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A genome sequencing array for time and cost-effective familial hypercholesterolemia mutation analysis

Ein Genomsequenzierungsarray als eine zeit- und kosteneffektive Alternative zur Diagnose der familiären Hypercholesterolämie

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Zusammenfassung

Familiäre Hypercholesterinämie (FH) ist eine häufig unterdiagnostizierte Erkrankung, die aufgrund hoher LDL-C-Werte zu schwerwiegenden klinischen Folgen wie atherosklerotischen Herz-Kreislauf-Erkrankungen, Myokardinfarkt und Schlaganfall in jungen Jahren führen kann. Bis heute wird die Diagnose anhand verschiedener klinischer Bewertungssysteme gestellt, die Punkte basierend auf dem Vorhandensein oder Fehlen klinischer und anamnestischer Zeichen und Symptome vergeben. Diskrepanzen in den Ergebnissen dieser Bewertungssysteme zeigen, dass es bei der aktuellen FH-Diagnose noch Verbesserungsbedarf besteht. Da FH häufig durch dominante Mutationen in einem der am Lipidstoffwechsel beteiligten Gene verursacht wird, könnten Gentests im Vergleich zur herkömmlichen klinischen Bewertung eine genauere Möglichkeit zur Diagnose von FH bieten oder zumindest eine ergänzende Maßnahme zur Erklärung klinischer Anzeichen und Symptome für Mutationen darstellen, deren biologische Auswirkungen noch nicht genau geklärt sind.

In der hier vorgestellten Studie wurde ein neuer DNA-Array namens „CARdioRENAL“ (CARRENAL)-Array entwickelt, um den Nachweis der häufigsten bei FH beschriebenen Mutationen zu ermöglichen. Er enthielt insgesamt 42615 SNPs, die in Genen lokalisiert waren, die am Lipidstoffwechsel beteiligt sind. Der Array wurde verwendet, um 70 Patienten mit Verdacht auf FH zu testen. Zusätzlich wurden die Patienten mit zwei verschiedenen klinischen Testsystemen getestet, nämlich den Simon-Broom-Kriterien und dem Dutch Lipid Clinic Network Score. 8 der 70 Patienten waren positiv für Mutationen gemäß dem CARRENAL-Array. Unter Verwendung der klinischen Bewertungsskala wurden einige, aber nicht alle dieser acht Patienten mit einem der beiden klinischen Bewertungssysteme als eindeutige FH eingestuft. Der Rest der acht Patienten wurde mit den beiden klinischen Bewertungen jeweils als „wahrscheinliche FH“ bewertet. Während sowohl die Simon-Broom-Kriterien als auch der Dutch Lipid Clinic Network Score mehrere Patienten fanden, bei denen das Vorliegen einer FH als unwahrscheinlich galt, war bei Verwendung des CARRENAL-Arrays keiner dieser Patienten positiv. Der Vergleich zwischen den CARRENAL-Daten und der DNA-Sequenzierung zeigte mehrere Diskrepanzen, die in einer zukünftigen Studie untersucht werden sollten.

Abstract

Familial hypercholesterolemia (FH) is a frequently underdiagnosed condition that can lead to severe clinical consequences related to high LDL-C levels such as atherosclerotic cardiovascular disease, myocardial infarction, and stroke at an early age. To date, diagnosis is established using several different clinical scoring systems that assign points based on the presence or absence of clinical and anamnestic signs and symptoms. Discrepancies in the results of these scoring systems show that there is room for improvement in the current diagnosis of FH. Since FH is often caused by dominant mutations in one of the genes involved in the lipid metabolism, genetic testing might provide to be a more accurate way to diagnose FH compared to conventional clinical scoring or, at the very least, provide a complementary measure to explain clinical signs and symptoms for mutations, the biological impact of which has not yet been well established.

In the study presented here, a new custom DNA array called the “CARdioRENAL” (CARRENAL) array was designed to allow for detection of the most common mutations described in FH. It contained a total of 42615 SNPs located in genes involved in the lipid metabolism. The array was used to test 70 patients suspected of FH. Additionally, the patients were tested using two different clinical testing systems, namely the Simon Broom criteria and the Dutch Lipid Clinic Network Score. Eight of the 70 patients were positive for mutations according to the CARRENAL array. Using the clinical scores, some but not all of these eight patients were scored as definite FH using either clinical scoring system, The remainder of the eight patients scored as ”probable FH” with the two clinical scores, respectively.

Accordingly, while both the Simon Broom Criteria and the Dutch Lipid Clinic Network Score found several patients who were considered unlikely to have FH, none of these were positive using the CARRENAL array. A comparison between the CARRENAL data and DNA sequencing showed several discrepancies that should be investigated in a future study.

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List of abbreviations

ABCG5/G8	Sterolin-1 and sterolin-2 heterodimer
APOB	Apolipoprotein B
APOE	Apolipoprotein E
ASCVD	Atherosclerotic cardiovascular disease
CAD	Coronary artery disease
CARRENAL	CARdioRENAL
DLCN	Dutch Lipid Clinic Network
DLCNS	Dutch Lipid Clinic Network Score
ECM	Extracellular matrix
ESC	European Society of Cardiologists
FGF	Fibroblast growth factor
FH	Familial hypercholesterolemia
FLD	Fisher’s linear discriminant
GWAS	Genome-wide association studies
HB-EGF	Heparin-binding epidermal growth factor
HetSO	Heterozygous Strength Offset
IFNg	Interferon gamma
IL	Interleukin
ISS	Ion-semiconductor sequencing
LCAM	Leukocyte cellular adhesion molecule
LDL	Low-density-lipoprotein
LDL-C	Low-density-lipoprotein cholesterol
LDLR	Low-density-lipoprotein receptor
LDLRAP1	LDLR adaptor protein 1
LIPA	Lysosomal acid lipase
MCP	Monocyte chemoattractant protein
MMPs	Matrix metallo- proteinases
NGS	Next generation sequencing
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin kexin type 9
PDGF	Platelet-derived growth factor
SBL	Sequencing by ligation
SBS	Sequencing by synthesis
SNPs	Single nucleotide polymorphisms
STAP1	Signal transducing adapting family member 1

TGF-□	Transforming growth factor-β
TH1	T-Helper Type 1 cells
TH17	T-Helper Type 17 cells
VCAM	Vascular cellular adhesion molecule

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1 Introduction

Hypercholesterolemia is a major risk factor for cardiovascular disease (1–3). It can be caused by lifestyle choices as well as by genetic predisposition. Atherosclerotic cardiovascular diseases that include coronary artery disease, ischemic stroke, and peripheral artery disease are among the most frequent causes of death in the general population (4). European statistics show that with 345.664 fatal cases in 2018, cardiovascular diseases are the most common cause of death in Germany and responsible for 36.2 % of all-cause mortality (5–7). Each year, more than four million people die from cardiovascular diseases in Europe. With about 55% of all cases of this disease, women are more frequently affected than men. However, cardiovascular deaths are much more common in men under 65 years of age, while women are affected more frequently a higher age (8–10). The direct financial impact of the treatment of cardiovascular diseases in Germany is about 40 billion Euros per year, which accounts for approximately one sixth of the total health care system expenses – without considering the indirect costs due to loss of productivity (9). Therefore, precise identification of lipid-related genetic risk factors for atherosclerotic cardiovascular diseases is an important factor to improve the accuracy of individual diagnostic and therapy options in addition to conventional risk factors such as life-style choice in primary and as well as in secondary prevention.

1.1 Familial hypercholesterolemia

1.1.1 Epidemiology and genetics

Severe hypercholesterolemia that runs in families is called familial hypercholesterolemia (FH) and is usually a monogenetic disease caused by mutation in several genes involved in cholesterol metabolism (3,11). It is frequently underdiagnosed which results in the undertreatment of the condition (12–14). Polygenetic causes of FH are also known (15). Although there is some data on prevalence available differences in methodology, they make it hard to compare the data across different regions; additionally, there are many gaps in existing data (14,16–22). The prevalence of the heterozygous form of FH is estimated to be somewhere between one in 200 to one in 500 (14,17,18,20,22).

The typical presentation of FH is an autosomal co-dominant disorder which’s predominant clinical sign is severely elevated serum low-density-lipoprotein cholesterol (LDL-C). As such it can be subclassified as a heterozygous, in which only one allele is affected, or in homozygous form in which both alleles are affected. Mutations in the genes encoding different proteins involved in the metabolism of cholesterol are known to cause FH. Among the more common mutations that have been identified are mutations in the genes encoding apolipoprotein B

(*APOB*), low-density-lipoprotein (LDL) receptor (*LDLR*), and proprotein convertase subtilisin kexin type 9 (*PCSK9*) (3). Less common mutations in the genes encoding apolipoprotein E (*APOE*) and signal transducing adapting family member 1 (*STAP1*) have been more recently identified using next generation sequencing (NGS) techniques (3).

The most common mutations are point mutations, but insertions and deletions which typically cause frameshifts leading to missense mutations and premature stop codons; copy number variations have also been reported (23,24). In addition to these dominantly inherited forms of FH, rare autosomal recessive forms have also been recognized. In these cases of FH, no phenotypical changes can be detected in heterozygous individuals, however, homozygous individuals show the tell-tale elevation of LDL-C serum concentrations. Mutations that can cause recessive forms of FH have been identified in the genes encoding LDLR adaptor protein 1 (*LDLRAP1*), lysosomal acid lipase (*LIPA*) and the sterolin-1 and sterolin-2 heterodimer (*ABCG5/G8*), which is also associated with cholesterol ester storage disease (Wolman disease) in its homozygous form and sitosterolemia in heterozygous individuals (3).

As Goldberg and Gidding pointed out in a commentary to a publication in 2016, many physicians in their daily practice severely underestimate the prevalence of FH and, therefore, undertreat the condition. Accurate estimates of the prevalence are, therefore, vital for efficient prevention (16). Newer estimates range from one in 80 in founder populations such as French Canadians and south Africans with a high frequency of the mutated alleles before a population bottle neck to about 1:800 in equally genetically isolated population. However, the risk alleles were of a low frequency in the founder population such as Icelanders (Figure 1). The overall worldwide average is estimated to be one in 300 (17).

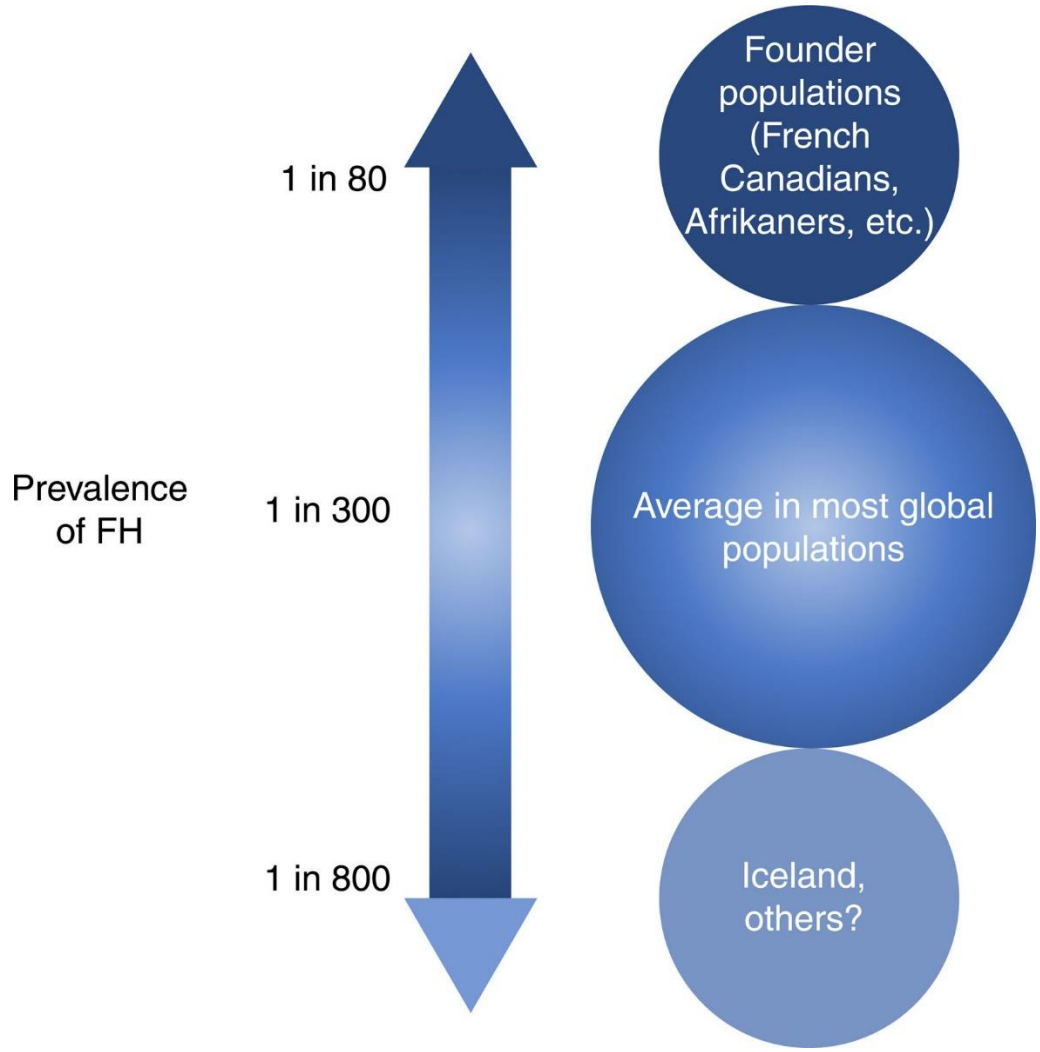

Figure 1: Estimated average prevalence of FH in different populations (17)

Bérard et al. (2019) investigated the prevalence in France. They examined a sample of 7928 participants from the general population with an age between 35 to 74 years and equal distribution between the genders. The found a prevalence of 0.85% of probable (as defined by Dutch Lipid Clinic Network (DLCN) criteria) or definite FH (as defined by the presence of a known mutation), which is a higher prevalence than estimated worldwide average of about 1 in 300 (14,17,18). Their study also revealed that while about 97% of the FH patients were aware of their hypercholesterolemia, only 70% received treatment for the condition (14).

A meta-analysis published in 2020 estimated the average worldwide prevalence of FH to be 0.32% or about one in 300 people (20). The study used date from 44 independent studies with a total of 10,921,310 study subjects of which 33,036 were diagnosed with FH. This prevalence was significant higher in individuals with coronary artery disease (3.2%) and even higher in patients with premature coronary artery disease (6.7). The highest incidence of FH was detected in people with severe hypercholesterolemia (7.2%). While data is impressive, overall

FH prevalence data is only known from a total of 17 countries (9%), which means no data is available from 178 (91%) of countries worldwide (20).

Hu et al. also published a meta-analysis of publications reporting on FH prevalence in 2020 (21). Their analysis included data from 42 independent studies and analysed a total of 7,297,363 individuals of which 24,636 were diagnosed with FH corresponding to a prevalence one in 311 or 0.32 %. A separate population of 48,158 patients with atherosclerotic coronary artery disease revealed 2827 cases of FH corresponding to a FH prevalence in this patient population of one in 16 (6.25%) The authors concluded that these prevalent data put FH into the group of the most commonly seen genetic diseases by general practitioners and that general practitioners, therefore, need to be more aware of this in order to provide the best possible treatment for their patients (21).

Considering that hypercholesterolemia is a major risk factor for atherosclerosis, it is not surprising that the prevalence of FH is much higher in populations with different diseases linked to atherosclerosis. For example, in a study with 225 patients from China with premature myocardial infarction, Cui and coworker found mutation in one of the LDLR, APOB, PCSK9, and LDLR adaptor protein 1 (*LDLRAP1*) genes in 10 patients, accordingly, the prevalence of mutations in these genes in this population was 4.4%. Four of these mutations had not been previously described. Using the diagnostic criteria of the Dutch Lipid Clinic Network for definite and probable FH was 8.0% and 23.6%, respectively (19).

