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FAMILIAL HYPERCHOLESTEROLEMIA IN SWEDEN: GENETIC AND CLINICAL STUDIES

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Familial hypercholesterolemia in Sweden – Genetic and clinical studies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Peter Benedek

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To my mother, Karin Benedek, who inspired me to always try my best!
I miss you every day.

Student: Professor, Aren´t these the same questions as last year´s final exam on Genetics?

Professor: Yes; But this year the answers are different!

ABSTRACT

Prevention of premature disease and death from cardiovascular complications of atherosclerosis is an important goal for public health, and the early identification of individuals with increased risk is an important goal of modern medicine. Familial hypercholesterolemia (FH) is one of the most common monogenic diseases (1/250 to 1/300) where strong evidence of positive health effects of intervention has been established. In afflicted families, 50% of children or siblings of a carrier are expected to inherit the disease. FH is generally the consequence of reduced function of the LDL receptor pathway, either due to mutations in the *LDLR* gene itself or to mutations in the *APOB* or *PCSK9* genes. Screening in a family where the molecular defect is known is relatively simple, but the large number of different FH-causing mutations makes genetic analysis complex. Consequently, most patients with FH are currently not diagnosed, and thus excluded from life-saving therapy.

Aims of this thesis:

(a) Investigate if genotyping could be a simpler and less expensive alternative to sequencing when searching for the molecular defect in Swedish patients with suspected FH, and to describe the mutation pattern in this population including the frequency of mutation-negative patients with this phenotype.
(b) Study the prevalence of FH in hypercholesterolemic patients with acute coronary syndrome.
(c) Evaluate the possible role of polygenic inheritance in patients with the FH phenotype.
(d) Use deep exome sequencing to explain a unique aggregation of homozygous FH in a family.

Results:

(a) We constructed a genotyping panel by selecting 113 FH-causing mutations and investigated 300 unrelated Swedish patients with suspected FH. Complementary sequencing was performed in those not diagnosed by genotyping. Altogether 81% of those with mutations were identified using the platform; and ten mutations accounted for more than 50% of those patients.
(b) In 116 patients with acute coronary syndrome and cholesterol ≥ 7 mmol/l, 8 (6.9%) had a mutation in the *LDLR* gene causing FH. This was more frequent at higher LDL levels.
(c) We evaluated the influence of a polygenic 12-SNP score for LDL cholesterol in 88 mutation-negative patients and 57 mutation-positive patients with probable FH. Although the negative cohort had a slightly higher score, there was no correlation between LDL cholesterol and SNP score in either cohort.
(d) With the use of haplotype construction from the heterozygous mother and her four homozygous children we made the postmortem diagnosis of homozygous FH in the father, explaining the extreme aggregation of this rare condition. Measurements of plasma markers of cholesterol and bile acid production showed that they are both normal in homozygote FH.

Conclusions:

Genotyping can be used as a cost-effective first step in the molecular diagnosis of patients with suspicion of FH. The prevalence of FH is high among hypercholesterolemic patients with acute coronary syndrome. Polygenic mechanisms of inheritance are not relevant in explaining the FH phenotype where no mutation in the *LDLR*, *APOB* or *PCSK9* genes can be found. Deep exome sequencing is useful to characterize genotype/phenotype patterns in rare forms of dyslipidemia and should now be applied in the search for novel mechanisms behind the "mutation-negative" FH phenotype in the Swedish population.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to be their Roman numerals:

I. P. Benedek, H. Jiao, K. Duvefelt, T. Skoog, P. Kiviluoma, J. Kere, M. Eriksson, B. Angelin.
Founder effects facilitate the use of a genotyping-based approach to molecular diagnosis in Swedish patients with familial hypercholesterolaemia.
Journal of Internal Medicine, 2021, 290: 404-415.

II. P. Benedek, M Eriksson, K Duvefelt, A Freyschuss, M Frick, P Lundman, L Nylund, K Szummer.
Genetic testing for familial hypercholesterolemia among survivors of acute coronary syndrome.
Journal of Internal Medicine 2018, 284:674-684.

III. P. Benedek, H. Jiao, K. Duvefelt, P. Kiviluoma, J. Kere, M. Eriksson, and B. Angelin.
Defining molecular defects in patients with phenotypic familial hypercholesterolemia: minor influence of polygenic SNP score on LDL cholesterol levels in Swedish patients.
Manuscript

IV. H. Jiao, P. Benedek, T. Skoog, M. Ghosh Laskar, J. Brinck, J. Kere, M. Rudling, M. Eriksson and B. Angelin.
Aggregation of homozygous familial hypercholesterolemia in a family: Explanation through genetic autopsy using deep exome sequencing.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette 1
ACAT 1	acetyltransferase 1
ACMG	American College of Medical Genetics
ACS	acute coronary syndrome
ADH	autosomal dominant hypercholesterolemia
ANGPTL3	angiopoietin-like protein 3 inhibitors
APO-A1	apolipoprotein A1
APO-B	apoB gene
Apo-C-III	apolipoprotein C-III
ARH	autosomal recessive hypercholesterolemia
ASCVD	atherosclerotic cardiovascular disease
CM	chylomicrons
CNV	copy number variations
CV	cardiovascular event
ddNTP	dideoxynucleotide triphosphates
DLCN	Dutch Lipid Clinic Network
ER	endoplasmic reticulum
ESC	European Society of Cardiology
FH	familial hypercholesterolemia
FLPC	Fast liquid protein chromatography
GLGC	Global Lipid Genetic Consortium
GWAS	Genome Wide Associations Studies
HDL	high density lipoproteins
HGP	Human Genome Project
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-coenzyme A
ICAM-1	intercellular adhesion molecule 1
LOF	loss of function
Lp(a)	Lipoprotein a
LPL	lipoprotein lipase
M-	mutation negative
M+	mutation positive
MALDI-TOF	matrix-assisted laser desorption-ionization time of flight
MCP-1	monocyte chemoattractant protein-1
MTP	microsomal triglyceride transfer protein
NGS	next generation sequencing
NPC1L	Niemann-Pick C1 Like 1
PCR	polymerase chain reaction
PCSK-9	proprotein convertase subtilisin/kexin type 9
PRR	pattern recognition receptor
SNP	single nucleotide polymorphism
SR-A1	scavenger receptor A1
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low-density lipoproteins

1 INTRODUCTION

Atherosclerosis is one of the major causes of vascular disease in the Western world. The impact of LDL-cholesterol on the vessel wall together with the following inflammatory cascade that takes place in the endothelial cells are the key elements in its formation. Even if the mortality and incidence of its manifestations, ischemic heart disease, ischemic stroke and peripheral arterial disease have decreased since the middle of the 20th century, cardiovascular disease is still the major cause of death globally [1, 2].

Familial hypercholesterolemia (FH) is an autosomal dominant inherited disorder closely associated with early-onset cardiovascular disease and death [3]. Phenotypically, FH is characterized by elevated LDL cholesterol, in some cases tendon xanthomas and/or arcus senilis. Early detection and treatment are crucial in preventing these complications. It has been estimated that 50 % of individuals with untreated FH will suffer an early myocardial event and only 30 % will survive their 70th birthday [4, 5]. However, with early initiation of lipid-lowering therapy and effective LDL-cholesterol reduction, cardiovascular events and early death can be reduced by approximately 40 %. [6]. When diagnosed and effectively treated already in childhood the risk of an early cardiovascular event is not different from that of a healthy sibling [7]. FH is a common disorder in its heterozygous form (1/250-300) [8]. The disease is under-diagnosed, and most cases are discovered after the first cardiovascular event (CV). Even then, the patients´ hypercholesterolemia may not arouse the suspicion of FH. The most effective way to find patients with FH is "cascade screening" within the family after a proband is diagnosed or when patients are diagnosed in primary care [9]. Only approximately 10% of individuals with FH are thought to have been identified in Sweden at present [10].

It has been estimated that 40 % of all deaths in Sweden are related to cardiovascular disease [11] and the latest guidelines from Socialstyrelsen (the Swedish National Board of Health and Welfare) strongly recommend that efforts should be made to improve treatment and diagnosis of FH. Swedish healthcare has been urged to identify 80 % of the FH-population before the year 2025. Furthermore, it is being emphasized that genetic testing is of great relevance as a diagnostic tool and should be offered at least to the proband within the family [12].

The genetical aspects of FH are quite complex compared to many other autosomal dominant disorders. Most often, FH is caused by a mutation in the *LDLR*-gene, more rarely in the genes coding for *APO-B 100* or *PCSK-9*. More than 1500 different mutations in the *LDLR*-gene alone have been described [13] or at least more than 1000 when applying the classification according to American College of Medical Genetics (ACMG) [14]. Moreover, even in the case of a well characterized phenotype and very high clinical probability of FH, a mutation is identified only in approximately 60-70% of the cases [15]. There are probably several explanations for this. The patient may be suffering from familial combined dyslipidemia but having triglyceride levels within the normal range at the time of testing. The patient's phenotype could over-emphasized due to a family history of early cardiovascular event that

may have other causes than hypercholesterolemia. Recently, there are also studies suggesting that even some forms of FH may have polygenic causes [16]. Finally, FH could be caused by mutations in genes that have so far not been recognized to be associated with cholesterol metabolism. Thus, importantly, genetic diagnostics in FH can only be used to confirm the disease, not to exclude it.

2 BACKGROUND

Cholesterol and the regulation of its metabolism

Cholesterol is of vital importance as a structural component of all cellular membranes, and serves as a precursor of steroid hormones and bile acids[17]. In the nervous system, cholesterol is important in the formation of myelin. Most of the body cholesterol (90%) is found intracellularly, while a smaller proportion circulates in plasma lipoproteins (see below). The total amount of cholesterol in the body is determined by the balance between the input as dietary cholesterol plus de novo synthesis and the output mainly through excretion via the bile, either directly or after conversion to bile acids (fig.1). In the gut, dietary and biliary cholesterol is mixed with bile acids and approximately 30-50% is absorbed and transported to the liver in lymph lipoproteins, while the rest is lost in feces [18]. In each cell, there is a delicate regulation of cholesterol levels by sensing the intramembranous free cholesterol levels via the SCAP-SERBP2 pathway which controls the expression of both LDL receptors and HMG-CoA reductase [19] .

