid lowering:* in this section, physicians reported if the patient wass taking some concomitant treatments other than the lipid lowering drugs, such as antidepressant, antihypertensive, antithrombotic, hypoglycemic or immunosuppressive drugs
- *Changes in lipid lowering therapy 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).

Additional parameters were collected for two sub-studies: Achilles tendons ultrasonography measurement for the ACTUS-FH sub-study and proteomic analysis for the Proteomic sub-study. Methodology is described in details within the paragraphs 3.3.1 and 3.4.1 respectively.

Comparisons among the adult cohort were performed stratifying by lipid-lowering therapy 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 (%).

Chapter III

RESULTS

I. *Descriptive analysis of the Italian and Russian FH cohorts*

For the current enhanced and interpopulation analysis, two cohorts from north cities of Italy and Russia - Milan (Milan_c) and Saint-Petersburg (SaintPet_c), were sorted out from the national registries.

3.1. Clinical data analysis

SaintPet genetic study cohort included at the moment of starting of the analysis 107 patients, with a mean age at the first visit - 40.7±17.6 years, Milan genetic study - 369 patients, with a mean age at the first visit – 39.9±19.5 years (Table 3.1.1). It should be noticed the difference in the mean age when FH was diagnosed first time in SaintPet cohort in comparison with the Milan ones (39.4±17.2 vs 24.6±15.1 years old respectively).

Table 3.1.1. Basic characteristics of Milan and SaintPet cohorts

	Milan_c	SaintPet_c	p
Male, N (%)	181(49.1%)	43(40.2%)	0.106
Age [years], mean±SD	39.9±19.5	40.7±17.6	0.663
Age groups: Below 18	67 (18.3%)	14 (13.2%)	0.582

18-30 years old, N (%) 31-50 years old, N (%) 51-70 years old, N (%) >70 years old, N (%)	47 (12.8%) 129 (35.2%) 112 (30.6%) 11 (3.0%)	16 (15.1%) 40 (37.7%) 33 (31.1%) 3 (2.8%)	
BMI [kg/m^2], mean±SD	24.4±4.8	25.6±5.7	0.045
<u>BMI classes:</u> <25, N (%) 25-30, N (%) >=30 N (%)	 191 (56.0%) 118 (34.6%) 32 (9.4%)	 51 (48.1%) 33 (31.1%) 22 (20.8%)	0.035
Age when FH was diagnosed [years], mean±SD	24.6±15.1	39.4±17.2	<0.0001

Within the analysis of biochemical parameters there wasn't observed significant differences in the levels of the lipid profiles and other biochemical parameters (Table 3.1.2), however the analysis of pre-treatment LDL-C levels uncovered it (Table 3.1.2).

Table 3.1.2. Biochemical data of Milan and SaintPet cohorts

	Milan_c	SaintPet_c	p
Total Cholesterol [mg/dL], mean±SD	308.0±83.9	281.5±93.7	0.009
LDL-C [mg/dL], mean±SD	228.0±79.4	208.2±90.6	0.042
Pre-treatment LDL-C [mg/dL], mean±SD	296.0±73.5	310.5±91.0	<0.0001
Triglycerides [mg/dL], median [IQR]	104.0 [71.0 – 140.0]	96.5 [69.1 – 135.5]	0.248
HDL-C [mg/dL], mean±SD	56.7±14.0	51.4±13.4	<0.0001

Glucose [mg/dL], mean±SD	92.1±15.6	93.4±19.2	0.525

On the two histograms presented below (Figures 3.1 A and B) we can observe the distribution of pre-treatment LDL-C levels in Milan and SaintPet cohort that demonstrate the higher mean values in SaintPet ones [304.63±70.41 vs 323.68±88.96 mg/dL respectively].

Figure 3.1 A. The distribution of pre-treatment LDL-C levels in Milan cohort.

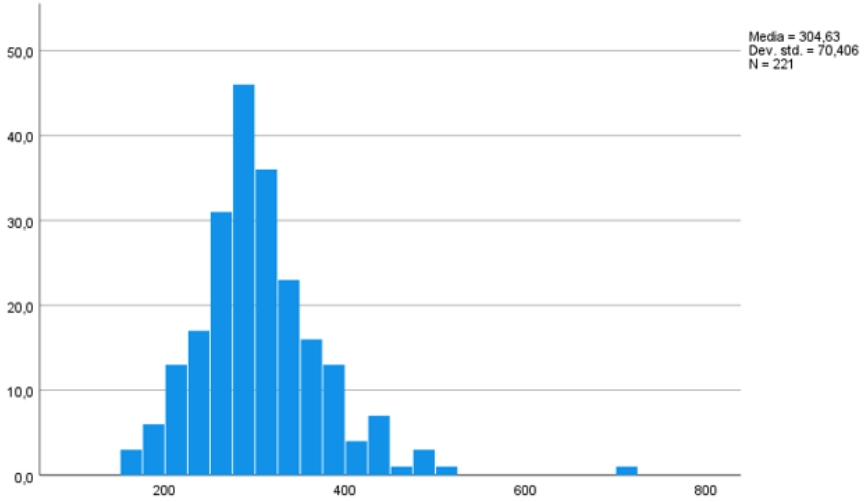
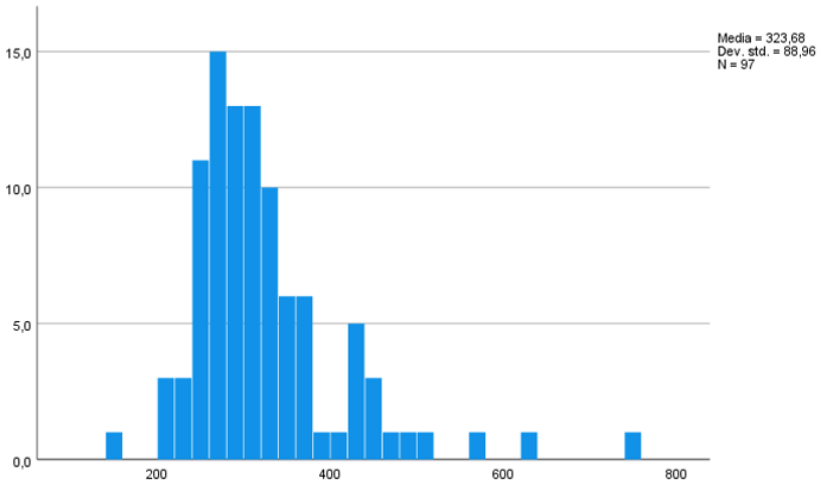


Figure 3.1 B. The distribution of pre-treatment LDL-C levels in SaintPet cohort.



Pre-treatment LDL-C levels (mg/dL)

The analysis of the DCLN score components in two cohorts (table 3.1.3) demonstrated the higher frequency of clinical history of premature CHD as well as of first-degree relative with premature CHD in SaintPet cohort, while the Milan ones – the higher frequency of increase of LDL-C above 95th percentile in children and first-degree relatives.

Table 3.1.3. DLCN score components for Milan and SaintPet cohorts

	Milan_c	SaintPet_c	p
Family history			
First-degree relative with premature CHD; N (%)	117 (31.7%)	54 (50.5%)	<0.0001
First-degree relative with LDL-C >95th percentile; N (%)	317 (85.9%)	63 (58.9%)	<0.0001
First-degree relative with tendon xanthoma and/or arcus cornealis; N (%)	31 (8.4%)	8 (7.5%)	0.759
Children <18 years with LDL-C >95th percentile; N (%)	81 (22.0%)	15 (14.0%)	0.072

Anamnesis of the ischemic CV diseases			
Clinical history of premature CHD; N (%)	26 (7.0%)	15 (14.0%)	0.024
Clinical history of premature cerebral or peripheral vascular disease; N (%)	23 (6.2%)	4 (3.7%)	0.484
Clinical examination / Biochemical data			
Tendon xanthoma; N (%)	39 (10.6%)	11 (10.3%)	0.932
Arcus cornealis before the age 45 years; N (%)	18 (4.9%)	12 (11.2%)	0.018
Pre-treatment LDL-C value; N (%) 155-190 mg/dL 191-250 mg/dL 251-325 mg/dL >325 mg/dL	 44 (11.9%) 120 (32.5%) 112 (30.4%) 68 (18.4%)	 3 (2.8%) 15 (14.0%) 52 (48.6%) 36 (33.6%)	<0.0001
Lipid lowering therapy; N (%)	96(26.0%)	44(41.1%)	0.003

However, after the adjustment for the pre-treatment LDL-C levels in both cohorts discrepancies in frequency of premature CHD and arcus cornealis before 45 had a trend to its evening out despite the small numbers of patients within the groups (Table 3.1.4).

Table 3.1.4. Frequency of clinical history of premature CHD and arcus cornealis before 45 in Milan and SaintPet cohorts after the adjustment for the pre-treatment LDL-C levels

		Premature CHD, N (%)	Arcus cornealis before the age 45, N (%)

		Milan_c	SaintPet_c	Milan_c	SaintPet_c
Pre-treatment LDL-C levels, mg/dL	250-299	5(6.3%)	1(2.7%)	2(2.5%)	3(8.1%)
	300-349	5(8.5%)	4(16.0%)	6(10.2%)	3(12.0%)
	350-399	2(6.9%)	3(27.3%)	2(6.9%)	1(9.1%)
	Overall	12(7.2%)	8(11.0%)	10(6.0%)	7(9.6%)

Sub-analysis stratified by the age

The sub-analysis for Milan and SaintPet cohorts stratified by age (Tables 3.1.5 A and B) demonstrated the very low frequency of young patients under the lipid-lowering treatment despite the high levels of LDL-C that indicate its necessity in both cohorts.

Table 3.1.5 The sub-analysis of basic clinical parameters stratified by age

A. Milan cohort

	Age groups				
	< 18	18 - 24	25 - 44	45 - 64	>65
	Basic characteristics / Biochemical data				
Male, N(%)	7 (35.0)	3(42.9)	40 (58.8)	47 (50.0)	7 (22.6)
Age, mean±SD	8.85± 8.07	21.00± 1.41	37.31± 5.14	53.74± 5.55	69.16± 4.03
Total cholesterol, [mg/dL], mean±SD	363.10± 117.38	327.57± 77.32	349.68± 84.30	332.20± 87.36	307.90± 89.78
LDL-C, [mg/dL],	285.35±	258.14±	270.93±	245.65±	225.94±

mean±SD	120.61	73.41	79.44	79.70	82.07
Pre-treatment LDL-C, [mg/dL], mean±SD	297.06± 120.40	316.56± 83.83	312.05± 58.58	299.45± 69.51	307.04± 53.06
TG, [mg/dL], median [IQR]	69.0[53.0-89.0]	90.0[70.0-124.0]	112.0[77.0-139.0]	117.0[84.0-175.0]	118.5[92.0-139.5]
HDL-C, [mg/dL], mean±SD	57.95± 18.73	47.71± 7.65	52.56± 13.57	58.32± 13.05	60.65± 17.99
On LLT, N(%)	1(5.0)	2(28.6)	21 (30.9)	34 (36.2)	18 (58.1)
	DCLN score components				
Tendon xanthoma, N(%)	6(30.0)	1(14.3)	13(19.1)	12(12.8)	7(22.6)
Arcus cornealis before 45 years, N(%)	0(0.0)	0(0.0)	6(8.8)	8(8.5)	4(12.9)
Clinical history of premature CHD, N(%)	0(0.0)	0(0.0)	6(8.8)	13(13.8)	2(6.5)
Clinical history of premature cerebral or peripheral vascular disease, N(%)	1(5.0)	0(0.0)	6(8.8)	13(13.8)	2(6.5)
DLCN score, number of points, N(%) 3 – 5 6 – 8 >9	 4(20.0) 8(40.0) 8(40.0)	 0(0.0) 3(42.9) 4(57.1)	 8(11.8) 27(39.7) 33(48.5)	 19(20.2) 41(43.6) 34(36.2)	 2(6.5) 19(61.3) 10(32.3)

Table 3.1.5 B. SaintPet cohort

	Age groups				
	< 18	18 - 24	25 - 44	45 - 64	>65
	Basic characteristics / Biochemical data				
Male, N(%)	5 (45.5)	0(0.0)	22 (57.9)	10 (24.4)	1 (16.7)
Age, mean±SD	9.36± 4.13	24.00	33.92± 5.06	53.73± 5.54	72.67± 8.24
Total cholesterol, [mg/dL], mean±SD	322.00± 67.50	376.53	302.87± 115.69	257.17± 77.68	254.15± 90.70
LDL-C, [mg/dL], mean±SD	257.87± 65.15	318.00	230.05± 112.27	181.93± 71.05	173.67± 86.71
Pre-treatment LDL-C, [mg/dL], mean±SD	297.64± 77.52	334.00	328.84± 103.96	319.30± 74.96	366.92± 104.36
TG, [mg/dL], median [IQR]	83.3[67.3-86.8]	64.6[51.4-77.9]	88.3[59.3-124.9]	106.3[80.6-146.1]	109.8[96.5-173.6]
HDL-C, [mg/dL], mean±SD	53.00± 10.15	48.00	50.46± 12.50	51.98± 14.12	46.83± 14.74
On LLT, N(%)	0(0.0)	0(0.0)	16 (42.1)	22 (53.7)	5 (83.3)
	DCLN score components				
Tendon xanthoma, N(%)	11(100.0)	1(100.0)	35(92.1)	34(82.9)	5(83.3)
Arcus cornealis before 45 years, N(%)	0(0.0)	0(0.0)	3(7.9)	7(17.1)	1(16.7)

Clinical history of premature CHD, N(%)	1(9.1)	0(0.0)	4(10.5)	7(17.1)	0(0.0)
Clinical history of premature cerebral or peripheral vascular disease, N(%)	0(0.0)	0(0.0)	5(13.2)	9(22.0)	0(0.0)
DLCN score, number of points, N(%)					
<5	0(0.0)	0(0.0)	0(0.0)	4(9.8)	0(0.0)
6 – 8	2(18.2)	0(0.0)	5(13.2)	3(7.3)	1(16.7)
>9	6(54.5)	0(0.0)	18(47.4)	19(46.3)	3(50.0)

Sub-analysis stratified by the components of DLCN score

The sub-analysis for Milan and SaintPet cohorts stratified by DLCN score is presented within the tables 3.1.6 A and B.

Table 3.1.6 The sub-analysis of basic clinical parameters stratified by DLCN score

A. Milan cohort

	DLCN score, number of points		
	3-5	6-8	>9
	Basic characteristics / Biochemical data		
Male, N(%)	13 (39.4)	46 (46.5)	45(50.6)
Age, mean±SD	47.09±16.19	47.71±17.78	43.00±17.05
Total cholesterol, [mg/dL], mean±SD	284.00±67.77	338.39±70.61	354.70±107.80

LDL-C, [mg/dL], mean±SD	200.12±60.63	253.27±64.44	276.79±102.72
Pre-treatment LDL-C, [mg/dL], mean±SD	231.75±31.38	283.63±36.38	355.00±74.21
TG, [mg/dL], median [IQR]	93.0[65.0-125.0]	120.0[84.0-175.0]	112.0[84.0-140.0]
HDL-C, [mg/dL], mean±SD	60.64±15.21	58.34±15.16	52.81±13.26
On LLT, N(%)	10(30.3)	26(26.3)	40(44.9)
	DCLN score components		
Tendon xanthoma, N(%)	0(0.0)	2(2.0)	37(41.6)
Arcus cornealis before 45 years, N(%)	0(0.0)	2(2.0)	16(18.0)
Clinical history of premature CHD, N(%)	1(3.0)	6(6.1)	14(15.7)
Clinical history of premature cerebral or peripheral vascular disease, N(%)	6(18.2)	6(6.1)	10(11.2)

Table 3.1.6 B. SaintPet cohort

	DLCN score, number of points		
	3-5	6-8	>9
	Basic characteristics / Biochemical data		
Male, N(%)	5 (45.5)	21 (45.7)	12(30.0)

Age, mean±SD	39.55±17.88	41.87±18.79	42.35±15.44
Total cholesterol, [mg/dL], mean±SD	263.54±61.84	277.63±78.42	295.68±120.81
LDL-C, [mg/dL], mean±SD	200.12±60.63	253.27±64.44	276.79±102.72
Pre-treatment LDL-C, [mg/dL], mean±SD	247.36±45.42	290.36±37.12	382.99±103.93
TG, [mg/dL], median [IQR]	86.8[67.3-151.0]	102.3[76.2-136.4]	95.6[64.2-128.0]
HDL-C, [mg/dL], mean±SD	56.82±14.82	51.43±12.43	49.24±12.83
On LLT, N(%)	3(27.3)	19(41.3)	21(52.5)
	DCLN score components		
Tendon xanthoma, N(%)	0(0.0)	0(0.0)	11(27.5)
Arcus cornealis before 45 years, N(%)	1(9.1)	0(0.0)	11(27.5)
Clinical history of premature CHD, N(%)	0(0.0)	6(13.0)	8(20.0)
Clinical history of premature cerebral or peripheral vascular disease, N(%)	0(0.0)	1(2.2)	3(7.5)

Sub-analysis stratified by the primary / secondary prevention groups

The current sub-analysis demonstrated that 68.9% of patients within the Milan cohort were referred to the primary prevention, and 31.1% - to the secondary ones, while 76.3% and 23.7% within the SaintPet cohort

respectively. The percentage of patients under the lipid-lowering therapy was higher within the secondary prevention groups in both cohorts despite the comparable levels of pre-treatment LDL-C levels (Tables 3.1.7 A and B).

Table 3.1.7 The sub-analysis of basic clinical parameters stratified by the primary / secondary prevention groups

A. Milan cohort

	Primary prevention	Secondary prevention	p
Male, N(%)	85(48.9)	30(58.8)	0.211
Age, mean±SD	32.81±21.16	51.31±16.00	<0.0001
Total cholesterol, [mg/dL], mean±SD	306.84±85.24	274.00±86.91	0.020
LDL-C, [mg/dL], mean±SD	226.0±78.25	194.46±83.17	0.018
Pre-treatment LDL-C, [mg/dL], mean±SD	244.42±79.72	287.21±88.26	0.003
TG, [mg/dL], median [IQR]	93.0[65.0-138.0]	113.0[87.0-128.0]	0.050
HDL-C, [mg/dL], mean±SD	57.69±13.67	54.72±12.38	0.156
On LLT, N(%)	28(16.1%)	35(68.6%)	<0.0001
	DCLN score components		
Tendon xanthoma, N(%)	18(10.3%)	8(15.7%)	0.295
Arcus cornealis before 45 years, N(%)	5(2.9%)	7(13.7%)	0.002

Clinical history of premature CHD, N(%)	0(0.0%)	26(51.0%)	<0.0001
Clinical history of premature cerebral or peripheral vascular disease, N(%)	0(0%)	20(39.2)	<0.0001
DLCN score, number of points, N(%) <5 6 – 8 >9	 69(51.1%) 37(27.4%) 29(21.5%)	 15(30.0%) 12(24.0%) 23(46.0%)	<0.0001

Table 3.1.7 B. SaintPet cohort

	Primary prevention	Secondary prevention	p
Male, N(%)	31(37.3)	12(50.0)	0.268
Age, mean±SD	37.29±17.29	52.83±13.43	<0.0001
Total cholesterol, [mg/dL], mean±SD	297.89±90.91	225.14±81.87	0.001
LDL-C, [mg/dL], mean±SD	223.63±89.12	154.92±75.21	<0.0001
Pre-treatment LDL-C, [mg/dL], mean±SD	308.61±92.15	330.89±85.97	0.280
TG, [mg/dL], median [IQR]	98.3[74.4-129.3]	93.9[67.3-164.8]	0.788
HDL-C, [mg/dL], mean±SD	52.61±13.20	47.29±13.60	0.092

On LLT, N(%)	25(30.1%)	19(79.2%)	<0.0001
	DCLN score components		
Tendon xanthoma, N(%)	7(8.4%)	4(16.7%)	0.244
Arcus cornealis before 45 years, N(%)	10(12.0%)	2(8.3%)	0.613
Clinical history of premature CHD, N(%)	0(0.0%)	15(62.5%)	<0.0001
Clinical history of premature cerebral or peripheral vascular disease, N(%)	0(0.0%)	4(16.7)	<0.0001
DLCN score, number of points, N(%) <5 6 – 8 >9	 17(21.0%) 36(44.4%) 28(34.6%)	 2(8.3%) 10(41.7%) 12(50.0%)	0.073

Key messages:

1. Basic clinical and biochemical data of both cohorts look relevant to each other. The higher pre-treatment LDL-C levels in SaintPet cohort probably determine the higher frequency of premature CHD and arcus cornealis before 45 and have to be discussed further based on the genetic background of both cohorts.
2. The very low frequency of young patients under the lipid-lowering treatment at the moment of enrolment to the registry despite the high levels of LDL-C in both cohorts, the percentage of patients within the secondary prevention area (31.1% and 23.7% in Milan and SaintPet cohorts respectively) demonstrate a need to enhance efforts in FH

identification and management especially in primary health care system.

3. The late age when FH was diagnosed first time in SaintPet cohort in comparison with the Milan ones (39.4±17.2 vs 24.6±15.1 years old) probably may reveal organizational difficulties in the lipid disorders identification system.

3.2. Genetic data and phenotype – genotype analysis

Genetic analysis for the Milan cohort was performed for 209 patients among 221 ones due to technical reasons, identifying positive mutation in 78.3% (173 patients), while negative one – in 12,7% and inconclusive ones – in 3,6%.

Genetic analysis for the SaintPet cohort was performed with the 1 exemption (due to technical reasons) - 106 patients of 107, identifying positive mutation in 43.9% (47 patients), while negative one – in 28.0% and inconclusive ones – in 27.1%.

Clinical and biochemical comparison description

based on the genetic background

The analysis of the basic characteristics of two cohorts demonstrated the late age when FH was diagnosed in all genetic type groups within SaintPet cohort in comparison with the Milan ones (Tables 3.2.1 A and B). However,

there was also the higher percentage of patients under lipid-lowering therapy in all groups.

The higher prevalence of DLCN components within the positive genetic groups in both cohorts confirm the relevance of DLCN score to the genetic background. A higher prevalence of DLCN components within the inconclusive group of SaintPet cohort in comparison with the Milan ones should be noticed.