Similar findings were detected in a study published by Harada-Shiba et al. in 2018. The authors investigated the prevalence of FH in patients from Japan with acute coronary syndromes such as myocardial infarction and unstable angina. The multicenter study analyzed data from 1944 patients with a mean age of 66 from 59 clinical sites. With 80.3%, the study population was predominantly male. Fifty-two of the patients had FH corresponding to a prevalence of 2.7% or one in every 37 patients. This incidence was even higher in patients with premature acute coronary syndrome which was defined as being younger than 55 years of age for males or younger than 65 years of age for women at the age of onset of acute coronary syndrome. Accordingly, the average age of patients with FH was younger than that of patients without FH (25).

A similar study used DLCN scoring to phenotypically diagnose FH retrospectively in a patient population from Poland suffering from acute coronary syndrome (26). The study found five cases of definite FH (DCLN score of >8) in a convenience sample of 341 consecutive patients corresponding to a prevalence of 1 in 68 or 1.5%. When probable FH cases (DCLN score >6) were included, a total of 15 patients were identified in the total sample of 341 patients (1 in 23 or 4.8%). The authors did not perform any genetic testing, so these numbers are based on

phenotype only (26). Nevertheless, these data show that prevalence can be quite high in certain patient populations and can differ significantly in the general population based on ethnic background.

1.1.2 Polygenic FH

Though the most severe cases of FH are monogenetic caused most by a mutation in the genes for the *LDLR*, *APOB*, or *PCSK9*, polygenetic causes of FH are also known. The phenotype of polygenic hypercholesteremia is typically milder compared to monogenetic FH, however, even in these cases, cardiovascular sequelae are unavoidable, if left untreated (15). There are currently about 95 known gene loci that can affect LDL cholesterol levels (27). In 2013, the Global Lipid Genetic Consortium defined a panel of 12 single nucleotide polymorphisms (SNPs) for the most common lipid raising mutations found using data from numerous genome-wide association studies (GWAS) (28). These 12 SNPs are used to calculate a score that measures the additive effect of the present lipid-raising SNPs in a single individual (Table 1). Its numerical values are normally distributed within given population and lower deciles differ significantly from higher deciles in their average LDL-C values with higher deciles corresponding to higher LDL-C levels (28). This association is apparent in all populations that have so far been examined (28,29) The score can predict this individual's risk to suffer atherosclerosis and cardiovascular sequelae, if the condition is left untreated (27,29,30).

Table 1: The 12 SNP LDL-C Score of the Global Lipid Genetic Consortium (28)

SNP	Gene	Chromosome number	Start position (GRCh37)	Common allele	Alternate allele	GLGC weight for score calculation
rs2479409	*PCSK9*	1	55504650	G	A	0.052
rs629301	*CELSR2*	1	109818306	G	T	-0.15
rs1367117	*APOB*	2	21263900	G	A	-0.1
rs4299376	*ABCG8*	2	44072576	G	T	0.071
rs1564348	*SLC22A1*	6	160578860	T	C	0.014
rs1800562	*HFE*	6	26093141	G	A	0.057
rs3757354	*MYLIP*	6	16127407	C	T	0.037
rs11220462	*ST3GAL4*	11	126243952	G	A	-0.050

Abbreviations: SNP (single nucleotide polymorphism), GLGC (Global Lipid Genetics Consortium)

1.1.3 Clinical sequelae - atherosclerosis

FH is associated with a more than ten-fold increased risk of early-onset cardiovascular disease (3). Statin treatment can reduce LDL-C serum concentration and combination therapy of statins and other drugs such ezetimibe or proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors allows the treatment to achieve LDL-C target concentration. Nevertheless, the disease is frequently underdiagnosed and, therefore, left untreated leading to premature atherosclerosis and severe cardiovascular sequelae (18). Cutaneous manifestations are also common in FH and are one of the clinical signs used in diagnosis of the condition (31).

That atherosclerosis is fundamentally an inflammatory disease has been accepted since more than two decades (32–34). For a long time, it has been less clear how hypercholesterolemia mediates the chronic inflammation and endothelial dysfunction that stands at the beginning of a chain of events that culminate in full-blown atherosclerosis and its cardiovascular complications. More recent research has been able to shed some light on this question.

At the beginning of the formation of an atherosclerotic plaque lies the development of a fatty streak in the lining of the affected artery (Figure 2). As a first step, which is exacerbated by hyperlipidemia, LDL transverses the endothelium and binds proteoglycans in the interstitial space of the artery. There, it can be modified by glycosylation oxidation and other enzymatic pathways. Modified LDL can activate endothelium cells, which then express selectins which allow monocytes to adhere to the endothelium in a process called rolling. Activated into celium cells, such as the vascular cellular adhesion molecule (VCAM)-1 and the leukocyte cellular adhesion molecule (LCAM)-1 are also expressed. These molecules can bind monocytes with higher affinity than selections. These stronger interactions enable monocytes to extravasate and enter the interstitial space of the blood vessel. In addition, activated endothelial cells can express several chemo attractive factors such as Monocyte chemoattractant protein (MCP)-1 that can attract more monocytes into the area. (35–39)

The monocytes in the interstitial space differentiate into fully activated macrophages which secrete more chemoattractants and other pro inflammatory signaling molecules which further exacerbate the inflammation of the area. Activated macrophages also express several different lipid receptors that allowed them to internalize native and modified lipids in the tissue by phagocytosis which eventually leads to the formation of foam cells. These are even more inflammatory cells than the activate that the macrophages they develop from. They are recruits from other pro inflammatory cells such as dentritic cells, mast cells and different kinds of T-cells into the area. (35–39)

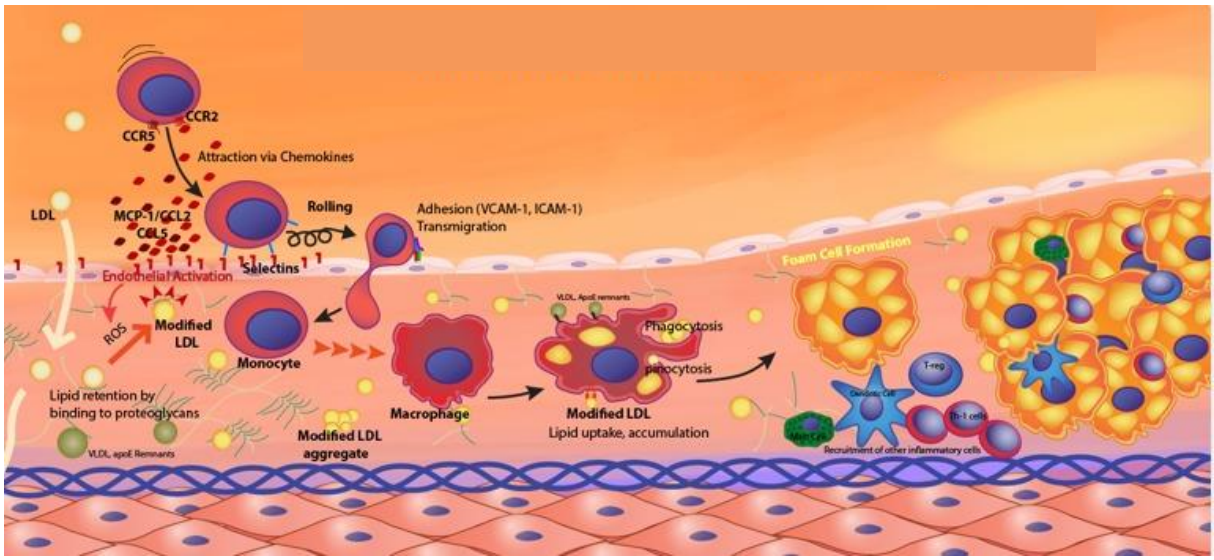

Figure 2: The initial steps in the development of a fatty streak (35)

Foam cell in the fatty streaks express several signaling molecules that function as chemoattractants and proliferative factors not only for inflammatory cells that are recruited from the blood stream, but also for smooth muscle cells from the tunica media (Figure 3). These signaling molecules include platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), heparin-binding epidermal growth factor (HB-EGF), and a number of different matrix metalloproteinases. In response to the presence of these proteins, smooth muscle cells migrate from the tunica media into the subluminal space directly underneath the endothelial cell layer where they start secreting extracellular matrix (ECM) molecules (34,35,37,40,41).

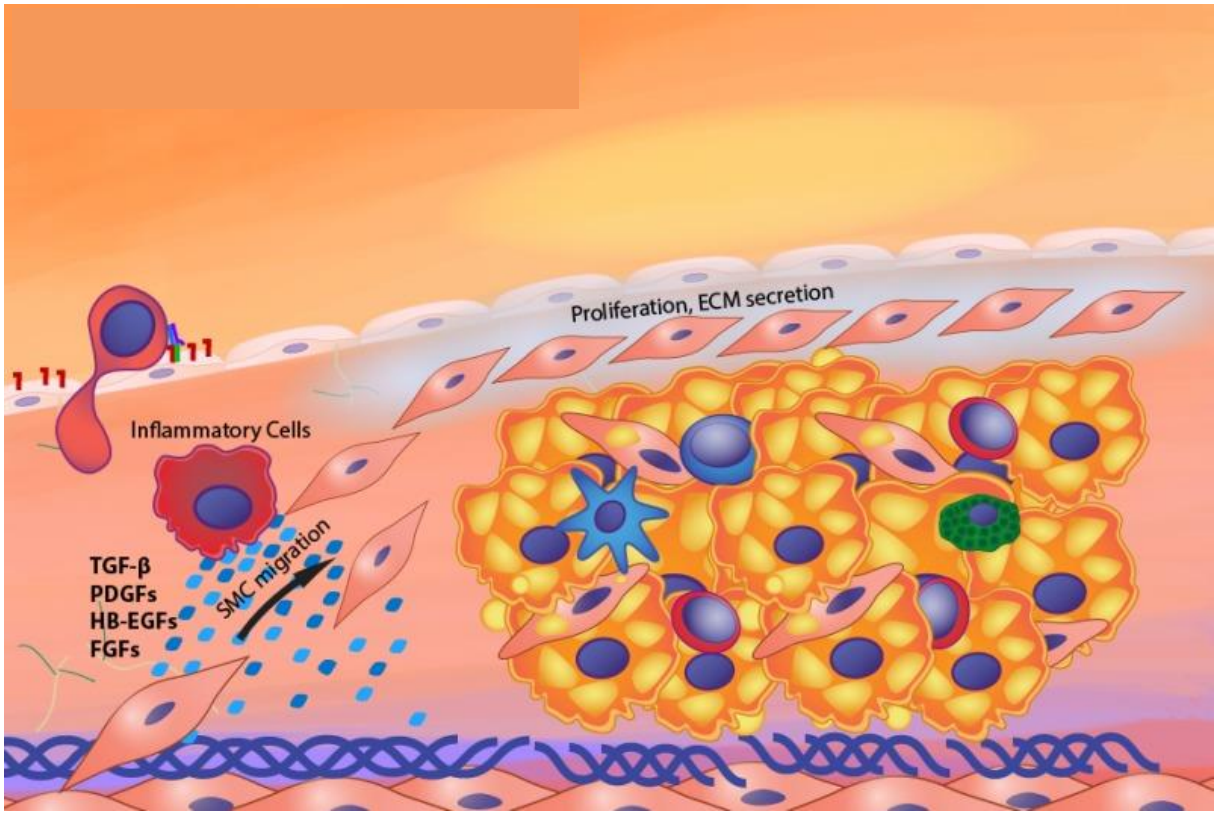

Figure 3: Progression of the fatty streak to a fully developed atherosclerotic plaque (35)

Among the T-cell populations that migrated to the forming atherosclerotic plaque are regulatory T-cells (T-reg) that produce anti-inflammatory cytokines such as TGF-β and Interleukin (IL)-10 (Figure 4). These cytokines play a crucial role in the formation of a stable atherosclerotic plaque with a thick fibrous cap consisting of smooth muscle cells and ECM that sequesters prothrombotic factors present in the foam cell area of the plaque from platelet in the blood stream and thus significantly reduces the likelihood of clinical events. The anti-inflammatory cytokines also reduce proinflammatory actions of foams cells and keep the overall size of the

plaque relatively small leading to a modest development of a necrotic core in the center of the plaque (34,35,37,40,41).

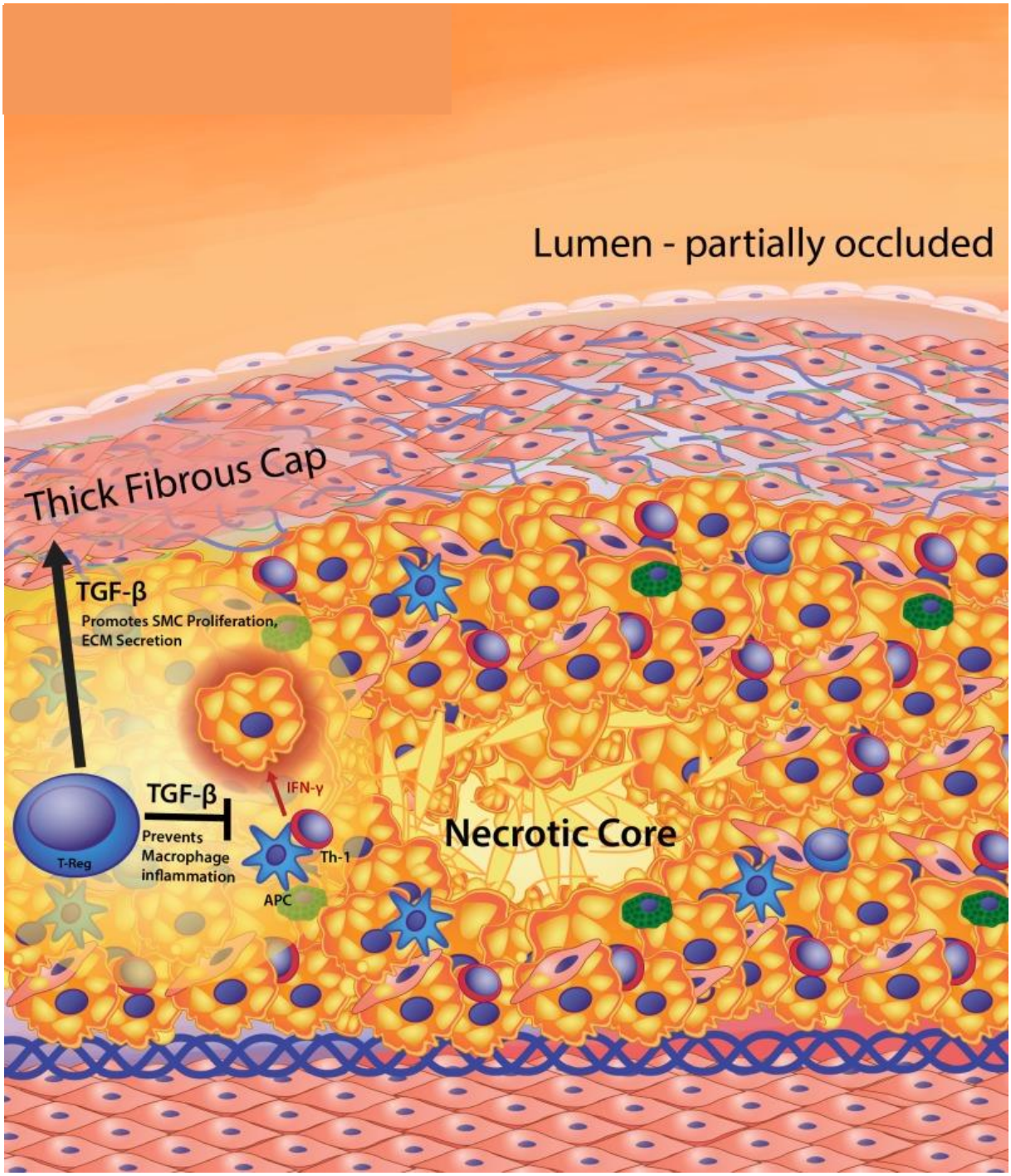

Figure 4: Schematic depiction of a stable atherosclerotic plaque (35)

Vulnerable plaques on the other side, pro-inflammatory influence keep the upper hand in this delicate balance in which antigen-specific T-Helper Type 1 cells (TH1) and Type 17 (TH17)

cells are the prevailing T-cell populations (Figure 5). These produce, among others, the strongly pro-inflammatory cytokines Interferon gamma (IFNg) and IL-17, respectively (35,42). Conversely, anti-inflammatory cytokines like IL-10 and TGF-β are less abundant in this milieu.