Fig. 1 Overview of cholesterol metabolism [20]

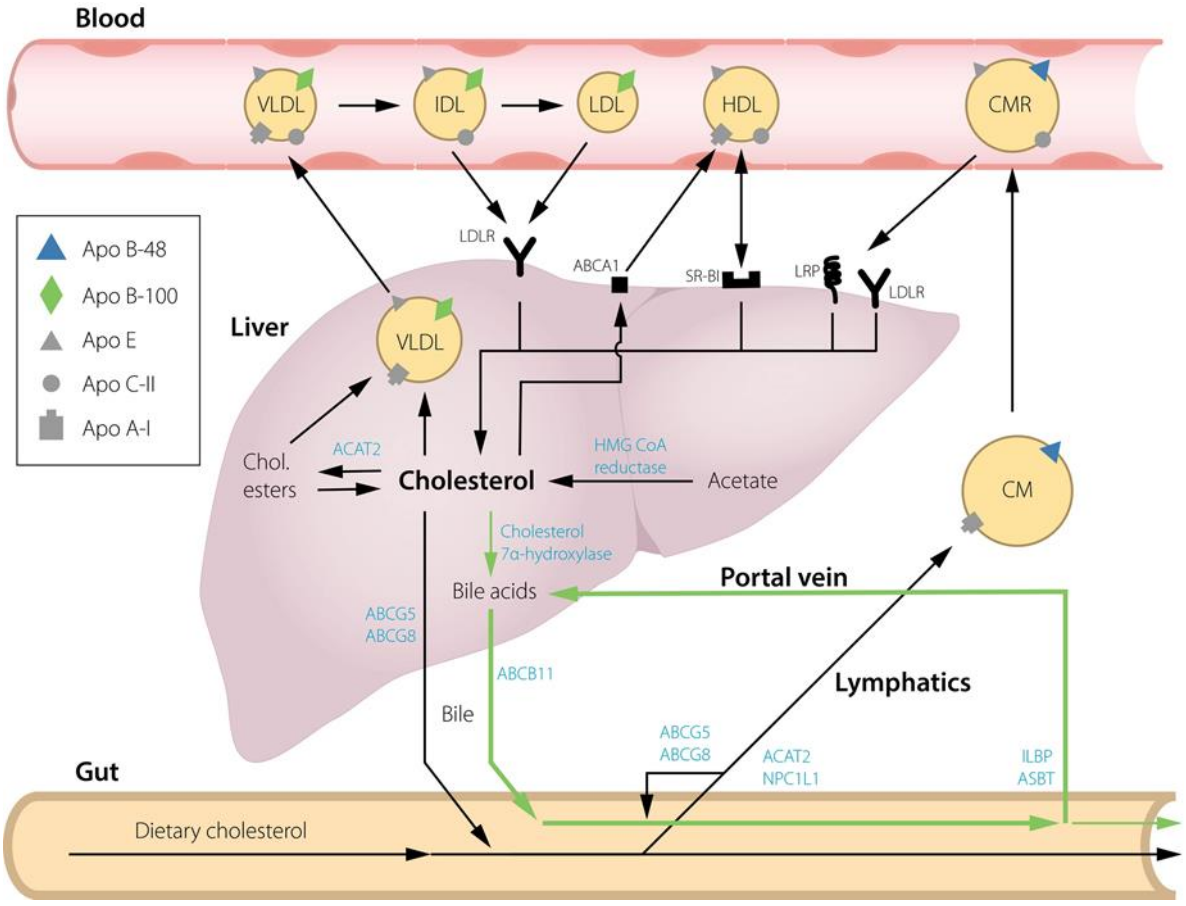
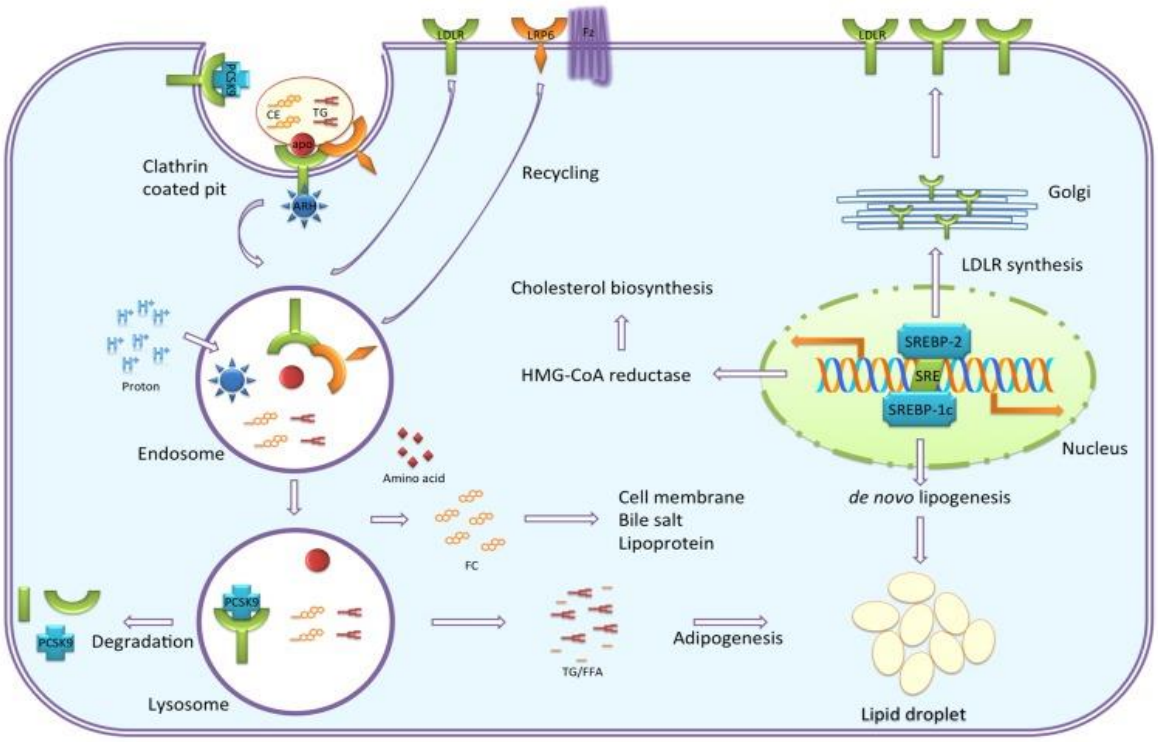


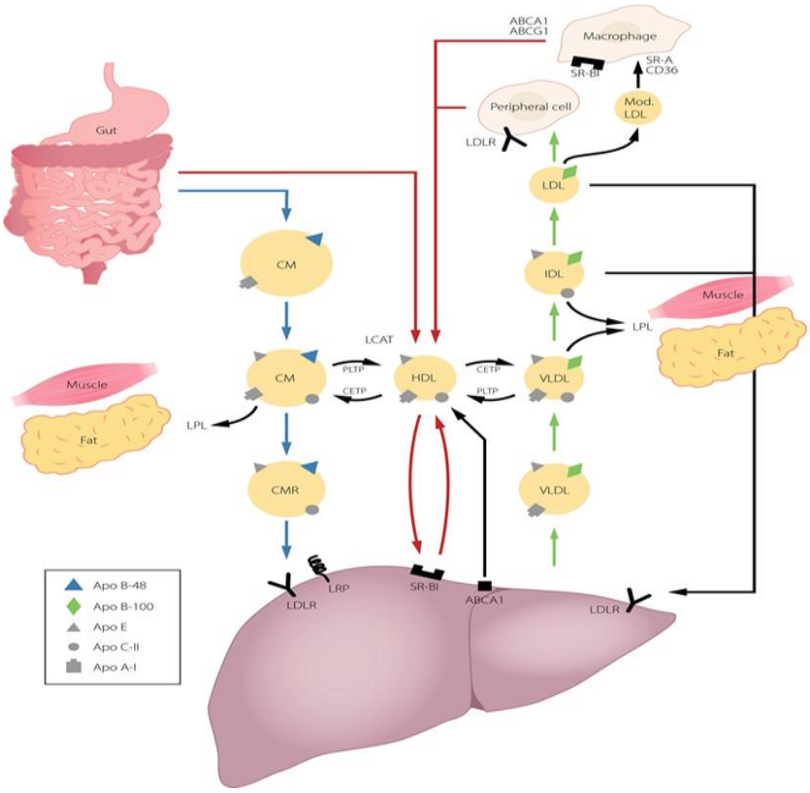
Fig. 2 Cellular homeostasis of cholesterol [21]



The *LDLR-gene* is located on chromosome 19p13.1-13.3 and consists of ~45 kb and 18 exons. It encodes the LDLR, an 860-amino acid type I, transmembrane protein which includes a signal sequence of 21 amino acids [22]. LDL-receptors are present in all cells but are more abundant in cells with steroid hormone production and in hepatocytes [20]. The LDLR mediates the uptake of the cholesteryl-ester-containing LDL-particles via clathrin-mediated endocytosis. After the internalization of the LDLR/LDL-particle complex, the ligand is liberated in the endosome and while the LDLR recirculates back to the cell surface, cholesterol is made available from the cholesteryl esters by hydrolysis in the lysosome. The expression of LDLR is dependent on the level of intracellular cholesterol. As a response to low levels, sterol regulatory element-binding proteins (SREBPs) are transported from the ER to the Golgi [23]. When released from the Golgi membrane and translocated to the nucleus, SREBP activates the transcription of the *LDLR*-gene as well as HMG-CoA reductase. Folding of newly synthesized LDLR-molecules are initiated in the ER before being transported via the Golgi complex to the plasma membrane (Fig 2).

Proprotein convertase subtilisin/kexin type 9 (PCSK-9) promotes LDLR degradation by binding to the LDLR. The PCSK-9/LDLR complex is internalized and directed to the lysosome in which the LDLR is degraded. PCSK-9 thereby inhibits the recycling capacity of LDLR, reducing the number of functional LDLR at the cell surface. “Gain of function” mutations in the *PCSK-9 gene*, expressed mainly in the liver, is a well-known cause of FH [24].

Fig. 3 Overview of lipoprotein metabolism [20]



Lipoproteins and their transport

Cholesterol and other lipids are water insoluble and are therefore transported in lipoproteins in the circulation.[25] The lipoproteins have an outer layer which is hydrophilic and mainly consists of free cholesterol and phospholipids, while its core is hydrophobic, consisting of mainly triglycerides and cholesterol esters. The main lipoproteins are divided according to their density, size and physiological function into: chylomicrons (CM), very low-density lipoproteins (VLDL), LDL and high density lipoproteins (HDL). Lipoprotein transport can be divided into the exogenous and endogenous pathways. In the exogenous pathway, the intestinal chylomicrons are formed by the interactions of triglycerides, esterified and non-esterified cholesterol, phospholipids and apoB-48, a process facilitated by the actions of microsomal triglyceride transfer protein (MTP) [26]. Chylomicrons are the largest lipoproteins, with a core consisting of apolar triglycerides and cholesterol esters while their surface is made up of free cholesterol, phospholipids, apoB-48, and apoA-1. Through the lymphatic vessels and the thoracic duct, chylomicrons enter the blood vessels where lipoprotein lipase (LPL) hydrolyzes the triglycerides. The remaining cholesterol-rich chylomicron remnants are removed from the blood by the liver (Fig 3).

In the endogenous pathway, apoB-100 rich VLDL particles are hydrolyzed to IDL and LDL by LPL after they have been secreted by the liver. IDL particles have a short half-life in the circulation and LDL represents the end stage lipoprotein after conversion of VLDL. LDL

particles have a half-life of about 2,5 days and are cholesterol rich lipoproteins with an outer coating of apoB-100 [18].

Since excess cholesterol cannot be metabolized in peripheral tissues, its removal is critically dependent on the HDL-pathway of reverse cholesterol transport. HDL particles contain mainly apoA-I, apoA-II and apoE. HDL originates from the liver and the intestine as lipid-poor nascent discoid particles. The cell surface protein ABCA1 mediates the efflux of unesterified cholesterol and phospholipids from the cell to the lipid-poor apoA-I [27]. As the cholesterol within HDL is esterified by LCAT in plasma, the particle increases in size and becomes spherical. Cholesterol-loaded HDL-particles bind to the SR-B1 receptor which mediates selective uptake of cholesterol esters into cells, particularly in the liver [28]. In the circulation, CETP mediates the transfer of cholesterol esters from HDL to VLDL and chylomicrons in exchange for triglycerides. In the liver, hepatic lipase hydrolyzes the triglycerides which may regenerate nascent HDL-particles[18].

Lipoprotein (a) [Lp(a)] is an LDL-like particle containing the unique apolipoprotein (a) bound covalently to apoB-100 on the surface. It has a highly variable size dependent on the number of kringle repeats of the protein. The number of Lp(a) particles and their concentration are inversely related to the number of kringle-coding repeats in the *LPA*-gene [29]. The Lp(a) particles are synthesized in the liver, and the level of Lp(a), which is under tight genetic control, is an independent risk factor for ASCVD [30, 31]. Apart from its atherogenic properties, Lp(a) is also considered as being prothrombotic due to its structural resemblance to plasminogen and high contents of platelet activating factor [32]. Studies has shown that up to 1/3 of patients with heterozygous FH also have elevated levels of Lp(a), which add on to the cholesterol burden and the risk of ASCVD [33].