Table 3.2.1 Clinical and biochemical description based on the genetic background

A. Milan cohort

	Genetic results		
	Positive	Inconclusive	Negative
Male, N(%)	116(46.2%)	19(50.0%)	38(59.4%)
Age [years], mean±SD	38.9±19.2	38.4±18.3	44.8±17.4
Under 18, N(%)	51(20.4%)	8(21.1%)	5(7.8%)
Age when FH was diagnosed [years], mean±SD	23.0±14.5	24.9±14.2	31.0±16.6
BMI [kg/m^2], mean±SD	24.4±4.8	23.7±4.4	25.4±4.7
	Biochemical data		
Total cholesterol, [mg/dL], mean±SD	317.8±86.8	294.9±69.4	280.4±74.3
LDL-C, [mg/dL], mean±SD	240.5±81.6	208.2±65.7	194.1±66.4
Pre-treatment LDL-C, [mg/dL], mean±SD	279.6±78.2	213.8±62.5	218.1±61.4

TG, [mg/dL], median [IQR]	83.3 [65.5-108.1]	117.5 [84.0-141.0]	113.0 [87.5-156.5]
HDL-C, [mg/dL], mean±SD	55.0±13.7	60.2±13.4	59.9±14.9
On LLT, N(%)	71(28.3%)	3(7.9%)	18(28.1%)
	DLCN score components		
Tendon xanthoma, N(%)	33(13.1%)	0(0.0%)	3(4.7%)
Arcus cornealis before 45 years, N(%)	16(6.4%)	0(0.0%)	0(0.0%)
Clinical history of premature CHD, N(%)	17(6.8%)	1(2.6%)	7(10.9%)
Clinical history of premature cerebral or peripheral vascular disease, N(%)	18(7.2%)	0(0.0%)	3(4.7%)

Table 3.2.1 B. SaintPet cohort

	Genetic results		
	Positive	Inconclusive	Negative
Male, N(%)	19(40.4%)	15(51.7%)	8(26.7%)
Age [years], mean±SD	37.8±18.3	37.3±14.4	49.0±17.5
Under 18, N(%)	10(21.3%)	3(10.3%)	1(3.3%)
Age when FH was diagnosed [years], mean±SD	36.0±17.4	36.7±14.3	47.4±17.6
BMI [kg/m^2], mean±SD	24.4±7.1	26.0±4.0	27.0±3.9

	Biochemical data		
Total cholesterol, [mg/dL], mean±SD	283.3±111.0	289.9±85.5	272.8±71.9
LDL-C, [mg/dL], mean±SD	216.1±107.8	216.1±78.8	190.0±70.2
Pre-treatment LDL-C, [mg/dL], mean±SD	327.8±97.7	318.5±105.2	292.0±50.7
TG, [mg/dL], median [IQR]	83.3 [65.5-108.1]	106.3 [74.4-146.1]	109.9 [76.2-151.0]
HDL-C, [mg/dL], mean±SD	48.8±11.5	52.1±14.4	55.4±14.5
On LLT, N(%)	20(42.6%)	12(41.4%)	12(40.0%)
	DLCN score components		
Tendon xanthoma, N(%)	6(12.8%)	4(13.8%)	1(3.3%)
Arcus cornealis before 45 years, N(%)	5(10.6%)	6(20.7%)	1(3.3%)
Clinical history of premature CHD, N(%)	9(19.1%)	3(10.3%)	3(10.0%)
Clinical history of premature cerebral or peripheral vascular disease, N(%)	4(8.5%)	0(0.0%)	0(0.0%)

LDL-C levels analysis based on the genetic data *

Under the current analysis based on the genetic data there was demonstrated a trend for higher level of pre-treatment levels of LDL-C within

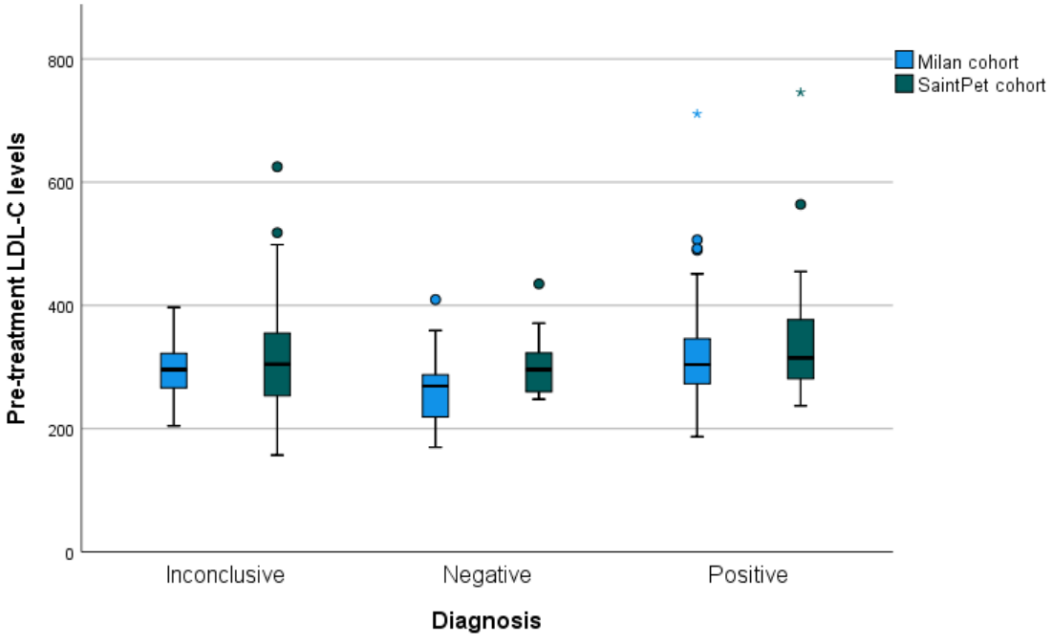
the SaintPet cohort in comparison with the Milan ones in all groups stratified by the genetic background (Table 3.2.2).

Table 3.2.2. LDL-C levels based on the genetic data in Milan and SaintPet cohorts

	Milan cohort, mg/dL	SaintPet cohort, mg/dL	p-value
LDL-C levels			
Positive	259.5±87.1	218.5±111.8	0.030
Inconclusive	283.3±76.1	215.8±80.2	0.050
Negative	233.6±68.5	191.9±73.8	0.035
Overall	258.8±77.2	208.7±88.6	<0.0001
Pre-treatment LDL-C levels			
Positive	**313.4**±68.8	**340.4**±95.5	0.091
Inconclusive	**296.2**±56.8	**321.8**±105.6	0.376
Negative	**265.4**±54.4	**299.6**±46.8	0.015
Overall	291.6±60.0	320.6±82.7	0.064

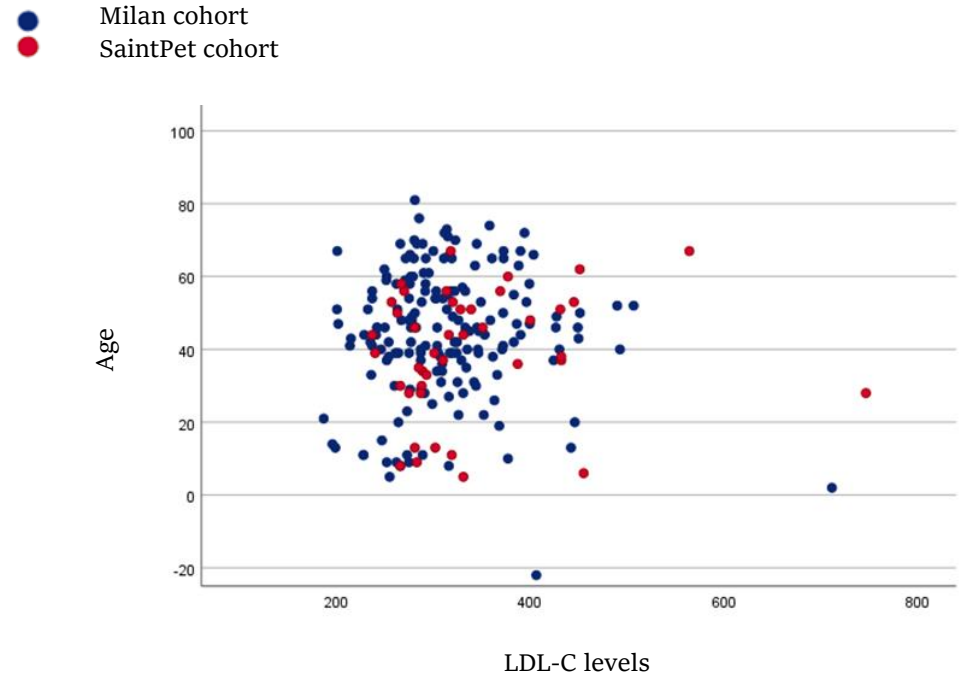
* - for those who reached 5 and more points according to DLCN score

Picture 3.2.1. LDL-C levels based on the genetic result (positive / inconclusive / negative) in Milan and SaintPet cohorts

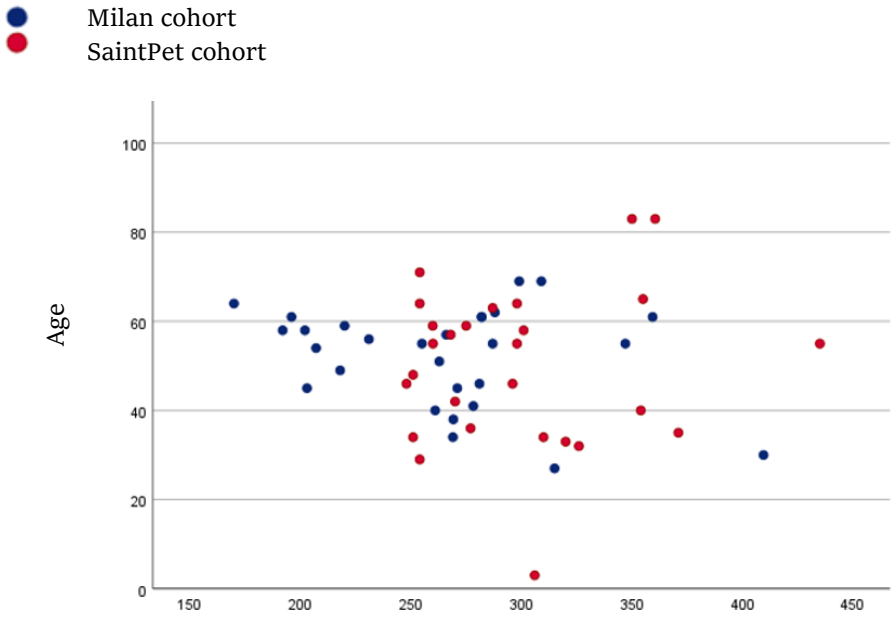


Picture 3.2.2. The dispensation of LDL-C levels based on the genetic data and age. Separate analysis for genetically positive FH (3.2.2 A) and negative (3.2.2. B) patients.

3.2.2 A. Sub-analysis for genetically positive patients in both cohorts.



3.2.2 A. Sub-analysis for genetically negative patients in both cohorts.



The analysis of the relevance of DLCN score points to the FH genetic background demonstrated the high percentage of positive results (in both cohorts) and inconclusive results (in SaintPet cohort) in patients who reached 3-5 points (Table 3.2.3).

Table 3.2.3. Relevance of DLCN score points to the genetic data results in both cohorts.

		Milan cohort, N (%)			SaintPet cohort, N (%)		
		Positive	Inconclusive	Negative	Positive	Inconclusive	Negative
DLCN score, N of points	3-5	**18(54.5%)**	1(3.0%)	9(27.3%)	**4(36.3%)**	**4(36.4%)**	3(27.3%)
	6-8	75(75.8%)	6(6.1%)	16(16.2%)	10(21.7%)	**16(34.8%)**	20(43.5%)
	>9	**80(89.9%)**	1(1.1%)	3(3.4%)	**14(35.0%)**	7(17.5%)	19(47.5%)

Description of the genetic causative FH mutations in both cohorts

The most frequent causative FH mutations in both cohorts was LDLR heterozygous form (Table 3.2.4).

Table 3.2.4. Description of genetically positive FH patients by causative mutation genes in both cohorts.

	Number of patients, N	
	Milan cohort	SaintPet cohort
LDLR hetero	160	36
APOB hetero	7	5
PCSK9 hetero	2	
double LDLR-APOB	2	
LDLR homo	1	
double LDLR-PCSK9	1	1

The comparative analysis of pre-treatment LDL-C levels in both cohorts stratified by the gene type mutation demonstrated the higher levels in SaintPet cohorts than in Milan ones within the same monogenic mutations (Table 3.2.5).

Table 3.2.5. Comparison on pre-treatment LDL-C levels based on the genetic causative mutated gene variants in both cohorts.

	Milan cohort		SaintPet cohort	
	Pre-treatment	Number of patients	Pre-treatment	Number of patients

	LDL-C, mg/dL		LDL-C, mg/dL	
LDLR homo	**324,0**	1	-	-
LDLR hetero	**282,0**	160	**335.6**	40
double LDLR-APOB	272.5	2	-	-
PCSK9 hetero	263,0	2	-	-
double LDLR-PCSK9	242,0	1	240,0	1
APOB hetero	248,4	7	**290,2**	6

The most frequent causative mutation in *LDLR* gene in both cohorts are presented in Tables 3.2.6 A and B. With the green colour a similar mutation for Milan and SaintPet cohort is marked.

Table 3.2.6 A. Frequent causative mutation in *LDLR* gene in Milan cohort.

Twenty nine patients with these mutations
c.662A>G p.Asp221Gly
Eighteen patients with these mutations
c.1646G>A p.Gly549Asp
Fourteen patients with these mutations
c.1775G>A p.Gly592Glu
Twelve patients with these mutations
c.682G>A p.Glu228Lys
Eight patients with these mutations
c.858C>A p.Ser286Arg
Seven patients with these mutations
c.941-12G>A
Six patients with these mutations

c.1567G>A p.Val523Met
c.1027G>A p.Gly343Ser
Five patients with these mutations
c.1735G>T p.Asp579Tyr
c.1246C>T p.Arg416Trp
c.1090T>C p.Cys364Arg
c.1567G>A p.Val523Met
Four patients with these mutations
c.1135T>C p.Cys379Arg
c.1846-?_2311+?del p.Asp616Leufs*17
c.418G>T p.Glu140*
Three patients with these mutations
c.304C>T p.Gln102*
c.1061-2A>G
c.1618G>A p.Ala540Thr
c.662A>G p.Asp221Gly
c.191-?_313+?del
c.530C>T p.Ser177Leu
c.2312-3C>A p.Ala771_796del
c.1207_1209del p.Phe403del
c.1576C>T p.Pro526Ser
c.1694G>T p.Gly565Val
Two patients with these mutations
c.1897C>T p.Arg633Cys
c.828C>G p.Cys276Trp
c.10580G>A p.Arg3527Gln
c.313+1G>A p.Leu64_Pro105delinsSer
c.1474G>A p.Asp492Asn
c.7615G>A p.Val2539Ile
c.1-?_67+?del
c.1463T>A p.Ile488Asn
c.1414G>T p.Asp472Tyr

c.1257C>G p.Tyr419*
c.1415_1418dupACAT p.Gln474Hisfs
c.2215C>T p.Gln739*
c.1846-?_2583+?del
One patient with these mutations
c.661G>A p.Asp221Asn
c.1415_1418dup p.Gln474Hisfs*63
c.1195G>A p.Ala399Thr
c.337G>T p.Glu113*
c.1739C>T p.Ser580Phe
c.539G>A p.Trp180*
c.7285T>A p.Ser2429Thr
c.(190+1_191-1)_(940+1_941-1)dup
c.2054C>T p.Pro685Leu
c.1846-1G>A
c.862G>T p.Glu288*
c.1846-?_2140+?del
c.898A>G p.Arg300Gly
c.1-?_2583+?del
c.352G>T p.Asp118Tyr
c.939C>A p.Cys313*
c.2096C>T p.Pro699Leu
c.1187-10G>A
c.420G>C p.Glu140Asp
c.818-2A>G p.Val273Glufs*31
c.907C>T p.Arg303Trp
c.1171delG p.Ala391Profs*22
c.1187-?_1586+?del
c.888C>G p.Asp296Glu
c.1778delG p.Gly593Alafs*72
c.68-?_313+?del
c.884delT p.Val295Alafs*75

c.401G>A p.Cys134Tyr
c.1478_1479del p.Ser493Cysfs*42
c.1056C>G p.Cys352Trp
c.825_826delCT p.Cys276Argfs*24
c.418G>T p.Glu140*
c.2000G>A p.Cys667Tyr
c.1951G>A p.Asp651Asn
c.1118G>A p.Gly373Asp
c.1618G>A p.Ala540Thr
c.1846-?_2311+?del
c.1491C>T p.Gly497=
c.2311+1G>A

Table 3.2.6 B. Frequent causative mutation in *LDLR* gene in SaintPet cohort.

DNA change for all 40 patients with causative mutation in LDLR gene
Two patients with these mutations
c.761A>C p.Gln254Pro
c.2416dup p.Val806Glyfs*11
c.361T>G p.Cys121Gly
c.(940+1_941-1)_(1845+1_1846-1)del
c.1202T>A p.Leu401His
c.(?_-187)_(190+1_191-1)del
c.355_356insTTCC p.(Gly119Valfs*12)
c.1775G>A p.(Gly592Glu)
c.654_656del p.(Gly219del)
c.1246C>T p.(Arg416Trp)
c.894G>A p.(Gln298=)
One patient with these mutations
c.661G>A p.Asp221Asn
c.1328G>A p.Trp443*

c.1202T>A p.Leu401His
c.2001_2002del p.Cys667*
c.1801G>T p.Asp601Tyr
c.(313+1_314-1)_(940+1_941-1)del .
c.(?_314)_(940_?)del .
c.361T>G p.(Cys121Gly)
c.301G>A p.(Glu101Lys)
c.1979dup p.(Gln660_659dup)
c.320_332del N2
c.2389G>A p.(Val797Met)
c.666C>A p.(Cys222*)
c.534T>G p.(Asp178Glu)
c.2416dup p.(Val806Glyfs*11)
c.2389G>A p.(Val797Met)
c.1750T>C p.(Ser584Pro)
c.1491C>T p.(Gly497=)

Phenotype (A) and genotype (B) characteristics of patients with the highest pre-treatment LDL-C levels and LDLR heterozygous causative mutations in Milan and SaintPet cohort are presented in tables 3.2.7 and 3.2.8.

Table 3.2.7 Phenotype (A) and genotype (B) characteristics of patients with the highest pre-treatment LDL-C levels and LDLR heterozygous causative mutations, Milan cohort

A. Phenotype characteristics

	Sex	Age, yrs	Age when FH was done, yrs	Pre-treatment LDL-C, mg/dL	TX	AC before 45	Premature CHD	Family history
N 16110	F	2	1	711	+	-	-	+

N 638	F	52	10	507	+	+	-	+
N 632	M	52	45	490	+	-	+	+
N 607	M	50	18	451	-	+	-	+
N 8312	M	43	29	450	-	-	+	+
N 634	F	46	18	449	-	-	-	+
N 570	M	20	3	446	-	-	-	+

TX – tendon xanthomas
AC – arcus cornealis

B. Genotype characteristics

	Sex	Pre-treatment LDL-C, mg/dL	Causative mutation in *LDLR* gene
N 16110	F	711	c.1775G>A p.Gly592Glu
N 638	F	507	c.941-12G>A
N 632	M	490	c.1171delG p.Ala391Profs*22
N 607	M	451	c.1646G>A p.Gly549Asp
N 8312	M	450	c.662A>G p.Asp221Gly
N 634	F	449	c.2312-3C>A p.Ala771_796del
N 570	M	446	c.941-12G>A

Table 3.2.8 Phenotype (A) and genotype (B) characteristics of patients with the highest pre-treatment LDL-C levels and LDLR heterozygous causative mutations, SaintPet cohort

A. Phenotype characteristics

	Sex	Age, yrs	Age when FH was done, yrs	Pre-treatment LDL-C, mg/dL	IMT, mm	TX	AC before 45	Premature CHD	Family history
N 077	F	28	19	746	1.10	+	+	-	-
N 031	F	67	39	564	0.92	+	-	-	+
N 072	F	6	4	455	0.33	-	-	-	+
N 001	F	62	54	451	0.87	-	+	+	+
N 058	F	53	53	445	1.02	-	-	-	+
N 022	M	38	37	432	0.71	-	-	-	+
N 036	F	48	48	400	0.70	-	-	-	-

TX – tendon xanthomas
AC – arcus cornealis

B. Genotype characteristics

	Sex	Pre-treatment LDL-C, mg/dL	Causative mutation in *LDLR* gene
N 077	F	746	c.2416dup p.(Val806Glyfs*11)
N 031	F	564	c.(?_-187)_(190+1_191-1)del .
N 072	F	455	c.654_656del p.(Gly219del)
N 001	F	451	c.1328G>A p.Trp443*
N 058	F	445	c.301G>A p.(Glu101Lys)
N 022	M	432	c.2416dup p.Val806Glyfs*11
N 036	F	400	c.(?_-187)_(190+1_191-1)del .

Key messages:

1. A trend for higher level of pre-treatment levels of LDL-C within the SaintPet cohort in comparison with the Milan ones in all groups stratified by the genetic background is identified.
2. The most frequent causative mutations in *LDLR* gene in both cohorts are identified.
3. The analysis of the relevance of DLCN score points to the FH genetic background demonstrates relatively high percentage of positive results (in both cohorts) and inconclusive results (in SaintPet cohort) in patients who reached 3-5 points (low probability of FH diagnosis based on DLCN score), that indicated gaps in knowledges that have to be considered for the clinical scores optimization.

II. *New approaches to improve FH identification and cardiovascular risk stratification*

3.3. Achilles tendons sub-study

The ACTUS-FH "AChilles Tendon UltraSonography in Familial Hypercholesterolemia" sub-study was performed by 4 LIPIGEN centres in Italy and the centre in Russia aiming to evaluate whether Achilles tendon xanthomata measured by ultrasonography may improve the identification of Familial Hypercholesterolemia patients as the physical examination of Achilles tendon xanthomata that is included in the current DLCN score presents poor sensitivity creating bias as it strongly depends on physician judgement.

3.3.1. Structure of the study and methods

A total of 769 adults with clinical diagnosis of FH according to DLCN score were enrolled to the current sub-study. Genetic test was performed to all the patients in order to identify the presence of monogenic FH background. The data collection included:

- *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 data:* 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.

- *Statistical methods:* 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 for those. 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.