Under the influence of the pro-inflammatory cytokines, more macrophages are recruited and consequently, the total number of pro-inflammatory foam cells in unstable plaques is much higher than in stable ones. Compared to regular tissue standing macrophages, foam cells are impaired in their ability to clear dead cells which, together with their increased susceptibility to apoptosis, leads to the development of a large necrotic core in the center of the plaque which exacerbates the pro-inflammatory conditions inside the plaque leading to increased death of the smooth muscle cells that form the protective cap on the lumen side directly beneath the endothelium. Increased production and secretion of ECM-degrading matrix metallo-proteinases (MMPs) and other ECM degrading enzymes by the remaining smooth muscle cells further destabilizes the protective cap. When the cap eventually ruptures, prothrombotic contents of the atherosclerotic plaque are exposed to platelets in the bloodstream culminating in thrombus formation and subsequent thromboembolic events such as myocardial infarction and ischemic stroke (35–38,40,43,44).

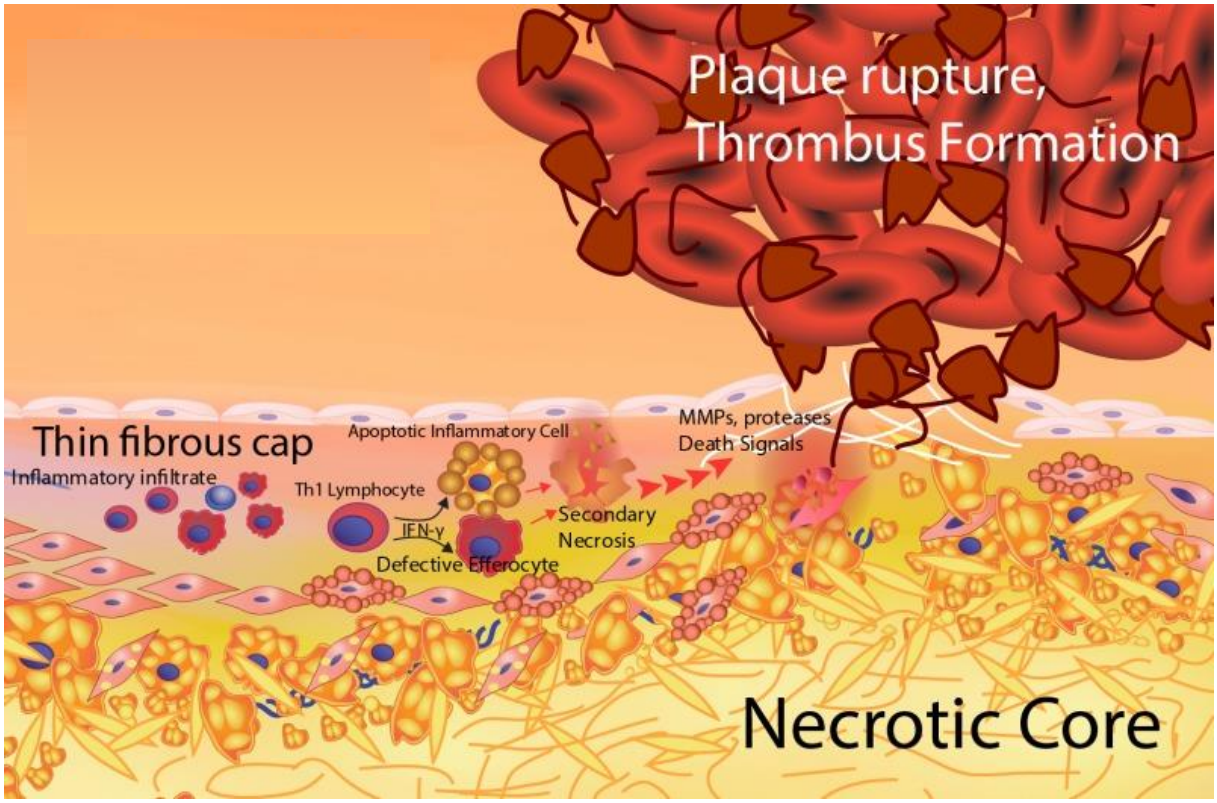

Figure 5: Events leading to plaque rupture and thrombus formation in a vulnerable, unstable plaque (35)

1.1.4 Diagnosis based in clinical signs

Elevated LDL-C serum levels are a hallmark of familial hypercholesterolemia and appear very early in life. Lipid screening of all children between the age of nine and 11 is, therefore, recommended by a number of American health organizations such as the American Heart Association, the American College of Cardiology, the American Academy of Pediatrics, the National Heart, Lung, and Blood Institute, and the National Lipid Association as an indispensable tool to combat the fact that familial hypercholesterolemia is widely underdiagnosed, in particular in children where treatment of hypercholesterolemia could have the most benefit for preventing later clinical consequences (45).

However, these recommendations are rarely followed by pediatricians and most patients are diagnosed with familial hypercholesterolemia in their fifties after they have experienced a related cardiovascular event (45). The European Society of Cardiologists (ESC) recommends stratifying patients with dyslipidemia according to their risk profile (46). According to the ESC risk criteria, patients with FH without other major risk factors are in the high-risk-category while FH-patients with atherosclerotic cardiovascular disease (ASCVD) or another major risk factor are categorized very high risk (Figure 6). The corresponding ESC treatment guidelines provide for lifestyle interventions in combination with drug treatment for virtually all FHJ patients (Figure 7).

Very-high-risk	People with any of the following: Documented ASCVD, either clinical or unequivocal on imaging. Documented ASCVD includes previous ACS (MI or unstable angina), stable angina, coronary revascularization (PCI, CABG, and other arterial revascularization procedures), stroke and TIA, and peripheral arterial disease. Unequivocally documented ASCVD on imaging includes those findings that are known to be predictive of clinical events, such as significant plaque on coronary angiography or CT scan (multivessel coronary disease with two major epicardial arteries having >50% stenosis), or on carotid ultrasound. DM with target organ damage,[a] or at least three major risk factors, or early onset of T1DM of long duration (>20 years). Severe CKD (eGFR <30 mL/min/1.73 m^2). A calculated SCORE ≥10% for 10-year risk of fatal CVD. FH with ASCVD or with another major risk factor.
High-risk	People with: Markedly elevated single risk factors, in particular TC >8 mmol/L (>310 mg/dL), LDL-C >4.9 mmol/L (>190 mg/dL), or BP ≥180/110 mmHg. Patients with FH without other major risk factors. Patients with DM without target organ damage,[a] with DM duration ≥10 years or another additional risk factor. Moderate CKD (eGFR 30–59 mL/min/1.73 m^2). A calculated SCORE ≥5% and <10% for 10-year risk of fatal CVD.
Moderate-risk	Young patients (T1DM <35 years; T2DM <50 years) with DM duration <10 years, without other risk factors. Calculated SCORE ≥1 % and <5% for 10-year risk of fatal CVD.
Low-risk	Calculated SCORE <1% for 10-year risk of fatal CVD.

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Figure 6: ESC risk categorization of patients with dyslipidemia.
Note that FH-patients are in high risk or very high risk categories depending on presence of additional risk factors (46)

	Total CV risk (SCORE) %	Untreated LDL-C levels					
		<1.4 mmol/L (55 mg/dL)	1.4 to <1.8 mmol/L (55 to <70 mg/dL)	1.8 to <2.6 mmol/L (70 to <100 mg/dL)	2.6 to <3.0 mmol/L (100 to <116 mg/dL)	3.0 to <4.9 mmol/L (116 to <190 mg/dL)	≥4.9 mmol/L (≥190 mg/dL)
Primary prevention	<1, low-risk	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	I/C	I/C	I/C	I/C	IIa/A	IIa/A
	≥1 to <5, or moderate risk (see *Table 4*)	Lifestyle advice	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	I/C	I/C	IIa/A	IIa/A	IIa/A	IIa/A
	≥5 to <10, or high-risk (see *Table 4*)	Lifestyle advice	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	IIa/A	IIa/A	IIa/A	I/A	I/A	I/A
	≥10, or at very-high risk due to a risk condition (see *Table 4*)	Lifestyle advice	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	IIa/B	IIa/A	I/A	I/A	I/A	I/A
Secondary prevention	Very-high-risk	Lifestyle intervention, consider adding drug if uncontrolled	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention	Lifestyle intervention and concomitant drug intervention
	Class[a]/Level[b]	IIa/A	I/A	I/A	I/A	I/A	I/A

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Figure 7: ESC treatment guidelines for patients with dyslipidemia according to risk stratification and untreated LDL-C levels (46)

Diagnosis of familial hypercholesterolemia based on clinical signs is usually accomplished using a validated set of defined clinical criteria such as the Dutch Lipid Clinical Network (DCLN) score (47). The criteria are a combination of family anamnesis and clinical signs in the patients, which are investigated. The family history examines the presence of premature cardiovascular events, LDL-C concentrations above the 95th percentile for age and gender as well as tendon xanthomata or corneal arcs in first degree relatives. Important clinical features in the patient are the presences of corneal arcs before the age of 45, the presence of tendon xanthomata, premature coronary artery disease (CAD) or peripheral or cerebral thromboembolic events. In the DCLN questionnaire, “premature” is defined as an age of less than 55 for men and less than 60 for women. Depending on their clinical importance, a certain number of points between 1 and 8 is assigned to each of these criteria. LDL-C serum levels garner different amounts of points based on severity. The highest possible score is 24, with scores of above 8 being considered a definite diagnosis of familial hypercholesterolemia. Scores between six and eight

are considered probable familial hypercholesterolemia, scores between three and five are possible and below three, they are unlikely (47). There are other similar questionnaires with defined clinical criteria to diagnose familial hypercholesterolemia, however the criteria of the DCLN are probably the most widely used in the world (48).

1.1.5 Genetic testing

Even though this disorder is typically caused by a mutation in a single gene, genetic testing is not commonly used in the diagnosis (49). A number of genetic tests have been developed in recent years and are increasingly used (50). A recent systematic review found that genetic testing improves diagnosis and clinical outcomes and the adherence both for suspected cases as well as their relatives, if they are subjected to testing – called cascade screening(51).

In 2018, therefore, the American College of Cardiology recommended genetic testing of the genes encoding the low-density lipoprotein receptor (LDLR), apolipoprotein B (APOB), and proprotein convertase subtilisin/kexin 9 (PCSK9) to be adopted as the standard of care for all suspected cases. However, the recommendation to this date is not universally followed by general practitioners (49). In 2019, the ESC published similar recommendations (46).

Using next generation sequencing (NGS) techniques, a mutation in one of the following four genes– *LDLR, APOB, PCSK9*, and *APOE* – can be identified in 20-40% of patients with a diagnosis of familial hypercholesterolemia based on clinical signs, indicating the existence of other mutations and in some patients an polygenic cause of the phenotype (52). The sensitivity of genetic testing depends on how many genes and possible variants are included in the test with sequencing array producing higher sensitivity than other more limited approaches (53). The principles of different genetic testing techniques will be presented in the following paragraphs. All these techniques use the DNA of the patient that can be extracted from a blood or even a saliva sample.

Next generation sequencing Next generation sequencing (NGS) comprises various sequencing methods, all of which were developed during the first decade of this millennium. The most used ones are pyrosequencing, sequencing by synthesis (SBS), sequencing by ligation (SBL) and ion-semiconductor sequencing (ISS) (54,55). All of these techniques have in common that they can sequence billions of DNA fragments simultaneously, making them a tool with high throughput potential that is useful for defining candidate genes in GWAS, whole genome sequencing or similar studies (55,56).

A particularly frequently used method is SBS, in which DNA fragments are immobilized on a chip using an adapter molecule. Immobilization is followed by an amplification step, in which

clusters of DNA fragments with different lengths but overlapping sequences are produced on the chip. The newly integrated terminator base can then be read in individual steps by new synthesis with labeled base pairs. The next step is the cleaving of the terminator bases followed by a new cycle of synthesis and detection (57). ISS is another form of the group of SBS DNA sequencing techniques in a wider sense. When using ISS, a template in a reaction vessel is flushed with a specific base pair triphosphate. If this is complementary to the template, it is incorporated into the newly synthesized strand. Consequently, an H+ ion is released, which can be detected using an ion-sensitive field effect transistor, which acts as an ion detector (58).

PCR-based techniques

Polymerase chain reaction (PCR)-based genetic testing techniques have been in use for several decades. They can employ sequence specific amplification were PCR-product yield only in the presence of a known mutation or where detection of the mutated allele is achieved by hybridization with a labeled sequence specific oligonucleotide (59,60). This technique combines the specificity of PCR with the ability to detect single nucleotide polymorphisms (SNPs) or mutations using hybridization probes (61). The SSAH technique involves three steps: target sequence amplification, hybridization, and detection. First, the target DNA sequence is amplified using PCR with primers that flank the region of interest. Then, the amplified product is denatured, and hybridization probes are added that bind specifically to the mutated or polymorphic site. Finally, the hybridization probes are detected using a colorimetric or chemiluminescent signal. SSAH has several advantages for the detection of FH, including its ability to detect mutations in a single copy of the LDLR gene and its specificity in distinguishing between different mutations. SSAH can also be used to detect mutations in other genes associated with hypercholesterolemia, such as the apolipoprotein B gene (*APOB*) and the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*) (59–61).

Microarrays

A microarray is a high throughput method that combines different molecular methods to allow for testing of many loci and/or patients simultaneously. It uses hybridization of small DNA fragments of the sample to DNA probes of known sequence to detect these sequences in the sample DNA. To detect the successful hybridization of the sample DNA to the immobilized probes on the microarray plate, the sample DNA is typically labelled with a fluorescent marker prior to the hybridization. The presence or absence of fluorescence is then indicative of the presence or absence of the specific sequence in the sample. Differential microarrays where a unknown sample is compared to a control is often used for tumor genetics (Figure 8). In this

case, the sample DNA is labelled with a different fluorescent dye, e.g. with red fluorescence than a control DNA with known sequence which might be labeled with a green fluorescent dye. The combined fluorescence which yields yellow indicates the presence of the target sequence in both the unknown sample as well as the control while red or green fluorescence indicates the presence of the sequence only the sample or control DNA, respectively (62).

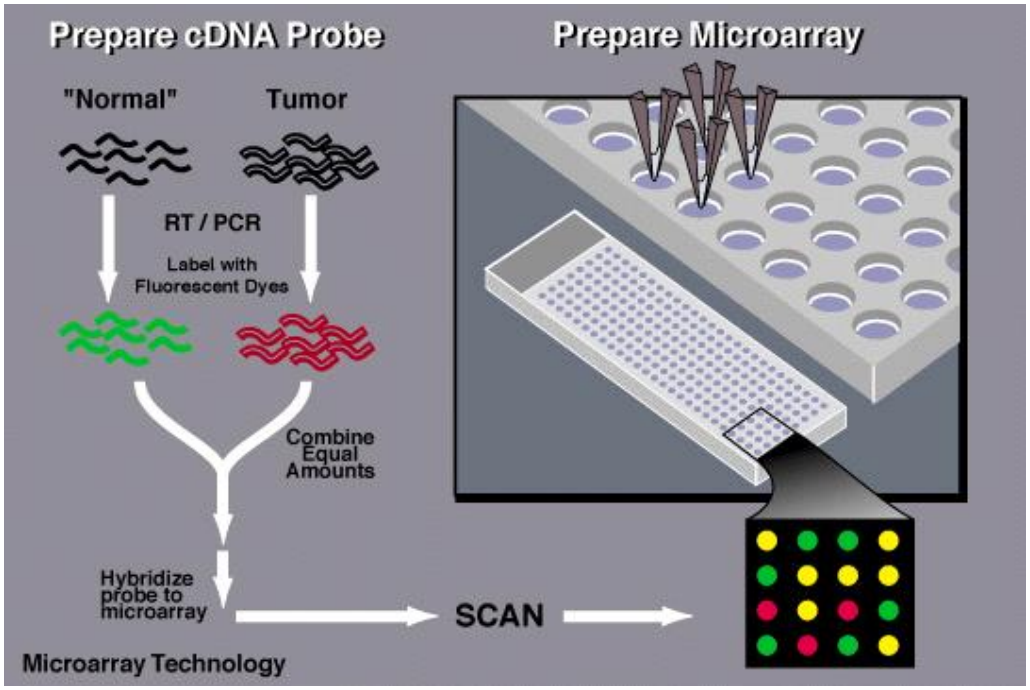

Figure 8: Schematic workflow of a differential microarray (62)

1.2 Causation in observation studies and Mendelian Randomization

Observational studies are a common research design used especially in epidemiology to examine the associations between exposure to certain environmental factors and diseases. However, one of the major problems with observational studies is that association does not mean causation and the assumption of causality can lead to erroneous conclusions (63).