The development of atherosclerosis

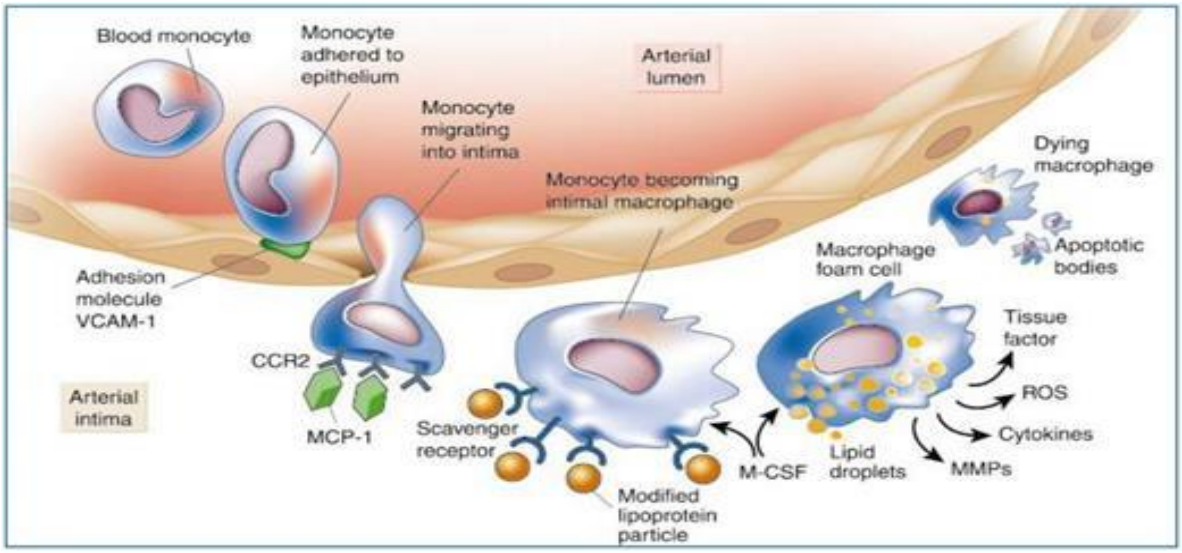
Atherosclerosis can be seen as a chronic inflammatory disease in which monocytes and macrophages together with oxidized cholesterol play a key role in the process of atherosclerotic plaque formation. Lipoproteins may attach to susceptible areas of the arterial wall where the laminar flow is disturbed and thereby influence the state of the endothelium [34]. While in the circulation, lipoproteins such as LDL are rather resistant to oxidative stress. However, it is generally thought that increased oxidative stress occurs in the artery wall and is responsible for the modification of LDL. As a response to the oxidized lipoproteins, endothelial cells secrete the cytokines TNF, IL-1 and IL-6 which generates the pro-inflammatory state and recruits more monocytes to the vessel wall. The modification of lipoproteins will also generate “damage” signals that can be recognized by PRRs (Pattern recognition receptors) expressed by macrophages.

In order to not only attract but also retain the recruited monocytes, activated endothelial cells increase their expression of several adhesion molecules (P-Selectin, VCAM-1, ICAM-1) and chemo attractants (MCP-1) which initiates the leukocyte adhesion cascade. In the intima the monocytes differentiate into macrophages and modified LDL-cholesterol is taken up via

macropinocytosis or by the scavenger receptor A1 (SR-A1) and CD 36 expressed by the macrophages. The resulting lipid-loaded macrophage is called a foam cell and will form the beginning of the atherosclerotic plaque. The inflammatory response is sustained by the foam cells due to their secretion of pro-inflammatory cytokines, IL-1, IL-6 and TNF but also due to the actions of the macrophage retention factors, Netrin 1 and Semaphorin 3 E [35]. SR-A1 and CD36 are two examples of scavenger receptors that in mice have been shown to be important factors in the atherosclerotic process. Mice deficient in both SR-A1 and CD36 showed reduced signs of inflammation and atherosclerosis [36].

In early stages of the atherosclerotic process, the foam cells form a so-called fatty streak lesion in the subendothelial space. As the foam cells grow in number, the degree of inflammation increases, smooth muscle cells and T-cells also infiltrate the intima which further amplifies the retention of oxidized lipoproteins. Cholesterol stored in the cell as esters is rather inert, whereas free cholesterol is considered to be cytotoxic. When the endoplasmic reticulum (ER) becomes enriched with free cholesterol, the esterification by acetyl-CoA acetyltransferase-1 (ACAT 1) in macrophages becomes impaired which leads to further accumulation of free cholesterol causing prolonged ER stress in the macrophages which eventually leads to apoptotic cell death (Fig 4).

Fig. 4 Illustration of the atherosclerotic process [37]



Due to the impaired function of phagocytic clearance (efferocytosis) in the atherosclerotic plaque, an accumulation of cellular debris eventually forms a necrotic core. This will increase the risk of plaque rapture and the formation of a thrombus that could occlude the lumen of the vessel leading to a cardiovascular event.

To counterbalance the accumulation of cholesterol in the foam cell, the efflux of lipids is facilitated by several cellular transporters, such as ATP-binding cassette (ABC)A1(ABCA1), ABCG1 and SR-B1. ABCA1 unloads cellular cholesterol to apolipoprotein A1 (APO-A1),

the precursor of HDL, while ABCG1 facilitates efflux to mature HDL particles. The genes coding for these transporters are upregulated by LXRs when the cellular cholesterol levels increase.

In addition to this mechanism, autophagy plays a crucial role in the efflux of cholesterol from macrophages. In this process called lipophagy, lipid droplets are targeted and hydrolyzed. The autophagosome fuses with the lysosome and degrades cholesterol esters to free cholesterol that becomes available for the above described ABCA1-dependent pathway. Studies have shown that autophagy also has an anti-inflammatory effect and is thereby considered to be atheroprotective.[35, 38, 39]

Familial hypercholesterolemia

Historical background

Between 1925-1938, the Norwegian pathologist Francis Harbitz (1867-1950) published several reports on his findings of the correlation between sudden death and xanthomatosis. Microscopically, he described that the so-called foam cells were more pronounced and characteristic in those patients compared to those suffering from senile arteriosclerosis. In 1937, the Norwegian professor Carl Müller (1886-1983) presented his paper "Angina pectoris in hereditary xanthomatosis" before the Nordic congress of internal medicine. This work was based on his observations in 17 families in Oslo in whom he described the correlation of early-onset cardiovascular events in family members with xanthomatosis and a dominant pattern of inheritance. He also thought the finding was rather common. [40]

Less than 50 years later, Goldstein and Brown received the Nobel prize for their work on describing the cholesterol synthetic pathway and the identification of the LDL-receptor and a defect in its internalization as the cause of familial hypercholesterolemia.

Epidemiology

FH in its heterozygous form is one of the most common monogenic disorders and a leading genetic cause of premature coronary heart disease. The prevalence was initially estimated to be 1/500 while recent studies indicate that a more accurately prevalence of heterozygous FH is 1/250, making it as common as Type 1 Diabetes [10]. World-wide, as many as 34 million people suffer from FH. In certain populations the condition is even more common due to a historical founder effect. Such founder effects are thought to be responsible for the high prevalence of FH amongst Finns, Icelanders, Christian Lebanese, Tunisians, Gujarati South African Indians, Ashkenazi Jews, South African Afrikaners and French Canadians.[41]

The homozygous form of FH is a rare disorder with a historical estimated global prevalence of 1/1 million. However, as suggested by recent studies, the prevalence could be as high as 1/160,000–300,000 [42].

Familial hypercholesterolemia reflects dysfunction of the LDLR pathway

In genetically verified cases of FH, the mutations generally affect the *LDLR*-gene. More rarely, mutations are located to the *APO-B* or the *PCSK-9* genes. In approximately 20-40 % of cases, the suspected diagnosis of FH, despite a well-defined phenotype, cannot be genetically verified. There are several plausible explanations to this which will be further addressed in the next chapter.

The LDL-receptor consists of a mosaic protein of 839 amino acids and is expressed on the cell surface of the hepatocytes. The receptor binds to the APO-B 100 protein of the LDL-particle and removes it from the circulation by internalization of the complex. The most common cause of FH is a mutation in the *LDLR*-gene. More than 1500 different disease-causing mutations in *LDLR*-gene have been described. The impact on the LDLR function of a specific mutation in the *LDLR*-gene can be divided into 5 different classes [43]:

I: The synthesis of the receptor in the ER is impaired
II: Impaired intracellular transport to the Golgi apparatus.
III: Binding of the LDL-particle is affected
IV: Dysfunctional internalization of the receptor-ligand complex
V: Mutations causing receptor recycling defects.

The consequence of the LDLR dysfunction is an increased level of LDL-cholesterol in the circulation and a prolonged half-life in plasma of individual LDL-particles. This in turn increases their exposure time to oxidative stress, rendering them more atherogenic [44].

The human *APO-B* gene, consisting of 29 exons is located on the short arm of chromosome 2 and codes for the formation of both full-length APO-B100 and (following editing of its mRNA) the shorter APO-B48 protein. In humans, APO-B100 is synthesized in the liver and APO-B48 solely in the intestine.

APO-B 100 acts as the ligand of LDL-particles when binding to the LDLR, and mutations affecting the LDLR-binding domain cause an increase in LDL-cholesterol levels in a similar manner as mutations affecting the ligand-binding sites of the LDLR. [45]. However, mutations in APO-B 100 have been described as having a milder phenotype compared with the more common mutations in the LDLR-gene. This may be due to reduced LDL production from VLDL and IDL secondary to clearing of APO-E-containing lipoproteins via the LDLR [46, 47].

A rare cause of FH is “gain of function” mutations in the *PCSK-9* gene. This gene encodes for the proprotein convertase subtilisin/kexin type 9, mainly expressed in the liver and intestine [48]. PCSK-9 binds to the epidermal growth factor (EGF)-like repeat A of the LDL receptor, a region that is crucial for recycling of the LDL receptor from endosomes to the cell surface.[49] Gain of function mutations resulting in high levels of the protease PCSK-9 decreases the number of functional LDL-receptors on the cell surface which leads to an increased level of circulating LDL-cholesterol [24].

Autosomal recessive hypercholesterolemia (ARH) is a rare disorder, caused by a complete loss of function of an adaptor protein (ARH protein) required for receptor-mediated hepatic uptake of LDL-cholesterol. This cytosolic protein, encoded by the *LDLRAP-1* gene, contains a phosphotyrosine binding (PTD) domain, important for the interaction with the cytoplasmic tail of the LDL-receptor in the liver [50]. ARH has previously been described as "pseudo-homozygous FH" and is associated with severely increased plasma LDL-cholesterol, tendon xanthomas and premature cardiovascular events [51].

The ***STAP-1-gene*** was previously implicated as the 4th cause of monogenic FH with an autosomal dominant pattern, presumably with a milder phenotype. The protein encoded by STAP-1 was thought to participate in a positive feedback pathway by upregulating the activity of tyrosine-protein kinase Tec[52]. However, recent studies have shown that *STAP-1* mutations are unlikely to be causative of monogenic FH [53, 54]

Clinical and genetic diagnosis of FH

In its "classic" form, FH is thus an autosomal dominant disease associated with mutations in the *LDLR, APO-B,* or *PCSK-9* genes. The rare recessive form of the disease is caused by mutations in the *LDLRAP-1* gene. However, in practice, genetic verification of FH is complex for several reasons.

Even when only analyzing patients with a very high clinical probability of FH, a mutation is detected in only 60-80 % of the cases [15]. In clinical practice as well as in most studies of FH and genetics, the decision to perform DNA analysis is based on the degree of clinical probability that the individual is suffering from FH. However, there is no international consensus on which patients should be genetically investigated [55]. The clinical diagnosis is based on the presence of high LDL-cholesterol, a family history of hypercholesterolemia, xanthomas or early onset cardiovascular events, presence of tendon xanthomas or arcus senilis on physical examination, as well as early onset cardiovascular disease in the patient. There are currently three generally accepted algorithms that can be used when clinically diagnosing FH, the Dutch lipid clinic network criteria (DLCN), the Simon Broome criteria and the MEDPED criteria.