3.3.2. **Results**

A total of 769 adults with clinical diagnosis of FH according to DLCN score were enrolled to the current sub-study. Clinical features of patients with clinically detected xanthoma, ultrasonography xanthoma or absence are presented in the table 3.3.1. 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 3.3.1). An increasing gradient in the other covariates (BMI, glucose and diabetes prevalence) was detected from No-ATX to CA-ATX subjects.

Table 3.3.1. Comparison of demographic and biochemical parameters among subjects with clinically detected xanthoma (CA-ATX), ultrasonography xanthoma (US-ATX) or absence (No-ATX).

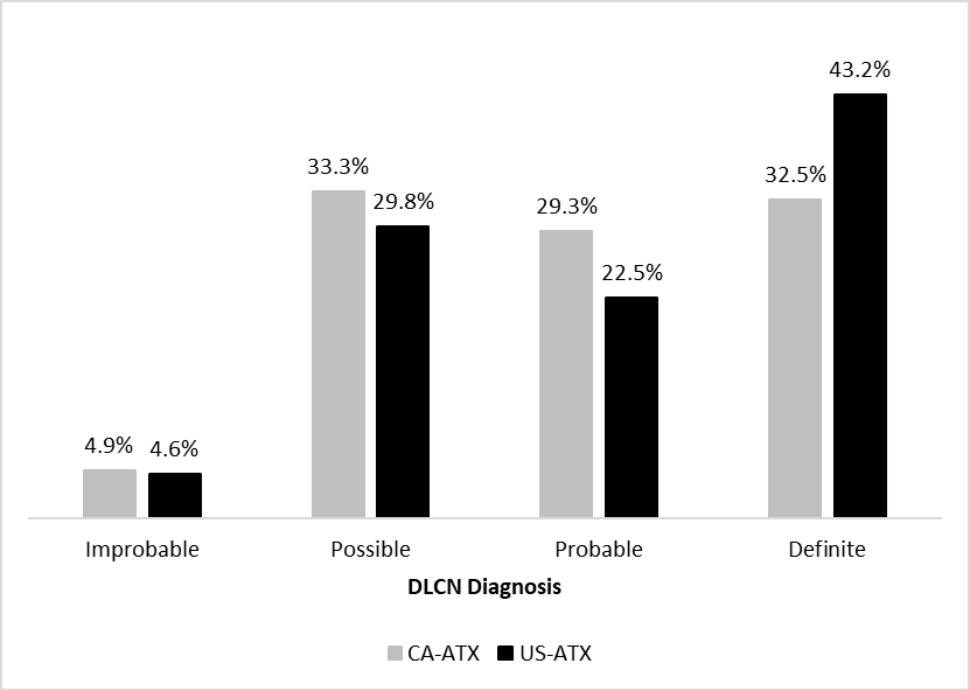
	CA-ATX		**US-ATX**	**No-ATX**	***p value***			
	N 75		**N 180**	**N 514**	***overall***	**CA-ATX vs US-ATX**	**US-ATX vs No-ATX**	**CA-ATX vs No-ATX**
Age [years], mean±SD	53.5±13.2		50.2±12.7	45.5±15.3	*<.0001*	*0.06*	*<.0001*	*<.0001*
Men, N (%)	32 (42.7)		83 (46.1)	266 (51.8)	*0.20*	*0.61*	*0.19*	*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*
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)	0 (0)		3 (1.7)	35 (6.8)	*<.0001*	*<.0001*	*<.0001*	*<.0001*
Possible (3-5)	0 (0)		27 (15.0)	229 (44.5)				
Probable (6-8)	0 (0)		56 (31.1)	169 (32.9)				
Definite (>8)	75 (100)		94 (52.2)	81 (15.8)				
Achilles tendon thickness [mm], mean±SD	10.3±4.2		7.0±1.8	5.4±1.0	*<.0001*	*<.0001*	*<.0001*	*<.0001*

DLCN score was calculated based on the clinical characteristics, family history and the lipid profile. 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% (Table 3.3.2).

Table 3.3.2. Reclassification of DLCN categories using the US-ATX instead of CA-ATX.



Strafing 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 3.3.3).

Table 3.3.3. Classification by (A) CA-ATX and (B) US-ATX among FH/M- and FH/M+ subjects.

(A)

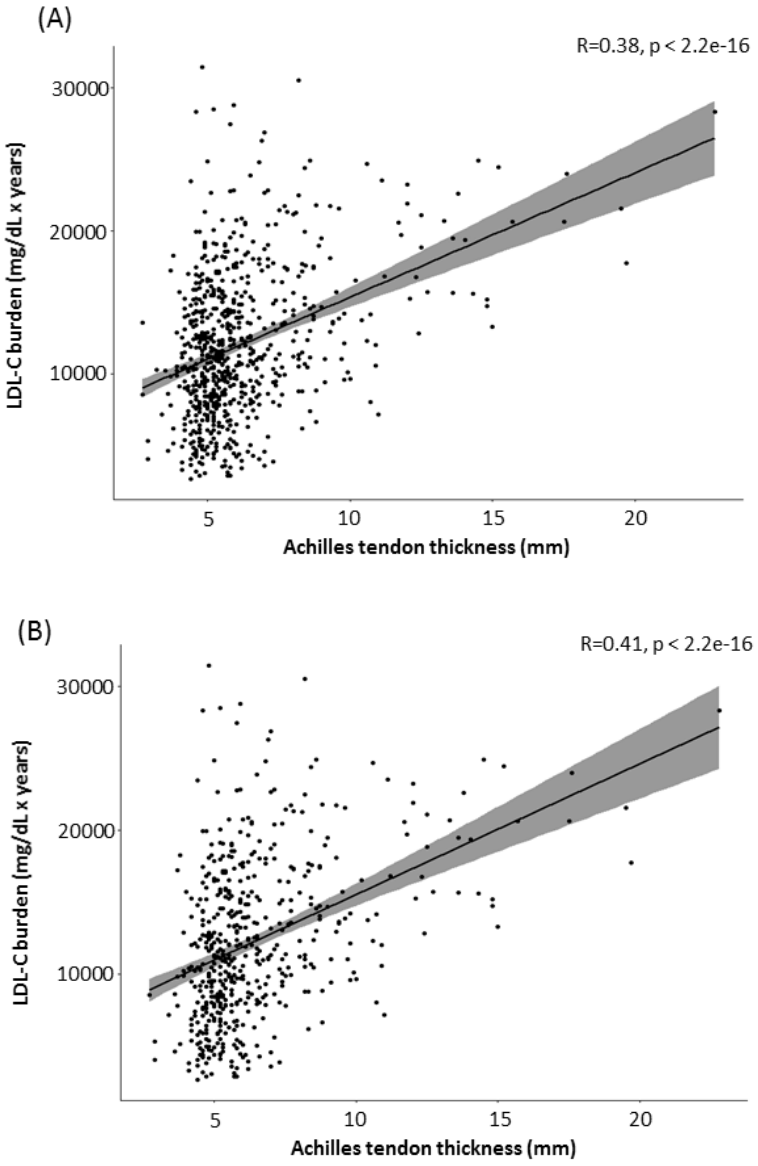
	FH/M-	FH/M+	Total
No	188	506	694
	27.1%	72.9%	
	98.4%	87.5%	
Yes	3	72	75
	4.0%	96.0%	
	1.6%	12.5%	
Total	191	578	769

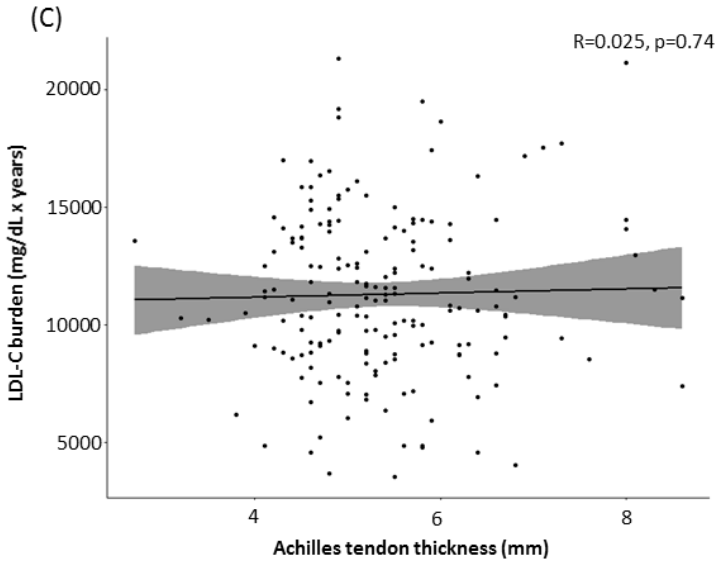
(B)

	FH/M-	FH/M+	Total
No	178	336	514
	34.6%	65.4%	
	93.2%	58.1%	
Yes	13	242	255
	5.1%	94.9%	
	6.8%	41.9%	
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 3.3.1).

Figure 3.3.1. 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).

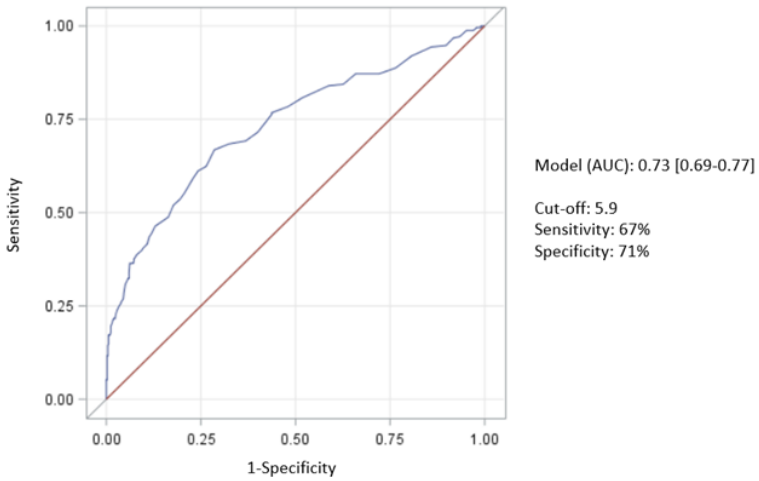

(A)
R=0.38, p < 2.2e-16
LDL-C burden (mg/dL x years)
30000
20000
10000
5
10
15
20
Achilles tendon thickness (mm)
(B)
R=0.41, p < 2.2e-16
LDL-C burden (mg/dL x years)
30000
20000
10000
5
10
15
20
Achilles tendon thickness (mm)



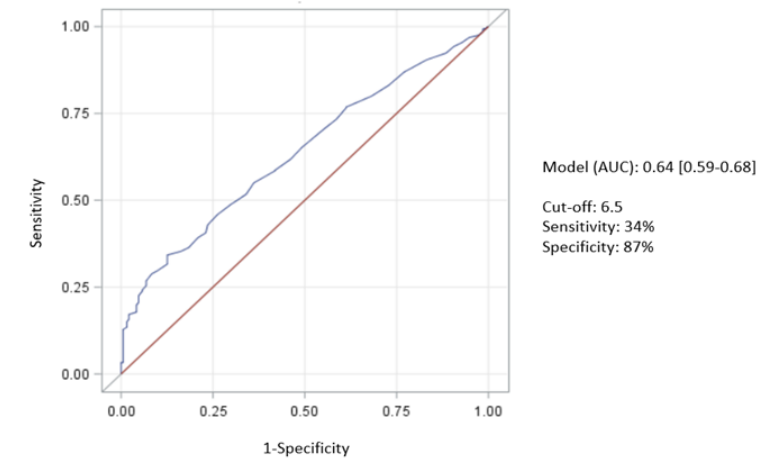
An increasing trend of maximum Achilles tendon thickness was also found taking into account the DLCN score classes: from 5.43 mm in the class with DLCN score < 6, to 5.76 mm in the class with 6-8 points and 7.60 mm in the class with DLCN score ≥9. Finally, the ROC analysis was performed to evaluate the 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 3.3.2 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 3.3.2 B).

Figure 3.3.2. 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.

(A)



(B)



Key messages:

1. In 769 subjects with clinical diagnosis of FH, ultrasonography improved xanthomata detection to 33.2% vs. 9.8% at physical examination, and the proportion of definite FH according to DLCN increased from 32.5% to 43.2%.
2. A higher proportion of subjects with xanthomata detected only by ultrasound carried FH-associated mutations (94.4%) versus subjects without Achilles tendon xanthomata (65.4%, p<.0001).
4. Ultrasonography identifies tendon xanthomata that cannot be detected

by physical examination, improving the identification of both DLCN-based definite FH and genetically-confirmed FH.

3.4. Proteomic analysis sub-study

3.4.1. Structure of the study and methods

For the purposes of this study, we included in the analysis 173 statine – naive FH patients from Milan and SaintPet cohort described in details in Chapter 1.

As a control group 586 subjects in primary prevention for CVD from the population-based study representative of the general population of the northern area of Milan ("PLIC", "Progressione delle Lesioni Intimali Carotidee"), which has been extensively described elsewhere [*Baragetti 2021, Baragetti 2016*], were sorted out. Subjects were screened at the Center for the Study of Atherosclerosis at E. Bassini Hospital (Cinisello Balsamo, Milan, Italy) for personal and familial clinical history, without previous CVD (either ischemic heart disease, ST elevation or non-ST elevation myocardial infarction, aortic-coronary by-pass grafting, angioplasty, transient ischemic attack, stroke, heart failure from Class II to IV according to New York Heart Association (NYHA) definition or documented peripheral arteriopathy. Subjects with Chronic Kidney Disease (Glomerular Filtration Rate, GFR < 60 ml/min or documented albuminuria > 30 mg/g), with reported malignant pregnancies as well as pregnant women were excluded from the study.

Data management and statistical analyses were performed with the coordination of the Epidemiology and Preventive Pharmacology Centre

(SEFAP) of the University of Milan. The study was approved by the Scientific Committee of the University of Milan (SEFAP/Pr.0003).

Biochemical parameters

Data on personal and familial clinical, pathological and pharmacological history for each subject was collected. Information about lifestyle habits (physical activity, alcohol consumption and smoking habit) was collected by validated questionnaire used in the PLIC as described elsewhere [*Baragetti 2021*]. Systolic and diastolic blood pressure and Body Mass Index (BMI), waist and waist/hip ratio were measured. Blood samples were collected from antecubital vein after 12 hours fasting on NaEDTA tubes (BD Vacuette) and, then, centrifuged at 3,000 rpm for 12 minutes (Eppendorf 580r, Eppendorf, Hamburg, Germany) for biochemical parameters profiling including: total cholesterol, HDL-C, triglycerides, ApoB, ApoA-I, glucose, liver enzymes, creatinine and creatinine-phospho kinase (CPK). Measurements were performed using immuno-turbidimetric and enzymatic methods thorough automatic analyzers (Randox, Crumlin, UK). LDL-C was derived from Friedewald formula.

Carotid Ultrasound Analysis

Carotid ultrasound determinations were performed by a single expert sonographer, blinded on subject's identity and clinical history, using a with a linear ultrasound probe (4.0e13.0 MHz frequency, 14X48 mm footprint, 38 mm field of view, Vivid S5 GE Healthcare®, Wauwatosa, WI, USA). A detailed description of the ultrasound methodology, based on validated protocol for the PLIC study, was published before [*Hoogeveen 2020*] and has been validated by comparison with that of studies with which PLIC participated in large epidemiological consortia [*Lorenz 2012*]. Briefly, IMT was determined using in the lateral projection at five standardized points (5, 10, 20, 25 and 30 mm from

the bulb dilatation) in both arteries and averaged to calculate the mean IMT for each subject. In two scans performed by the same operator in 75 subjects, the mean difference in IMT was 0.005±0.002 mm and the coefficient of variation (CV) was 1.93%. The correlation between two scans was significant (r = 0.96; P < 0.0001). Faster common carotid IMT progression was considered for IMT above the 75th percentile of the median IMT for a Caucasian population according to ASE guidelines [*Stein 2008*]. Development of Subclinical Carotid Atherosclerosis (SCA) was defined for presence of focal intimal thickening (caliper >1.3 mm at a single point, in longitudinal resolution, lateral or medial angle).

Proteomics analysis

Proteins were measured by Proximity Extension Assay (PEA) strategy as previously described [*Hoogeveen 2020*], from the CV II, CV III, Cardiometabolic, and Inflammation panels of the Olink™ platform. The complete list of the proteins that are included in the panels provided by the Manufacturer have been previously indicated [*Hoogeveen 2020*]. Proteins were measured in 200 uL of plasma samples by Proximity Extension Assay (PEA). Plasma samples were separated by whole blood after centrifugation and aliquoted in 96-wells plates at -80 degrees, the final assay read-out is given in Normalized Protein eXpression (NPX) [*Enroth 2018*], which is an arbitrary unit on log2-scale where a high value corresponds to a higher protein expression. Each PEA measurement has a lower detection limit (LOD) calculated based on negative controls that are included in each run, and measurements below LOD were removed from further analysis. Using an internal extension control and an interpolate control, data quality is controlled and normalized. All assay characteristics including detection limits and measurements of assay performance and validations are available from the manufacturers webpage (http://www.olink.com).

3.4.2. Results

Basic characteristics, including biochemical parameters, of genetically positive (FH+) and negative (FH-) patients are presented in the Table 3.4.1. A reasonable difference in TC, LDL-C and ApoB levels between genetically positive FH in comparison with genetically negative ones and controls should be noticed.

Table 3.4.1. Basic characteristics analysis of clinically suspicious FH (divided by genetic background) and controls

	Patients cohorts				p-value			
	Control group	FH with clinical diagnosis (both FH+ and FH-)	FH with negative genetic test (FH-)	FH with positive genetic test (FH+)	controls/ FH all	controls / FH-	controls / FH+	FH/ FH+
Age [years], median [IQR]	55 (50-61)	32 (14-46)	39 (29; 54)	30 (13-45)	p<0,001	p<0,001	p<0,001	p=0,001
Female, N(%)	416 (66%)	83 (48%)	21 (52%)	62 (46%)	p<0,001	p=0,079	p<0,001	p=0,514
BMI [kg/m2], median [IQR]	26 (23-28)	24 (21-27)	24 (5)	24 (5)	p<0,001	p=0,015	p<0,001	p=0,464
Systolic blood pressure [mmHg], median [IQR]	130 (120-140)	115 (100-125)	122 (100-130)	110 (100-120)	p<0,001	p<0,001	p<0,001	p=0,384
Diastolic blood pressure [mmHg], median [IQR]	80 (80-90)	70 (65-80)	70 (70-80)	70 (60-80)	p<0,001	p<0,001	p<0,001	p=0,674
Glucose [mg/dL], median [IQR]	88 (82-95)	89 (84-95)	89 (86-93)	89 (82-96)	p=0,200	p=0,896	p=0,462	p=0,752
Total cholesterol, [mg/dL], median [IQR]	220 (196-246)	307 (269-356)	279 (53)	324 (73)	p<0,001	p<0,001	p<0,001	p<0,001
LDL-C, [mg/dL], median [IQR]	142 (119-166)	234 (196-273)	197 (50)	245 (62)	p<0,001	p<0,001	p<0,001	p<0,001
Pre-treatment LDL-C, [mg/dL], median [IQR]	NA*	252 (210-298)	238 (187-264)	261 (215-307)	NA*	NA*	NA*	p=0,003
TG, [mg/dL], median [IQR]	88 (63-127)	95 (69-139)	115 (81-143)	92 (67-136)	p=0,040	p=0,365	p=0,081	p=0,948
HDL-C, [mg/dL], median [IQR]	54 (45-66)	51 (45-61)	56 (47-66)	50 (44-60)	p=0,095	p=0,726	p=0,052	p=0,193
Non-HDL-C [mg/dL], median	161 (137-190)	250 (209-295)	223 (54)	271 (70)	p<0,001	p<0,001	p<0,001	p<0,001

[IQR]								
ApoB [mg/dL], median [IQR]	110 (93-127)	181 (150-226)	167 (35)	196 (62)	p<0,001	p=0,001	p<0,001	p=0,094
ApoA1 [mg/dL], median [IQR]	149 (27)	149 (18)	171 (24)	147 (17)	p=0,907	p=0,168	p=0,629	p=0,143
Xanthomas, N (%)	NA*	16 (10%)	2 (5%)	14 (11%)	NA*	NA*	NA*	p=0,149
Previous CV events, N (%)	0	9 (5%)	1 (2%)	8 (6%)	NA*	NA*	NA*	p=0,353
"Unlikely FH" (DLCNS), N(%)	NA*	10 (9%)	7 (21%)	3 (3%)	NA*	NA*	NA*	p=0,009
Possible FH (DLCN), N(%)	NA*	24 (22%)	8 (25%)	16 (20%)	NA*	NA*	NA*	p=0,009
"Probable FH" (DLCNS), N(%)	NA*	41 (37%)	12 (37%)	29 (37%)	NA*	NA*	NA*	p=0,009
"Definite FH" (DCLNS), N(%)	NA*	34 (31%)	5 (15%)	29 (37%)	NA*	NA*	NA*	p=0,009
Statin treatment, N(%)	0	14 (8%)	2 (5%)	12 (9%)	NA*	NA*	NA*	p=0,413
Anti-hypertensives, N(%)	151 (23%)	19 (15%)	6 (25%)	13 (13%)	p=0,039	p=0,908	p=0,019	p=0,149
Anti-platelets, N(%)	9 (1%)	12 (18%)	4 (20%)	8 (18%)	p<0,001	p<0,001	p<0,001	p=0,863

NA* - not applicable

Analysis of basic characteristics of Milan and SaintPet cohorts demonstrate its comparability (Table 3.4.2). The only significant difference (p<0,001) is related to the higher pre-treatment LDL-C levels in SaintPet cohort that is relevant to the general FH analysis (Part 3.1. of the current study).