Another problem that can occur with observational studies is confounding, which describes when a third variable is related to both the environmental factor and the associated outcome and distorts the true association between them. Confounding variables can be difficult to identify and control for in observational studies, leading to inaccurate estimates of the environmental factor-outcome relationship. A related problem is reverse causation, which occurs when the perceived outcome is the cause of the association. The complex relationship

between all-cause-mortality and physical activity within a population can be viewed as an example of the confounding effect due to reverse causation. While there is a strong negative correlation between physical activity levels and mortality in middle aged and older adults, it is not immediately clear whether physical activity protects from mortality or whether people who are at higher risk of dying are less physically active due to existing health problems (64,65)

Deducing causation from observational studies can also be problematic due to the possibility of selection bias, which can arise due to the non-random selection of study participants. For example, participants in observational studies may be more likely to be healthier or have better access to healthcare, which can bias estimates of the exposure-outcome relationship (66–69).

Finally, observational studies are often subject to measurement error, which can lead to inaccurate estimates of exposure-outcome relationships. Measurement error can occur due to self-reported measures of exposure or outcome, misclassification of exposure or outcome, or inaccurate measurement instruments (70–74).

To address these problems, researchers may use techniques such as propensity score matching, sensitivity analysis, or regression adjustment to control for confounding and selection bias in observational studies. However, these methods are not foolproof, and it is still challenging to draw causal inferences from observational studies (75–79).

Another way to address confounding and other problems when trying to establish causation using data from observational studies is the statistical technique known as Mendelian Randomization, which exploits the random allocation of genetic variants at conception to create an unbiased sample estimating the causal effect of genetic variant to an outcome. The use of genetic variants as instrumental variables in Mendelian Randomization assumes that the variant is associated with target outcome. When using Mendelian Randomization in a study, the first step is to identify genetic variants that are robustly associated with target outcome. This can be done through genome-wide association studies (GWAS) or by selecting known genetic variants that are associated with the outcome. Using data from multiple GWAS the allows to establish the effect of the genetic variant on the outcomes of interest and the respective effect size (80,81).

One of the main advantages of Mendelian Randomization is that it can reduce the effects of confounding and reverse causation. It can overcome these issues by utilizing genetic variants as instrumental variables, which are less susceptible to confounding and because genetic variants are present at birth, and therefore cannot be influenced by later developments of the outcome. Additionally, Mendelian Randomization can provide insights into the underlying biological mechanisms that link the genetic variant, the exposure, in our case the

hypercholesterolemia to the outcome of atherosclerosis and premature cardiovascular events (82,83).

However, Mendelian Randomization also has its own limitations which include the need for Additionally, these randomizations can be affected by the possibility of pleiotropy, which happens when a genetic variant affects multiple phenotypes or outcomes. This can lead to biased estimates, if not accounted for properly (80,81,84–86).

Despite these limitations, Mendelian Randomization has been widely used in epidemiology and genetics to investigate the causal relationships between a range of exposures and outcomes, including cardiovascular disease, cancer, and different mental illnesses (87–95).

1.3 Aim of the study, research question and hypotheses

Several studies have shown that genetic testing has the potential to improve health outcomes of patients suffering from familial hypercholesterolemia. That testing, however, is not commonly adopted as standard of care by general practitioners due to the lack of fast, simple, reliable and validated arrays and because the prevalence of the disease is frequently underestimated (49,51–53).

There are three well-known clinical diagnostic tools for familial hypercholesterolemia, which were developed by The Simon Broome Register Group in the United Kingdom and the Dutch Lipid Clinic Network (96). To the diagnose familial hypercholesterolemia a DNA test should be performed to detect the mutations in the *LDLR*, *APOB* and *PCSK9* gene. However, all conventional methods for new generation sequencing are expensive, laborious, and highly time-consuming. An accepted gold standard method to diagnose FH DNA testing is not available worldwide. Cost effective genetic testing methods may facilitate the diagnosis of FH and thus improve public health and clinical outcomes for many families.

1.3.1 Aim of the study and research question

The aim of this study was to test a new designed array as a time and cost-effective way for genetic testing by assessing effectiveness, sensitivity, specificity and costs for the identification of patients with familial hypercholesterolemia and an alternative or complement of traditional testing based on clinical signs and symptoms (97). There are studies which directly estimate sensitivity and specificity of different clinical diagnostic criteria for familial hypercholesterolemia to validate results of molecular genetic findings of index patients which showed a weak phenotype-genotype concordance(98). This pilot study was conducted to assess the accuracy

of the array (CARRENAL ARRAYS) in diagnosing the best known and the most frequent mutations for FH mutations and compare the results to those obtained with two of the most commonly used clinical FH-screening tools: the Simon Broome Registry Criteria and the criteria of the Dutch Lipid Network Clinic.

1.3.2 Hypotheses

The study tested the following hypotheses:

1. The CARRENAL ARRAY detects pathological mutations in one of its targeted genes in at least one third of suspected patients.
2. In a small number of patients, the CARRENAL ARRAY detects previously unknown mutations.
3. No pathologic mutation can be detected with the CARRENAL ARRAY in a subset of patients with definite familial hypercholesterolemia based on clinical symptoms

2 Material and Methods

2.1 Patients

Seventy patients with LDL-Hypercholesterolemia and suspected FH from the lipid outpatient clinic of the Clinic of Internal Medicine III (Cardiology, Angiology and Intensive Care) of the Saarland University Hospital were included in this study. This study was conducted according to the Declaration of Helsinki. All participants gave written informed consent, and the Ethics Committee of the Saarland Medical Association (Kenn-Nr. 162-15) approved the study.

2.2 Sample collection and DNA extraction

Whole blood was collected with EDTA as anticoagulant from the 70 patients. DNA was extracted from these samples using QIAamp DNA Mini Kit 250 (Qiagen, Hilden) according to manufacturers' instructions (99). Briefly, 200 µl whole blood was mixed with an equal amount of lysis buffer and incubated room temperature for 5 min. The sample was then applied to a spin column, allowed to bind, and subsequently washed twice (Figure 9). The DNA was removed from the spin column using the appropriate elution buffer and stored at 4°C until further use.

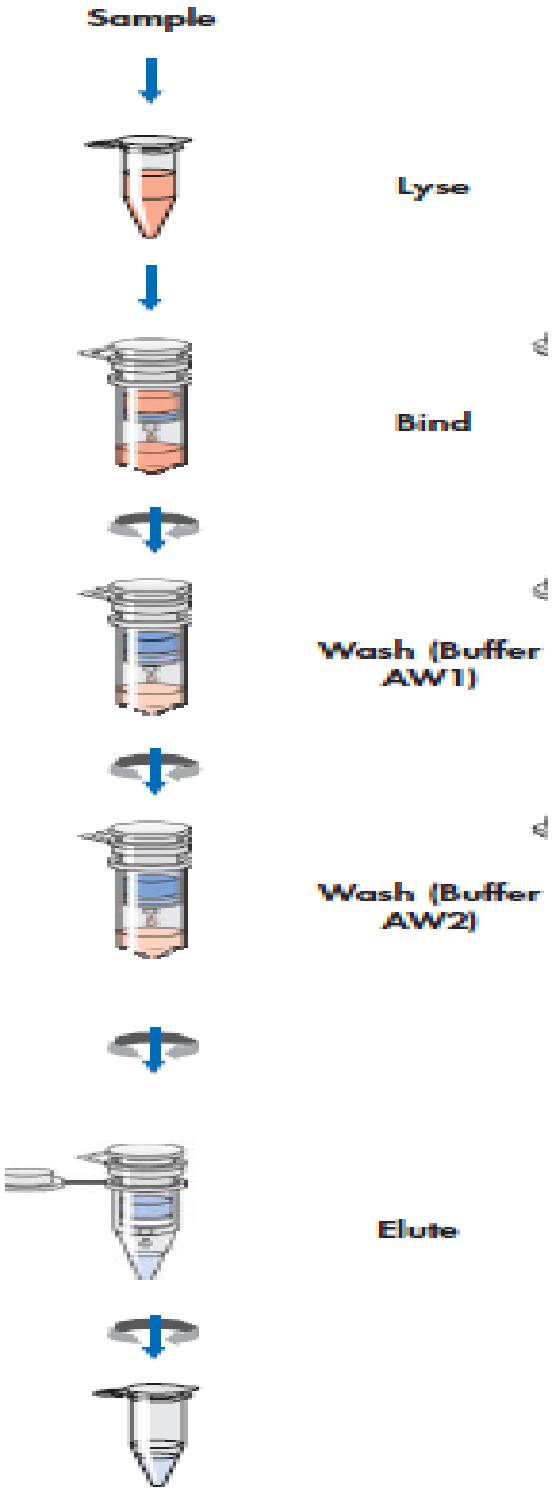

Figure 9: DNA extraction procedure (99)

2.3 CARRENAL custom array

The Axiom® 384HT myDesign™ Custom Array is a commercial DNA array from Affymetrix that is fully customizable. It uses a 384-well array plate that can contain a minimum of 1500

and a maximum of 50000 single nucleotide polymorphisms (SNP) markers as chosen for the specific application. The customization of the array allows its use for genotyping in GWAS and similar applications such as candidate gene studies. It can include both de novo SNPs as well as established SNPs from published databases (100)

The custom array that was designed for this study was called the “CARdioRENAL” (CARRENAL) array. Its purpose was to detect SNPs in patients with suspected FH that might be implicated in the disease process. In this pilot study, the accuracy of the CARRENAL array was assessed in terms of its ability to diagnose the best known and most frequent mutations associated with FH. Accordingly, the CARRENAL array was designed to cover published SNPs in the coding regions of the *LDLR, APOB*, and *PCSK9* genes, which are the three genes most mutated in FH patients (51,52,101). The published SNPs that were included were taken from the HGMD® Human Gene Mutation Database (accessed May 21, 2015).

Additionally, SNPs from the LOVD database were selected(102). These included SNPs located both in splice sites as well as regulatory regions. To account for more common genetic variants, SNPs known to be associated with blood lipid levels from a GWAS meta-analysis published in 2013 were also added (103). Additional SNPs located in the *APOE* gene used in a polygenic score published by Talmud et al in 2013 were also added to the custom array (104). Other SNPs included were located in the *APOB* gene (105,106), as well as ten novel mutation in the *LDLR* gene (107). 223 SNPs in the *LPA* gene submitted by a collaborator as well as SNPs in the *MYBPC3* and *MYH7* genes that are associated with cardiomyopathy were also added to the array design.

The effect size of common variants detected in GWAS is usually small, which means it only explains a relatively small proportion of the estimated heritability of the associated phenotypes. But the same genes that are detected to be associated with the phenotype of interest in GWAS analyses might contain rare variants that can affect the respective phenotypes in a more profound way. Because of this, rare variants from 157 gene loci that have previously been shown to affect blood lipid levels (103) and other rare variants in genes that have been detected as genes of interest in monogenic dyslipidemias (108) were included. Additional rare SNPs located in genes associated with coronary artery disease (109), diabetes mellitus (110), uric acid (111), chronic kidney disease (112), and hsCRP (110) were also included (Figure 10). The total number of proposed SNPs was 45298 (Table 2). A complete list of then in the array included genes is shown in Table 3.

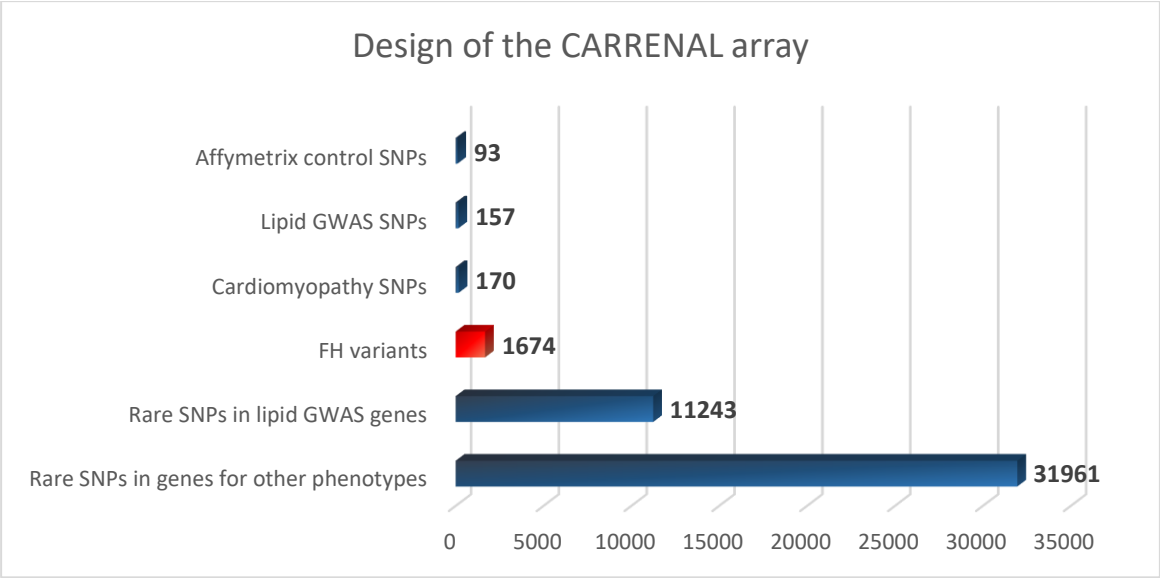

Figure 10: Number of genetic variants included in the design of the CARRENAL custom array.

Table 2: SNPs included into the final design of the CARRENAL array

LDLR/APOB/PCSK9 SNPs HGMD	1594
LDLR/PCSK9 SNPs LOVD	70
LDLR SNPs Synlab	10
Lipid associated SNPs from GWAS (Willer et al. 2013)	157
SNPs in cardiomyopathy genes	170
Lp(a) SNPs	223
ApoE-SNPs + ApoB SNP (Talmud et al.)	3
ApoB-SNPs (Motazacker et al., Alves et al.)	3
SNPs in genes from lipid GWAS	11014
SNPs in genes from other phenotypes	31961
Affymetrix control SNPs	93
Total number of SNPs	45298

Table 3: Selected Genes for CARRENAL ARRAY for the Mutation Detection for significant lipid metabolism disorders

Monogenic hypercholesterolemia
MTTP,LMNB2,STAP1,SAR1B,BSCL2,AGPAT2,CAV1,PTRF,PLIN1,CIDEC,GPD1,LIPE,AKT2,LPIN1,DYRK1B,POLD1,ZMPSTE24,GPIHBP1,LMF1,APOC2,WRN
Lipids (LDL-C, HDL-C, TC, TG)
SCARB1,HNF4A,PABPC4,PPP1R3B,FTO,IRS1,KLF14,MC4R,ABCA1,ABCA8,ADH5,AKT1,ALOX5,AMPD3,ANGPTL1,ANGPTL4,ANGPTL8,ARL15,ATG7,C4orf52,CITED2,CMIP,COBLL1,DAGLB,DGAT2,FAM13A,GALNT2,GSK3B,HAS1,HDGF,IKZF1,KAT5,LACTB,LCAT,LILRA3,LIPC,LIPG,LRP4,MARCH8,MOGAT2,MVK,NR0B2,OR4C46,PDE3A,PGS1,PIGV,PLTP,PMVK,RBM5,RSPO3,SBNO1,SETD2,SLC39A8,SNX13,STAB1,STARD3,TMEM176A,TRPS1,TTC39B,UBE2L3,ZBTB42,ZNF648,ZNF664,APOE,ABO,APOB,FN1,LDLR,LPA,PCSK9,SORT1,ACAD11,ANXA9,APOH,BRCA2,CERS2,CMTM6,CSNK1G3,EHBP1,HFE,INSIG2,LINC01101,MIR148A,MTMR3,MYLIP,NYNRIN,OSBPL7,PLEC1,PRKCA,SNX5,SOX17,SPTLC3,ST3GAL4,TOP1,HNF1A,CILP2,A2ML1,ABCB11,ASAP3,BRAP,C6orf106,CUBN,CYP7A1,DLG4,DNAH11,ERGIC3,EVI5,FAM117B,FRK,GPAM,GPR146,HBS1L,HLADRA,HMGCR,HPR,IRF2BP2,KCNK17,LDLRAP1,MAFB,MAMSTR,MOSC1,NPC1L1,PHC1,PHLDB1,PPARA,PXK,RAB3GAP1,RAF1,SPTY2D1,TIMD4,TOM1,UBASH3B,UGT1A1,VIM,VLDLR,APOA1,LPL,LRP1,TRIB1,PEPD,AKR1C4,ANGPTL3,CAPN3,CYP26A1,FADS1,FADS2,FRMD5,INSR,JMJD1C,KLHL8,LRPAP1,MAP3K1,MET,MLXIPL,MPP3,MSL2L1,NAT2,PDXDC1,PINX1,PLA2G6,TYW1B

Several of the proposed variants encountered design problems. Accordingly, only 43,094 SNPs were ultimately included in the final array. Out of these, 41,852 variants were successfully detected in the analyses. However, analytics thresholds like call rate < 99.5, Fisher's linear discriminant (FLD) < 3.9, a Heterozygous Strength Offset (HetSO) < 0.165, or the marker having an off-target variant were also employed as exclusion criteria from the final analysis.