The DLCN-criteria grade the likelihood that the patient is suffering of FH into unlikely, possible, probable, and definitive, based on a point-scale where a score of ≥ 6 represents probable FH and ≥ 8 definitive FH. LDL-cholesterol levels above 6,5 mmol/l generates 5 p and presence of xanthomas 6 p. 2 p are a given if the patient has suffered an early cardiovascular event or if there is a family history of xanthomas, corneal arcus, or children with high LDL-cholesterol. 1 p is added if there is positive family history of early onset cardiovascular event or of hypercholesterolemia (Fig.5).

Fig. 5 Dutch Lipid Clinic Network (DLCN) score [10]

Criteria	Score
Family History	
First-degree relative with premature coronary and/or vascular disease (men ≤55 years, women ≤60 years), OR	1
First-degree relative with tendon xanthomata and/or arcus cornealis, OR	2
Clinical History	
Patient with premature coronary artery disease (age as above)	2
Patient with premature cerebral or peripheral vascular disease (age as above)	1
Physical Examination	
Tendon Xanthomas	6
Arcus cornealis at age ≤45 years	4
LDL Cholesterol (mmol/L)	
LDL-C ≥8.5	8
LDL-C 6.5 - 8.4	5
LDL-C 5.0 - 6.4	3
LDL-C 4.0 - 4.9	1
DNA Analysis – functional mutation *LDLR*, *APOB* and *PCSK-9*	8
Stratification	Total Score
Definite Familial Hypercholesterolemia	>8
Probable Familial Hypercholesterolemia	6-8
Possible Familial Hypercholesterolemia	3-5
Unlikely Familial Hypercholesterolemia	<3

The Simon Broome criteria only differentiate between possible and definitive diagnosis of FH without using a point-scale, and instead emphasizes the presence of xanthomas in the patient or his/her family, together with LDL-cholesterol > 4,9 mmol/l as its definition of a definitive diagnosis of FH, the same level of LDL-cholesterol is applied for the probable FH diagnosis when occurring together with a positive family history of early cardiovascular events or hypercholesterolemia. The MEDPED criteria are based only on the level of the patient´s total cholesterol or LDL-cholesterol but applies different levels of LDL-cholesterol in diagnosing FH in relation to the age of the patient and whether there are known cases of FH in the family.

The probability of identifying mutations associated with FH in patients with “definitive” FH according to either DLCN or Simon Broome criteria has shown to be very high [15]. However, when analyzing patients with “possible” FH according to the Simon Broome criteria, a mutation was only detected in approximately 13 % [56]. A much higher detection rate was found in patients who were clinically categorized as “probable” FH scored by the DLCN-criteria. Studies have shown a strong correlation between the finding of tendon xanthomas and a positive genetic testing [55]. However, this clinical finding is rather rare in the normal clinical setting and is furthermore dependent on the skills and experience of the physician. In the more detailed DLCN criteria, difficulties in obtaining all relevant

information needed to correctly assess the patient may result in an incorrect estimation of the probability that the patient is suffering from FH [55].

Despite the rapid technological progress of genetic testing paralleled with the decline in cost of analyzing DNA, it is still important to make a thorough clinical investigation before deciding on genetic testing. When considering the high prevalence of FH, the cost of genetic testing still becomes substantial even if only those with a high clinical probability of FH are analyzed. When a mutation is detected in a patient with a clinical diagnosis of FH, it facilitates the further cascade screening of the family, especially in children. However, cascade screening by measuring LDL-cholesterol in relatives of a mutation negative proband is still a useful diagnostic tool to detect those at risk of being affected [9].

Other types of hereditary dyslipidemias with similar phenotypes as FH

Familial combined hyperlipidemia (FCHL)

FCHL is probably an even more common inherited dyslipidemia than FH with an estimated prevalence of 1/100 [57]. The disease is associated with an increased risk of ASCVD but, in contrast to FH, the pathogenesis of FCHL is not well understood [58, 59]. The lipid phenotype in patients with FCHL is variable over time but is generally associated with a combination of raised LDL-cholesterol, hypertriglyceridemia, and low HDL-cholesterol. The metabolic pathophysiology of FCHL is complex, and most probably not the same in all families. Combinations of dysfunctional adipose tissue, delayed clearance of TG-rich lipoproteins as well as overproduction of VLDL, apo-B, and hepatic fat have been described[59]. Since the lipid phenotype is variable, FCHL is sometimes misdiagnosed as FH if the patient at the time of testing has normal levels of triglycerides while having an increased level of LDL-cholesterol.

Polygenic hypercholesterolemia

Genome Wide Associations Studies (GWAS) have so far revealed more than 50 genomic loci associated with increased LDL-cholesterol levels[60]. Based on these loci, several studies have been done using variable numbers of single nucleotide polymorphisms (SNPs), each of which is contributing to a small increase in LDL-cholesterol, to calculate a polygenic score.

As mentioned, in ~20-40 % of patients with a phenotype and a family history strongly suggestive of FH, no causative mutations can be detected by DNA analysis. Over the recent years, the question has been raised whether many of these patients have a polygenic rather than monogenic cause of their hypercholesterolemia. Could a phenotype not distinguishable from that seen in classic FH develop when a certain number of the LDL-cholesterol raising SNP´s is inherited from both parents? [61]. Talmud et al [16], used 12 SNP´s selected by the Global Lipid Genetic Consortium (GLGC) and suggested that up to 60 % of patients with a clinical diagnosis of FH but without a detectable monogenic cause instead suffered from

polygenic familial hypercholesterolemia. Futema et al used a reduced 6-SNP score model and claimed that up to 88 % of mutation negative patients with FH may have a polygenic cause [62]. There is currently little consensus regarding the proportion of mutation-negative patients that may instead have a polygenic explanation for their phenotype. Recent studies indicate that patients with polygenic FH in general may have a lower risk of ASCVD compared to FH-patients with a detectable mutation in either the *LDLR*, *APOB* or *PCSK-9* gene [63-65]. One explanation for this may be that the phenotype seen in polygenic FH is often milder compared with monogenic FH, especially regarding the level of untreated LDL-cholesterol [66] which is one of the major elements in the risk stratification [67]. Another important point is that little is known about the time of onset of the hypercholesterolemia seen in polygenic FH patients, which would affect the level of "cholesterol burden", another crucial factor in the risk of ASCVD [68]. In two smaller studies done on children with a clinical diagnosis of FH without a detectable monogenic cause, there was no relation between the SNP-score and the level of LDL-cholesterol [69, 70].

The estimated difference in LDL-cholesterol between the 1st and 10th decile when using the 12 SNP-panel applied in several of the studies on polygenic FH is 1,1 mmol/l or 24 % of the range [16].This indicates that a top polygenic score can only explain the hypercholesterolemia in patients with a mild phenotype that in fact does not always meet the criteria for a clinical diagnosis of FH, depending on which algorithm used. Several studies have shown a weak correlation between the SNP-score and LDL-cholesterol in mutation negative FH-patients which indicates that further studies on this topic are needed [71, 72].

Sitosterolemia

Sitosterolemia is a rare autosomal recessive metabolic disorder associated with loss of function (LOF) mutations in the ATP-binding cassette (ABC) subfamily G, 5 or 8 (*ABCG5* or *ABCG8* genes) resulting in increased absorption and decreased secretion of plant sterols, mainly sitosterol. Recent studies indicate that sitosterolemia may be more common than previously thought [73]. Homozygous sitosterolemia is associated with tendinous xanthomas, consisting of plant sterols, hypercholesterolemia and premature ASCVD. However, the phenotype is variable, and some carriers may have normal LDL-cholesterol while instead suffering from hemolysis, splenomegaly, platelets abnormalities and arthralgias [74]. Normally, more than 95 % of the dietary plant sterols, (sitosterol, campesterol and stigmasterol) absorbed by the intestine via the Niemann-Pick C1 Like 1 (NPC1L1) protein are re-excreted from the enterocytes by the ABCG5/G8 complex [75]. Biallelic (homozygous) compound heterozygous or double heterozygous (LOF) mutations in ABCG5 and ABCG8 lead to decreased excretion into the intestine and via the bile, and accumulation of plant sterols in plasma. Levels of sitosterol are measured by high-sensitive gas chromatography, and normal levels in males is between 0.99-3.88 µg/mL and 1.03-4.45 µg/mL in females. Levels > 10 µg/mL is suggestive of sitosterolemia and the diagnosis can be confirmed by gene sequencing of the *ABCG5/8* gene.

Ezetimibe together with bile acid sequestrants (resins) are first line therapies [76, 77]. Resins reduce the levels of sitosterol with up to 30% by promoting increased excretion while Ezetimibe, an inhibitor of NPC1L1, decrease the absorption of sitosterol in the intestine and reduces the plasma levels by approximately 20 % in sitosterolemic patients. It has also been shown to act favorable on the platelet count [78]. Statin treatment in sitosterolemia has little effect since the condition is associated with a strong inhibition of the HMG-CoA reductase activity [77].

Cerebrotendinous xanthomatosis (CTX)

CTX is a rare autosomal recessive lipid storage disease with an estimated prevalence of 1/50 000 Caucasians and is caused by mutations in the *CYP27A1*-gene which codes for the mitochondrial enzyme sterol 27-hydroxylase. The mutations lead to decreased synthesis of bile acid and over-production of cholestanol with its subsequent accumulation in tissues. Patients usually develop neurological symptoms such as ataxia, dementia, and epilepsy as well as tendon xanthomas and premature atherosclerosis, despite normal levels of cholesterol. Early diagnosis and treatment with chenodeoxycholic acid improve neurological symptoms and the prognosis of the disease [79].

Methods of genetic analysis and their role in the investigation of hereditary dyslipidemias.

The rapid technological development and lower costs of analyzing individual genes, exomes or whole genomes, allow us to use genetic analysis as a diagnostic tool to a much larger extent than before. However, in a common disease such as FH, which can be caused by many different mutations in more than one gene, the cost of genetic screening becomes substantial. This is not only because of the high prevalence of the disease but also because gene sequencing is most often used as the primary method of analysis. Sequencing of genes is 5-20 times more expensive compared to genotyping for pre-defined mutations.