Table 3.4.2. Basic characteristics analysis divided by genetic background of Milan and SaintPet individuals

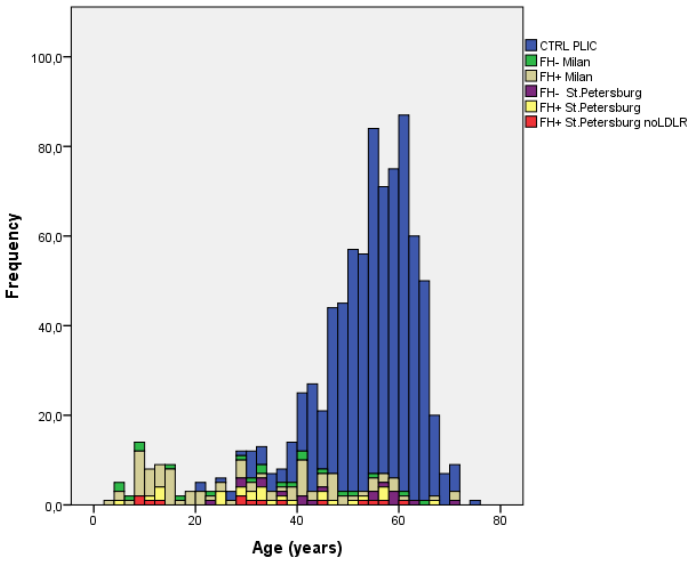
		Patients cohorts				p-value	
	Control group	**FH with negative genetic test (FH-) (Milan)**	**FH with positive genetic test (FH+) (Milan)**	**FH with negative genetic test (FH-) (St.Petersburg)**	**FH with negative genetic test (FH+) (St.Petersburg)**	**FH-Milan/FH-St.Petersburg**	**FH+Milan/FH+St.Petersburg**
Age [years], median [IQR]	55 (50-61)	32 (12-45)	29 (13-45)	43 (32-58)	32 (24-48)	p=0,011	p=0,277
Female, N(%)	416 (66%)	8 (36%)	40 (43%)	13 (72%)	22 (53%)	p=0,024	p=0,277
BMI [kg/m2], median [IQR]	26 (23-28)	22 (5)	23 (4)	26 (3)	24 (5)	p=0,017	p=0,281
Systolic blood pressure [mmHg],	130 (120-140)	97 (91-100)	110 (100-120)	127 (110-131)	120 (110-130)	p=0,005	p=0,007

median [IQR]							
Diastolic blood pressure [mmHg], median [IQR]	80 (80-90)	62 (48-68)	70 (60-75)	76 (70-80)	75 (65-80)	p=0,007	p=0,084
Glucose [mg/dL], median [IQR]	88 (82-95)	87 (84-92)	90 (82-96)	90 (86-98)	89 (83-96)	p=0,294	p=0,971
Total cholesterol, [mg/dL], median [IQR]	220 (196-246)	276 (40)	327 (73)	282 (67)	317 (73)	p=0,782	p=0,467
LDL-C, [mg/dL], median [IQR]	142 (119-166)	201 (150-234)	237 (205-277)	189 (158-246)	246 (213-295)	p=0,744	p=0,900
Pre-treatment LDL-C, [mg/dL], median [IQR]	/	200 (150-234)	242 (205-283)	264 (250-298)	288 (256-334)	p<0,001	p<0,001
TG, [mg/dL], median [IQR]	88 (63-127)	117 (81-134)	93 (68-140)	107 (79-151)	86 (67-113)	p=0,937	p=0,348
HDL-C, [mg/dL], median [IQR]	54 (45-66)	57 (49-68)	53 (45-61)	55 (43-59)	48 (43-55)	p=0,321	p=0,061
Non-HDL-C [mg/dL], median [IQR]	161 (137-190)	219 (44)	271 (70)	227 (66)	/	p=0,710	NA*
Xanthomas, N (%)	/	1 (4%)	9 (10%)	1 (5%)	5 (11%)	p=0,911	p=0,823
Previous CV events, N (%)	0	1 (4%)	3 (3%)	0	5 (12%)	p=0,360	p=0,059
"Unlikely FH" (DLCNS), N(%)	/	6 (40%)	3 (6%)	1 (5%)	0	p<0,001	p=0,033
Possible FH (DLCN), N(%)	/	8 (53%)	14 (29%)	0	2 (6%)		
"Probable FH" (DLCNS), N(%)	/	0	15 (31%)	12 (70%)	14 (46%)		
"Definite FH" (DCLNS), N(%)	/	1 (6%)	15 (31%)	4 (23%)	14 (46%)		
Statin treatment, N(%)	0	1 (4%)	8 (8%)	1 (5%)	4 (9%)	p=0,884	p=0,844
Anti-hypertensives, N(%)	151 (23%)	1 (16%)	3 (5%)	5 (27%)	10 (24%)	p=0,586	p=0,005
Anti-platelets, N(%)	9 (1%)	1 (50%)	2 (66%)	3 (16%)	6 (14%)	p=0,264	p=0,024

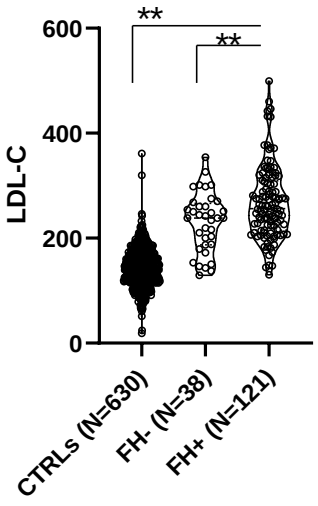
The distribution of the study cohorts according to age is presented within Figure 3.4.1 A. The distribution of the study cohorts according to LDL-C levels predictably demonstrated higher LDL-C levels in genetically positive statin-naive FH patients in comparison with negative ones (Figure 3.4.1 B), in genetically positive statin-naive SaintPet FH patients in comparison with Milan positive ones (Figure 3.4.1 C) and all genetically positive SaintPet FH patients in comparison with Milan positive ones (Figure 3.4.1 D).

Figure 3.4.1. The distribution of the study cohorts according to age (A) and LDL-C levels (B, C, D).

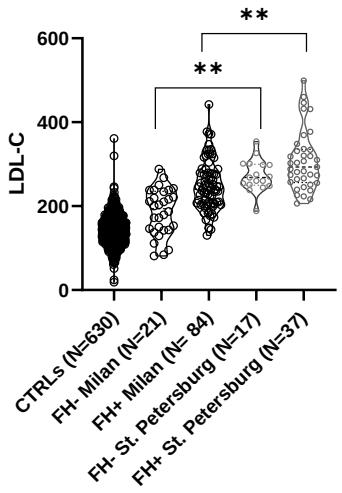
(A) The distribution of the study cohorts according to age.



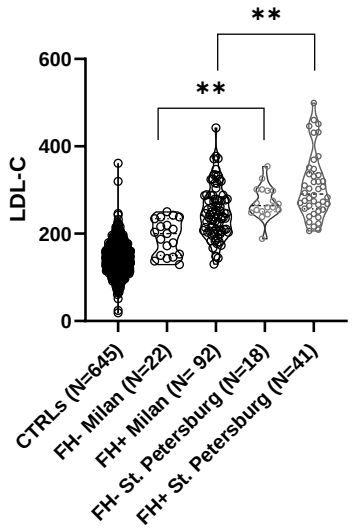
(B) The distribution of the study cohorts according to LDL-C levels for all statin-naive patients.



(C) The distribution of the study cohorts according to LDL-C levels for all statin-naive patients: sub-analysis for Milan and SaintPet cohorts.

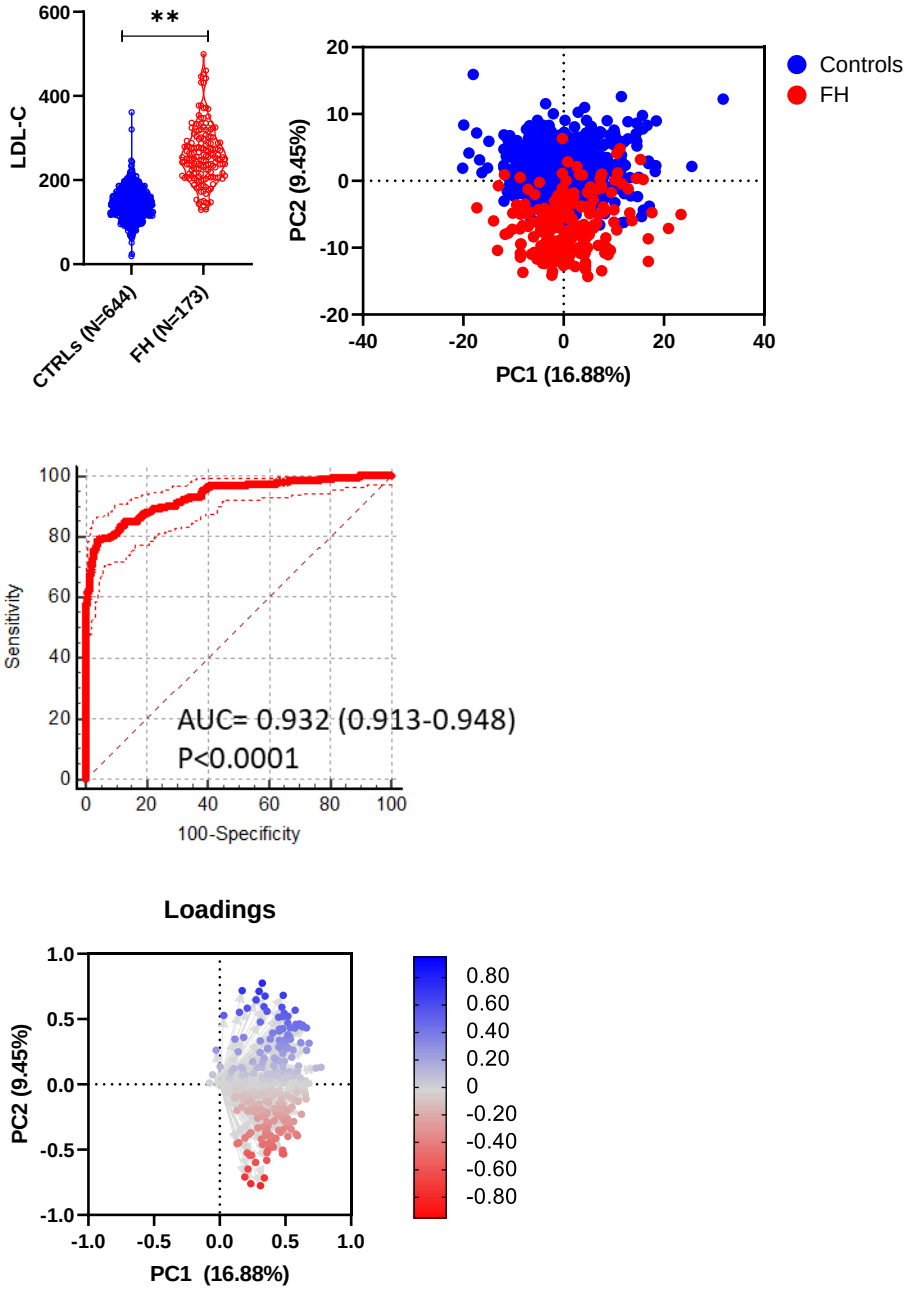


(D) The distribution of the study cohorts according to LDL-C levels for all patients (independently on the therapy): sub-analysis for Milan and SaintPet cohorts.



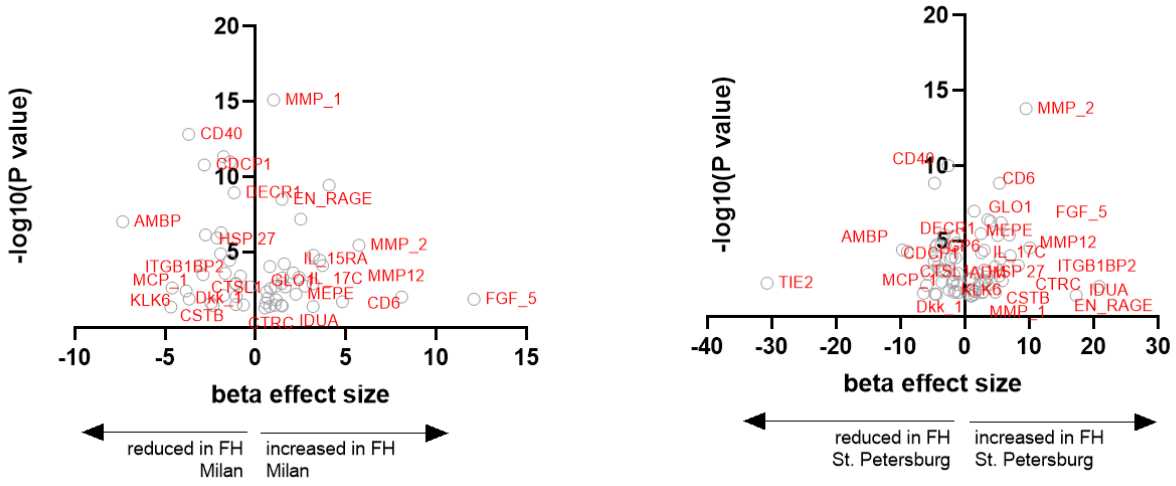
A significant difference was observed since whole proteomics (n=264 proteins listed in Table 2 of the Supplementary materials) robustly discriminate clinically defined FH vs controls in addition to LDL-C-levels (Figure 3.4.2). Area under the receiver operating characteristic curve, estimating the predictive accuracy of a proteomic set with FH clinically defined FH individuals.

Figure 3.4.2. Association of plasma proteomics with CV risk in clinically defined FH individuals (both Milan and SaintPet cohorts).



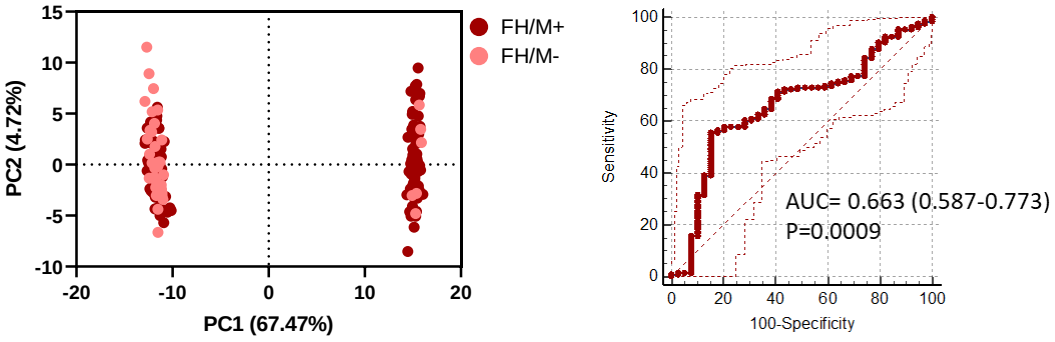
Within the analysis of the variations in the single proteins in Milan and SaintPet cohorts a few proteins are mutually found, despite important inter-center variation (Figure 3.4.3).

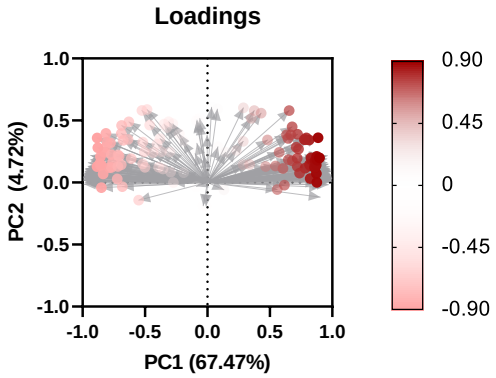
Figure 3.4.3. The analysis of proteins similar for the Milan and SaintPet cohorts in clinically defined FH individuals.



A significant difference was observed since whole proteomics robustly discriminate also genetically confirmed FH vs controls in addition to LDL-C-levels (Figure 3.4.4).

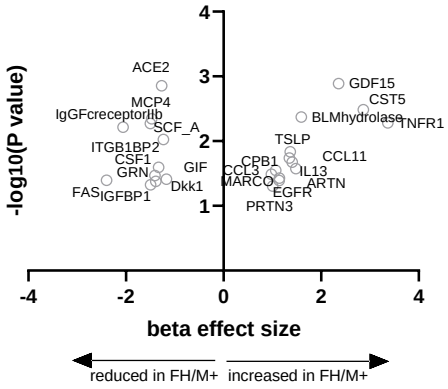
Figure 3.4.4. Association of plasma proteomics with cardiovascular risk in genetically confirmed FH individuals (both Milan and SaintPet cohorts).





The beta effect size demonstrating how much the proteins associates with the phenotype/genotype per each standard deviation changes in its absolute value is plotted within Figure 3.4.5.

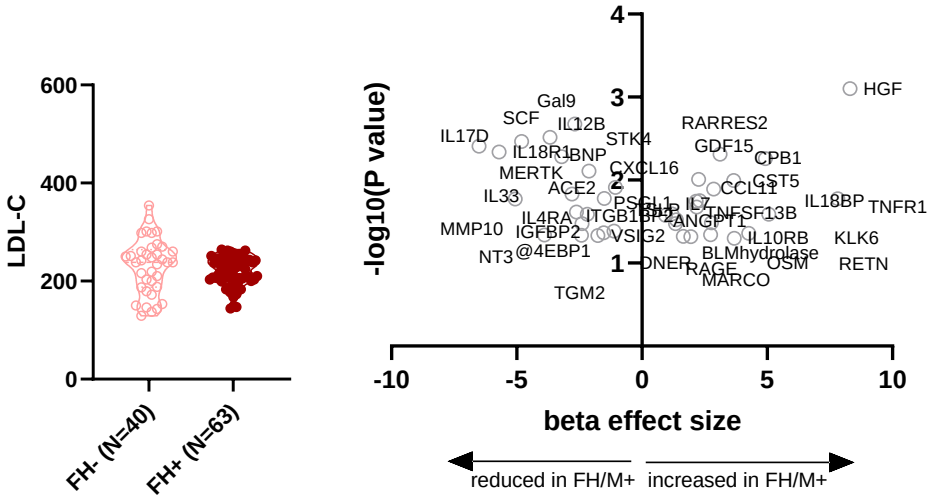
Figure 3.4.5. Association of the proteins with the phenotype/genotype per each standard deviation changes.



A further analysis pairing by LDL-C has been performed. When comparing genetically confirmed FH vs genetically negative FH individuals by LDL-C levels, further proteins have been significantly found (Figure 3.4.6). Even when paired for LDL-C, genetically positive and negative FH patients still displayed a significant amount of immune-inflammatory proteins diverse. The list of significant proteins (sorted by P value) is attached to the “Supplementary materials” tables.

Figure 3.4.6 Comparison of the protein profile in genetically confirmed FH vs

genetically negative FH individuals by LDL-C levels.



Key messages:

1. We identified that a set of immune-inflammatory proteins associated with increased CVD risk, significantly characterize the clinically determined FH phenotype.
2. Crossing the clinical phenotype with genetic analysis, allowed to identify FH/M+ subjects that, in addition to a higher LDL-C burden, are also characterized by a peculiar set of immune-inflammatory proteins as compared to FH/M-.
3. By pairing FH/M+ and FH/M- for LDL-C we still appreciated a number of proteins significantly different, thus suggesting that the prognostic value of these proteins should be longitudinally addressed.

Chapter IV

DISCUSSION

Since 2015, the European Atherosclerosis Society project, the EAS FH Studies Collaboration [*Vallejo-Vaz 2015*], has been successfully in operation, aiming consolidation of the efforts to identify and to treat patients with familial FH that belongs to high or very high CV risk. The maintenance of a register allowed to draw attention to this problem and significantly increase the percentage of FH detection. The six-year results of the project provided the registry with more than 62,000 patients from 62 countries, among which 41,000 patients are participants with a definite or probable diagnosis according to clinical and/or genetic FH criteria.

Along with this initiative a range of national registries, such as the SpAnish Familial HypErcHolEsterolaemiA CohoRt Study (SAFEHEART) in Spain [*Mata 2011, de Isla 2017*], Canadian FH registry in the British Columbia of Canada [*Brunham 2018*] and the CAscade SCreening for Awareness and DEtection of Familial Hypercholesterolemia registry (CASCADE FH) in the United States [*Duell 2019*], demonstrated its extremely important role in FH identification and increase of management effectiveness.

Thus, first national Italian and Russian Genetic Networks brought a big impact to the better understanding of a state of the disease, especially in the area of molecular diagnosis. The reason of first focus among all genetically determined dyslipidemias on FH, was based of the fact that it is the most common form of genetic lipid disorders, i.e. has the biggest impact on cardiovascular morbidity and mortality on the national level.

If to discuss the pheno- and genotype characteristics of the Italian and Russian FH cohort next points might be mentioned. Basic clinical and biochemical data of both cohorts looked relevant to each other. The higher pre-treatment LDL-C levels in SaintPet cohort probably determined the higher frequency of premature CHD and arcus cornealis before 45. The very low frequency of young patients under the lipid-lowering treatment at the moment of enrolment to the registry despite the high levels of LDL-C in both cohorts, the percentage of patients within the secondary prevention area (31.1% and 23.7% in Milan and SaintPet cohorts respectively) demonstrated a need to enhance efforts in FH identification and management especially in primary health care system. The late age when FH was diagnosed first time in SaintPet cohort in comparison with the Milan ones probably may reveal organizational difficulties in the lipid disorders identification system.

A trend for higher level of pre-treatment levels of LDL-C within the SaintPet cohort in comparison with the Milan ones in all groups stratified by the genetic background was identified. The most frequent causative mutations in *LDLR* gene in both cohorts were identified with just one similar mutation within both Italian and Russian cohorts. The analysis of the relevance of DLCN score points to the FH genetic background demonstrated relatively high percentage of positive results (in both cohorts) and inconclusive results (in SaintPet cohort) in patients who reached 3-5 points (low probability of FH diagnosis based on DLCN score), that indicated gaps in knowledges that might be useful for the clinical scores optimization.

National genetic network registries might potencially bring its impact in lipid-lowering therapy adherence and goals achievement, providing also innovative drugs classes and therapeutic strategies into real clinical practice. However, the impact of modifying and setting an efficient high-intensity therapy might be evaluated only in future when the follow up data will be available. The compilation of follow up information will be crucial also to

obtain a real-world snapshot of the Italian and Russian cohorts in terms of achievement of LDL-C goals, type of prescribed therapy and incidence of CV events and deaths, trying to fill the treatment gap in care.

Along with the descriptive analytics that might serve as an important tool to overcome organizational lowlights, the joint interpopulation registry helped to address issues related to the scienficil gaps in FH identification.