One of the goals of the study was to test the positive predictive value of the CARRENAL array for familial hypercholesterolemia covering hypercholesterolemia associated mutations among 70 patients with LDL-Hypercholesterolemia and suspected clinic for familial

hypercholesterolemia from our lipid clinic in the Cardiology Clinic of the Saarland University Hospital. For each patient, at least 30 µl DNA with a minimum concentration of 10 ng/µl and sufficient purity as indicated by a 260nm/280nm absorption quotient between 1.65 and 2.1 was necessary for successful genotyping using the CARRENAL array.

Genotyping was performed at the Oxford Genomics Centre (Wellcome Centre for Human Genetics, Roosevelt Drive, Oxford OX3 7BN, UK) following standard protocol for Axiom Affymetrix platform. Quality control of genotype data was carried out using Axiom Analysis Suite ver. 2.0.0.35 software following the Best Practices procedures recommended by Affymetrix. A schematic depiction of the study flowchart is shown in Figure 11:

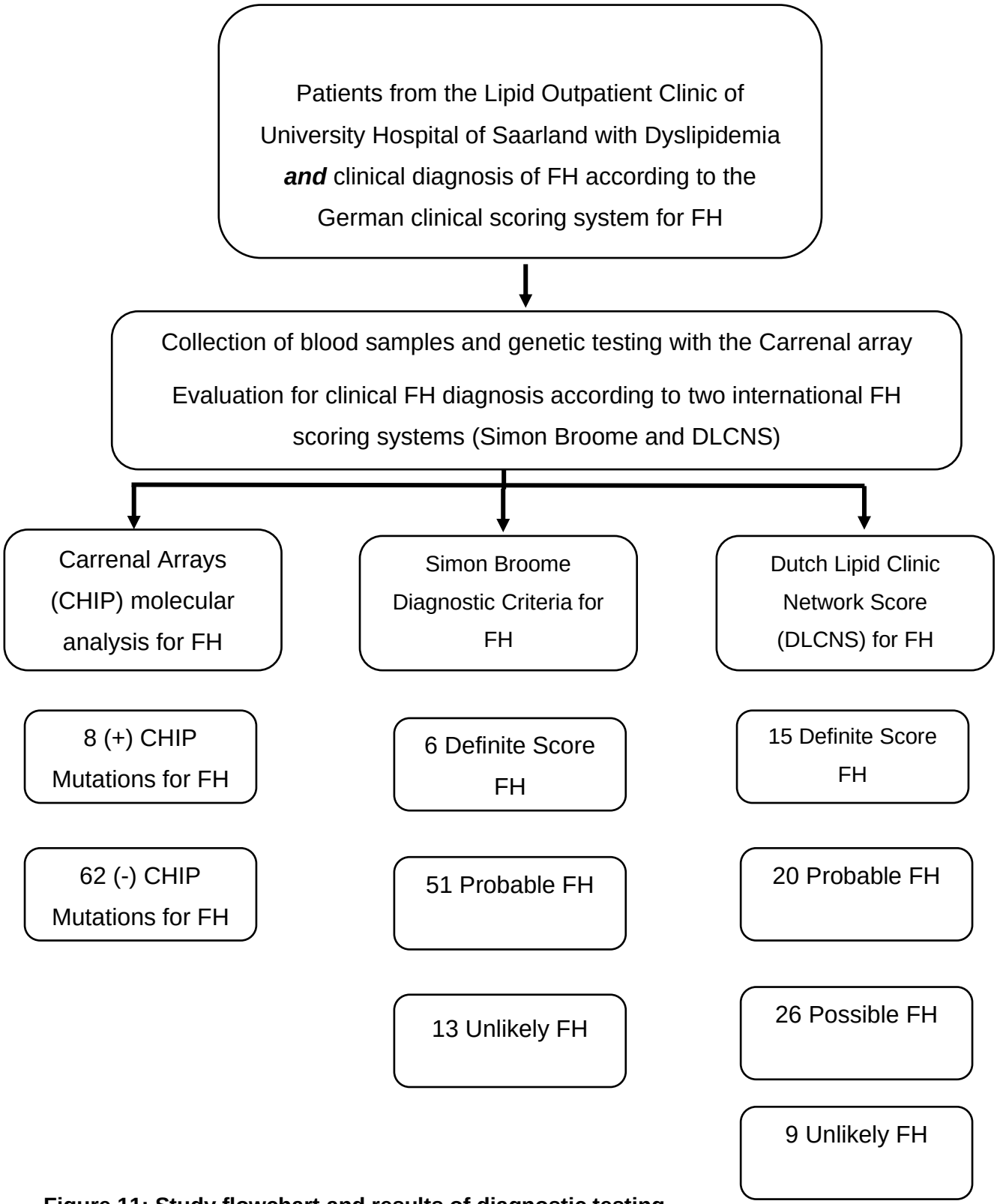

Figure 11: Study flowchart and results of diagnostic testing.

2.4 Clinical Screening Tools

After receiving informed consent, we screened patients via two clinical diagnostic tools, namely The Simon Broome Register Group in the United Kingdom and the Dutch Lipid Clinic Network and German Clinical Lipid Criteria for FH (113–115).

The German Clinical Lipid Criteria (Figure 12) was used for patient selection only, i.e. all patients enrolled in this study were positive for FH using these criteria

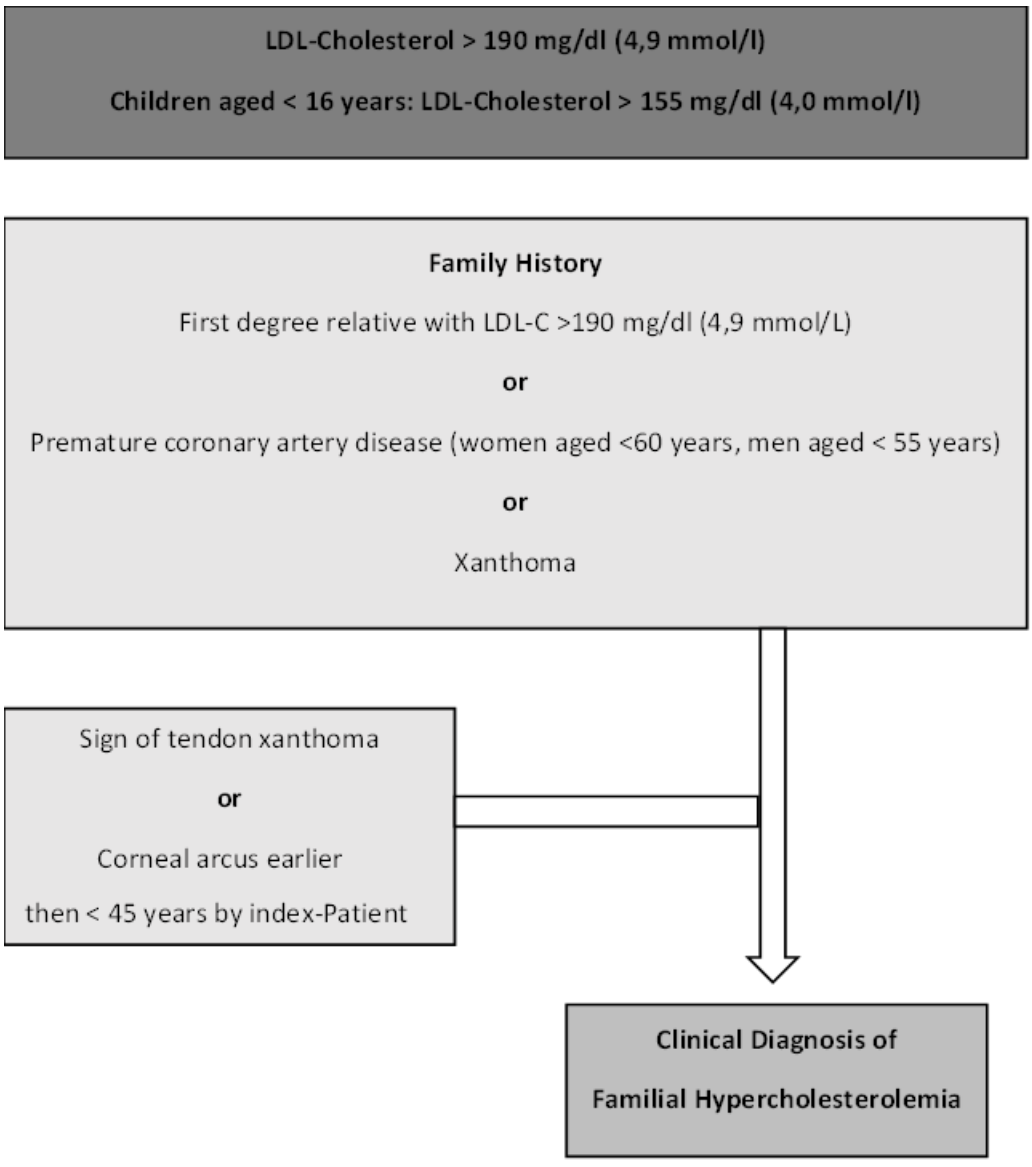

Figure 12: German Clinical Lipid Criteria for FH (50)**.**

The Simone Broome Criteria define definite FH in an adult as total cholesterol levels > 290 mg/dL (7.5 mmol/L) or LDL-C > 190 mg/dL (4.9 mmol/L) or in a child aged 15 or younger as

total cholesterol levels > 260 mg/dL (6.7 mmol/L) or LDL-C > 155 mg/dL (4.0 mmol/L) (. Additionally, for both children and adults, at least one of the two following physical findings must also be present: tendon xanthomas, or tendon xanthomas in first or second degree relative, or DNA-based evidence of a mutation in the gene for the LDL-receptor, the apo B-100 gene, or the PCSK9 gene. Possible FH is defined as the same high serum cholesterol levels in adults or children, respectively with the additional presence of one of the two following criteria: either a first degree relative suffered a myocardial infarction at age 60 or younger or age 50 or younger in a second degree relative or adult first or second degree relative has elevated total cholesterol of more than 290 mg/dL (7.5 mmol/L) or more than 260 mg/dL (6.7 mmol/L), if the first-degree relative is 15 or younger (114,115).

Table 4: Simone Broome criteria and scoring (114,115)

Definite Familial Hypercholesterolemia	
	PLUS at least one of the following
Adult: **Total cholesterol levels > 290 mg/dL (7.5 mmol/L) or LDL-C > 190 mg/dL (4.9 mmol/L)** **Child less than 16 years of age:** **Total cholesterol levels > 260 mg/dL (6.7 mmol/L) or LDL-C > 155 mg/dL (4.0 mmol/L)**	Tendon xanthomas, or tendon xanthomas in first or second degree relative
	OR
	DNA-based evidence of an LDL-receptor mutation, familial defective apo B-100, or a PCSK9 mutation.
Possible Familial Hypercholesterolemia	
	PLUS at least one of the following
Adult: **Total cholesterol levels > 290 mg/dL (7.5 mmol/L) or LDL-C > 190 mg/dL (4.9 mmol/L)** **Child less than 16 years of age:** **Total cholesterol levels > 260 mg/dL (6.7 mmol/L) or LDL-C > 155 mg/dL (4.0 mmol/L)**	Family history of myocardial infarction at: Age 60 years or younger in first degree relative Age 50 years or younger in second-degree relative
	OR
	Family history of elevated total cholesterol > 290 mg/dL (7.5 mmol/L) in adult first- or second-degree relative > 260 mg/dL (6.7 mmol/L) in child, brother or sister aged younger than 16 years

The DLCN criteria assign a certain number of points when a certain criterium based on family history, clinical history, physical signs, laboratory findings, or DNA evidence is present in a patient (113)t. If a patient reaches a total score of more than 8 points a definite FH diagnosis is made. A score of 6-8 points yields a “probable FH” diagnosis according to the DLCN criteria. 3-5 points correspond to “possible FH”, while less than 3 points correspond to a diagnosis of “unlikely FH”. The criteria and corresponding point scores are listed in Table 5.

Table 5: The Dutch Lipid Clinic Network criteria and scoring (113)

Group 1: family history	**Points**
First-degree relative with known premature (<55 years, men; <60 years, women) coronary heart disease (CHD) OR	1
First-degree relative with known LDL cholesterol >95th percentile by age and gender for country	1
First-degree relative with tendon xanthoma and/or corneal arcus OR	2
Child(ren) <18 years with LDL cholesterol >95th percentile by age and gender for country	2
Group 2: clinical history	
Subject has premature (<55 years, men; <60 years, women) CHD	2
Subject has premature (<55 years, men; <60 years, women) cerebral or peripheral vascular disease	1
Group 3: physical examination	
Tendon xanthoma	6
Corneal arcus in a person <45 years	4
Group 4: biochemical results (LDL cholesterol)	
>8.5 mmol/L (>325 mg/dL)	8
6.5–8.4 mmol/L (251–325 mg/dL)	5
5.0–6.4 mmol/L (191–250 mg/dL)	3
4.0–4.9 mmol/L (155–190 mg/dL)	1
Group 5: molecular genetic testing (DNA analysis)	
Causative mutation shown in the *LDLR*, *APOB*, or *PCSK9* genes	8

Subsequently, the predictivity of the Carrenal Array was compared to that of these clinical screening tools.

2.5 Statistics

Statistical analysis was carried out using the statistical program SPSS (IBM, version 25.0.0.2). Briefly, results using DLCNS, Simon Broome, and the German Clinical Lipid Criteria for FH were compared to results using the Carrenal Array. Chi- Square and Fisher's Exact Tests were employed to detect statistical significance between the frequencies of the different diagnoses according to the different test.

Positive predictive value (PPV) was calculated using the following formula:

Number of true positives divided by (Number of true positives + false positives)

True positives were defined as those that were considered definite or probable FH by DLCNS or Simon Broome, while false positives were defined as mutation positive and either “unlikely” (Simon Broome and DLCNS) or “possible” (DLCNS only)

Negative predictive value (NPV) was calculated using the following formula:

Number of true negatives divided by (Number of true negatives + false negatives)

True negatives were defined as mutation negatives that also had a score of “unlikely FH (DCLNS or Simon Broome) or “possible FH (DLCNS), while false negatives are mutation negatives with a Simon Broome or DLCNS score of “definite or probable FH”

3 Results

This study was undertaken to compare the results of two different clinical screening tools with a newly developed genetic screening array. A comparison of the genetic screening array to sequencing data was also undertaken.

3.1 Patients

Clinical and genetic screening data from a total of 70 patients of the Lipid Outpatient Clinic of University Hospital of Saarland with dyslipidemia and suspected FH were analyzed as part of this study. The baseline demographic and clinical characteristics of these 70 patients all of whom suffered from dyslipidemia are shown in Table 6 and Table 7.