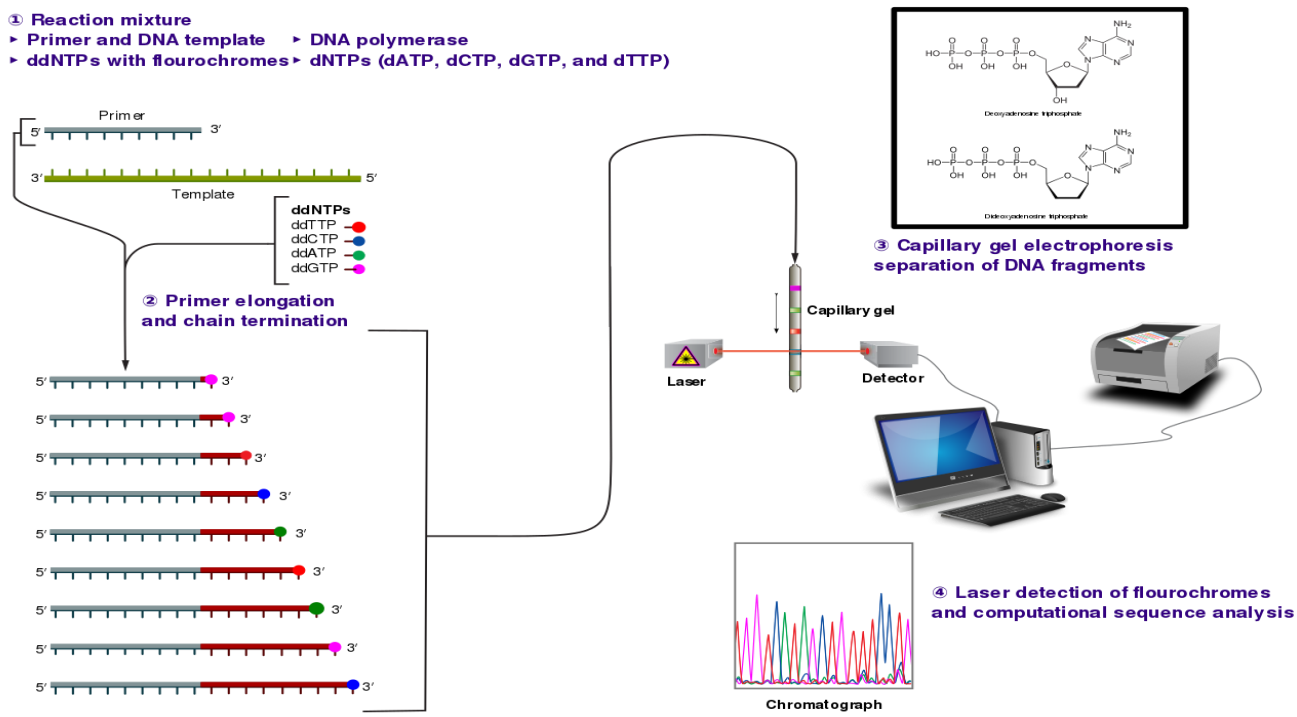
Sequencing of genes

In 2001, the results of the Human Genome Project (HGP) were first published. At a cost of 2,7 billion dollars and after 13 years the human genome had been sequenced for the first time [80]. Since then, sequencing of both individual genes and the whole genome has been repeated millions of times and today there is a large number of databases containing information of the human genome. Genome wide associations studies (GWAS) have greatly increased our knowledge and understanding of many diseases and how the interactions of single nucleotide polymorphisms (SNP´s) in several genes may lead to a higher risk of developing a certain disease [81].

In the sequencing process, labelled dideoxynucleotide triphosphates (ddNTP´s) are added to the reaction mixture containing primer, DNA template, DNA polymerase and normal deoxynucleotide triphosphates. The modified ddNTPs lack a 3´-OH group which prevents the

formation of phosphodiester bonds between two nucleotides. This causes the DNA polymerase to stop the extension av DNA when a ddNTP is incorporated. The labelled ddNTPs can later be detected in automated sequencing machines. In modern sequencing technology, Next Generation Sequencing (NGS), a computer reads the sequence by detecting the unique fluorescent signal being emitted from each base in the segment of DNA (Fig 6). Before the development of NGS, Sanger sequencing used radiolabelled nucleotides and the fragments of the sequences were read manually. By knowing the sequence of the complement strand, the sequence of the template DNA can be read. The resulting sequence is compared with the corresponding sequence in a bioinformatic database, for example NCBI to see if the investigated gene sequence contains a mutation that could be disease causing.

Fig. 6 The principle of gene sequencing (https://commons.wikimedia.org/wiki/File:Sanger-sequencing.svg)



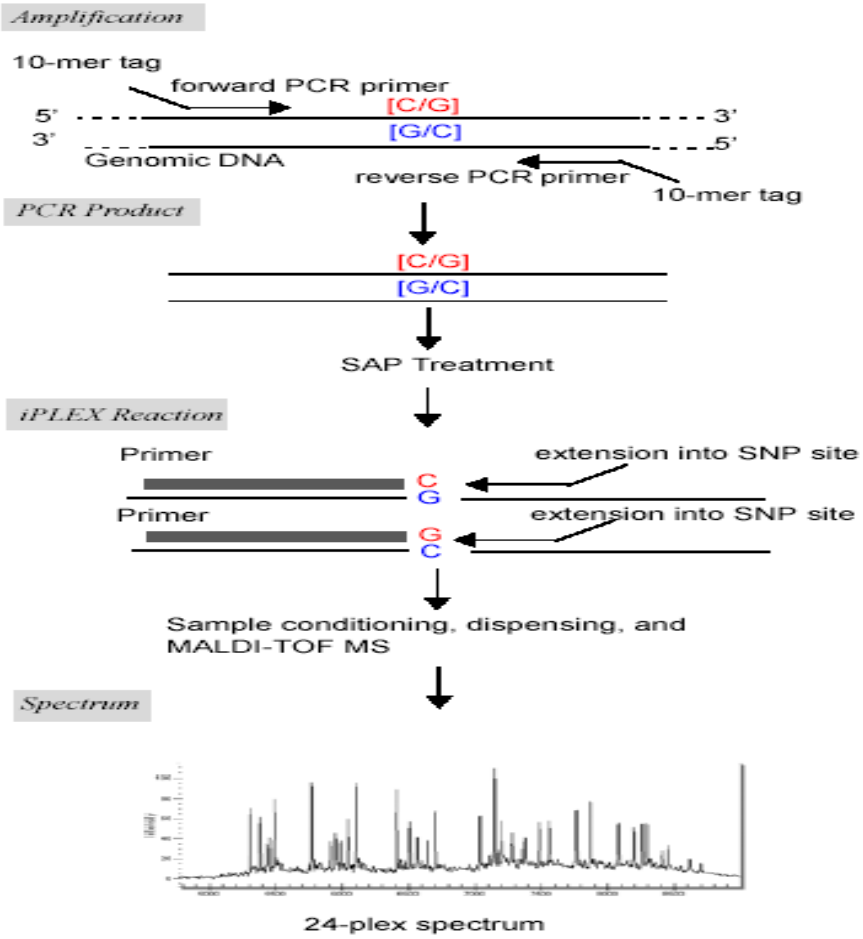
Genotyping

In genotyping, the investigated DNA is directly compared to a pre-defined genotype, for example a disease-causing mutation. By knowing the mutation´s exact location in the gene sequence, an assay can be designed to test the patient's DNA for that particular mutation. The extracted DNA is fragmented using restriction enzymes and then amplified using polymerase chain reaction (PCR). In this stage, the fragmented DNA-strands are separated into single strands. Each strand can then be re-paired using exogenic complementary strands, a process called hybridization. By adding polymerase, an enzyme promoting synthesis of complementary strands, the fragmented DNA is amplified. The DNA fragments for which the sequence is known, make up the probe. The target consists of DNA from the sample to be

investigated. Only strands that carry the complementary sequence of the probe are able to hybridize. By using target sample markers, the result of the analysis can then be read by different techniques depending on the labelling of the sample.

In publications I and II, assays for 112 known FH-causing mutations had been designed and assembled on a panel, based on Single Nucleotide Polymorphism (SNP) genotyping. A SNP is a single base pair mutation at a specific locus that usually consist of two alleles. The genotyping in our studies was based on Primer extension technique during which the probe is hybridized to the bases immediately adjacent to the SNP nucleotide being investigated. DNA polymerase then extends the hybridized primer by adding a base complementary to the targeted SNP nucleotide. The incorporated base can then be detected and determines the SNP-allele [82]. In our studies, Matrix-assisted laser desorption-ionization time of flight (MALDI-TOF) mass spectrometry [83] was used as the detection method. This technique is based on the fact that the different extension nucleotides have different molecular weight (Fig. 7).

Fig. 7 Genotyping based on primer extension and detection by MALDI-TOF mass spectrometry.



Treatment of familial hypercholesterolemia and other dyslipidemias

Early pharmaceutical intervention as well as achieving effective LDL-cholesterol lowering are the main keystones in preventing premature atherosclerotic cardiovascular disease (ASCVD) in individuals suffering from FH [84, 85]. Untreated genetically verified FH-subjects have a 22-fold increased risk of developing ASCVD [86]. In the latest guidelines by European Society of Cardiology (ESC) from 2019, a therapeutic target of 1,8 mmol/l for LDL-cholesterol in primary prevention and 1,4 mmol/l in secondary prevention is recommended. In addition to dietary and life-style changes, statins are the mainstay of treatment. In many cases, despite high-intensity statin therapy the patients fail to reach the treatment target. By combining a statin with Ezetimibe, the LDL-cholesterol may be decreased with another 24% [87]. In children with FH, European guidelines recommend that statin treatment may be initiated from the age of 6 and that enzetimibe may be added from the age of 10 if target level of LDL-cholesterol (< 3,5 mmol/l) is not reached [88]. PCSK-9-antibodies (Evolocumab and Alirocumab) may be prescribed to patients with established ASCVD and/or with heterozygous FH that fail to reach target levels of LDL-cholesterol despite being on maximum tolerated dose of statin in combination with ezetimibe. In double-blinded placebo-controlled trials, PCSK-9 antibodies have been shown to decrease LDL-cholesterol by approximately 60 % in patients already on a combination with a statin and ezetimibe [89, 90].

While most lipid-modulating therapies do not influence Lp(a), the presence of elevated levels (> 125 mmol/l) (ref) are generally taken as an indication for intensified LDL-lowering treatment. Statin treatment has little or no effect in lowering levels of Lp(a); in fact, the treatment may increase the levels by 8-24% [91]. Treatment with PCSK-9 antibodies has been shown to decrease the levels of Lp(a) by 15-30 % [92]. Anti-sense oligonucleotides (ASO) have shown promising results in drug trials and may lower Lp(a) levels with up to 90 % by interfering with LPA mRNA [93]. Both LDL-apheresis and specific LP(a)-apheresis efficiently lower Lp(a) in patients with homozygous FH [94].

New and up-coming cholesterol-lowering drugs

Lomitapide

Inhibits microsomal triglyceride transfer protein leading to decreased VLDL and chylomicron synthesis, resulting in LDL-cholesterol reduction, independent on the LDLR-function. Lomitapide has shown efficient LDL-cholesterol lowering in patients with homozygous FH [95].

Bempedoic acid

Inhibits adenosine triphosphate citrate lyase (ACL) that is an essential enzyme in cholesterol biosynthesis. When hepatic ACL is inhibited intracellular cholesterol becomes depleted, leading to upregulation of LDLR which in turn result in an increased LDL-cholesterol clearance [96]. Outcome trials with bempedoic acid are ongoing.

Inclisiran

Inhibits the translation of PCSK-9 by the action of an interfering ribonucleic acid (SiRNA) that induce PCSK-9 mRNA degradation. In the ORION-9 trial that included 482 heterozygous FH-patients, an LDL-cholesterol reduction by 39,7% was seen when Inclisiran was administrated twice a year, as add on therapy to maximum tolerated dose of statin ± ezetimibe [97].

Angiopoietin-like protein 3 inhibitors (ANGPTL3)

ANGPTL3 is synthesized in the liver and inhibits lipoprotein lipase and endothelial lipase thereby decreasing VLDL and HDL clearance [98]. Naturally occurring loss of function mutations in ANGPTL3 had been noted to be associated with lower levels of triglycerides, LDL-cholesterol and HDL-cholesterol as well as with a lower risk of ASCVD [99]. Evinacumab is a monoclonal antibody against ANGPTL 3 which has been shown to reduce LDL-cholesterol levels of up to 23% and triglyceride levels up to 76 % [100]. ANGPTL 3 inhibition reduces LDL-cholesterol independently of LDLR and is therefore a promising therapeutic agent in patients with homozygous FH. Evinacumab has been granted orphan drug designation for homozygous FH by FDA. Preliminary results from phase 3 trials have shown LDL-cholesterol reductions of up to 49 % in homozygous FH-patients [101].

Gemcabene

A peroxisome proliferation-activated receptor (PPARα) agonist that acts by interfering with mRNA of apolipoprotein C-III (apo-C-III) which in turn results in an enhanced VLDL-clearance and inhibition of the production of fatty acids and cholesterol in the liver. Gemcabene as add-on to stable statin therapy has demonstrated additional reductions in LDL-cholesterol of up to 39% [102] and is given per oral once daily.