Nowadays, among the wide range of scales, such as The Simon Broome criteria, The US MEDPED Criteria and others, DLCN score is recommended in Europe as an optimal tool for a clinical FH diagnosis, however, several limitations might be considered that in some cases decrease its sensitivity [*Casula 2018*]. Several attempts have been done in order to 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. 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 sub-study with the proteomic dataset was performed in order to evaluate its ability to improve accuracy of FH diagnosis and, thus, cardiovascular risk stratification to promote personalized approach in the future. As was mentioned before there are just few data demonstrating the impact of protein biomarkers in risk prediction for FH patients. In 2017 Sven Bos and colleagues aimed to check a hypothesis whether proteomics is useful to identify novel protein biomarkers that differentiate genetically confirmed

heterozygous patients with FH at high CAD risk from those at low CAD risk [*Bos 2017*]. The study has identified 6 potencial novel protein biomarkers that were associated with more advanced stages of atherosclerotic disease and subsequent coronary events in patients with heterozygous FH. Hovewer, the several limitations of the study included the crosssectional study design and inclusion of a highly selected population with a relatively small sample size (20 patients in each of three groups). And so, within the current sub-study a relatively larger sample size from Italian and Russian FH patients was sorted out in order to check the hypothesis whether a set of immune-inflammatory proteins may be an indicator of FH and non-FH pheno- and genotype. The key results demonstrated that a set of immune-inflammatory proteins associated with increased CVD risk, significantly characterize the clinically determined FH phenotype. Crossing the clinical phenotype with genetic analysis, allowed to identify FH/M+ subjects that, in addition to a higher LDL-C burden, are also characterized by a peculiar set of immune-inflammatory proteins as compared to FH/M-. By pairing FH/M+ and FH/M- for LDL-C we still appreciated a number of proteins significantly different, thus suggesting that the prognostic value of these proteins should be longitudinally addressed. Hovewer, to become a generally useful tool for medical decision-making, the assays and associated predictive models will have to be robust to sex, various ethnicities and racial groups, in the young as well as in the elderly and across geographic regions. The risk predictions will need to be sensitive to a variety of risk factors and reflect changes in risk from behavioural, pharmacological, and surgical interventions. Data accumulating in ongoing studies will establish whether the great potential of proteomics to improve lipid disorders identification and management is fulfilled. More importantly, machine learning technology further will facilitate the use of complex, massive data, such as proteomics, in clinical decision making [*Deo 2015, Rajkomar 2019*]. The need for better discrimination of subjects at highest CV event risk is underscored by the advent

of expensive medication in CVD preventive therapy beyond generic statins, among which PCSK9-antibodies [*Schwartz 2018,Sabatine 2017*] and others. Whereas a high-risk proteomic panel holds a promise to help identify higher-risk subjects, it is tempting to speculate that pathway analysis of the proteomic signature may also allow for the guidance of what medication to use in specific patient categories [*Lindsey 2015*]. This concept is underscored by the CANTOS study, where predominantly CRP responders demonstrated CV benefit of interleukin 1 beta-antibody administration [*Ridker 2017*]. However, this concept needs further validation with special emphasis on relationships between biomarkers and protein network analysis [*Lindsey 2015, Johnson 2018*]. Hypothetically, the development of a targeted-proteomic based risk score might enable a more patient tailored approach for CV prevention.

In conclusion, this thesis provided a full clinical and genetic characterization of FH individuals in two north cities of Italy and Russia – Milan and Saint Petersburg. Pheno- and genotype discrepancies in both cohorts were discriminated in order to find out the key gaps for the future FH management reorganization and to address scientific questions to improvement of the current clinical FH scores. Based on these characteristics, critical issues were discussed in the second part of the project in order to analyse new approaches for FH identification improvement. Thus, this thesis evaluated reasonability of the inclusion of the Achilles tendon ultrasonography in the current diagnostic algorithm, identifying that the ultrasound detected xanthoma as a valuable marker for clinical practice and a support for physician in the identification of FH subjects with higher LDL-C burden, who require to be earlier and more aggressively treated. And also, a proteomic sub-study revealed that a set of immune-inflammatory proteins associated with increased CVD risk and significantly characterize the clinically determined FH phenotype. The prognostic value of significantly different in genetically positive and

negative FH individuals proteins adjusted for LDL-C levels might be considered further.

Creation of Italian and Russian Genetic Networks contributed to improving the knowledge of FH pheno- and genotypes in Italy and Russia by providing genetic testing access also for different regions of the countries. Unique and collaborative work of national registries create an opportunity to enhance an international standards of FH identification, risk stratification and management that strongly affect CV morbidity and mortality on a population level.

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SUPPLEMENTARY MATERIALS

Table 1. Lipid lowering therapy correction factors for the retrocalculation of pre-treatment LDL-C levels.

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
Alirocumab	70	1.67
	150	2.22
Evolocumab	140	2.22

Table 2. Descriptive of the population by subclinical carotid atherosclerosis (SCA) occurrence.

	No SCA occurred (N=361)	SCA occurred (N=225)	P
Age (years)	55 (49-60)	56 (51-61)	0,191
Women (n,%)	247 (68,4)	141 (62,6)	0,139
Current smoking habit (n,%)	57 (16,5)	48 (21,6)	0,059
Former smoking habit (n,%)	79 (21,8)	58 (26,1)	0,120
BMI (Kg/m^2)	26,02 (23,64-28,68)	26,86 (24,24-29,37)	0,041
Waist circumference (cm)	89 (80-96)	91 (82-98)	0,086
Systolic blood pressure (mmHg)	130 (120-140)	130 (120-140)	0,010
Diastolic blood pressure (mmHg)	80 (80-90)	80 (80-90)	0,015
Anti-hypertensive treatments (n,%)	78 (21,8)	53 (23,9)	0,600
Fasting glucose (mg/dL)	88,0 (82,0-96,0)	88,0 (82,0-96,0)	0,816

Glucose lowering drugs (n,%)	2 (0,5)	1 (0,4)	0,854
Total cholesterol (mg/dL)	219,0 (196,0-245,0)	223,0 (199,0-247,0)	0,273
HDL-C (mg/dL)	57,0 (47,0-68,0)	52,0 (44,0-62,5)	0,002
Triglycerides (mg/dL)	87,0 (63,0-119,5)	103,0 (65,5-149,0)	0,003
LDL-C (mg/dL)	141,0 (116,8-164,2)	143,0 (120,9-166,9)	0,229
ApoB (mg/dL)	109,0 (92,0-125,5)	112,0 (96,0-128,5)	0,082
ApoA-I (mg/dL)	152,97 (27,27)	146,27 (26,84)	0,006
Lipid lowering drugs (n,%)	22 (6,1)	22 (9,9)	0,103
Hs-CRP (mg/L)	1,78 (0,66-3,41)	1,64 (0,82-3,69)	0,336
Previous CVD (n,%)	0 (0)	0 (0)	-
Common carotid IMT (mm)	0,60 (0,53-0,68)	0,62 (0,55-0,71)	0,054
SCA at basal visit (n,%)	0(0)	0(0)	-
Anti platelet drugs (n,%)	4 (1,1)	2 (0,9)	0,794

Table 3. Comparison of the proteins NPX and their changes, as z-scores, between subjects by SCA occurrence.

	Mean NPX (SCA occurrence)	**S.D. NPX (SCA occurrence)**	**Mean NPX (no SCA occurrence)**	**S.D. NPX (no SCA occurrence)**	**P value**	**mean Z-score**	**S.D. Z-score**
CSTB	4,87	0,57	4,65	0,52	2,39E-06	-0,22	1,65
IL18	7,86	0,79	7,56	0,74	2,99E-06	-0,05	3,34
CCL18	6,18	0,64	5,94	0,68	3,11E-05	-0,14	1,94
IL18_bis	8,07	0,76	7,81	0,75	3,84E-05	-0,36	0,97
HGF	8,39	0,43	8,24	0,46	8,77E-05	-0,29	1,05
KIM1	7,01	0,69	6,79	0,66	1,72E-04	-0,07	2,35
GDF_15	5,10	0,41	4,96	0,42	1,75E-04	-0,22	1,84
TR_AP	1,80	0,42	1,67	0,43	2,93E-04	-0,36	1,02
RARRES2	11,12	0,28	11,03	0,30	3,73E-04	-0,26	1,13
IGFBP_7	6,80	0,31	6,69	0,37	3,84E-04	-0,16	2,42

CEACAM8	3,27	0,69	3,06	0,68	4,11E-04	-0,24	1,19
TNFRSF10A	2,42	0,36	2,33	0,28	4,58E-04	-0,21	0,93
PRSS8	8,67	0,45	8,54	0,39	4,65E-04	0,15	3,15
PIgR	5,77	0,22	5,70	0,23	7,42E-04	-0,09	2,23
IL16	5,47	0,69	5,27	0,72	7,49E-04	-0,16	1,91
IL6	3,19	0,63	3,02	0,62	9,68E-04	-0,17	1,12
U_PAR	4,47	0,38	4,36	0,40	1,05E-03	-0,13	1,61
PON3	4,78	0,81	5,00	0,83	1,46E-03	0,40	0,92
IL_1ra	4,20	0,83	3,98	0,79	1,63E-03	-0,09	1,67
PGF	7,15	0,32	7,07	0,31	1,75E-03	-0,27	1,00
IL_1RT2	5,85	0,29	5,77	0,31	2,09E-03	7,78	2,16
AP_N	4,78	0,31	4,70	0,34	2,16E-03	0,07	1,67
PAI	5,90	0,84	5,68	0,85	2,21E-03	0,05	2,05
TNFSF13B	6,94	0,33	6,86	0,33	2,50E-03	0,13	2,39
CASP_8	5,43	1,03	5,18	1,00	3,72E-03	-0,21	0,97
IL1RL2	3,59	0,41	3,49	0,42	4,29E-03	-0,08	1,61
CTSD	4,32	0,42	4,22	0,41	4,30E-03	-0,04	1,59
MMP_9	4,81	0,76	4,62	0,80	4,41E-03	-0,02	1,70
IL7R	1,27	0,40	1,37	0,43	5,03E-03	0,14	1,10
CCL16	6,18	0,53	6,05	0,55	5,08E-03	-0,18	0,96
CHI3L1	6,44	0,85	6,24	0,82	5,44E-03	0,06	2,23
CST3	6,67	0,37	6,58	0,38	5,52E-03	-0,02	2,32
MMP_12_bis	5,37	0,66	5,22	0,62	5,77E-03	-0,07	1,86
LOX_1	6,81	0,83	6,62	0,85	7,10E-03	-0,02	2,34
ICAM1	5,70	0,34	5,61	0,40	7,11E-03	-0,02	2,05
IL6_2	2,70	0,64	2,55	0,67	8,16E-03	-0,18	1,08
ACE2	2,87	0,64	2,74	0,56	8,95E-03	-0,08	1,20
TNF_R1	5,81	0,32	5,74	0,33	9,71E-03	-0,27	1,00
FCGR2A	1,91	0,77	1,74	0,76	9,96E-03	-0,18	1,14
CCL20	5,65	1,08	5,43	0,99	1,08E-02	-0,16	1,09
C1QTNF1	3,31	0,72	3,18	0,56	1,19E-02	0,00	1,37
SOD2	8,60	0,31	8,54	0,32	1,23E-02	0,04	3,14
CPB1	5,12	0,65	4,99	0,58	1,26E-02	0,04	1,84
CA1	5,92	0,82	5,74	0,90	1,30E-02	0,04	2,15
CD163	7,19	0,49	7,09	0,51	1,33E-02	0,09	2,38
TNFRSF11A	4,90	0,40	4,81	0,41	1,37E-02	-0,09	1,83
hOSCAR	9,70	0,29	9,64	0,27	1,38E-02	0,29	3,47
MERTK	4,69	0,37	4,61	0,40	1,51E-02	0,03	1,79
HB_EGF	3,75	0,93	3,56	0,92	1,52E-02	-0,04	1,45
IDUA	3,77	0,98	3,57	0,94	1,53E-02	-0,07	1,33
TFPI	9,22	0,32	9,15	0,35	1,56E-02	0,20	3,30
LDL_receptor	4,43	0,59	4,31	0,58	1,58E-02	0,11	1,66
IL_18R1	7,23	0,40	7,15	0,41	1,63E-02	-0,11	1,06

TRAIL_R2	4,59	0,63	4,47	0,50	1,64E-02	-0,04	1,43
TGM2	8,62	0,51	8,50	0,60	1,65E-02	0,22	3,31
TIMP1	4,92	0,28	4,86	0,33	1,72E-02	-0,08	1,99
CX3CL1	5,83	0,40	5,91	0,46	1,77E-02	0,28	1,16
CCL25	5,25	0,68	5,11	0,76	1,95E-02	-0,21	0,98
OPG	3,53	0,29	3,47	0,32	1,97E-02	-0,03	1,44
Gal_9	8,17	0,33	8,10	0,35	2,01E-02	0,13	2,85
PCOLCE	5,31	0,39	5,23	0,40	2,01E-02	0,06	1,91
EN_RAGE	2,75	0,80	2,59	0,80	2,05E-02	-0,12	1,09
REN	7,13	0,81	6,98	0,75	2,09E-02	0,16	2,45
CXCL11	7,85	0,83	7,66	1,00	2,10E-02	-0,10	1,13
FAS	5,05	0,28	4,99	0,33	2,22E-02	-0,01	1,83
CD5	4,24	0,38	4,16	0,37	2,22E-02	-0,22	0,94
PSGL_1	4,07	0,27	4,01	0,28	2,31E-02	-0,07	1,51
CPA1	4,54	0,71	4,42	0,59	2,44E-02	0,09	1,60
CDCP1	3,12	0,58	3,01	0,58	2,57E-02	-0,16	1,02
Gal_3	5,60	0,31	5,54	0,35	2,58E-02	0,01	1,91
SHPS_1	2,95	0,36	2,88	0,38	2,67E-02	-0,12	1,31
IL_27	4,02	0,46	4,11	0,46	3,03E-02	0,33	1,67
SORT1	7,78	0,34	7,71	0,37	3,14E-02	0,16	2,81
CNDP1	2,31	0,58	2,43	0,74	3,16E-02	0,39	1,39
IL2_RA	3,80	0,42	3,72	0,43	3,27E-02	0,00	1,40
CCL15	7,07	0,42	6,99	0,42	3,41E-02	0,06	2,38
PLTP	1,61	0,23	1,69	0,56	3,41E-02	0,45	2,99
IL_18BP	5,72	0,32	5,65	0,38	3,52E-02	-0,17	1,02
NID1	2,86	0,41	2,78	0,41	3,59E-02	-0,12	1,27
STK4	3,85	1,21	3,63	1,20	3,65E-02	-0,03	1,60
TNF_R2	4,94	0,32	4,89	0,33	3,82E-02	0,00	1,73
SOD1	1,23	0,47	1,14	0,54	3,85E-02	-0,07	1,18
TGFBI	7,60	0,38	7,52	0,44	3,89E-02	0,12	2,76
uPA2	9,08	0,32	9,02	0,35	3,93E-02	-0,24	1,23
SELE	12,25	0,65	12,14	0,63	4,07E-02	0,33	4,37
PGLYRP1	6,96	0,62	6,86	0,64	5,09E-02	0,17	2,41
TNFB	4,02	0,43	4,09	0,48	5,12E-02	0,16	1,14
TGA_alpha_bis	3,86	0,72	3,74	0,69	5,27E-02	-0,17	0,91
CCL3	5,59	0,73	5,46	0,82	5,33E-02	0,02	1,98
COL18A1	2,27	0,35	2,21	0,36	5,35E-02	-0,16	1,13
CA5A	2,32	0,73	2,20	0,73	5,44E-02	-0,02	1,03
PDGF_subunit_B	10,26	0,74	10,13	0,82	5,44E-02	0,32	3,91
TNF_SF14	3,88	0,68	3,76	0,75	5,64E-02	-0,15	1,03
CFHR5	7,62	0,43	7,55	0,46	5,64E-02	0,19	2,72
CCL3_bis	5,07	0,63	4,96	0,68	5,71E-02	-0,15	1,26
E4_BP1	5,40	1,44	5,17	1,45	5,90E-02	-0,08	0,94

OSM	5,14	0,89	5,00	0,93	5,96E-02	-0,15	0,97
ST2	3,75	0,44	3,67	0,48	5,97E-02	-0,06	1,43
NT_proBNP	1,50	0,79	1,63	0,77	6,01E-02	0,18	0,97
uPA	5,05	0,33	4,99	0,41	6,11E-02	-0,02	2,06
HSP_27	9,64	0,54	9,55	0,62	6,26E-02	0,08	3,46
PDGF_subunit_A	4,01	0,83	3,88	0,90	6,26E-02	-0,09	1,12
MCP_4	14,01	0,70	13,89	0,72	6,28E-02	-0,10	0,98
TM	8,79	0,33	8,74	0,34	6,30E-02	0,13	3,21
PRSS2	1,74	0,47	1,68	0,38	6,51E-02	-0,01	0,86
PAR_1	6,94	0,70	6,82	0,79	6,62E-02	0,08	2,68
ANG	7,09	0,40	7,03	0,39	6,68E-02	0,10	2,46
TIE1	1,24	0,22	1,21	0,20	6,76E-02	-0,14	0,83
NRTN	0,38	0,30	0,45	0,57	6,86E-02	0,40	2,36
CCL17	8,27	0,88	8,12	0,97	7,26E-02	0,26	3,08
MMP_10	5,65	0,66	5,75	0,63	7,96E-02	0,20	0,93
SELP	9,64	0,75	9,53	0,76	8,00E-02	0,27	2,99
CXCL5	10,88	1,29	10,68	1,40	8,09E-02	-0,07	1,15
ALCAM	5,07	0,25	5,03	0,26	8,77E-02	0,15	1,77
LIF_R	2,63	0,23	2,66	0,28	8,80E-02	0,15	1,17
CTSZ	5,24	0,41	5,18	0,38	8,84E-02	-0,13	0,98
CD59	1,94	0,32	1,90	0,33	8,85E-02	-0,14	1,21
TR	5,83	0,37	5,76	0,48	8,85E-02	0,09	2,14
MEGF9	1,86	0,28	1,82	0,31	8,98E-02	0,06	1,16
MPO	3,40	0,36	3,34	0,44	9,10E-02	0,05	1,50
CD40	10,46	0,50	10,38	0,55	9,21E-02	-0,13	1,18
MMP7	4,29	1,69	4,53	1,71	9,23E-02	0,63	2,00
t_PA	6,82	0,58	6,73	0,64	9,35E-02	0,22	2,44
FABP4	5,58	0,67	5,49	0,67	9,92E-02	0,13	1,90
EFEMP1	2,27	0,63	2,35	0,63	1,01E-01	0,42	0,86
CCL5	3,00	0,93	2,86	0,99	1,01E-01	0,11	1,39
G__T	1,55	0,69	1,64	0,72	1,02E-01	0,25	1,24
GP6	2,02	0,71	1,93	0,70	1,02E-01	-0,11	1,08
ITGAM	0,82	0,32	0,88	0,44	1,02E-01	0,29	1,52
IL_6RA	12,20	0,38	12,15	0,39	1,03E-01	0,40	4,37
TNFRSF14	4,25	0,31	4,21	0,33	1,05E-01	0,02	1,65
IL4	0,04	0,39	0,11	0,58	1,11E-01	0,27	1,79
QPCT	1,67	0,33	1,63	0,32	1,11E-01	-0,09	1,12
TNFRSF9	6,01	0,40	5,96	0,40	1,11E-01	-0,19	0,95
ICAM3	1,92	0,27	1,87	0,34	1,13E-01	-0,03	1,39
PLXNB2	0,83	0,26	0,80	0,22	1,13E-01	-0,06	0,90
PI3	3,55	0,55	3,43	0,96	1,13E-01	-0,19	2,83
GAS6	3,33	0,44	3,39	0,52	1,17E-01	0,82	1,71
CASP_3	6,73	1,12	6,58	1,17	1,18E-01	0,12	2,13

PCSK9	3,20	0,36	3,15	0,38	1,19E-01	0,14	1,45
BLM_hydrolase	5,30	0,41	5,24	0,44	1,20E-01	0,10	1,80
CXCL9	7,28	0,71	7,19	0,70	1,20E-01	-0,30	0,79
Dkk_1	9,05	0,72	8,95	0,75	1,20E-01	0,26	3,51
TF	5,58	0,32	5,54	0,32	1,22E-01	0,03	2,08
DCN	3,95	0,24	3,91	0,27	1,24E-01	-0,12	1,75
LILRB1	0,58	0,25	0,54	0,25	1,24E-01	-0,22	1,05
MCP_1	3,46	0,66	3,39	0,48	1,28E-01	0,02	1,18
HAOX1	2,62	1,28	2,46	1,22	1,30E-01	0,08	1,18
IGFBP_1	5,40	1,05	5,54	1,05	1,32E-01	0,18	1,80
FCGR3B	3,33	0,53	3,27	0,51	1,33E-01	0,06	1,32
COMP	6,46	0,42	6,40	0,47	1,37E-01	-0,17	1,24
PAM	1,09	0,43	1,14	0,42	1,42E-01	0,22	0,75
THPO	1,76	0,31	1,72	0,34	1,43E-01	-0,05	1,15
LAP_TGF_beta_1	6,31	0,60	6,24	0,65	1,44E-01	-0,11	1,07
GP1BA	4,03	0,84	3,95	0,62	1,48E-01	0,07	1,30
ENG	1,41	0,26	1,38	0,26	1,51E-01	-0,09	1,15
CES1	1,23	0,54	1,16	0,57	1,51E-01	0,00	1,08
MCP_3	1,77	0,45	1,71	0,51	1,54E-01	-0,01	1,30
AZU1	3,63	0,81	3,52	0,92	1,57E-01	-0,02	1,34
PROC	3,95	0,38	3,90	0,43	1,66E-01	0,06	1,67
PD_L2	2,23	0,40	2,18	0,36	1,68E-01	-0,08	0,96
ITGB2	6,51	0,34	6,46	0,36	1,68E-01	0,09	2,18
DLK_1	5,87	0,57	5,80	0,66	1,69E-01	0,10	2,26
VSIG2	2,81	0,46	2,75	0,43	1,72E-01	-0,06	1,18
DPP4	5,07	0,33	5,03	0,40	1,74E-01	0,01	1,95
CD46	2,35	0,68	2,29	0,42	1,75E-01	-0,02	0,78
GRN	5,86	0,29	5,83	0,34	1,76E-01	0,06	2,17
ITGB1BP2	3,29	1,12	3,17	1,05	1,76E-01	0,07	1,26
MARCO	5,48	0,43	5,43	0,44	1,79E-01	0,23	1,88
PARP_1	2,42	0,99	2,31	1,01	1,79E-01	0,04	1,13
CCL4	5,86	0,71	5,78	0,77	1,81E-01	-0,13	0,97
OPG2	9,74	0,30	9,71	0,34	1,83E-01	-0,05	1,11
CHL1	2,58	0,33	2,55	0,31	1,84E-01	0,00	1,13
CXCL16	4,60	0,27	4,57	0,30	1,89E-01	0,15	1,74
STAMBP	4,15	0,99	4,04	0,98	1,89E-01	0,01	1,08
SCGB3A2	2,89	1,01	2,79	0,89	1,93E-01	-0,03	1,10
PLC	6,45	0,33	6,41	0,33	1,94E-01	0,12	2,28
IFN_gamma	0,33	0,18	0,37	0,41	2,00E-01	0,29	2,42
PRELP	5,53	0,32	5,49	0,40	2,00E-01	0,25	2,01
FGF_21	5,22	1,35	5,08	1,27	2,05E-01	0,24	2,23
TLT_2	5,00	0,38	4,96	0,41	2,07E-01	0,13	1,87
MMP_2	2,88	0,34	2,92	0,36	2,11E-01	0,15	1,23