Table 6: Baseline characteristics of the 70 panel patients I (n=70)

Variable	Percentage (number) Median (range)
General	
N=70	
Age (years)	58 (15-84)
Male %	48.5 (34/70)
Female %	51.4 (36/70)
Medical History	
Coronary artery disease	68.6 (48)
Previous myocardial infarction	41.4 (29)
Previous stent	64.3 (45)
Bypass Surgery	14.3 (10)
Previous Stroke	4.3 (3)
Peripheral Artery Disease	32.9 (23)
Family History for premature ASCVD	
1. Degree (N: 66)	92.4 (61/66)
2. Degree (N:59)	57.1 (32/56)
Lipid lowering medication	
Statin (daily dose in mg)	
Simvastatin	12.8 (9/70)
Atorvastatin	62.8 (44/70)
Fluvastatin	0.85 (6/70)
Pravastatin	0.57 (4/70)
Rosuvastatin	(0/70)
Non-Statin (daily dose in mg)	
Ezetimib	62.8 (44/70)
Fibrates	0.14 (1/70)
Cholastagel	0.28 (2/70)

Table 7: Baseline characteristics of the 70 panel patients II (n=70)

PCSK9 Inhibitors (daily dose in mg)	
Evolucumab	25.7 (18/70)
Alirocumab	18.5 (13/70)
Clinical characterictics	
Smoking (active)	17.6 (12/68)
Smoking (ex)	43.2 (29/67)
Arterial Hypertension	77.1 (54/70)
Diabetes mellitus	24.2 (17/70)
Metabolic Syndrom	31.4 (22/70)
Systolic blood pressure (mmHg)	140 (100-195)
Diastolic blood pressure (mmHg)	85 (60-110)

Heart rate (min-1)	67 (49-103)
Heigt (cm)	1.72 (1.51-1.96)
Weigt (kg)	85 (45-125)
Body mass index (kg/m2)	28.4 (18-42)
Cutaneous Symptoms	13.6 (8/60)
Laboratory values	
Fasting glucose (mg/dl)	100 (77-309)
HbA1c (%)	5.7 (4-11)
eGFR (ml/min)	88.2 (28-141)
CK (U/L)	108.5 (51-427)
GOT (U/L)	27.5 (16-83)
GPT (U/L)	27.0 (5-105)
GGT (U/L)	27.5 (7-330)
Total cholesterol (mg/dl)	230 (91-548)
HDL cholesterol (mg/dl)	50.5 (16-103)
Untreated LDL-Cholesterl (mg/dl)	222 (84-499)
LDL cholesterol (mg/dl)	162 (18-459)
Non-HDL cholesterol (mg/dl)	174.5 (40-491)
Fasting triglycerides (mg/dl)	138.5 (43-2646)
Lipoprotein (a) (nmol/l)	59.10 (7-677)
Apo A1 (mg/dl)	166.5 (78-334)
Apo B (mg/dl)	131.0 (32-307)

3.2 Clinical Screening

Using the Simon Broome criteria, out of the 70 patients in our panel, n=6 (8.6%) were diagnosed with definite FH, n=51 (72.6%) with probable FH, and n=13 (18.6%) were diagnosed with unlikely FH (Table 8).

15 of the 70 patients (21.4%) from our panel were diagnosed with definite FH using the DLCNS criteria. Probable FH was the DLCNS diagnosis for 20 patients (28.6%), 26 (37.1%) were diagnosed with possible FH, and another 9 (12.9%) were found to be unlikely to have FH.

Table 8: Overview of clinical diagnoses in our panel (n=70)

Diagnosis	Simon Broome N (%)	DLCNS N (%)
Definite	6 (8.6)	15 (21.4)
Probable	51 (72.6)	20 (28.6)
Possible	Not part of criteria	26 (37.1)
Unlikely	13 (18.6)	9 (12.9)

3.3 CARRENAL ARRAY

3.3.1 Design of the array

After the inception of the original design idea, an Affymetrix account manager was contacted to facilitate the design of the array (Figure 13). At this point, a list of target genes (Table 9), sequences, SNP types, and species information was provided to Affymetrix (Figure 14). Affymetrix proposed an original design which was evaluated by our group and subsequently modified. After several rounds of design proposal by Affymetrix and modifications by our group, a final design was approved, followed by the manufacture of the array that included a total number of 45298 individual SNP from the loci listed in Table 3. Due to design problems with some of the oligonucleotides, only 43,094 SNPs were ultimately included in the production array (Figure 15). Out of these, 41,852 variants could be detected successfully in the analyses.

944 of these SNPS were located within the *LDLR*, *APOB* and *PCSK9* genes and had previously determined as FH mutations in the Human Gene Mutation Database (HGMD) or the Leiden Open Variation Database (LOVD), respectively.

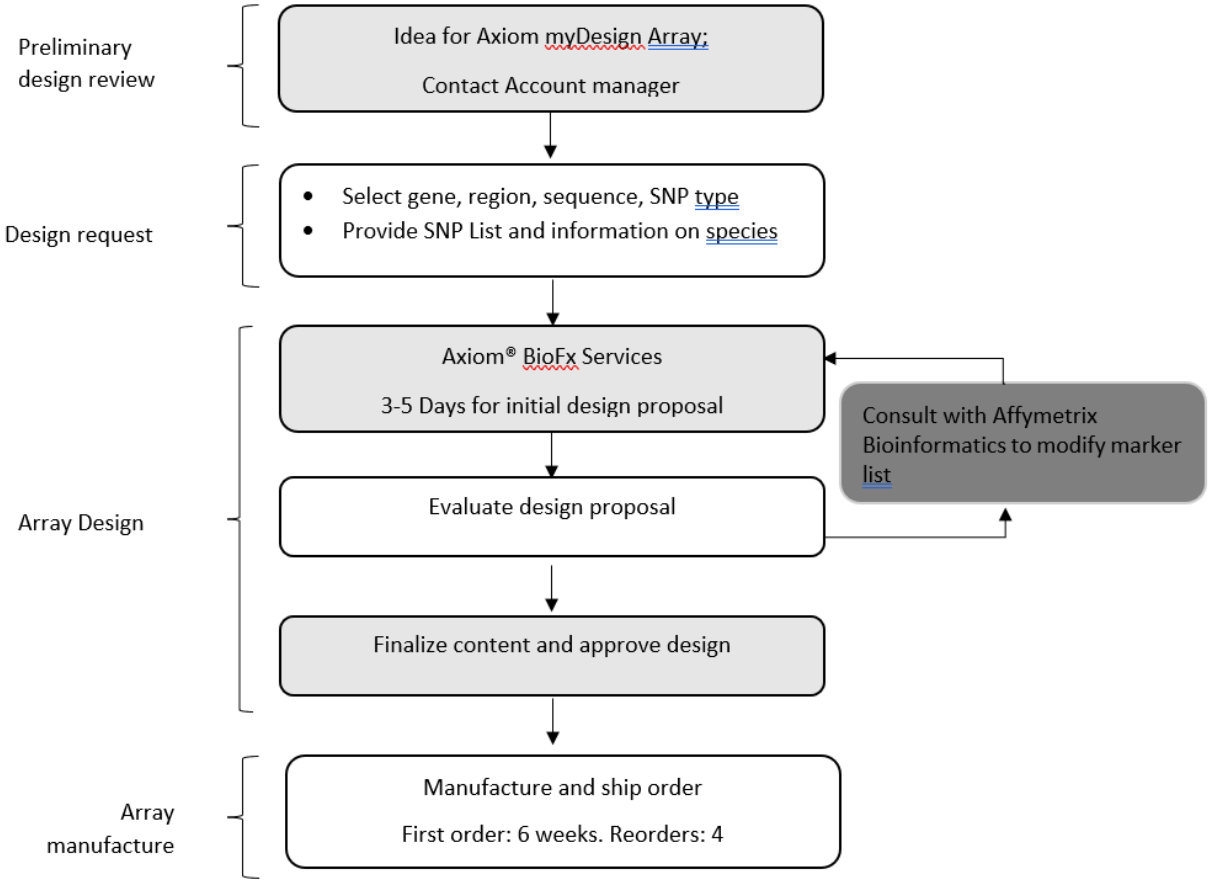

Figure 13: Workflow for the design of the Carrenal Array.

Table 9: First selection of genetic variants for early Design of CARRENAL Array (CHIP)

		Number
FH-Gene (LDLR, APOB, PCSK9)		
	biallelic	1314
	multiallelic	360
SNPs from Lipid GWAS		157
SNPs of lipid associated genes		11016
SNPs in genes for further phenotypes		29768
	Total	**42615**

Table 10: The number of SNPs that have been selected for inclusion into the final Design CARRENAL array (CHIP)

LDLR/APOB/PCSK9 SNPs HGMD	1594
LDLR/PCSK9 SNPs LOVD	70
LDLR SNPs Synlab	10
Lipid associated SNPs from GWAS (Willer et al. 2013)	157
SNPs in cardiomyopathy genes	170
Lp(a) SNPs	223
ApoE-SNPs + ApoB SNP (Talmud et al.)	3
ApoB-SNPs (Motazacker et al., Alves et al.)	3
SNPs in genes from lipid GWAS	11014
SNPs in genes from other phenotypes	31961
Affymetrix control SNPs	93
Total number of SNPs	**45298**

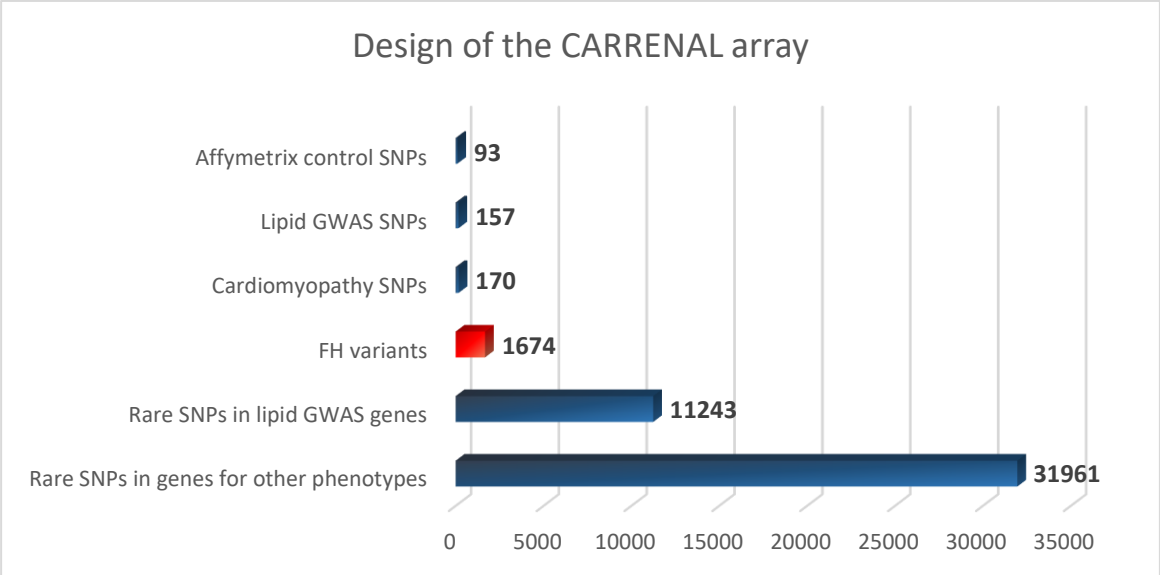

Figure 14: Number of genetic variants included in the design of the CARRENAL custom array.

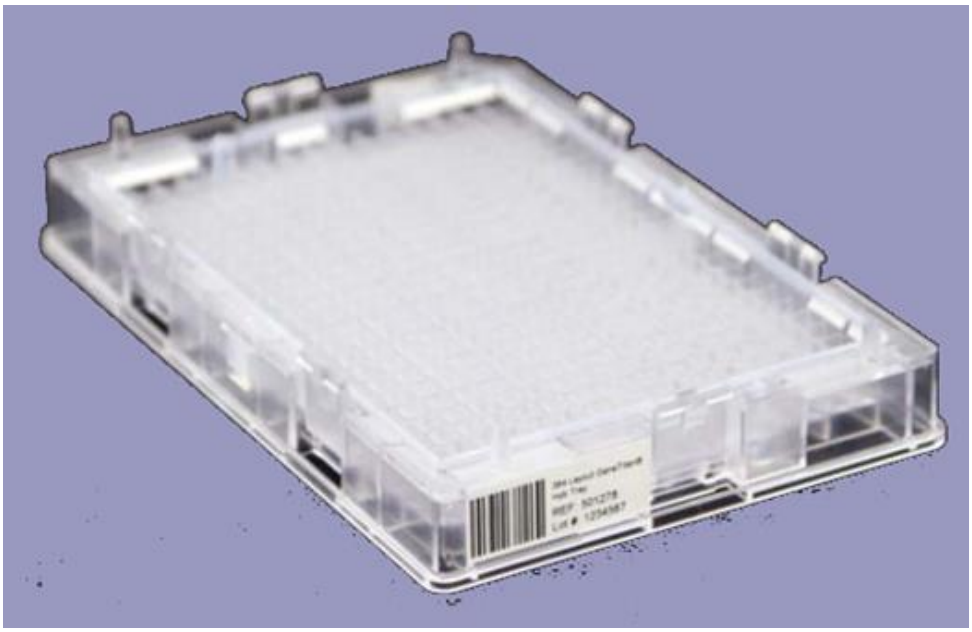

Figure 15: Axiom® myDesign™ Custom Array 384well plate.

3.3.2 Genotyping

DNA from the 70 patients of our suspected FH patient panel were genotyped using the Axiom® 384HT myDesign™ Custom Array from Affymetrix (CARRENAL array) that included a total of 41,852 SNPs. Markers with an off-target variant, a call rate < 99.5, an FLD < 3.9, or a Heterozygous Strength Offset (HetSO) < 0.165 were excluded from the subsequent analysis. Additionally, cluster plots of markers with an FLD between 3.9 and 6 were examined by hand and markers that did not appear well-clustered were excluded from further analyses. Using these exclusion criteria, a total of 30,655 SNPs were available for final analysis. The Axiom Analysis Suite ver. 2.0.0.35 software was used to classify SNPs s into seven different categories (Figure 16).

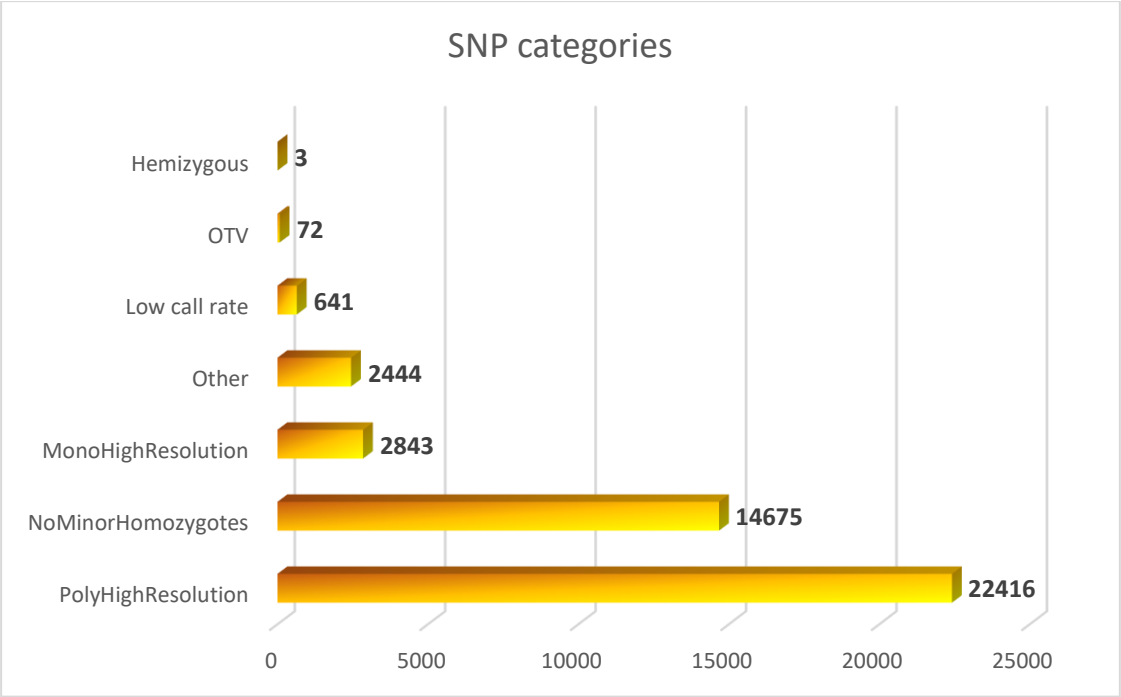

Figure 16: Classification of SNPs by the Axiom Analysis Suite.