Treatment of homozygous FH

The majority of lipid lowering drugs in clinical use are dependent on residual LDLR-function to have any effect on the LDL-cholesterol levels. In some patients with true homozygous FH, compound heterozygotes and double heterozygous, the remaining LDLR-activity enables the use of statins and PCSK-9 inhibitors. However, in many cases, therapeutic target levels cannot be reached and as for homozygous FH patients with no residual LDLR-function, apheresis is the only effective treatment to achieve LDL-cholesterol lowering. Apheresis treatment is apart from being costly, associated with an impact on quality of life and is not always readily available [103]. Liver transplant corrects the genetic defect in the organ most vital for LDL-cholesterol clearance but has disadvantages such as surgical complications and the need for life-long immunosuppressive therapy [103]. Lomitapide, ANGPTL 3 inhibitors and Gemcabene may prove useful in the treatment of patients with homozygous FH, either as an alternative therapy or to widening the intervals between apheresis treatments.

3 AIMS OF THE PRESENT STUDY

The overall aim of this thesis is to gain a deeper understanding of the molecular and clinical aspects of patients with familial hypercholesterolemia in Sweden. With the acquisition of new knowledge and insights in the field of FH, a clinical update concerning diagnostics and treatment can be provided to clinicians on a nation-wide basis.

Specific aims are:

1) To investigate if genotyping, a simpler and more cost-effective method compared with NGS, could be used as a first-step method in genetic testing in patients with a clinical diagnosis of FH in clinical practice.

2) To investigate the prevalence of FH among hypercholesterolemic ACS patients and study if specific clinical parameters may be helpful to discriminate which patients should be referred for genetic testing.

3) To investigate the correlation between level of LDL-cholesterol and polygenic SNP-score in mutation negative FH-patients fulfilling the Swedish clinical criteria for FH.

4) To investigate the unusual aggregation of children with homozygous FH in a family with a heterozygous mother and a deceased father, and to characterize the influence of homozygous FH on cholesterol and bile acid metabolism.

4 MATERIAL AND METHODS

DNA analysis

In papers 1-4, three different methods of DNA-analysis were used:

Exome sequencing

Performed at Science for Life Laboratory, Stockholm, Sweden. DNA libraries for each sample were prepared from genomic DNA and SureSelect Human All Exon V5 target enrichment kit (Agilent Technologies, Santa Clara, CA, USA) was used. The sequencing runs were performed on an IlluminaHiSeq2500 instrument (Illumina, San Diego, California, USA) according to manufacturer's instructions. Major computations were performed at UPPMAX (Uppsala Multidisciplinary Center for Advanced Computational Science, Uppsala, Sweden).

Amplicon sequencing

Performed with Progenikas's Familial Hypercholesterolaemia Genetic Analysis (SEQPRO LIPO S® platform (Progenika Biopharma, Derio,Spain) according to manufacturer's instructions, with the exception that the *STAP1* gene was not analyzed.

Devyser´s FH kit that enables analysis of sequence variants associated with autosomal dominant hypercholesterolemia (ADH). The assay detects substitutions and INDELS in exons and intron-exon boundaries in the LDLR gene (NM_000527.5) exon 1-18, the PCSK9 gene (NM_174936.4) exon 1-12 and the APOB gene (NM_000384.3) exon 26-29. The assay also detects copy number variations (CNV) in the LDLR gene associated with FH. Elaborate proprietary algorithms were used to detect the presence of genomic deletions and duplications in all 18 exons and the promotor region in LDLR. In addition, the method also analyzed the 12 SNPs associated with polygenic hypercholesterolemia. The polygenic SNP-score was calculated as described by Talmud et al [16].

Genotyping

Genotyping was performed at the Mutation Analysis Facility, Clinical Research Centre at Karolinska University Hospital Huddinge, using the Agena MassARRAY (MassARRAYAgena Bioscience, San Diego, California, USA) technology with iPLEX® Gold chemistry, according to the manufacturer's instructions. Of the 124 chosen variants, 117 were straightforward to implement. The FH-Helsinki deletion, was analyzed using a previously published design, with the addition of a positive control assay that enables discrimination of homozygous deletions versus no amplification of the target region. During the study it became evident that five mutations originally reported to be pathogenic had been re-classified as benign, resulting in a total of 112 mutations being analyzed. The genotyping was validated using a set of 14 trio families, in total 42 individuals, with genotype data available through the HapMap consortium. Concordance analyses with the HapMap data as

well as analysis of the parent-offspring-compatibility were performed. The genotyping platform was also validated by analyzing DNA-samples from 21 FH individuals where mutations represented on the platform had been identified using NGS at the Department of Medicine, Sahlgrenska University Hospital, Göteborg (positive controls). All those were identified using our genotyping procedure.

Patients and Ethical considerations

Study subjects in papers I-IV were recruited from various collaborating FH-clinics in Sweden. Each subject was scored according to the DLCN-scale and graded accordingly (Fig 5). All patients had signed an informed consent to participate in the studies after receiving oral and written information. All studies were performed according to the declaration of Helsinki and had been approved by the Regional Ethics committee, Stockholm.

Paper 1

300 unrelated subjects of Swedish origin were included in the study. 119 with a DLCN-score of 3-5 (possible FH), 151 with a score of 6-8 (probable FH) and 30 scoring > 8 (definitive FH). All included subjects were analyzed with genotyping. Subjects in whom no mutation was detected were further analyzed with either Exome sequencing or Amplicon sequencing (Progenika) depending on when they had been included in the study.

Paper II

116 subjects with a personal history of ACS and hypercholesterolemia were included in the study (Fig 12). A total cholesterol of ≥ 7 mmol/l or ≥ 4,9 mmol/l if on lipid lowering medication as well as a triglyceride level ≤ 2,6 mmol/l were set as the definition of hypercholesterolemia. Patients fitting the inclusion criteria were identified using the SWEDEHEART-registry [104] and were invited to enroll into the study via an invitation mail. After enrollment, each subject was interviewed concerning family history of either hypercholesterolemia or premature onset of cardiovascular disease in a first-degree relative. All subjects were scored according to the DLCN-scale and analyzed with genotyping. Those negative for a FH-causing mutation were further analyzed with Amplicon sequencing (Progenika).

Paper III

The 12-SNP-score (Fig 8) were calculated as described by Talmud et al, in 88 mutation negative (M-) and 57 mutation positive (M+) subjects with a DLCN-score of ≥ 6. All subjects were analyzed using Devyser´s FH Kit, Amplicon sequencing as described above. The correlation between LDL-cholesterol level and SNP-score (defined as deciles, 1-10) was studied in both M- and M+ group.

Fig. 8 SNPs used for the calculation of LDL cholesterol score

SNP	Gene	Score
rs2479409	PCSK9	0,052
rs629301	CELSR2	0,15
rs1367117	APOB	0,1
rs4299376	ABCG8	0,071
rs1564348	SLC22A1	0,014
rs1800562	HFE	0,057
rs3757354	MYLIP	0,037
rs11220462	ST3GAL4	0,05
rs8017377	NYNRIN	0,029
rs6511720	LDLR	0,18
rs429358	APOE	0,1
rs7412	APOE	0,1

Paper IV

This paper describes the unusual aggregation of homozygosity for the c.313+5 G>A, *LDLR*-mutation in 4 children to a heterozygous mother of Afghani origin. The daughter, 19 years old at the time, was referred to the outpatient Lipid Clinic of the Department of Endocrinology due to widespread xanthomas and elevated plasma cholesterol. The father and the older brother had been killed in the war at a young age. There were several reports about consanguinity in the family. Further clinical examination revealed the presence of xanthomas in the three living brothers to the index patient, while none were seen in the mother. To identify a FH-causing mutation, the mother and all four children were analyzed with Exome sequencing. To investigate whether the hypothesis that the deceased father had been homozygous for the c.313+5 G>A mutation was true, haplotype construction was done by using Merlin software. To explore if homozygous FH would influence cholesterol and bile acid metabolism, fasting levels of lathosterol and 7alpha-hydroxy-4-cholesten-3-one (C4), biomarkers of cholesterol synthesis and bile acid production were measured.

Biochemical assays

Fast protein liquid chromatography (FPLC) was used to analyze the individual lipoprotein profiles in the four homozygous siblings, before and after treatment with PCSK-9 antibodies [105].

Cholesterol and bile acid metabolism were assessed by measuring fasting levels of lathosterol and 7 alpha-hydroxy-4-cholesten-3-one (C4). C4 was determined by high-performance liquid chromatography and the values were normalized for total cholesterol. Unesterified lathosterol levels were measured by dilution mass spectrometry [106].

Statistics

In paper I, unequal sample size *t*-tests were used to calculate significance of differential age distribution and *p* values were based on two-tailed hypothesis.

In paper II, test of significance was calculated using Wilcoxon rank-sum test for continuous variables and with Fisher´s exact test for categorical variables. Receiver operating curve (ROC) was used to assess the accuracy for discrimination of mutation positive cases.

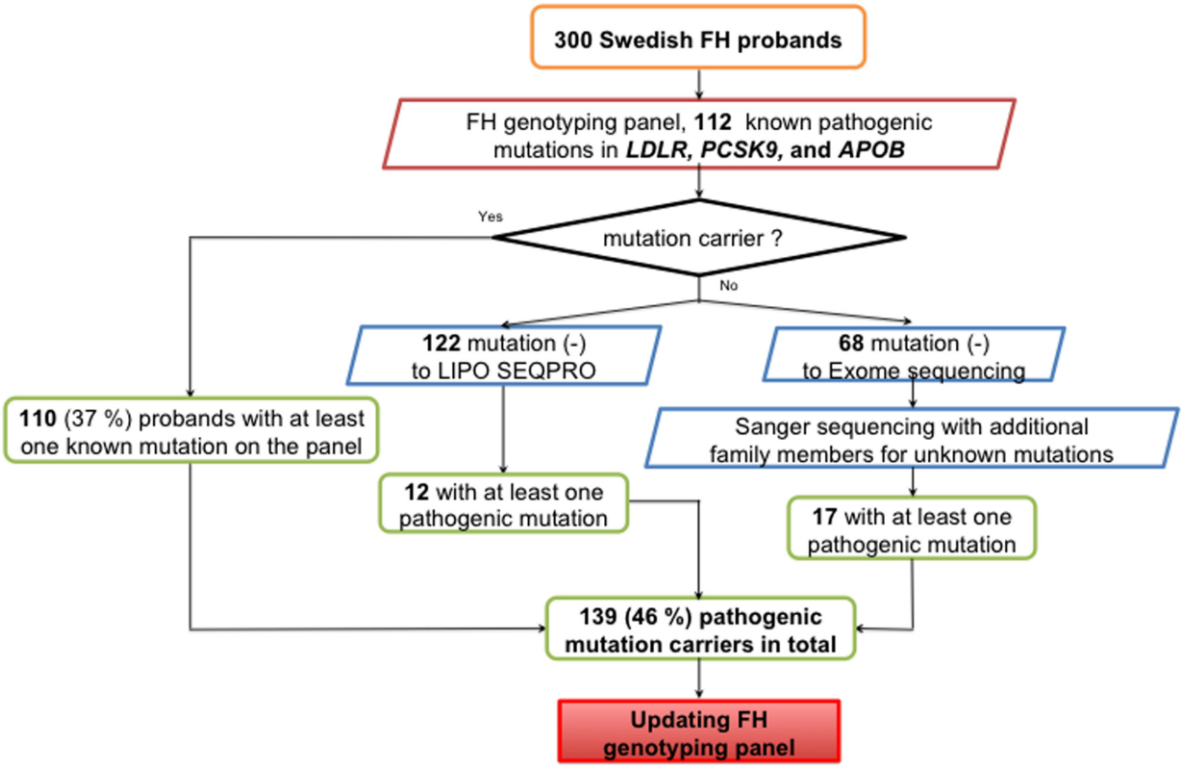
In paper III, unequal sample size *t*-tests were applied to calculate the statistical significance of mean value difference of SNP-scores between the different groups. *P*-values are based on two-tailed hypothesis. Differences in frequency distribution between groups were tested using Pearson´s Chi-square test with Yates´ correction. Linear regression model with response (LDL-cholesterol) and predictor (SNP-score) was analyzed by using the standard least squares method, with figures plotted using R.