BNP	0,37	0,24	0,34	0,22	2,12E-01	1,40	1,11
SIRT2	3,89	1,19	3,77	1,20	2,19E-01	0,00	1,08
GH	8,61	2,02	8,83	2,15	2,20E-01	0,42	3,34
Gal_4	2,74	0,38	2,70	0,39	2,20E-01	0,00	1,23
CST5	6,25	0,50	6,30	0,52	2,22E-01	0,12	1,00
ANG_1	7,95	0,91	7,85	0,97	2,24E-01	0,35	3,15
REG1A	5,82	0,53	5,76	0,55	2,28E-01	0,04	2,04
IGFBP6	3,91	0,31	3,87	0,35	2,33E-01	0,05	1,48
THBS2	5,14	0,22	5,12	0,21	2,35E-01	0,09	1,85
ARNT	1,27	0,34	1,30	0,41	2,47E-01	0,23	1,36
AXIN1	2,06	1,10	1,96	1,07	2,51E-01	-0,04	1,10
OPN	4,56	0,67	4,49	0,75	2,55E-01	0,07	1,64
CNTN1	3,87	0,31	3,84	0,33	2,55E-01	0,00	1,49
TNC	1,59	0,42	1,55	0,46	2,60E-01	-0,05	1,07
PRTN3	4,80	0,61	4,74	0,68	2,61E-01	0,12	1,65
LILRB2	2,90	0,34	2,86	0,34	2,64E-01	0,33	1,44
IGFBP_2	7,34	0,69	7,40	0,68	2,68E-01	-0,06	0,96
UMOD	0,72	0,26	0,74	0,28	2,68E-01	0,15	1,00
TNFRSF10C	5,74	0,51	5,69	0,48	2,73E-01	0,22	1,90
MMP_3	5,77	0,70	5,70	0,71	2,73E-01	-0,11	0,95
IL_17_C	1,11	0,30	1,15	0,52	2,76E-01	0,24	2,17
ST6GAL1	2,80	0,31	2,76	0,44	2,76E-01	-0,17	1,58
MB	6,02	0,50	5,97	0,54	2,77E-01	0,06	2,08
DEFA1	2,11	0,18	2,14	0,49	2,89E-01	0,33	2,66
TGFBR3	1,15	0,34	1,12	0,33	2,99E-01	-0,02	1,05
IGLC2	6,05	0,50	6,01	0,49	3,00E-01	1,09	3,22
KLK6	5,16	0,34	5,18	0,33	3,07E-01	0,15	0,89
SELL	7,25	0,31	7,22	0,37	3,10E-01	1,31	3,52
SERPINA12	2,95	1,26	2,84	1,26	3,10E-01	0,04	1,17
Ep_CAM	5,80	1,02	5,72	1,02	3,11E-01	0,17	2,11
ADA	1,71	0,53	1,66	0,60	3,11E-01	0,01	1,12
CCL_11	6,79	0,56	6,74	0,67	3,15E-01	0,05	0,95
SAA4	4,59	0,78	4,52	0,74	3,16E-01	0,06	1,53
SCF_bis	9,97	0,46	10,01	0,50	3,17E-01	0,15	0,88
CXCL10	9,68	0,71	9,61	0,93	3,18E-01	-0,19	0,95
NEMO	3,25	0,96	3,17	0,93	3,21E-01	-0,02	1,13
IL1_alpha	0,50	0,69	0,57	0,84	3,23E-01	0,06	1,29
ANGPTL3	2,75	0,40	2,71	0,52	3,25E-01	-0,04	1,54
MCP1	10,20	0,47	10,16	0,52	3,32E-01	-0,02	1,06
LIF	0,78	0,35	0,82	0,51	3,34E-01	0,17	1,73
AXL	8,40	0,32	8,38	0,34	3,39E-01	0,28	3,06
IL_24	1,28	0,50	1,33	0,72	3,44E-01	0,28	1,82
AMBP	7,24	0,23	7,22	0,23	3,45E-01	0,34	2,54

EPHB4	4,52	0,25	4,50	0,27	3,46E-01	0,07	1,69
MEPE	3,81	0,68	3,76	0,69	3,52E-01	-0,12	1,09
RETN	6,27	0,49	6,24	0,47	3,61E-01	0,16	2,09
IL_10	2,17	0,53	2,22	0,67	3,62E-01	0,13	1,34
IL_10RB	5,97	0,30	5,94	0,36	3,62E-01	0,04	1,07
EGFR	2,57	0,19	2,55	0,22	3,64E-01	0,08	1,37
VEGFD	6,26	0,51	6,29	0,43	3,79E-01	0,30	2,33
IL_22RA1	2,14	0,40	2,18	0,64	3,82E-01	0,09	1,91
CD4	3,83	0,43	3,80	0,43	3,90E-01	0,23	1,32
CR2	5,10	0,47	5,07	0,49	3,99E-01	0,10	1,76
CCL14	4,84	0,34	4,82	0,34	4,00E-01	0,12	1,80
FGF_23	1,69	0,46	1,66	0,48	4,00E-01	0,00	1,19
MMP_1	10,91	1,41	11,01	1,44	4,03E-01	0,26	1,01
MFAP5	1,16	0,24	1,18	0,34	4,04E-01	0,18	1,66
CXCL6	8,14	0,90	8,08	0,98	4,12E-01	0,00	1,01
TFF3	4,85	0,59	4,90	0,87	4,23E-01	0,46	2,33
FABP2	7,49	0,77	7,44	0,72	4,29E-01	0,10	2,92
VEGFA	9,07	0,53	9,04	0,60	4,31E-01	-0,02	1,08
SPON1	0,80	0,24	0,78	0,25	4,32E-01	-0,08	1,14
NCAM1	2,46	0,28	2,45	0,27	4,34E-01	-0,12	1,04
TSLP	1,23	0,46	1,27	0,70	4,39E-01	0,18	1,84
FGF21	4,18	1,27	4,10	1,17	4,40E-01	0,05	0,94
CCL19	9,02	0,94	8,95	1,06	4,52E-01	0,00	0,97
FGF23	1,83	0,44	1,80	0,54	4,63E-01	-0,01	1,46
CXCL1	8,72	0,95	8,65	1,01	4,65E-01	0,33	3,17
TNXB	0,62	0,19	0,63	0,19	4,65E-01	0,11	0,97
CDH1	3,74	0,33	3,72	0,44	4,66E-01	0,02	1,80
TNFRSF13B	8,32	0,40	8,29	0,46	4,75E-01	0,18	2,94
FETUB	0,66	0,35	0,68	0,43	4,76E-01	0,17	1,34
CSF_1	8,72	0,26	8,71	0,28	4,77E-01	-0,11	1,08
CD93	10,72	0,32	10,70	0,36	4,81E-01	0,47	3,94
CD244	6,61	0,45	6,58	0,50	4,84E-01	0,00	1,03
GIF	6,12	0,92	6,06	0,85	4,89E-01	0,07	2,02
HO_1	10,56	0,64	10,53	0,67	4,89E-01	0,40	4,07
FAP	1,22	0,23	1,23	0,28	4,98E-01	0,07	1,32
SERPINA7	4,01	0,38	3,99	0,39	4,99E-01	0,15	1,45
GLO1	3,93	0,87	3,88	0,92	5,02E-01	0,30	1,49
IL_13	1,24	0,52	1,27	0,53	5,14E-01	0,11	1,09
THBS4	4,12	0,53	4,09	0,57	5,20E-01	0,17	1,58
MCP_2	8,27	0,63	8,23	0,73	5,27E-01	-0,08	1,06
OSMR	1,09	0,22	1,08	0,21	5,31E-01	-0,09	0,96
BMP_6	2,54	0,82	2,50	0,82	5,47E-01	0,04	1,17
CA4	1,17	0,24	1,16	0,31	5,52E-01	-0,06	1,54

MBL2	8,21	1,31	8,14	1,33	5,53E-01	1,43	3,86
PTPRS	0,56	0,21	0,57	0,20	5,59E-01	0,06	0,96
IGFBP3	4,17	0,37	4,15	0,40	5,65E-01	0,13	1,57
LYVE1	4,10	0,33	4,08	0,34	5,69E-01	0,04	1,48
IL_17RA	3,51	0,52	3,49	0,49	5,72E-01	0,10	1,23
DNER	8,72	0,27	8,70	0,25	5,77E-01	-0,06	0,89
NT_3	1,19	0,39	1,21	0,54	5,82E-01	0,21	1,73
PDL_1	4,69	0,44	4,67	0,45	5,92E-01	-0,07	1,10
CCL23	9,86	0,44	9,84	0,46	5,94E-01	-0,13	1,08
F7	3,72	0,34	3,71	0,36	5,95E-01	0,13	1,51
CTSL1	6,63	0,52	6,61	0,56	5,95E-01	-0,01	2,19
IL_10RA	0,56	0,71	0,53	0,75	5,97E-01	0,09	1,31
ADAM_TS13	5,09	0,25	5,08	0,30	5,97E-01	0,19	1,97
CCL24	5,62	0,95	5,57	0,96	6,04E-01	0,27	2,00
PRCP	0,95	0,31	0,93	0,45	6,11E-01	0,15	1,81
TRANCE	4,22	0,52	4,19	0,61	6,18E-01	-0,17	1,32
C2	4,69	0,70	4,66	0,69	6,20E-01	0,14	1,56
SLAMF7	3,02	0,48	3,00	0,48	6,22E-01	0,11	1,52
IL5	1,47	1,19	1,43	1,07	6,26E-01	-0,05	0,92
PTX3	1,98	0,43	2,00	0,40	6,29E-01	0,14	0,88
SRC	6,46	1,01	6,42	1,06	6,31E-01	0,17	2,29
TIMP4	2,71	0,52	2,69	0,48	6,33E-01	0,12	1,09
CA3	-0,21	0,25	-0,23	0,34	6,34E-01	-0,20	1,35
PECAM_1	4,19	0,55	4,17	0,57	6,34E-01	-0,10	1,03
ADM	4,65	2,00	4,57	2,05	6,35E-01	0,33	2,44
PRSS27	8,35	0,51	8,37	0,48	6,36E-01	0,40	3,21
IL_2B	4,35	0,58	4,37	0,60	6,38E-01	0,05	0,96
REG3A	0,78	0,21	0,77	0,22	6,46E-01	-0,07	1,06
VASN	1,19	0,27	1,18	0,25	6,56E-01	-0,05	0,99
GNLY	0,87	0,33	0,85	0,35	6,57E-01	-0,01	1,06
TCN2	2,65	0,39	2,63	0,44	6,60E-01	0,08	1,22
IL_1RT1	6,00	0,25	5,99	0,28	6,62E-01	0,21	2,14
F11	6,65	0,30	6,64	0,32	6,62E-01	0,29	2,43
LEP	5,93	1,11	5,89	1,19	6,63E-01	0,32	2,02
CRTAC1	1,00	0,48	1,02	0,49	6,63E-01	0,14	0,97
IgG_Fc_receptor_II_b	2,02	0,71	2,04	0,68	6,67E-01	0,08	1,05
SCF	10,09	0,44	10,11	0,50	6,72E-01	0,52	3,65
PAPPA	1,81	0,39	1,80	0,37	6,75E-01	0,00	0,98
GDF_2	8,32	0,57	8,34	0,57	6,80E-01	0,23	3,01
IL2	0,82	0,24	0,83	0,37	6,81E-01	-1,18	1,88
CD40_L	4,01	1,30	3,96	1,32	6,83E-01	0,34	2,20
CXCL1_bis	9,85	0,93	9,82	1,01	6,91E-01	0,01	1,15
FS	10,37	0,60	10,39	0,65	6,95E-01	0,74	3,67

JAM_A	5,05	0,93	5,02	0,96	7,08E-01	0,15	1,62
LCN2	1,66	0,35	1,64	0,46	7,09E-01	-0,04	1,16
LILRB5	3,32	0,77	3,34	0,77	7,23E-01	0,11	0,90
LTBR	3,53	0,28	3,52	0,30	7,26E-01	0,16	1,40
Flt3L	9,19	0,44	9,17	0,41	7,27E-01	-0,13	0,88
IL_20	0,12	0,38	0,13	0,48	7,27E-01	0,15	1,55
vWF	6,92	0,71	6,90	0,76	7,34E-01	-0,01	1,19
IL_15RA	0,63	0,20	0,62	0,25	7,40E-01	-0,02	1,53
ST1A1	3,28	1,35	3,25	1,32	7,43E-01	0,05	1,09
ICAM_2	4,89	0,38	4,88	0,40	7,51E-01	0,02	1,07
Notch_3	3,98	0,35	3,99	0,39	7,53E-01	0,14	1,50
CHIT1	6,19	1,63	6,23	1,39	7,60E-01	0,20	1,77
XCL1	3,89	0,80	3,91	0,77	7,62E-01	0,26	1,36
BOC	3,58	0,32	3,58	0,35	7,68E-01	0,02	1,57
NRP1	1,16	0,16	1,15	0,16	7,70E-01	-0,14	0,99
AOC3	2,95	0,36	2,94	0,34	7,73E-01	-0,08	1,33
TIE2	7,96	0,27	7,97	0,29	7,78E-01	0,36	2,87
IL33	0,61	0,26	0,61	0,40	7,81E-01	0,25	2,01
IL_20RA	0,72	0,34	0,71	0,40	7,93E-01	0,01	1,45
IL7	2,39	0,75	2,41	0,84	7,94E-01	0,21	1,22
AGRP	2,85	0,61	2,86	0,60	7,96E-01	0,30	1,19
MET	1,83	0,22	1,82	0,23	8,11E-01	0,01	1,13
IL_2RB	1,34	0,36	1,35	0,42	8,21E-01	0,07	1,50
IL8	5,60	0,64	5,59	0,79	8,24E-01	-0,01	1,19
SERPINA5	8,66	0,82	8,67	0,37	8,30E-01	0,39	3,02
COL1A1	3,02	0,39	3,03	0,39	8,32E-01	-0,04	1,11
CD6	5,08	0,56	5,09	0,55	8,36E-01	-0,04	0,99
SPON2	9,40	0,28	9,41	0,30	8,42E-01	0,47	3,52
SLAMF1	2,13	0,39	2,13	0,61	8,42E-01	-0,01	1,82
APOM	5,72	0,40	5,73	0,56	8,43E-01	0,20	2,38
RAGE	4,45	0,33	4,45	0,36	8,63E-01	0,18	1,70
SPARCL1	1,17	0,25	1,18	0,30	8,68E-01	0,05	1,22
FCN2	5,19	0,69	5,18	0,73	8,71E-01	0,30	1,90
IL_4RA	1,50	0,40	1,50	0,30	8,72E-01	0,11	0,87
CTRC	9,43	0,67	9,42	0,63	8,78E-01	0,44	3,22
DECR1	5,11	1,66	5,13	1,63	8,78E-01	0,18	1,60
KIT	3,31	0,37	3,31	0,35	8,79E-01	0,21	1,61
CD8A	8,35	1,06	8,34	1,06	8,81E-01	8,02	0,78
TIMD4	3,03	0,45	3,02	0,48	8,84E-01	0,11	1,27
CDH5	3,85	0,37	3,85	0,41	8,87E-01	0,15	1,34
TRAIL	7,21	0,39	7,21	0,42	8,98E-01	0,07	1,05
VCAM1	3,81	0,31	3,81	0,36	9,00E-01	0,07	1,52
IL_17A	1,05	0,48	1,05	0,54	9,04E-01	0,08	1,38

NOTCH1	2,16	0,21	2,16	0,23	9,05E-01	0,12	1,26
FGF_19	7,34	0,84	7,33	0,94	9,22E-01	0,00	0,98
CCL28	1,89	0,44	1,89	0,46	9,22E-01	0,05	1,14
CD84	4,37	0,57	4,37	0,53	9,33E-01	0,25	1,57
TWEAK	8,94	0,55	8,94	0,62	9,38E-01	0,16	1,10
IL_17D	2,01	0,29	2,01	0,32	9,46E-01	-0,02	0,90
LPL	7,28	0,77	7,29	0,89	9,51E-01	0,41	2,71
LTBP2	1,01	0,26	1,01	0,25	9,60E-01	-0,04	0,95
BETA_NGF	0,38	0,53	0,38	0,42	9,60E-01	0,07	0,78
TNF	1,00	0,46	1,00	0,49	9,62E-01	0,05	1,30
PLA2G7	1,25	0,25	1,25	0,46	9,67E-01	0,09	2,08
FGF5	0,05	0,28	0,05	0,37	9,72E-01	0,14	1,48
PSP_D	2,53	0,59	2,53	0,63	9,90E-01	0,02	1,14
GDNF	1,61	0,34	1,61	0,50	9,97E-01	0,10	1,69

Table 4. Hit immune-inflammatory proteomics predictors of SCA occurrence.

	Beta	S.E.	Wald F	H.R. (95% C.I.)	Lower limit	Upper limit	P value
CX3CL1	-0,906	0,337	7,215	0,404	0,209	0,783	**0,007**
SORT1	-1,385	0,580	5,707	0,250	0,080	0,780	**0,017**
FCGR2A	0,332	0,156	4,539	1,393	1,027	1,891	**0,033**
CSTB	0,704	0,348	4,091	2,021	1,022	3,996	**0,043**
PON3	-0,389	0,193	4,081	0,677	0,464	0,988	**0,043**
IL1RL2	0,587	0,309	3,610	1,799	0,982	3,297	0,057
AP_N	0,827	0,457	3,277	2,287	0,934	5,601	0,070
CHI3L1	0,304	0,170	3,196	1,355	0,971	1,892	0,074
DBP_1	0,030	0,017	3,169	1,031	0,997	1,066	0,075
ICAM1	0,802	0,463	3,005	2,231	0,901	5,527	0,083
KIM1	0,300	0,181	2,739	1,350	0,946	1,926	0,098
REN	0,251	0,158	2,518	1,285	0,943	1,751	0,113
PLTP	-0,897	0,593	2,288	0,408	0,128	1,304	0,130
CEACAM8	0,462	0,308	2,250	1,588	0,868	2,904	0,134
PIgR	1,015	0,685	2,196	2,760	0,721	10,573	0,138
CTSD	-0,587	0,403	2,118	0,556	0,252	1,226	0,146
TNFRSF13B	-0,496	0,344	2,079	0,609	0,310	1,195	0,149
TNFSF13B	0,653	0,455	2,061	1,921	0,788	4,685	0,151
CNDP1	-0,345	0,242	2,036	0,708	0,441	1,138	0,154
IL_1ra	-0,346	0,256	1,824	0,708	0,429	1,169	0,177
FAS	-0,712	0,556	1,640	0,491	0,165	1,459	0,200
SELE	-0,277	0,220	1,584	0,758	0,492	1,167	0,208

TNFRSF10A	0,587	0,476	1,522	1,799	0,708	4,572	0,217
CCL25	0,238	0,196	1,486	1,269	0,865	1,862	0,223
TIMP1	-0,889	0,740	1,443	0,411	0,096	1,753	0,230
MERTK	-0,504	0,437	1,330	0,604	0,257	1,422	0,249
IL_18BP	-0,790	0,686	1,327	0,454	0,118	1,741	0,249
CXCL11	0,193	0,174	1,230	1,213	0,862	1,708	0,267
CA1	0,309	0,279	1,226	1,363	0,788	2,356	0,268
LOX_1	-0,338	0,308	1,207	0,713	0,390	1,303	0,272
IL18	0,254	0,232	1,196	1,289	0,818	2,033	0,274
IGFBP_7	0,602	0,583	1,065	1,826	0,582	5,730	0,302
PAI	-0,247	0,246	1,013	0,781	0,482	1,264	0,314
TGM2	-0,362	0,362	1,001	0,696	0,342	1,415	0,317
OPG	0,466	0,491	0,901	1,594	0,609	4,174	0,342
EN_RAGE	0,216	0,231	0,872	1,241	0,789	1,953	0,350
CASP_8	0,265	0,296	0,804	1,304	0,730	2,330	0,370
TNF_R2	-0,595	0,747	0,635	0,552	0,128	2,383	0,426
GDF_15	0,298	0,378	0,625	1,348	0,643	2,825	0,429
PSGL_1	-0,377	0,487	0,599	0,686	0,264	1,781	0,439
U_PAR	-0,405	0,524	0,596	0,667	0,239	1,865	0,440
SHPS_1	0,258	0,339	0,577	1,294	0,666	2,515	0,447
CCL20	0,087	0,119	0,537	1,091	0,864	1,377	0,464
IL_27	-0,197	0,274	0,516	0,821	0,480	1,405	0,473
PGF	0,416	0,586	0,504	1,516	0,480	4,786	0,478
hOSCAR	0,421	0,595	0,500	1,523	0,475	4,887	0,480
CPA1	0,226	0,361	0,392	1,254	0,618	2,545	0,531
NID1	0,324	0,521	0,388	1,383	0,498	3,837	0,533
IDUA	0,084	0,153	0,302	1,088	0,806	1,469	0,582
TGFBI	0,213	0,387	0,301	1,237	0,579	2,641	0,583
SBP_1	0,005	0,009	0,298	1,005	0,987	1,024	0,585
HB_EGF	0,125	0,240	0,271	1,133	0,708	1,815	0,603
TFPI	-0,196	0,437	0,202	0,822	0,349	1,934	0,653
ACE2	-0,110	0,250	0,193	0,896	0,549	1,462	0,661
CD163	0,129	0,295	0,191	1,138	0,638	2,030	0,662
TNFRSF11A	0,190	0,437	0,188	1,209	0,513	2,847	0,664
SOD2	0,272	0,689	0,155	1,312	0,340	5,061	0,693
IL6_2	-0,083	0,220	0,141	0,920	0,598	1,417	0,707
IL_1RT2	0,178	0,483	0,136	1,195	0,464	3,082	0,712
PCOLCE	0,147	0,411	0,128	1,158	0,518	2,590	0,721
RARRES2	0,213	0,620	0,118	1,237	0,367	4,171	0,731
HGF	0,180	0,553	0,106	1,197	0,405	3,536	0,745
CST3	-0,180	0,577	0,097	0,835	0,270	2,588	0,755
CD5	0,137	0,441	0,096	1,146	0,483	2,722	0,757

SOD1	-0,120	0,453	0,070	0,887	0,365	2,154	0,791
C1QTNF1	0,059	0,229	0,067	1,061	0,678	1,660	0,796
CCL15	0,070	0,298	0,055	1,072	0,598	1,922	0,815
STK4	0,035	0,178	0,040	1,036	0,730	1,470	0,842
TR_AP	0,064	0,340	0,036	1,066	0,548	2,076	0,850
CCL16	-0,040	0,252	0,026	0,960	0,586	1,573	0,872
CPB1	0,055	0,369	0,022	1,056	0,512	2,178	0,882
IL_18R1	-0,046	0,323	0,020	0,955	0,507	1,800	0,887
IL16	-0,039	0,312	0,016	0,962	0,521	1,774	0,900
Gal_9	0,061	0,513	0,014	1,062	0,389	2,903	0,906
LDL_receptor	0,036	0,307	0,013	1,036	0,568	1,892	0,908
MMP_12_bis	0,021	0,214	0,010	1,022	0,672	1,553	0,920
PRSS8	0,025	0,377	0,004	1,025	0,489	2,148	0,948
Gal_3	-0,020	0,449	0,002	0,981	0,407	2,365	0,965
TRAIL_R2	-0,007	0,216	0,001	0,993	0,650	1,517	0,975
uPA2	-0,016	0,510	0,001	0,984	0,362	2,674	0,975
MMP_9	-0,004	0,321	0,000	0,996	0,531	1,870	0,990

Table 5. Comparison of the proteins NPX and their changes, as z-scores, between subjects by faster carotid IMT progression.