In our sample of 70 patients, 8 were positive for LDL-Receptor associated mutations (Table 11). These patients were younger and had a higher female proportion compared to the entire panel (Table 12). Clinical baseline characteristics including laboratory findings, however were similar between these 8 patients and the entire panel (compared Table 6 and Table 7, above, to Table 12 and Table 13, below).

Table 11: List of the LDLR mutations for each CHIP (+) individual

Patient ID with (+)ve Mutation	FH Mutations
CH-01	Gln33Term/LDLR
CH-17	Asp221Gly/LDLR
CH-26	Ala50Thr/LDLR
CH-31	Glu140Gly/LDLR
CH-46	Val827Ile/LDLR
CH-48	Ala50Thr/LDLR
CH-58	Asp221Gly/LDLR
CH-64	Phe655Leu/LDLR

Table 12: Comparison of baseline characteristics of the CARRENAL ARRAY (CHIP) positive and negative populations

Variable	CHIP positive (n=8) Percentage (number) Median (range)	CHIP negative (n=62) Percentage (number) Median (range)	p-Value (T-Test)
General			
Age (years)	45 (36-66)	56 (15-84)	0.122
Male%	37.5	53.2	0.190
Female%	62.5	46.8	
Medical History			
Coronary artery disease %	62.5	65.6	0.211
Previous myocardial infarction	37.5	37.7	0.255
Previous stent	62.5	60.0	0.242
Bypass Surgery	0	15.0	0.851
Previous Stroke	0	4.9	0.521
Peripheral Artery Disease	37.5	0.33	0.815
Family History for premature ASCVD			
1. Degree	100	85.2	0.919
2. Degree	87.5	45.9	0.521
Clinical characterictics			
Smoking (active)%	12.5	19.0	0.675
Smoking (ex)%	25	44.8	0.695
Arterial Hypertension%	50	81.9	0.039
Diabetes mellitus%	25	26.2	0.397
Metabolic Syndrom%	12.5	38.9	0.202
Systolic blood pressure (mmHg)	135 (125-160)	138 (100-195)	0.868
Diastolic blood pressure (mmHg)	87.5 (70-95)	84.4 (110-60)	0.973
Heart rate (min-1)	64.5 (51-79)	67.8 (49-103)	0.649
Height (cm)	167 (156-186)	170 (151-196)	0.505
Weight (kg)	88 (55-96)	81 (45-125)	0.424
Body mass index (kg/m2)	28.9 (21.0-35.3)	29 (18-42)	0.737
Cutaneous Symptoms	12.5	13.5	0.582

Table 13: Baseline laboratory values of the CARRENAL ARRAY (CHIP) positive population

Laboratory values	CHIP positive (n=8) Median (range)	CHIP negative (n=62) Median (range)	P-value
Fasting glucose (mg/dl)	94 (86-189)	101 (77-309)	0.198
HbA1c (%)	6 (4-8)	5.7 (4.5-10.8)	0.680
eGFR (ml/min)	99 (73-112)	85 (28-141)	0.699
CK (U/L)	181 (59-259)	108 (51-427)	0.732
GOT (U/L)	27 (17-62)	28 (16-83)	0.801
GPT (U/L)	27 (20-105)	27 (5-69)	0.267
GGT (U/L)	26 (14-330)	29 (7-159)	**<0.001**
Total cholesterol (mg/dl)	259 (142-548)	259 (142-548)	0.395
HDL cholesterol (mg/dl)	59 (34-81)	59 (34-103)	0.248
Untreated LDL-Cholesterol (mg/dl)	330 (88-459)	330 (219-459)	0.254
LDL cholesterol by the 1.Visit (mg/dl)	176 (88-459)	176 (18-339)	0.780
Non-HDL cholesterol (mg/dl)	194 (104-491)	165 (40-348)	0.146
Fasting triglycerides (mg/dl)	155 (77-244)	138 (43-516)	0.925
Lipoprotein (a) (nmol/l)	86 (11-677)	53 (7-516)	**0.003**
Apo A1 (mg/dl)219-459)	175 (122-207)	166 (78-334)	0.194
Apo B (mg/dl)	141 (88-305)	131 (32-307)	0.521

Each of the 8 CARENNAL-positive patients had a unique LDLR-mutation (Table 11 and Table 14). Seven were point mutations that lead to an amino acid change while one point mutation introduced a premature stop codon.

Table 14: Summary of FH Mutations in the study population

CH-01	CH-17	CH-26	CH-31	CH-46	CH-50	CH-58	CH-64	HGMD_ID	Affy_SNP_ID
0	0	1	0	0	0	1	0	CM920420	Affx-80260140
0	0	0	0	1	1	0	0	CM994424	Affx-89015917
0	1	0	0	0	0	0	0	CM013558	Affx-89015785
1	0	0	0	0	0	0	0	CM920404	Affx-82462358
0	0	0	1	0	0	0	0	CM920471	Affx-52198252
0	0	0	0	NA	NA	NA	1	CM960938	Affx-89022326

The following images (Figure 17 to Figure 22) show the cluster plots of six of the eight mutations across the 70 genotyped patients.

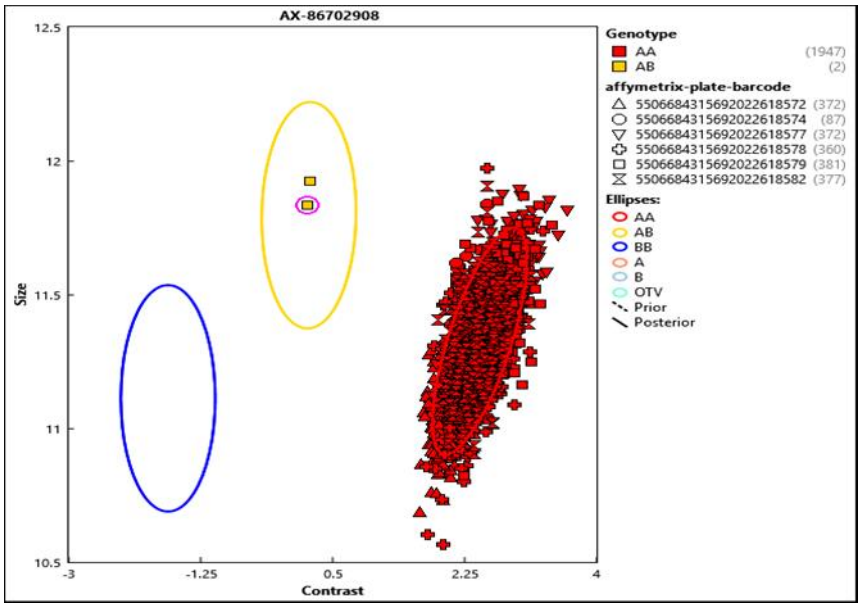

Figure 17: Clusterplot Affx-80260140
Data of six individual CARENNAL plates for the SNP 80260140. Expected plot points for genotypes AA, AB, and BB are denoted by red, yellow, and blue ellipses, respectively. Different plot symbols denote data from different CARENNAL plates.

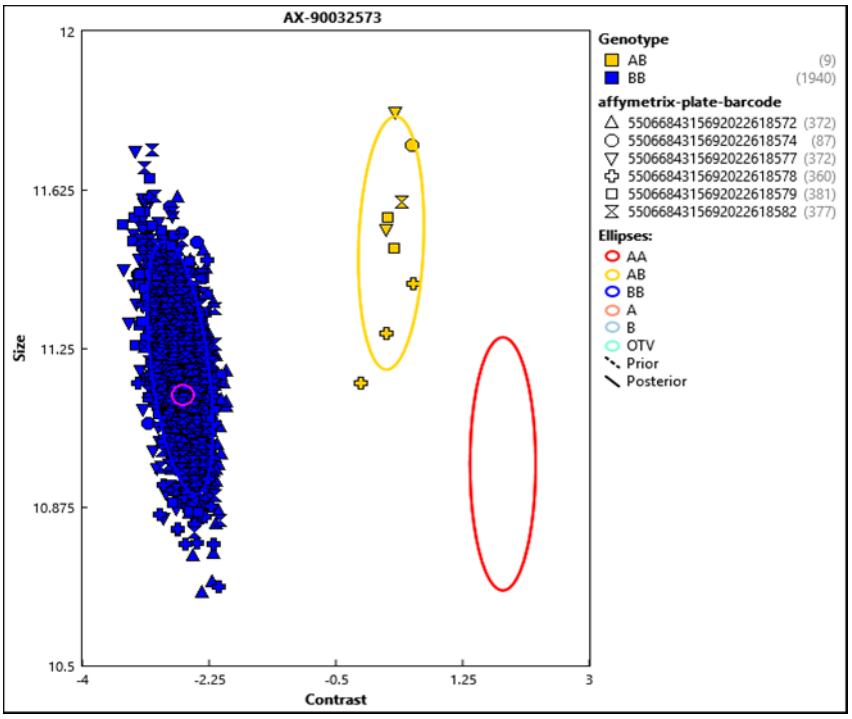

Figure 18: Clusterplot Affx-89015917
Data of six individual CARENNAL plates for the SNP 89015917. Expected plot points for genotypes AA, AB, and BB are denoted by red, yellow, and blue ellipses, respectively. Different plot symbols denote data from different CARENNAL plates.

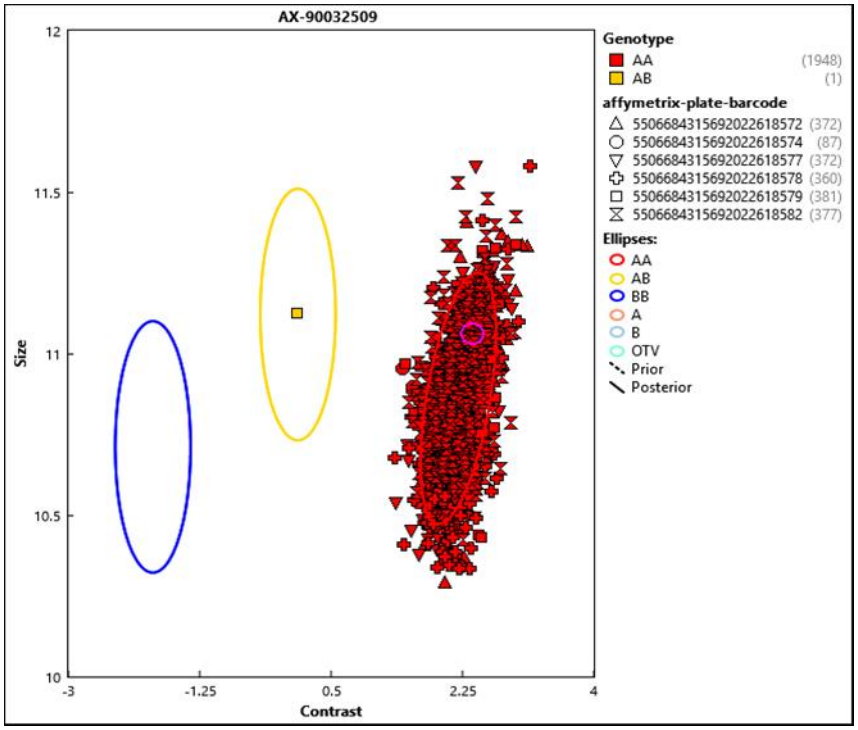

Figure 19: Clusterplot Affx-89015785
Data of six individual CARENNAL plates for the SNP 89015785. Expected plot points for genotypes AA, AB, and BB are denoted by red, yellow, and blue ellipses, respectively. Different plot symbols denote data from different CARENNAL plates.

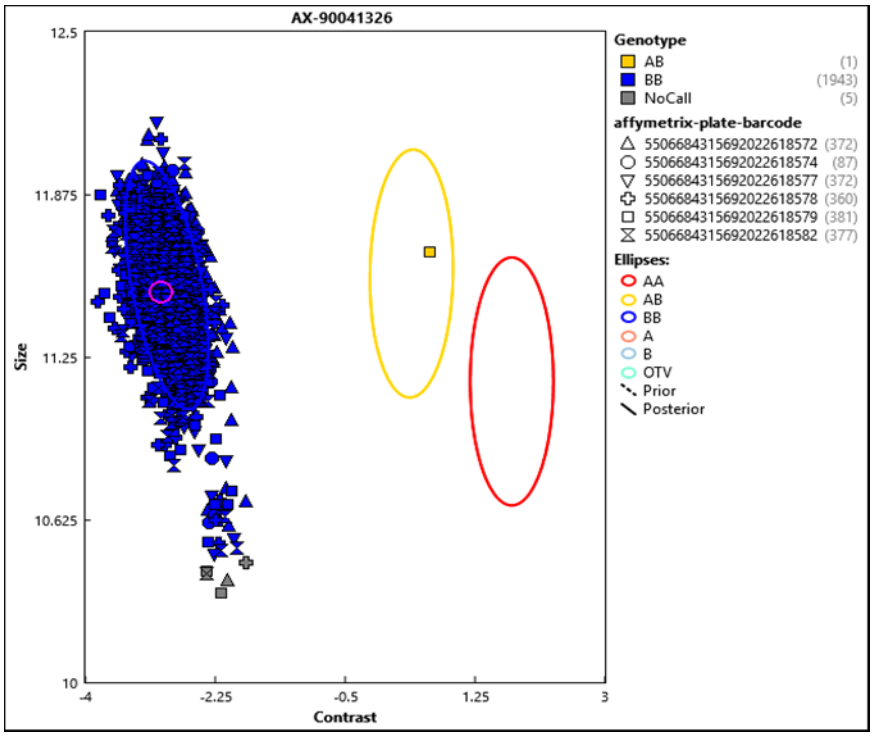

Figure 20: Clusterplot Affx-82462358
Data of six individual CARENNAL plates for the SNP 82462358. Expected plot points for genotypes AA, AB, and BB are denoted by red, yellow, and blue ellipses, respectively. Different plot symbols denote data from different CARENNAL plates.

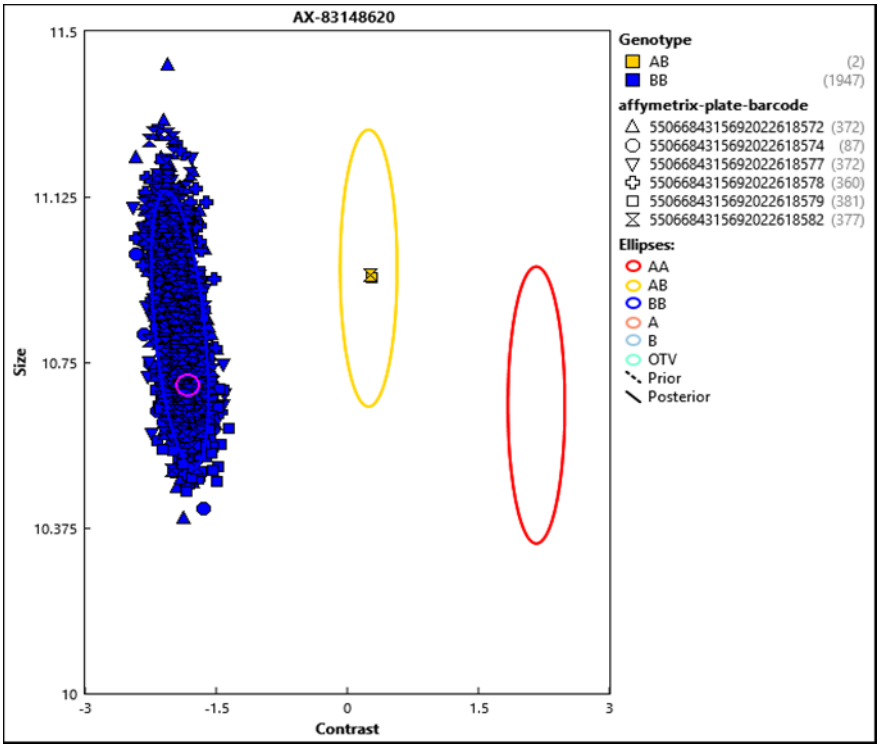

Figure 21: Clusterplot Affx-52198252
Data of six individual CARENNAL plates for the SNP 52198252. Expected plot points for genotypes AA, AB, and BB are denoted by red, yellow, and blue ellipses, respectively. Different plot symbols denote data from different CARENNAL plates.