5 RESULTS

Paper I

Of the 300 investigated subjects, a FH-causing mutation was identified in 139 cases (46 %). The genotyping panel detected the causative mutation in 79 % of the cases (110/139) (Fig 9)

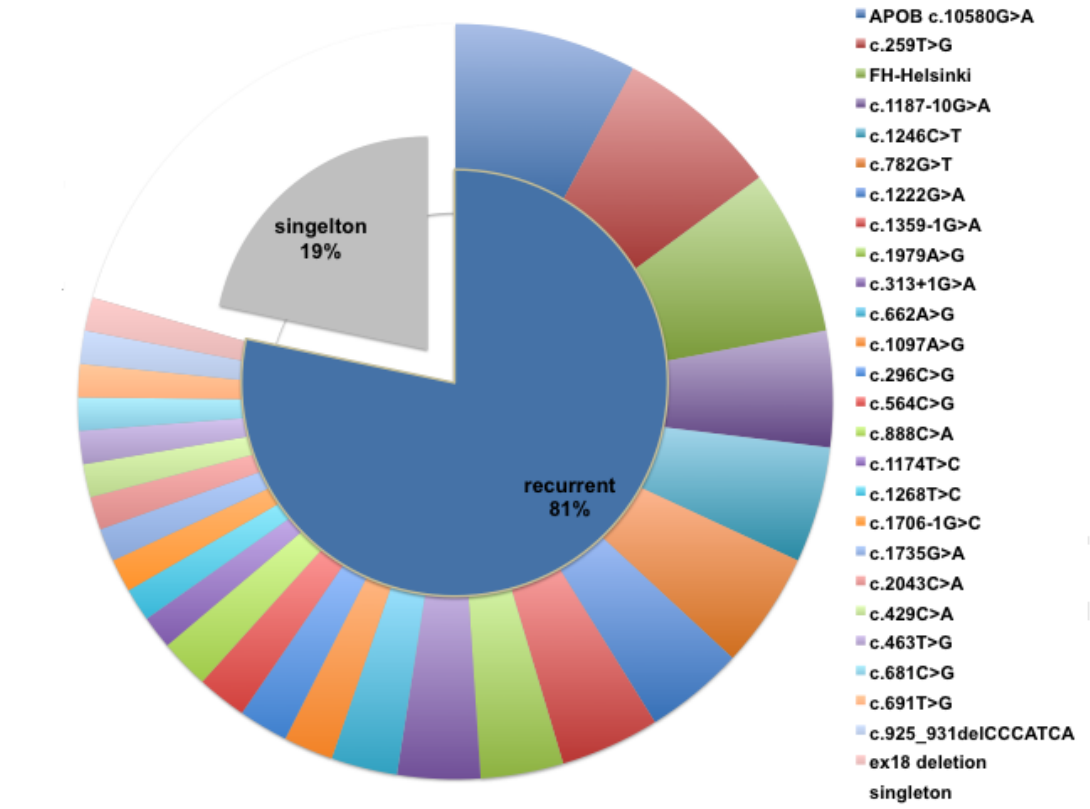
Fig. 9 Flow chart of 300 Swedish FH probands



A total of 55 different mutations were identified among the 139-mutation positive subjects. 34 of which were detected by the genotyping panel and 21 by NGS.

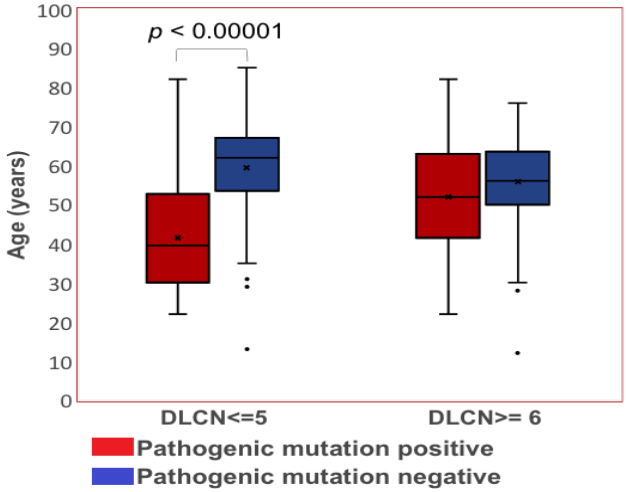
26 out of the 55 different mutations were recurrent and explained the genetic cause in 81 % of the mutation carriers, strongly indicating the presence of founder effects in the Swedish population (Fig 5). Ten of the most frequently occurring mutations (≥5 subjects) accounted for 53% of all mutation positive FH-patients (Fig 10).

Fig. 10 Twenty-six recurrent mutations found in Swedish FH patients



The overall detection rate was much higher in patients with "probable/Definitive" FH (61%) compared to patients with "possible" FH (20%). However, there was a clear age-dependent influence on the detection rate in the group with "possible" FH as shown in Fig 11.

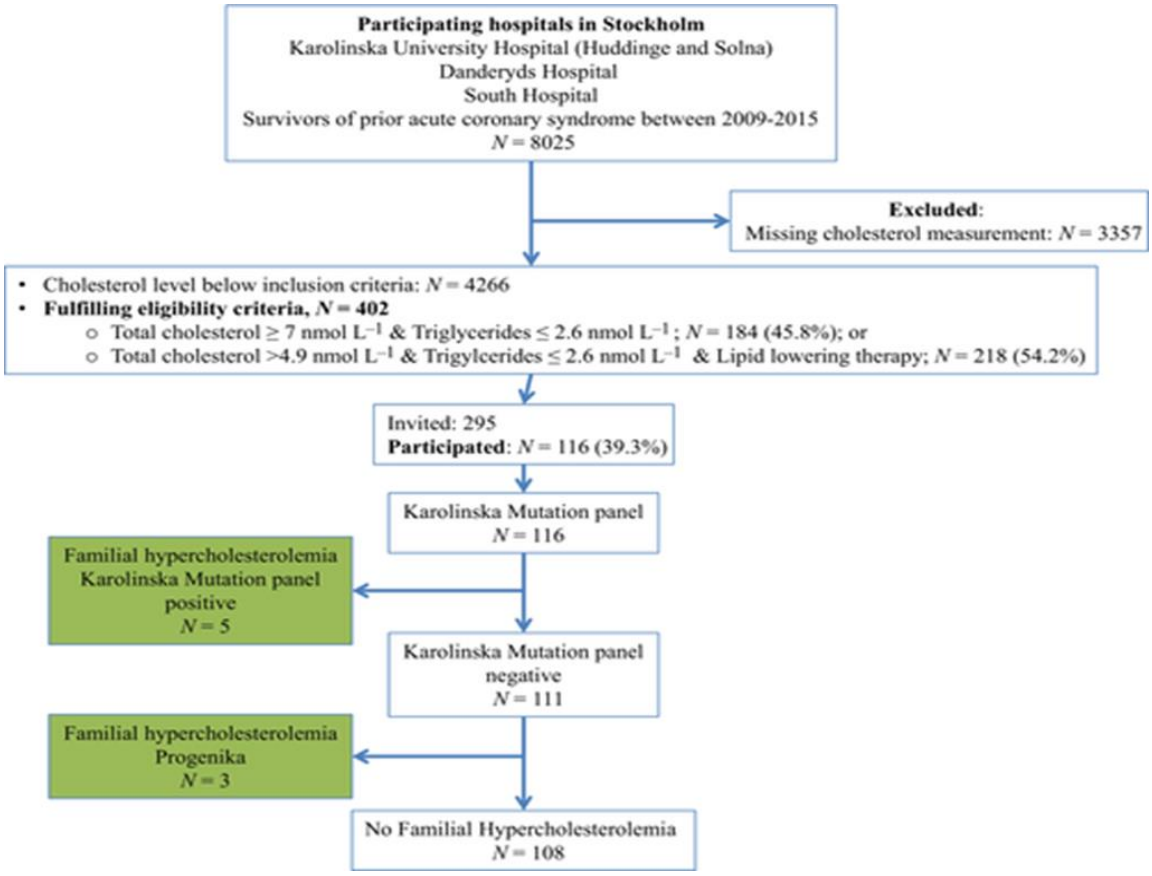
Fig.11 Age distribution of pathogenic mutation carriers according to DLCN



Paper II

A FH-causing mutation was identified in 8 out of the 116 (6,9%) investigated subjects, a result in line with previous similar studies. The mutation positive patients had a higher mean LDL-cholesterol and scored higher on the DLCN-scale than mutation negative subjects. A positive family history of either premature ASCVD or/and hypercholesterolemia was found in ~ 45 % of investigated cohort but was lacking in 2 of the 8 mutation positive subjects (Fig 12). The study illustrates the importance in defining the patient´s phenotype as much as possible before deciding to investigate the genotype.

Fig. 12 Flow chart of included patients



Paper III

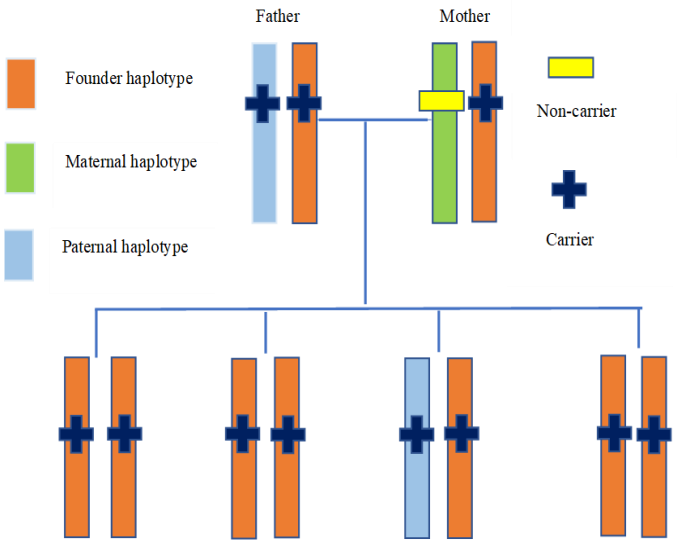
The mean polygenic score in the 88 (M-) patients was 1,05. Out of these, 16 (18%) scored within the 10th decile and 51 (63%) had a polygenic score corresponding to the 8th-10th decile. In the (M+) group, the mean polygenic score was 0,95, and 6 out of 57 (11%) scored within the 10^{th} decile. 19 (33%) of the (M+) patients were within the 8th -10th decile [$P<0.01$ compared to (M-)]. However, despite a higher mean polygenic score in the (M-) group, there was no difference in the LDL-cholesterol levels between the groups (Fig 7 and 8). In fact, the mean LDL-cholesterol level in the (M+) group with a high polygenic score was lower than in the M (+) group with a score below the 8th decile (7,3 mmol/l and 8,0 mmol/l, respectively).

In the (M-) group, the average LDL-cholesterol level was the same for patients that scored below the 8th decile when compared to those within the 8th-10th decile (7,1 mmol/l). The 16 patients in the (M-) group that scored within the 10th decile had the same average LDL-cholesterol as (M-) patients scoring below the 8th decile (6,9 mmol/l and 7,1 mmol/l, respectively.

Paper IV

One haplotype made up of 10 variants (CG**A**GCCAAGAC) around the c.313+5G>A FH-causing mutation was present in both parents, confirming their kinship. The father's haplotypes were inferred based on genotypes of the offspring and the mother. Three of the homozygous brothers inherited a copy from their mother and another copy of the founder haplotype from the father. The index patient (daughter) however, had inherited a different copy from her father indicating him as homozygous for the c.313+5G>A mutation (fig 13).

Fig.13 Illustration of haplotype segregation within the investigated family.