	Mean NPX (faster IMT progression)	S.D. NPX (faster IMT progression)	Mean NPX (slower IMT progression)	S.D. NPX (slower IMT progression)	P value	mean Z-score	S.D. Z-score
FABP2	7,63	0,77	7,40	0,73	1,22E-03	0,19	3,05
TF	5,63	0,33	5,53	0,32	1,30E-03	0,02	2,61
MMP7	4,04	1,76	4,57	1,68	1,39E-03	0,52	1,81
EFEMP1	2,18	0,68	2,36	0,62	3,95E-03	0,34	1,07
CNDP1	2,24	0,60	2,42	0,71	6,94E-03	0,39	1,37
REG1A	5,89	0,62	5,75	0,50	1,14E-02	0,17	2,65
CXCL1	8,86	0,97	8,62	1,00	1,43E-02	0,36	3,69
FS	10,27	0,59	10,42	0,65	1,45E-02	0,96	4,58
LDL_receptor	4,25	0,59	4,39	0,58	1,76E-02	0,48	1,94
FGF21	3,91	1,24	4,19	1,20	1,84E-02	0,42	1,64
LEP	5,72	1,20	5,98	1,15	2,08E-02	0,61	2,49
IGFBP_2	7,49	0,68	7,34	0,69	2,10E-02	0,20	3,15
IL_17D	2,06	0,41	2,00	0,26	2,30E-02	-0,09	0,87
GAS6	3,29	0,44	3,40	0,51	2,54E-02	4,51	3,13
IL16	5,46	0,78	5,31	0,69	2,80E-02	0,14	2,24
TNFRSF13B	8,36	0,56	8,27	0,36	3,16E-02	0,39	3,63
IL1RL2	3,60	0,42	3,51	0,41	3,34E-02	-0,02	1,67
ADM	4,29	2,00	4,70	2,01	3,45E-02	0,50	1,95

TNFSF13B	6,84	0,33	6,91	0,33	3,68E-02	0,60	3,22
FCN2	5,07	0,72	5,22	0,72	3,81E-02	0,51	2,20
IL_13	1,18	0,24	1,27	0,53	3,84E-02	0,37	2,17
Dkk_1	9,11	0,71	8,96	0,75	4,00E-02	0,41	3,99
THPO	1,78	0,32	1,72	0,32	4,44E-02	-0,14	1,11
SCF	10,17	0,46	10,08	0,49	4,52E-02	0,46	4,39
PAM	1,06	0,46	1,14	0,41	4,79E-02	0,17	0,90
NT_3	1,14	0,40	1,22	0,47	5,06E-02	0,22	1,20
IL_1RT1	6,03	0,27	5,98	0,27	5,09E-02	0,13	3,00
CXCL1_bis	9,97	0,94	9,79	0,99	5,45E-02	0,39	3,96
SOD2	8,61	0,29	8,55	0,32	5,46E-02	0,30	4,23
CTSL1	6,69	0,54	6,59	0,55	6,41E-02	0,24	3,06
CCL20	5,64	1,13	5,47	0,98	7,00E-02	0,17	2,40
THBS4	4,03	0,56	4,13	0,56	7,10E-02	0,40	1,78
CASP_8	5,41	1,12	5,23	0,98	7,16E-02	0,13	2,08
OSMR	1,11	0,23	1,07	0,21	7,40E-02	-0,17	0,96
PAR_1	6,96	0,65	6,83	0,79	7,70E-02	0,24	2,94
PDGF_subunit_B	10,29	0,74	10,15	0,80	7,80E-02	0,53	4,51
PRSS27	8,43	0,51	8,34	0,48	7,93E-02	0,39	3,78
HAOX1	2,33	1,14	2,53	1,21	8,26E-02	0,27	1,31
ICAM_2	4,94	0,35	4,87	0,40	8,36E-02	0,04	2,24
FGF_21	4,95	1,36	5,17	1,28	8,51E-02	0,47	2,08
HO_1	10,62	0,63	10,51	0,67	8,86E-02	0,54	4,43
TWEAK	8,86	0,56	8,96	0,60	9,07E-02	0,66	3,58
XCL1	3,80	0,79	3,93	0,79	9,15E-02	0,36	1,54
CEACAM8	3,23	0,79	3,11	0,66	9,43E-02	0,03	1,39
IL_18R1	7,13	0,39	7,20	0,42	1,01E-01	0,53	3,13
GDF_2	8,40	0,54	8,31	0,57	1,01E-01	0,39	3,83
TNFRSF11A	4,90	0,40	4,83	0,40	1,02E-01	0,12	2,22
CCL17	8,29	0,88	8,14	0,96	1,04E-01	0,41	3,69
FETUB	0,63	0,34	0,69	0,42	1,07E-01	0,15	1,22
VSIG2	2,83	0,42	2,76	0,45	1,10E-01	-0,03	1,40
LOX_1	6,79	0,91	6,66	0,83	1,11E-01	0,32	2,96
SPON2	9,37	0,32	9,42	0,29	1,12E-01	0,71	4,43
PD_L2	2,24	0,35	2,18	0,39	1,13E-01	-0,08	1,29
hOSCAR	9,69	0,27	9,65	0,29	1,16E-01	0,41	4,78
IGFBP_7	6,78	0,37	6,72	0,35	1,18E-01	0,26	3,06
GLO1	3,79	0,84	3,92	0,91	1,22E-01	0,36	1,65
Gal_9	8,16	0,36	8,11	0,34	1,23E-01	0,36	3,77
MMP_12_bis	5,21	0,58	5,30	0,66	1,26E-01	0,49	2,44
STK4	3,85	1,17	3,67	1,22	1,26E-01	0,04	1,67
CNTN1	3,89	0,34	3,85	0,31	1,31E-01	0,06	1,81
IGFBP_1	5,62	0,97	5,46	1,07	1,31E-01	0,24	2,82

CXCL6	8,22	0,94	8,08	0,96	1,35E-01	0,31	3,22
MPO	3,41	0,42	3,35	0,41	1,36E-01	0,03	1,62
SLAMF7	3,06	0,55	2,99	0,44	1,42E-01	0,06	1,42
MFAP5	1,14	0,24	1,18	0,33	1,42E-01	0,19	1,41
TM	8,80	0,34	8,75	0,33	1,42E-01	0,40	4,10
IL_17_C	1,09	0,33	1,14	0,38	1,43E-01	0,15	1,15
TRAIL	7,17	0,42	7,23	0,41	1,45E-01	0,50	3,02
AGRP	2,79	0,63	2,87	0,60	1,50E-01	0,26	1,23
CCL14	4,80	0,33	4,84	0,34	1,51E-01	0,40	2,27
ANG_1	7,98	0,87	7,85	0,97	1,58E-01	0,39	3,43
LAP_TGF_beta_1	6,33	0,62	6,25	0,64	1,61E-01	0,20	2,55
CTRC	9,50	0,62	9,41	0,66	1,61E-01	0,50	4,20
AMBP	7,20	0,23	7,23	0,22	1,63E-01	0,50	3,75
MET	1,85	0,21	1,82	0,23	1,67E-01	-0,10	1,27
CCL19	8,87	0,93	9,00	1,04	1,71E-01	0,65	3,56
TNFB	4,02	0,44	4,08	0,46	1,73E-01	0,32	1,79
TIMP4	2,65	0,49	2,71	0,50	1,73E-01	0,25	1,35
SCF_bis	10,04	0,48	9,98	0,49	1,75E-01	0,42	4,13
ST6GAL1	2,82	0,35	2,77	0,41	1,76E-01	-0,03	1,59
GRN	5,87	0,32	5,83	0,32	1,79E-01	0,20	2,81
IL18	7,75	0,92	7,65	0,73	1,80E-01	0,42	3,28
ARNT	1,25	0,34	1,29	0,35	1,80E-01	0,14	1,06
PDGF_subunit_A	4,02	0,86	3,90	0,88	1,81E-01	0,07	1,72
SCGB3A2	2,93	1,01	2,81	0,92	1,84E-01	0,04	1,39
NEMO	3,30	0,96	3,18	0,94	1,90E-01	0,05	1,50
SORT1	7,77	0,36	7,73	0,36	1,90E-01	0,34	3,64
TNF_SF14	3,88	0,86	3,79	0,68	1,95E-01	0,08	1,53
NT_proBNP	1,51	0,78	1,61	0,78	1,97E-01	0,17	1,10
PRTN3	4,83	0,78	4,75	0,60	1,97E-01	0,19	2,02
PON3	4,83	0,90	4,94	0,81	2,01E-01	0,37	1,98
HB_EGF	3,73	0,95	3,61	0,93	2,01E-01	0,10	1,70
CXCL5	10,89	1,34	10,72	1,38	2,03E-01	0,53	4,25
MCP_2	8,31	0,73	8,22	0,69	2,03E-01	0,35	3,29
IL_22RA1	2,12	0,43	2,17	0,50	2,13E-01	0,20	1,31
MCP_4	14,01	0,64	13,92	0,73	2,13E-01	0,70	5,80
TSLP	1,20	0,41	1,26	0,57	2,19E-01	0,16	1,39
KLK6	5,15	0,37	5,18	0,32	2,22E-01	0,34	2,22
PAPPA	1,84	0,39	1,79	0,38	2,23E-01	-0,06	1,05
TIMP1	4,91	0,29	4,87	0,32	2,24E-01	0,13	2,44
GP1BA	4,05	0,73	3,97	0,71	2,29E-01	0,13	1,93
FABP4	5,47	0,65	5,55	0,68	2,37E-01	0,46	2,37
KIM1	6,94	0,71	6,86	0,68	2,39E-01	0,34	2,88
EN_RAGE	2,72	0,82	2,63	0,80	2,40E-01	0,01	1,33

TNXB	0,61	0,17	0,63	0,19	2,42E-01	0,08	1,15
CD8A	8,25	0,97	8,37	1,10	2,42E-01	8,88	2,06
FGF23	1,76	0,42	1,82	0,50	2,42E-01	0,17	1,27
PCSK9	3,14	0,34	3,18	0,39	2,44E-01	0,30	2,34
CFHR5	7,54	0,43	7,59	0,46	2,53E-01	0,59	3,49
IL_17RA	3,54	0,53	3,49	0,49	2,54E-01	0,08	1,55
TRANCE	4,15	0,55	4,21	0,57	2,54E-01	0,30	1,71
CCL5	3,00	0,97	2,89	0,97	2,54E-01	0,04	1,40
U_PAR	4,44	0,42	4,40	0,39	2,56E-01	0,14	1,95
F7	3,69	0,35	3,73	0,36	2,58E-01	0,29	1,81
TNFRSF10A	2,39	0,35	2,35	0,30	2,59E-01	-0,01	1,18
GIF	6,01	1,02	6,11	0,83	2,59E-01	0,49	2,51
IL_15RA	0,64	0,17	0,62	0,21	2,60E-01	-0,15	1,24
uPA2	9,07	0,29	9,03	0,36	2,63E-01	0,31	4,22
IL8	5,66	0,82	5,58	0,71	2,65E-01	0,21	2,20
MCP_1	3,47	0,56	3,41	0,57	2,65E-01	0,07	1,58
ITGAM	0,83	0,31	0,87	0,42	2,65E-01	0,12	1,36
DPP4	5,08	0,35	5,04	0,39	2,67E-01	0,16	2,42
HSP_27	9,63	0,59	9,57	0,60	2,69E-01	0,53	4,15
IL_24	1,26	0,45	1,32	0,60	2,71E-01	0,15	1,36
uPA	5,05	0,35	5,01	0,40	2,71E-01	0,16	2,35
NRTN	0,39	0,34	0,43	0,47	2,81E-01	0,09	1,41
GP6	2,02	0,71	1,95	0,71	2,81E-01	-0,02	1,20
SELP	9,63	0,74	9,55	0,77	2,82E-01	0,55	4,13
DLK_1	5,88	0,62	5,82	0,62	2,83E-01	0,26	2,49
IL_10	2,15	0,57	2,21	0,62	2,90E-01	0,18	1,24
GDF_15	5,06	0,40	5,01	0,42	2,93E-01	0,18	2,37
Ep_CAM	5,84	1,10	5,74	0,99	2,93E-01	0,28	2,41
AOC3	2,98	0,32	2,94	0,36	2,94E-01	0,02	1,63
MMP_2	2,88	0,36	2,91	0,35	2,97E-01	0,22	1,42
PLC	6,41	0,34	6,44	0,33	2,97E-01	0,47	3,00
PSGL_1	4,05	0,30	4,02	0,27	2,99E-01	0,11	1,91
BMP_6	2,58	0,77	2,50	0,84	3,00E-01	-0,01	1,25
TR_AP	1,76	0,45	1,71	0,43	3,04E-01	-0,04	1,05
IFN_gamma	0,33	0,20	0,36	0,31	3,08E-01	0,09	1,57
OPG	3,52	0,35	3,49	0,29	3,08E-01	0,08	1,62
CASP_3	6,73	1,15	6,62	1,16	3,15E-01	0,36	2,92
CD163	7,09	0,52	7,14	0,51	3,17E-01	0,55	3,14
MERTK	4,67	0,38	4,63	0,40	3,20E-01	0,16	2,18
PLA2G7	1,22	0,25	1,26	0,44	3,20E-01	0,15	1,81
PGF	7,12	0,33	7,09	0,32	3,23E-01	0,25	3,12
CD5	4,22	0,43	4,18	0,35	3,25E-01	0,11	1,77
NOTCH1	2,15	0,20	2,17	0,23	3,26E-01	0,16	1,46

COL18A1	2,21	0,34	2,24	0,36	3,36E-01	0,17	1,34
CA5A	2,19	0,71	2,25	0,72	3,46E-01	0,17	1,09
TNFRSF14	4,25	0,34	4,22	0,32	3,47E-01	0,14	1,99
BOC	3,60	0,32	3,57	0,35	3,48E-01	0,08	1,70
SLAMF1	2,15	0,51	2,11	0,46	3,54E-01	-0,02	1,04
NCAM1	2,47	0,28	2,45	0,28	3,58E-01	0,00	1,38
PLTP	1,63	0,22	1,67	0,53	3,59E-01	0,21	2,41
TFF3	4,84	0,49	4,91	0,86	3,64E-01	0,42	2,54
PTPRS	0,56	0,20	0,58	0,21	3,65E-01	0,05	1,07
DCN	3,94	0,23	3,92	0,27	3,69E-01	0,08	2,14
GH	8,89	2,11	8,71	2,10	3,78E-01	0,56	3,89
GNLY	0,84	0,35	0,87	0,34	3,82E-01	0,06	0,98
E4_BP1	5,16	1,39	5,29	1,47	3,83E-01	0,35	2,04
G__T	1,65	0,78	1,59	0,68	3,84E-01	-0,02	0,97
PRCP	0,91	0,39	0,95	0,41	3,85E-01	0,08	1,06
CHL1	2,59	0,30	2,56	0,33	3,86E-01	0,01	1,44
CES1	1,15	0,61	1,20	0,54	3,88E-01	0,08	0,92
LYVE1	4,07	0,32	4,10	0,34	3,91E-01	0,29	1,98
FAS	5,03	0,34	5,00	0,31	3,92E-01	0,20	2,33
SAA4	4,50	0,79	4,56	0,76	3,93E-01	0,36	2,01
CXCL9	7,18	0,68	7,24	0,71	4,02E-01	0,48	2,95
RARRES2	11,05	0,32	11,07	0,29	4,07E-01	0,64	5,01
CA1	5,76	0,90	5,83	0,85	4,18E-01	0,10	1,38
ALCAM	5,07	0,28	5,05	0,25	4,21E-01	0,19	2,42
PLXNB2	0,80	0,20	0,82	0,25	4,22E-01	0,06	1,24
SPARCL1	1,16	0,24	1,18	0,30	4,31E-01	0,09	1,27
SHPS_1	2,89	0,35	2,92	0,38	4,34E-01	0,21	1,54
ENG	1,41	0,21	1,39	0,27	4,35E-01	-0,08	1,38
APOM	5,70	0,41	5,74	0,54	4,39E-01	0,43	2,75
IL_20	0,10	0,36	0,13	0,43	4,44E-01	0,03	1,25
TNF_R1	5,79	0,35	5,77	0,32	4,46E-01	0,20	2,52
FAP	1,21	0,23	1,23	0,27	4,48E-01	0,09	1,20
IL_6RA	12,19	0,37	12,17	0,39	4,49E-01	0,71	5,75
FCGR2A	1,76	0,86	1,81	0,74	4,53E-01	0,13	1,01
IL_2B	4,34	0,60	4,38	0,58	4,53E-01	0,28	1,84
IL7R	1,31	0,44	1,34	0,42	4,54E-01	0,09	1,01
TGA_alpha_bis	3,83	0,77	3,78	0,67	4,57E-01	0,12	1,55
CCL25	5,20	0,70	5,15	0,75	4,57E-01	0,18	2,00
MCP1	10,21	0,52	10,17	0,50	4,61E-01	0,50	4,23
CRTAC1	0,98	0,48	1,02	0,49	4,68E-01	0,06	1,02
CCL15	7,01	0,37	7,04	0,44	4,77E-01	0,50	3,33
CCL3_bis	4,97	0,52	5,02	0,72	4,79E-01	0,33	2,22
IL6	3,12	0,63	3,08	0,63	4,81E-01	0,09	1,40

TNF_R2	4,93	0,33	4,90	0,33	4,82E-01	0,20	2,33
TCN2	2,62	0,40	2,65	0,44	4,88E-01	0,18	1,40
IL18_bis	7,95	0,91	7,90	0,71	4,89E-01	0,41	3,17
PDL_1	4,69	0,46	4,66	0,42	4,90E-01	0,16	2,06
MEPE	3,75	0,67	3,80	0,70	5,01E-01	0,24	1,62
REG3A	0,79	0,21	0,77	0,22	5,05E-01	-0,09	1,07
NRP1	1,16	0,15	1,15	0,16	5,17E-01	-0,08	1,20
AXL	8,40	0,34	8,38	0,33	5,22E-01	0,45	3,90
THBS2	5,14	0,20	5,12	0,22	5,22E-01	0,18	2,78
LCN2	1,63	0,44	1,66	0,42	5,25E-01	0,10	1,04
ITGB2	6,50	0,39	6,48	0,34	5,28E-01	0,33	2,89
CD40_L	3,91	1,28	3,99	1,33	5,30E-01	0,35	2,14
CCL28	1,91	0,47	1,88	0,41	5,34E-01	0,00	1,02
FCGR3B	3,27	0,51	3,30	0,52	5,36E-01	0,23	1,63
VASN	1,20	0,26	1,18	0,26	5,40E-01	-0,06	1,04
ITGB1BP2	3,27	1,00	3,20	1,12	5,41E-01	0,10	1,57
EGFR	2,57	0,20	2,56	0,22	5,47E-01	0,01	1,61
CD40	10,44	0,55	10,41	0,53	5,52E-01	0,54	4,38
IL_18BP	5,70	0,36	5,67	0,37	5,56E-01	0,21	2,42
SERPINA5	8,69	0,32	8,66	0,66	5,58E-01	0,41	4,60
VCAM1	3,82	0,31	3,81	0,35	5,59E-01	0,13	1,91
IGLC2	6,01	0,48	6,04	0,50	5,60E-01	9,20	6,10
IDUA	3,69	1,04	3,63	0,94	5,61E-01	0,13	1,52
CXCL10	9,61	0,81	9,65	0,86	5,64E-01	0,60	3,73
CDH5	3,84	0,35	3,86	0,41	5,64E-01	0,25	1,98
MBL2	8,11	1,43	8,19	1,30	5,68E-01	12,96	8,83
IL_2RB	1,32	0,22	1,34	0,36	5,68E-01	0,09	1,64
IL2	0,83	0,25	0,82	0,28	5,70E-01	1,02	1,16
CDCP1	3,03	0,61	3,07	0,57	5,70E-01	0,17	1,28
AZU1	3,60	1,04	3,55	0,83	5,76E-01	0,17	1,61
CTSZ	5,23	0,42	5,20	0,39	5,76E-01	0,20	2,19
PAI	5,80	0,89	5,76	0,84	5,81E-01	0,32	2,41
STAMBP	4,05	0,97	4,10	1,00	5,83E-01	0,25	1,75
RAGE	4,46	0,37	4,44	0,34	5,85E-01	0,19	1,96
TIE1	1,23	0,21	1,22	0,21	5,85E-01	-0,05	1,07
MMP_1	10,93	1,49	11,00	1,41	5,97E-01	0,72	4,30
TNF	0,98	0,34	1,00	0,45	6,02E-01	0,05	1,31
t_PA	6,74	0,58	6,77	0,64	6,08E-01	0,49	3,04
CCL16	6,09	0,54	6,11	0,55	6,10E-01	0,37	2,54
SIRT2	3,77	1,16	3,83	1,22	6,12E-01	0,23	1,65
NID1	2,80	0,39	2,82	0,42	6,13E-01	0,17	1,50
IL_10RB	5,94	0,28	5,96	0,36	6,13E-01	0,33	2,74
SERPINA7	3,98	0,37	4,00	0,39	6,14E-01	0,26	1,89