Metabolic studies

As expected, the response to treatment with statin in combination with Ezetimibe was poor. The limited decrease in LDL-cholesterol seen, indicates some residual LDLR-activity. The reduction in total cholesterol levels observed when the four homozygous patients were treated with a PSCK-9 inhibitor was related to a significant reduction in VLDL-level.

Investigation of biomarkers of cholesterol synthesis and bile acid production (lathosterol and 7alpha-hydroxy-4-cholesten-3-one (C4)) showed levels within the normal range supporting previous reports that the hepatic cholesterol balance can be maintained even in the absence of functional LDL receptors.

6 GENERAL DISCUSSION

Familial hypercholesterolemia is one of the most commonly occurring monogenic autosomal dominant diseases. As genetic testing has become more available in routine medical practice, its genotypic complexity has become more evident. In most studies a mutation detection rate of 60-80% is reported when the proband has the higher level of clinical suspicion of FH (probable or definitive FH according to DLCN). In less well-defined cohorts, the detection rate has been reported to be as low as 20-30%, which probably at least partly reflects difficulties in obtaining adequate or correctly interpreting medical data. It may also be due to the presence of other dyslipidemic conditions that mimic the FH phenotype, such as FCHL or ketogenic diet, which together with a family history of premature ASCVD may lead to a tentative diagnosis of FH. However, low yield of detected mutations may also be due to the decision by clinicians to send patients with a milder phenotype and lower clinical score for genetic testing. In paper I, we found a detection rate as low as < 10 % in patients with a DLCN-score of 4 that were > 45 yrs of age. Furthermore, due to the high prevalence of FH, the cost of the analysis still becomes substantial and therefore it is important to optimize clinical algorithms regarding whom to test and to use cost-efficient methods for detecting FH-causing mutations.

It is important to keep in mind that despite the rapidly declining cost of analysing DNA, as well as its increasing availability, genetic testing can only be used to confirm FH, not to rule it out. Furthermore, due to the high prevalence of FH, the cost of the analysis still becomes substantial and therefore it is important as much as possible to optimize clinical algorithms regarding whom to test and to use cost-efficient methods for detecting FH-causing mutations. In paper I, we investigated whether the presence of founder effects could allow us to use genotyping as a first step in the molecular diagnosis of FH in Sweden. Based on the knowledge from previous genetic FH-studies within Sweden and from our neighbouring Scandinavian countries, a genotyping platform consisting of 112 known FH-causing mutations was designed. In the study, 80 % of detectable mutations were captured by the platform. When NGS were performed in those patients negative on the platform, the total detection rate was increased by approximately 10%. Ten of the recurrent mutations in the study explained the genotype in about 50 % of the mutation positive patients, clearly indicating the presence of a founder effect. The genotyping panel has since the completion of the study been in clinical use, and currently 27 lipid clinics from all over Sweden send their samples to be genotyped. By continuously analysing samples negative on the panel with NGS other FH-causing mutations with a potential founder effect can be detected and added to the genotyping panel. In clinical practice genotyping cannot replace NGS but may serve as a cost-efficient first screening step for patients of Scandinavian origin. In patients with a more extreme phenotype, additional analysis with NGS should be considered even if a causative mutation is detected by the panel, in order not to fail to detect compound heterozygosity in a patient carrying an additional mutation not represented on the genotyping panel.

Patients with untreated familial hypercholesterolemia have a marked increased risk of ASCVD. To improve the identification of potential probands among patients treated for acute coronary syndrome, we investigated the prevalence of FH within such a cohort in paper II. While our results were in alignment with other similar studies, the efficacy could have been improved if more strict criteria for genetic testing had been applied. A family history of hypercholesterolemia or premature cardiovascular disease in a 1st degree relative, level of untreated LDL-cholesterol and age at which the cardiovascular event occurs are all important factors in the FH clinical risk score. Patients with hypercholesterolemia (defined as total cholesterol ≥ 7 mmol/l or ≥ 4,9 if on lipid lowering medication) with a history of an acute coronary event, regardless at what age it occurred, were eligible for inclusion. 17/116 (15%) of the patients in the studied cohort had a DLCN-score of ≥6 and thereby met the clinical diagnostic criteria for FH. Out of these, four were genetically confirmed (24%). Four of the patients with a detectable mutation had a DLCN score of less than 6. In three of them, LDL-cholesterol was > 4,9 but < 6,5 mmol/l and their myocardial event did not occur prematurely according to the criteria, but all three were below 70 years of age. In the fourth patient a family history of premature cardiovascular disease or of hypercholesterolemia could not be confirmed, but the patient had an LDL-cholesterol of 5,5 mmol/l and suffered his acute coronary syndrome at the age of 55 yrs. Even if the DLCN-score is a helpful tool in clinically grading the level of likelihood that the patient suffers from FH, it is important to consider the risk of both over- and underscoring. When in doubt whether to refer the proband for genetic testing or not, screening siblings of the probands for LDL-cholesterol levels, could be used to strengthen the clinical suspicion of FH. In Sweden, patients with acute coronary syndrome are registered in a quality register called SWEDEHEART. Data such as age, gender, lipid levels, blood pressure and current medication are noted but unfortunately family history is not one of the included parameters. Considering the high prevalence of FH among hypercholesterolemic patients, especially at younger age, routine screening in this patient group would most likely be cost-effective. By adding family history of premature cardiovascular disease and/or of hypercholesterolemia as a parameter in Swedeheart, patients could be scored according to DLCN and those with “probable” FH selected for genetic testing and subsequent cascade screening of relatives at risk.

In recent years much focus has been on patients with a clinical diagnosis of FH without a detected monogenic cause. In clinical practise this group is much larger than in the clinical studies and represents 70-80 % of FH-patients in comparison with 20-40 % in the studies. This has most likely a multifactorial explanation like difficulties in obtaining correct clinical data concerning the proband resulting in incorrect scoring, less strict cut-off levels for those tested as well as the physicians experience and local routines. Further cascade screening of the proband´s relatives is also greatly facilitated if a mutation is detected which may influence the decision to perform genetic testing even if the probability of a positive result is low. The proportion of subjects with a clinical diagnosis of FH that is found to be carriers of an FH-causing mutation may also depend on which clinical

algorithm is being used when assessing the patient. Many studies apply the concept of "extreme" phenotype as having an LDL-cholesterol > 4,9 mmol/l together with a family history of premature ASCVD and/or hypercholesterolemia. If the Simon Broome classification is applied to these clinical data, the proband will be scored as "possible FH", the same as the DLCN-scale (4p), if LDL-cholesterol is < than 6,4 mmol/l. When using the DLCN-scale and LDL-cholesterol exceeds 6,4 mmol/l, the proband will instead be scored as "probable" (6-8p) or "definite" (> 8p) FH if LDL-cholesterol is > 8,5 mmol/l. This clear distinction in LDL-cholesterol level is not applied in the Simon Broome classification. In paper I, we showed that the mutation detection rate in patients > 45 yrs of age with "possible" FH was only 10 % while it increased to 61 % in the "probable" FH group. In Sweden as well as in many other European countries using the DLCN-scale, genetic testing is generally not recommended in patients with a score of < 6 unless the proband is of younger age.

The concept of polygenic familial hypercholesterolemia has been widely studied and is considered by many to be the most common explanation as to why a monogenic cause cannot be detected in a proband clinically diagnosed with FH. It has also been suggested that cascade screening of relatives is not cost-efficient to probands with a high polygenic score. Furthermore, several studies have proposed that the risk of cardiovascular disease is much less pronounced in patients with a clinical diagnosis of FH without a detected monogenic cause [63-65]. However, polygenic hypercholesterolemia is generally associated with lower levels of LDL-cholesterol which is directly related to the cardiovascular risk, and little is known about the onset of hypercholesterolemia in these patients which in turn would influence the negative impact of cholesterol burden. In paper III we show the lack of correlation between a high polygenic SNP-score and the level of LDL-cholesterol both in mutation negative and in mutation positive patients that fulfill the criteria for FH in Sweden (DLCN ≥ 6p). Similar results have also been found in other studies, indicating that the concept of polygenic hypercholesterolemia cannot explain the phenotype in this group of patients, and it is important not to defer from cascade screening or active lipid-lowering treatment.

The prevalence of homozygous FH is probably higher than previously thought. Consanguineous families increase the risk of this severe form of FH and there is need for a better understanding of the metabolic consequences in the search for new therapeutic approaches. There are several upcoming pharmaceutical therapies, such as ANGPTL 3 inhibitors and Gemcabene, that may prove effective in the treatment of homozygous FH. Genetic and metabolic studies in affected members of the same family is a unique opportunity to gain further insights into this rare disease.

7 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In 2010, when the work was initiated that eventually led to this doctoral thesis, relatively little was known about the FH-population in Sweden. With a few exceptions, genetic testing for the disease was not used in clinical practice and there had only been a few reports on which mutations had been identified in the Swedish cohort [107].Clinical follow-up of patients with suspected FH was not widely established, and often occurred in a relatively unstructured manner. Less than 5 % of the Swedish FH-cohort had been identified. Whole genome/exome sequencing was available for research purposes in only a few locations and at a high cost. Selective NGS to identify mutations in the three classical FH-genes (*LDLR, APOB and PCSK-9*) was not available in the Stockholm area and was also considered costly. By studying previous reports on FH-mutation frequencies in Norway, Denmark, and Finland together with those from Sweden, the hypothesis that founder effects would be seen in the Swedish FH-population could be formulated and tested by the development of rapid mutation diagnostics using genotyping. The construction and subsequent application of a genotyping panel, together with further detailed genetic analysis in well-characterized patients with the FH phenotype without detectable mutations in *LDLR, APOB* and *PCSK-9* genes constituted the basis for this thesis.

Over the last ten years, the rapid technological development, both of genetic analysis and of how to analyze, store and share enormous amounts of data has led to many new insights in the field of medicine. GWAS analyses have led to the development of SNP-risk scores that can aid in predicting the risk of a certain disease, and to a better understanding of how variants in different genes may lead to polygenic disease. These improvements have developed at the same time as the costs have been continuously lowered.

In Sweden today, most regions have outpatient clinics that investigate probands with a clinical suspicion of FH and perform cascade screening of their relatives. Even if genetic testing is available at several other sites, the genotyping platform developed here is now used for analysis of ~ 500 samples/ year from 27 clinics around the country.

Familial hypercholesterolemia remains an under-diagnosed disease in Sweden, as well as in other countries, and there are still major new pathogenic mechanisms and treatment options to discover. Development of efficient screening programs is still an important issue. In paper II, we could show that 15 % of patients with hypercholesterolemia that was admitted to hospital with acute coronary syndrome fulfilled the criteria to be clinically diagnosed with FH. By integrating the parameter of "family history" into health care registries like SWEDEHEART, it would be possible to automatically score all patients according to DLCN, which would markedly increase the number of individuals being diagnosed with FH.

Further molecular studies in patients with a clinical FH phenotype without a detectable monogenic cause represent a continued scientific challenge. In paper III, we show that most of those cannot be explained by the polygenic SNP-score proposed, with a lack of correlation with LDL-cholesterol. It is important not to categorize the mutation negative FH-cohort as a homogenous group. Instead, it most likely consists of individuals with various degree of likelihood that their hypercholesterolemia is inherited and had an early onset in life. Probands with an extreme phenotype regarding the level of LDL-cholesterol and confirmed family history, especially if based on hypercholesterolemia in a 1st degree relative, should be offered an aggressive lipid-lowering treatment, and cascade screening of the relatives should be undertaken regardless of the result from genetic testing.

To identify novel mono- or oligogenic mechanisms of dominant hypercholesterolemia by studying members of families with well characterized metabolic phenotypes using deep whole genome sequencing will be an important focus for the coming years, also since discoveries of novel mechanisms of disease will most probably lead to improvements both in diagnosis and treatment of dyslipidemic patient.

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