LILRB2	2,89	0,32	2,87	0,35	6,17E-01	3,48	2,60
CD46	2,33	0,49	2,31	0,55	6,21E-01	0,05	1,39
F11	6,64	0,29	6,65	0,32	6,25E-01	0,42	3,29
VEGFD	6,29	0,55	6,27	0,44	6,32E-01	0,36	2,70
ACE2	2,80	0,60	2,77	0,59	6,36E-01	0,09	1,32
PCOLCE	5,25	0,39	5,27	0,41	6,38E-01	0,35	2,37
MB	5,97	0,51	5,99	0,53	6,42E-01	0,41	2,59
CCL23	9,84	0,44	9,86	0,46	6,47E-01	0,58	4,23
CHIT1	6,27	1,40	6,21	1,54	6,48E-01	0,35	2,61
FGF_23	1,65	0,46	1,68	0,48	6,52E-01	0,09	1,11
MMP_10	5,73	0,58	5,70	0,67	6,62E-01	0,25	2,44
CHI3L1	6,34	0,88	6,31	0,82	6,65E-01	0,38	2,73
IL_4RA	1,51	0,46	1,49	0,29	6,74E-01	0,00	0,74
LTBR	3,52	0,31	3,53	0,29	6,76E-01	0,20	1,72
IGFBP3	4,15	0,39	4,17	0,39	6,77E-01	0,26	1,99
SOD1	1,16	0,52	1,18	0,52	6,77E-01	0,05	1,02
TGM2	8,53	0,55	8,55	0,56	6,80E-01	0,60	3,75
PECAM_1	4,20	0,60	4,18	0,55	6,85E-01	0,17	1,80
BETA_NGF	0,36	0,61	0,38	0,38	6,87E-01	-0,02	0,67
CDH1	3,72	0,33	3,73	0,43	6,88E-01	0,23	1,96
MARCO	5,44	0,41	5,45	0,45	6,89E-01	0,35	2,27
OPG2	9,74	0,36	9,73	0,31	6,91E-01	0,47	4,29
IL_10RA	0,56	0,74	0,53	0,72	6,91E-01	-0,07	1,02
BNP	0,36	0,25	0,35	0,22	6,94E-01	1,31	1,04
CPB1	5,06	0,64	5,04	0,60	6,97E-01	0,27	2,20
CCL24	5,63	0,95	5,59	0,96	6,98E-01	0,33	2,43
KIT	3,32	0,35	3,31	0,36	7,08E-01	0,15	1,76
COMP	6,41	0,44	6,43	0,47	7,11E-01	0,36	2,69
CD6	5,10	0,63	5,08	0,52	7,12E-01	0,23	2,02
TR	5,80	0,44	5,78	0,45	7,15E-01	0,31	2,55
ST2	3,72	0,52	3,70	0,45	7,18E-01	0,17	1,62
CD93	10,70	0,39	10,71	0,33	7,19E-01	0,72	4,91
CXCL16	4,57	0,28	4,58	0,29	7,19E-01	0,26	2,26
PTX3	2,01	0,47	1,99	0,39	7,26E-01	0,04	1,01
IL_1RT2	5,81	0,30	5,80	0,31	7,28E-01	7,68	1,76
CR2	5,09	0,49	5,07	0,48	7,30E-01	0,27	2,27
AP_N	4,74	0,29	4,73	0,35	7,31E-01	0,21	2,30
SPON1	0,78	0,23	0,79	0,25	7,31E-01	0,01	1,09
TNFRSF10C	5,73	0,51	5,71	0,49	7,33E-01	0,32	2,55
SELL	7,25	0,29	7,24	0,37	7,36E-01	11,27	7,67
LPL	7,26	0,82	7,29	0,86	7,43E-01	0,49	2,87
UMOD	0,73	0,27	0,74	0,27	7,48E-01	0,00	1,00
TIMD4	3,01	0,41	3,03	0,48	7,49E-01	0,18	1,64

PROC	3,92	0,36	3,93	0,43	7,50E-01	0,23	2,02
IL_17A	1,03	0,46	1,04	0,46	7,51E-01	0,03	1,00
VEGFA	9,07	0,58	9,05	0,57	7,53E-01	0,48	3,75
TNC	1,56	0,43	1,58	0,45	7,58E-01	0,07	1,09
ADA	1,66	0,53	1,68	0,57	7,58E-01	0,07	1,16
CXCL11	7,76	0,89	7,73	0,96	7,62E-01	0,39	2,98
MCP_3	1,72	0,41	1,73	0,47	7,65E-01	0,07	1,23
ST1A1	3,23	1,26	3,27	1,35	7,67E-01	0,18	1,60
PI3	3,46	0,57	3,49	0,91	7,68E-01	0,23	2,07
IL_27	4,06	0,43	4,08	0,47	7,76E-01	0,25	1,89
CA3	-0,23	0,25	-0,22	0,32	7,76E-01	-0,05	1,37
PSP_D	2,52	0,66	2,53	0,61	7,76E-01	0,14	1,25
MEGF9	1,84	0,26	1,84	0,30	7,83E-01	0,08	1,30
PIgR	5,73	0,24	5,73	0,22	7,84E-01	0,27	2,90
CD244	6,60	0,53	6,59	0,46	7,89E-01	0,33	2,71
CX3CL1	5,87	0,44	5,88	0,42	7,93E-01	0,31	2,41
IL7	2,42	0,75	2,40	0,83	7,93E-01	0,06	1,30
CCL4	5,83	0,74	5,81	0,76	7,95E-01	0,28	2,31
ICAM1	5,66	0,34	5,65	0,40	7,98E-01	0,29	2,63
Flt3L	9,17	0,46	9,18	0,41	8,04E-01	0,52	3,86
CCL3	5,52	0,69	5,51	0,83	8,13E-01	0,31	2,42
Gal_3	5,56	0,38	5,57	0,32	8,15E-01	0,34	2,53
ANG	7,05	0,37	7,06	0,41	8,15E-01	0,45	3,30
LILRB1	0,55	0,25	0,56	0,25	8,16E-01	-0,02	1,03
C2	4,69	0,65	4,67	0,70	8,17E-01	0,27	2,25
GDNF	1,61	0,45	1,60	0,37	8,21E-01	0,01	0,89
MMP_3	5,73	0,79	5,72	0,68	8,26E-01	0,27	2,11
RETN	6,27	0,55	6,26	0,45	8,32E-01	0,38	2,70
MMP_9	4,69	0,86	4,71	0,76	8,36E-01	0,31	1,99
TIE2	7,96	0,24	7,96	0,30	8,41E-01	0,40	4,18
SERPINA12	2,91	1,24	2,88	1,28	8,42E-01	0,10	1,28
LTBP2	1,01	0,25	1,01	0,26	8,50E-01	-0,03	1,06
Notch_3	3,98	0,39	3,99	0,37	8,50E-01	0,23	1,77
vWF	6,91	0,68	6,92	0,77	8,50E-01	0,41	2,94
CCL18	6,03	0,67	6,04	0,68	8,51E-01	0,41	2,67
OPN	4,51	0,64	4,53	0,75	8,52E-01	0,28	2,02
DNER	8,71	0,25	8,71	0,26	8,53E-01	0,38	4,18
ADAM_TS13	5,08	0,26	5,09	0,29	8,55E-01	0,27	2,63
IgG_Fc_receptor_II_b	2,02	0,66	2,03	0,70	8,56E-01	0,09	1,18
CCL_11	6,76	0,56	6,77	0,66	8,58E-01	0,37	2,72
LIF	0,79	0,39	0,80	0,41	8,62E-01	0,00	1,06
CA4	1,16	0,21	1,17	0,31	8,62E-01	0,01	1,49
ICAM3	1,89	0,31	1,89	0,32	8,66E-01	0,04	1,20

CD4	3,81	0,42	3,81	0,44	8,66E-01	0,21	1,64
IL33	0,60	0,24	0,60	0,30	8,67E-01	-0,01	1,29
Gal_4	2,72	0,40	2,72	0,39	8,70E-01	0,10	1,34
IL_20RA	0,71	0,32	0,71	0,33	8,75E-01	-0,04	1,04
FGF5	0,05	0,32	0,04	0,29	8,77E-01	-0,07	0,97
TNFRSF9	5,97	0,37	5,98	0,40	8,81E-01	0,30	2,63
COL1A1	3,03	0,39	3,04	0,39	8,86E-01	0,13	1,48
TGFBR3	1,13	0,35	1,13	0,33	8,88E-01	-0,01	0,96
HGF	8,31	0,51	8,30	0,44	8,89E-01	0,44	3,46
SRC	6,45	1,04	6,43	1,02	8,90E-01	0,43	2,83
PARP_1	2,35	1,08	2,34	0,98	8,93E-01	0,08	1,18
C1QTNF1	3,23	0,49	3,23	0,68	8,93E-01	0,20	1,81
EPHB4	4,52	0,25	4,51	0,27	8,94E-01	0,20	2,32
CPA1	4,47	0,70	4,46	0,62	8,99E-01	0,26	1,93
CST5	6,28	0,54	6,28	0,50	9,10E-01	0,31	2,47
TGFBI	7,56	0,36	7,55	0,44	9,12E-01	0,44	3,57
IGFBP6	3,89	0,29	3,88	0,35	9,15E-01	0,18	2,02
CTSD	4,25	0,43	4,26	0,42	9,15E-01	0,24	1,92
PRSS8	8,59	0,45	8,59	0,42	9,16E-01	0,54	3,87
IL_1ra	4,06	0,89	4,07	0,78	9,18E-01	0,24	1,67
TLT_2	4,98	0,40	4,98	0,40	9,19E-01	0,29	2,26
QPCT	1,65	0,30	1,65	0,34	9,26E-01	0,05	1,20
SELE	12,18	0,64	12,18	0,63	9,28E-01	0,86	5,38
DECR1	5,14	1,57	5,13	1,66	9,30E-01	0,32	2,30
CD59	1,91	0,26	1,92	0,35	9,30E-01	0,05	1,55
OSM	5,06	1,05	5,07	0,87	9,33E-01	0,29	2,03
PRSS2	1,71	0,47	1,70	0,40	9,34E-01	0,04	0,95
LIF_R	2,65	0,25	2,65	0,26	9,34E-01	0,07	1,46
ANGPTL3	2,74	0,40	2,73	0,50	9,37E-01	0,11	1,60
CSF_1	8,72	0,28	8,72	0,27	9,39E-01	0,42	4,04
IL1_alpha	0,54	0,66	0,54	0,81	9,46E-01	-0,03	1,31
BLM_hydrolase	5,27	0,47	5,27	0,42	9,47E-01	0,32	2,30
PGLYRP1	6,90	0,74	6,90	0,60	9,54E-01	0,45	2,91
AXIN1	1,99	0,99	2,00	1,11	9,56E-01	0,08	1,35
IL2_RA	3,75	0,45	3,75	0,42	9,60E-01	0,19	1,70
FGF_19	7,34	0,83	7,34	0,92	9,60E-01	0,40	2,93
DEFA1	2,13	0,32	2,13	0,43	9,66E-01	0,07	1,50
PRELP	5,50	0,33	5,51	0,39	9,69E-01	0,32	2,47
LILRB5	3,34	0,78	3,34	0,76	9,70E-01	0,14	1,38
TFPI	9,18	0,34	9,18	0,34	9,73E-01	0,56	4,40
JAM_A	5,04	0,97	5,04	0,94	9,75E-01	0,33	2,25
REN	7,03	0,67	7,03	0,80	9,80E-01	0,46	3,08
CSTB	4,74	0,59	4,74	0,54	9,82E-01	0,28	2,09

IL6_2	2,61	0,66	2,61	0,65	9,84E-01	0,10	1,23
CD84	4,37	0,55	4,37	0,55	9,87E-01	0,25	1,91
TRAIL_R2	4,52	0,36	4,52	0,62	9,89E-01	0,24	2,65
CST3	6,62	0,37	6,62	0,39	9,91E-01	0,39	3,10
IL5	1,44	1,26	1,44	1,06	9,94E-01	0,03	0,88
IL4	0,07	0,46	0,07	0,45	9,97E-01	-0,06	1,04

Table 6. Hit immune-inflammatory proteomics predictors of faster common carotid IMT progression.

	Beta	**S.E.**	**Wald F**	**H,R, (95% C.I.)**	**Lower limit**	**Upper limit**	**P value**
IL_17D	0,960	0,438	4,801	2,613	1,107	6,168	**0,028**
CXCL1	0,353	0,173	4,152	1,423	1,014	1,997	**0,042**
TNFSF13B	-0,749	0,376	3,965	0,473	0,226	0,988	**0,046**
IL_13	-0,687	0,368	3,479	0,503	0,244	1,036	0,062
TF	0,729	0,406	3,221	2,074	0,935	4,599	0,073
FABP2	0,285	0,172	2,740	1,329	0,949	1,862	0,098
REG1A	0,322	0,206	2,450	1,380	0,922	2,064	0,118
FCN2	-0,234	0,167	1,969	0,791	0,571	1,097	0,161
MMP7	-0,141	0,105	1,793	0,869	0,707	1,067	0,181
FGF_21	-0,116	0,100	1,325	0,891	0,732	1,085	0,250
IL1RL2	0,341	0,306	1,243	1,406	0,772	2,560	0,265
FS	-0,239	0,228	1,101	0,787	0,504	1,231	0,294
GAS6	0,324	0,358	0,821	1,383	0,686	2,790	0,365
Dkk_1	-0,218	0,261	0,697	0,804	0,482	1,341	0,404
PAM	-0,280	0,402	0,485	0,756	0,343	1,662	0,486
SCF	0,190	0,309	0,379	1,210	0,660	2,217	0,538
EFEMP1	-0,163	0,298	0,300	0,849	0,473	1,524	0,584
TNFRSF13B	0,144	0,273	0,277	1,155	0,676	1,972	0,599
LEP	-0,061	0,119	0,268	0,940	0,745	1,187	0,605
IL16	-0,074	0,191	0,149	0,929	0,639	1,350	0,700
ADM	-0,022	0,079	0,076	0,978	0,838	1,142	0,783
CNDP1	-0,059	0,222	0,070	0,943	0,610	1,458	0,791
THPO	0,112	0,555	0,041	1,119	0,377	3,321	0,840
IGFBP_2	0,024	0,209	0,013	1,024	0,680	1,544	0,909
LDL_receptor	-0,012	0,271	0,002	0,988	0,581	1,681	0,965

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Warmest thanks to my family: parents and sister Madina, Nikolai and my friends for the support and inspiration to move forward.

PhD ACTIVITY REPORT

During my PhD programme, conducted at the Epidemiology and Preventive Pharmacology Service (SEFAP) of the Department of Pharmacological and Biomolecular Sciences, I have gain experiences and acquired/improved skills related to:

- Integrate and use the knowledge acquired in order to developing interdisciplinary and interpopulation studies in the field of atherosclerosis, lipid disorders with a genetic background, including FH.
- As a physician - performing physical and instrumental examination of FH patients within both registries, including traditional subclinical atherosclerosis methods (ultrasound measurement of carotid arteries, ankle brachial index, pulse wave velocity) as well as the new one (ultrasound measurement of the Achilles tendons, CAC score evaluation) and the analysis of the patient profiles based on geno- and phenotype data.
- Understanding of the molecular background of the FH, including a process of a clinical and genetic data relevance analysis for the 3rd type of the mutation patients based on the family tree data and the severity of the phenotype.
- Bibliographic search, review of scientific literature (through PubMed, EMBASE and Web of Science databases) and interpretation of FH data.
- Design, conduction (including enrolment, patient management) and analysis of the studies (using SPSS software) related to the atherosclerosis and genetic lipid disorders, including FH.
- Mentoring activity for student theses.

My research activity during the PhD was mainly focused on the approaches that could be considered perspective in terms of improvement the identification of FH above the current diagnostic algorithms and cardiovascular risk stratification, as well as pheno- and genotype particularites of FH patients in terms of interpopulation differences. To manage interpopulation and sufficient in a sense of numbers and representativeness FH study an official agreement between Milan University (Milan, Italy) and Almazov National Medical Research Centre (Saint Petersburg, Russia) was conducted in 2018. Having a possibility to work as a study coordinator and a physician in a clinical Centre of Atherosclerosis in Saint Petersburg and to perform my PhD project in the Department of Pharmacological and Biomolecular Sciences in Milan University that is focused not only in clinical aspects of FH but also in fundamental studies in this area, I have also enhanced my knowledge on gene and other fundamental aspects, such as omics technologies, related to the cardiovascular risk assessment in genetically predisposed lipid disorders, and experienced the conduction of study that was aimed to answer clinical questions to fill evidence gaps in the area of risk stratification and better identification of FH patients.

Besides this project, during my PhD course, I have also collaborated with:

- The research group of Professor Elena Vasichkina, MD, DSc, Chief Researcher of the Research Department for CVDs in Children (Almazov National Medical Research Centre) in order to promote the management of children before 18 years old within the registry and the first line degree analysis. In this context, I could gain experience in the practical issues on lipid-lowering therapy and treatment of children before 18 years old.
- The Chief cardiologists and lipid teams of 4 Russian regions (within the EAS “Lipid Clinics Network” project):

- Samara region, Samara, Chief Cardiologist Dmitriy Duplyakov
- Chuvashia Republic, city Cheboksary, Chief Cardiologist Natalia Svetlova
- Novgorod region, city Nizhny Novgorod, Chief Cardiologist, Yulia Gurianova
- Rostov region, city Rostov, Chief Cardiologist Alexey Hripun

in order to create the genetic network in Russian regions to promote genetic testing, a proper management of FH patients and to fulfil the national genetic registry.

- The Chief Veterinarian and all the department of the Almazov centre in order to establish the new scientific direction and work on experimental lipid disorders models in Almazov centre: LDLR and PCSK9 knockout mices, zebrafish to improve understanding of particularities in atherosclerosis process based on different genetic background and to create a background for a search of new pharmacological approaches in atherosclerosis treatment.

During my PhD programme, I attended a number of congresses and conferences (outlined below), at national and international level, in the belief that sharing experiences with other research groups working on the same topic of interest is a valuable key point to broaden knowledge and develop and optimize research practices.

Date	**Title of contribution**	**Site**
November 25th 2021	The Latest Russian Clinical Guidelines on Dyslipidemia. Basic principles ***Invited speaker***	World Heart Federation Roundtable on Cholesterol, online

November 25th 2021	Lipid Clinic Network in Russia. The Concept ***Invited speaker***	World Heart Federation Roundtable on Cholesterol, online
November 11th - 12th 2021	Lysosomal acid lipase deficiency – the disease that often masks by familial hypercholesterolemia ***Invited speaker***	Conference “Rare diseases: diagnosis, treatment and rehabilitation 2021”
June 30th May-2nd 2021	Lipid Clinics Network. Rationale and design of the EAS global project ***Poster presentation***	The 89th EAS Congress. Virtual Edition
June 30th May-2nd 2021	Prevalence and relationship between metabolic syndrome and risk of cardiovascular disease: Evidence from two population-based studies ***Poster presentation***	The 89th EAS Congress. Virtual Edition
April 11th - 14th 2021	Subclinical vascular damage in patients with severe dyslipidemia depending on blood pressure levels: 5-year follow-up results (ID:1575)	ESH-ISH *ON-AIR* Joint Meeting

	E-poster presentation	
October 21st - 23rd 2021	Cardivascular risk management in dyslipidemias within primary prevention. Role of decision support system. ***Oral presentation***	Russian Cardiology Congress 2021
December 18th 2020	Lysosomal acid lipase deficiency - genetically determined dyslipidemia ***Invited speaker***	Conference "Rare diseases: diagnosis, treatment and rehabilitation 2020"
September- October 29th – 01st 2020	Patients with lipid metabolism disorders. Strategic approaches to the problem solution ***Oral presentation***	Russian Cardiology Congress 2020
February 23rd 2020	Familial hypercholesterolemia – epidemiology, diagnosis, screening programmes ***Invited speaker***	Advanced Course in Rare Lipid Disease, EAS educational programme
May 26th - 29th 2019	Early subclinical atherosclerosis and Lp(a): is there an association in Russian population? ***Poster presentation***	The 87th EAS Congress. Maastricht, the Netherlands

September 24th – 26th 2019	Predictors of early vascular aging syndrome in a population sample (5-year follow-up results) ***Oral presentation***	Russian Cardiology Congress 2019

Moreover, in the last three years, I followed a series of seminars, conferences and educational courses (listed below), as a speaker and attendee in order to keep on training, sharing and updating my knowledge:

- International Atherosclerosis Research School 2019, Prague, August 17th – 23rd, 2019
- World Heart Federation Roundtable on Cholesterol, November 25th 2021
- Conference "Rare diseases: diagnosis, treatment and rehabilitation 2021", November 11th - 12th 2021
- The 89th EAS Congress. Virtual Edition, June 30th May-2nd 2021
- ESH-ISH *ON-AIR* Joint Meeting, April 11th - 14th 2021
- Russian Cardiology Congress 2021, October 21st - 23rd 2021
- Advanced Course in Rare Lipid Disease, EAS educational programme, February 23rd 2020
- Russian Cardiology Congress 2020, September-October 29th – 01st 2020
- The 87th EAS Congress. Maastricht, the Netherlands, May 26th - 29th 2019

Furthermore, I was involving in dissemination activity through RicercaMix blog of the Department of Pharmacological and Biomolecular Sciences of the University of Milan (www.ricercamix.org).

Finally, I am a reviewer of:

- "PLOS ONE" since 2020
- "Frontiers in Cardiovascular Medicine" since 2021

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