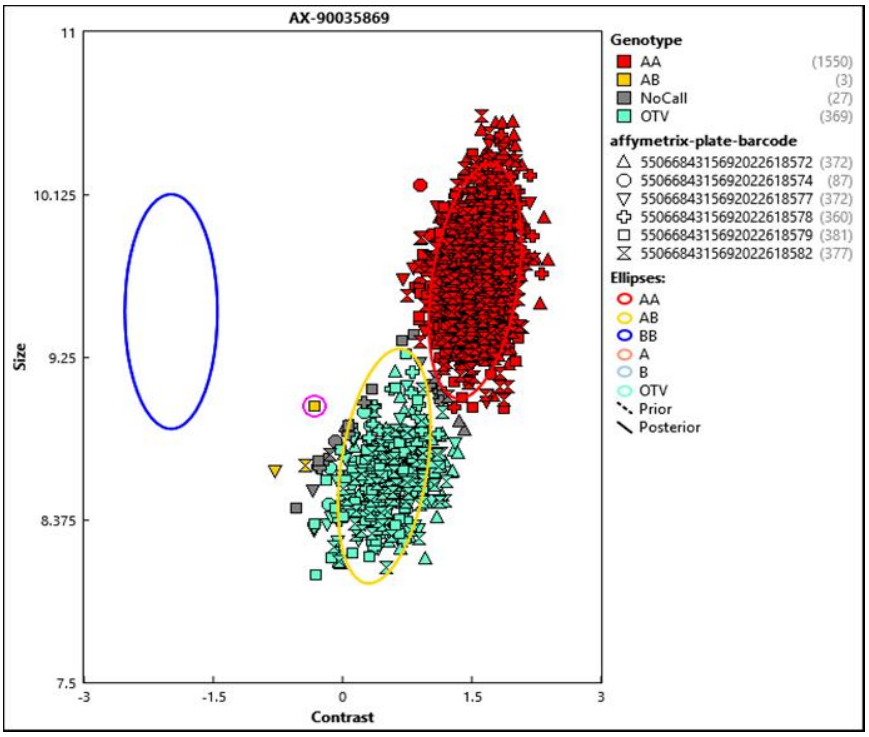

Figure 22: Clusterplot Affx-89022326
Data of six individual CARENNAL plates for the SNP 89015917. Expected plot points for genotypes AA, AB, and BB are denoted by red, yellow, and blue ellipses, respectively. Different plot symbols denote data from different CARENNAL plates.

The individuals who scored as highly likely to have FH using the different clinical score instruments most likely had a polygenetic variant of FH (Figure 23)

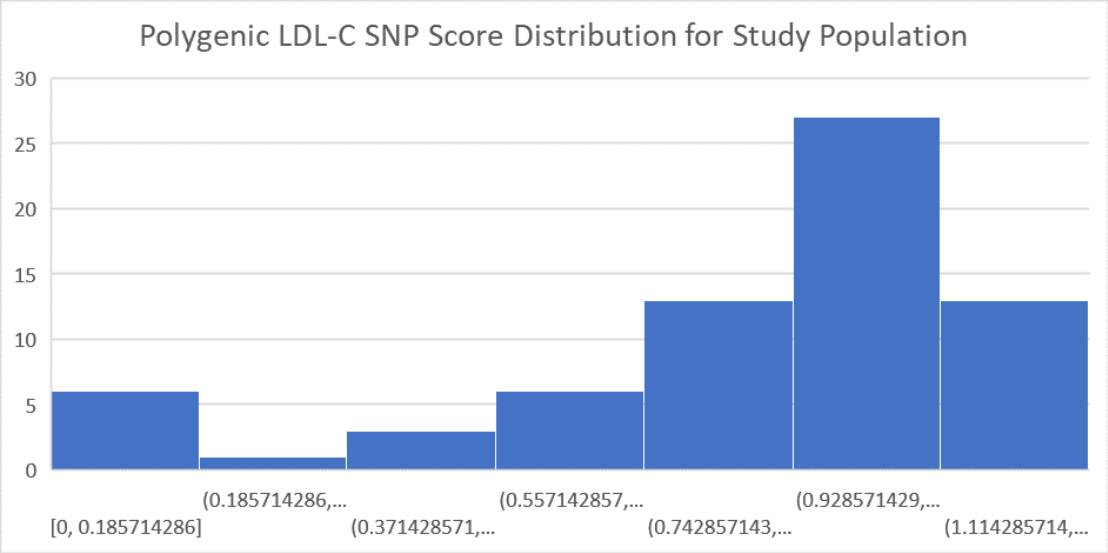

Figure 23: The polygenic explanation for the mutation negative individuals with highly clinical likelyhood for FH, FH/M-

Accordingly, a polygenetic score was calculated for each patient in the n=70 complete panel (Table 15)

Table 15: Calculated polygenetic score for each individual of the study population

Patient_ID	FH_polyscore		Patient_ID	FH_polyscore
CH-01	1.166		CH-44	0.792
CH-02	0.946		CH-45	0.976
CH-03	1.207		CH-46	NA
CH-04	0.577		CH-47	1.047
CH-05	1.3		CH-48	0.968
CH-06	1.134		CH-49	0.986
CH-07	0.737		CH-50	0.355
CH-08	0.777		CH-51	0.796
CH-09	0.825		CH-52	0.72
CH-10	0.741		CH-53	0.947
CH-11	1.095		CH-54	0.403
CH-12	NA		CH-55	1.138
CH-13	0.578		CH-56	NA
CH-14	1.033		CH-58	1.116
CH-15	0.477		CH-59	1.01
CH-16	0.855		CH-60	0.775
CH-17	1.107		CH-61	1.057
CH-18	0.964		CH-62	0.933
CH-19	0.857		CH-63	0.689
CH-20	0.972		CH-64	1.034
CH-21	0.976		CH-65	0.77
CH-22	1.105		CH-66	1.214
CH-23	0.436		CH-67	1.088
CH-24	1.02		CH-68	0.954
CH-25	1.226		CH-69	1.077
CH-26	NA		CH-70	1.076
CH-27	1.162			
CH-28	1.159			
CH-29	0.901			
CH-30	1.043			
CH-31	0.986			
CH-32	0.939			
CH-33	0.755			
CH-34	0.928			
CH-35	0.847			
CH-36	NA			
CH-37	1.268			
CH-38	1.162			
CH-39	1.025			
CH-40	1.184			
CH-41	NA			
CH-42	1.033			
CH-43	0.914			

Yellow highlight: chip-positive patient

3.3.3 Predictability and comparison between array and clinical scores

According to the Simon Broome Diagnostic Criteria, 50 patients were characterized as “definite FH”, of which seven had a mutation that was detected by the CARRENAL array (Table 18). The other seven CARRENAL positive patients scored as “probable FH” using the Simon Broome Diagnostic Criteria. None of the chip positive patients was rated unlikely using the Simone Broome Criteria. Of the 62 chip-negative patients, five scored as definite FH using Simon Broome Criteria while 44 more chip-negative patients scored “probable” using Simon Broome. 13 of the 62 CAREENAL-negative patients scored as “unlikely FH” using Simon Broome. The correlation between Simon Broome results and the CARRENAL array was not statistically significant (p=0.367, Table 17).

Table 16: Cross table Simon Broome Diagnostic Criteria by Molecular Diagnosis via Carrenal Arrays (CHIP) for FH

			Simon Bromm			Total
			definite FH	possible FH	unlikely FH	
Mutation	no	Number	43	5	13	61
		% within mutation	70,5%	8,2%	21,3%	100,0%
		% within Simon Bromm	86,0%	83,3%	100,0%	88,4%
	yes	Number	7	1	0	8
		% within mutation	87,5%	12,5%	0,0%	100,0%
		% within Simon Bromm	14,0%	16,7%	0,0%	11,6%
Total		Number	50	6	13	70
		% within mutation	72,5%	8,7%	18,8%	100,0%
		% within Simon Bromm	100,0%	100,0%	100,0%	100,0%

Table 17: Chi-Square Tests for Cross table Simon Broome Diagnostic Criteria by Molecular Diagnosis via Carrenal Arrays (CHIP) for FH

Chi-Square-Tests

	Wert	df	Asymptotic Significance (two-tailed)	Exact Significance (2-tailed)	Exact Significance (1-tailed)	Point-likelihood
Pearson’s Chi-square	2,138[a]	2	,343	,430		
Likelihood-Quotient	3,606	2	,165	,228		
Fisher’s Exact Test	2,117			,357		
Linear correlation	1,636[b]	1	,201	,234	,136	,072
Number of valid cases	1,636[b]	1	,201	,234		

The PPV and NPV of the CARRENAL array compared to the Simon Broom Diagnostic Criteria as gold standard were calculated these numbers an overview of which is shown in Table 18. PPV was:

True positives / (true positives + false positives) = 8/(8+0)=1=100%

NPV was:

True negatives/ (true negatives + false negatives) = 13 / (13 + 49) = 0.209 = 20.9%

Accordingly the test had a sensitivity of true positives/false negatives=8/49=16.3% and a specificity of true negatives/ false positives = 62/0 which is infinite.

Table 18: Comparision between predictive Values of Simon Broome Diagnostic Criteria and Molecular Diagnosis via Carrenal Arrays (CHIP) for FH

Simon Broome diagnostic criteria for FH	**Carrenal Arrays (CHIP) Genetic Analysis**	
N=70	**Mutation (+) ve (N= 8)**	**Mutation (-) ve (N=62)**
Definite FH (N=50)	7/8	43/62
Probable FH (N=6)	1/8	6/62
Unlikely FH (N= 13)	0/8	13/62
Predictivity	PPV: 100%	NPV: 20.9%

Using the DLCNS 15 patients were scored as “definite FH”. Out of these patients four were chip-positive (Table 19 and Table 21). The remaining four CARRENAL-positive patients were considered “probable FH” using the DLCNS scoring system. No DLCNS “possible FH” or “unlikely FH” patients were CARRENAL-positive. 11 of the 62 CARRENAL-negative patients scored as “definite FH” using the DLCNS criteria, while 16 scored as “probable FH”. “Possible FH” was the DLCNS diagnosis for 26 of the 62 CARRENAL-negative patients and nine additional CARRENAL-negative patients were scored “unlikely FH” using DLCNS criteria. The

correlation between the DLCNS and CARRENAL array results were statistically significant (p=0.18 Table 20).

Table 19: Cross table DLCNS by Molecular Diagnosis via Carrenal Arrays (CHIP) for FH

			DLCNS				Total
			definite FH	eprobable FH	possible FH	unlikely FH	
Mutation	no	Number	11	17	23	10	61
		% within mutation	18,0%	27,9%	37,7%	16,4%	100,0%
		% within DLCNS	73,3%	81,0%	100,0%	100,0%	88,4%
	yes	Number	4	4	0	0	8
		% within mutation	50,0%	50,0%	0,0%	0,0%	100,0%
		% within DLCNS	26,7%	19,0%	0,0%	0,0%	11,6%
Total		Number	15	21	23	10	69
		% within mutation	21,7%	30,4%	33,3%	14,5%	100,0%
		% within DLCNS	100,0%	100,0%	100,0%	100,0%	100,0%

Table 20: Chi-Square Tests for cross table DLCNS by Molecular Diagnosis via Carrenal Arrays (CHIP) for FH

Chi-Square-Tests

	Wert	df	Asymptotic Significance (two-tailed)	Exact Significance (2-tailed)
Pearson's Chi-square	8,791[a]	3	,032	,034
Likelihood-Quotient	11,661	3	,009	,011
Fisher's Exact Test	8,225			,018
Number of valid cases	69			

a. 4 Zellen (50,0%) haben eine erwartete Häufigkeit kleiner 5. Die minimale erwartete Häufigkeit ist 1,16.

The PPV and NPV of the CARRENAL array compared to the DLCNS as gold standard were calculated these numbers an overview of which is shown in Table 18. PPV was:

True positives / (true positives + false positives) = 8/(8+0)=1=100%

NPV was:

True negatives/ (true negatives + false negatives) = 35 / (35 + 27) = 0.565 = 56.5%

Accordingly the CARRENAL array had a sensitivity of true positives/false negatives=8/27=29.6% and a specificity of true negatives/ false positives = 62/0 which is infinite.

Table 21: Comparision/Relation between predictive Values of Dutch Lipid Clinic Network Score (DLCNS) and Molecular Diagnosis via Carrenal Arrays for FH

Dutch Lipid Clinic Network Score (DLCNS) for FH	**Carrenal Arrays (CHIP) Genetic Analysis**	
N=70	**Mutation (+) ve (N= 8)**	**Mutation (-) ve (N=62)**
Definite FH (N=15)	4/8	11/62
Probable FH (N=20)	4/8)	16/62
Possible FH (N=26)	0/8	26/62
Unlikely FH (N= 9)	0/8	9/62
Predictivity	PPV: 100%	NPV: 56.5%

In addition to the molecular analysis using the CARRENAL array, genetic data was obtained using DNA-sequencing. This analysis detected complete concordance in 2 of the eight CARRENAL-positive patients (Table 22, shaded green). Six patients had sequencing variants that were not included in the CARRENAL array and consequently not detected (Table 22, not shaded). In one of these, a different, adjacent mutation was detected using the CARRENAL array (patient ID CH-46). Five variants detected by CARRENAL array did not show a corresponding sequence variation using DNA sequencing (Table 22, shaded red).

Table 22: Comparision between sequencing and CARRENAL data

Patient ID	Sequencing	CARRENAL-array
CH-01	autosomal dominant (heterozygous FH c.97C Mutation) /LDL-R	(heterozygot FH c.97C Mutation) Gln33Term/LDL-R
CH-26	Asp221Gly/LDL-R	Asp221Gly/LDL-R
CH-03	Apo-B Mut, (Arg/Gln) (SLC-O1-B1 (T52C) heterozygous T/C	Not included in chip design
CH-04	LDL-c679_680insCGGTATGGACT heterozygous	Not included in chip design
CH-29	c.463T LDL-R mutation	Not included in chip design
CH-45	c.589-T>G and Cyc197Gly	Not included in chip design
CH-46	pGlu48Lys in LDLR gene	pGlu48Lys not included in chip design /detected Ala50Thr/LDL-R
CH-50	c.257_265del, p.(Phe86_Arg88del) LDL-R Gen	Not included in chip design
CH-65	Apo-B Mut, R-3500Q, heterozygous	Not included in chip design
CH-17		Glu140Gly/LDL-R
CH-31		Val827Ile/LDL-R
CH-48		Ala50Thr/LDL-R
CH-58		Asp221Gly/LDL-R
CH-64		Phe655Leu/LDL-R

4 Discussion

4.1 Patients

A total of 70 patients with dyslipidemia that were previously scored probably suffer from FH according to the German clinical scoring system for FH that were analyzed during this study. Their baseline characteristics lead to to a diagnosis of FH according to the German clinical criteria which was the reason why these patients were included in this study. In particular, the very high rate of first- and second-degree relatives with a premature ASCVD, their own medical history with high rates of coronary artery disease, previous myocardial infarctions, and peripheral artery disease in combination with dyslipidemia, made this population an ideal test population for the newly developed CARRENAL array.

4.2 Clinical Screening

As a first step the patients were evaluated with two widely accepted clinical scoring systems, the Simon Broome Criteria (114,115) and the DLCNS (113). The two scoring systems, however, showed considerable discrepancies in the results, highlighting the need for a more accurate and faster system, such as the newly developed CARRENAL arrays that, if used in routine diagnostics, would allow the fast and unambiguous detection of the most common FH-mutations. The discrepancies between the Simon Broome Criteria diagnoses and the DLCNS were apparent not only indifferent rates of “definite FH” which was less than half for the DLCNS compared to the Simon Broome Criteria, but also for “probable FH”. Since it might lead to undermedication and subsequent clinical consequences in the case of false negatives, even more importantly, Simon Broome Criteria considered 13 of the 70 patients to be “unlikely FH” while the DLCNS considered only 9 patients to fall into this category. This is especially striking since this patient population was selected because of a suspicion of FH when using the German clinical scoring criteria. Undermedication can have serious clinical sequalae while proper medical treatment can lead to good goal attainment and subsequent better long-term prognosis (116,117)

4.3 CARRENAL ARRAY

4.3.1 Screening data

The CARRENAL array found eight patients with mutations suggestive of FH. In accordance with the gene for the LDLR being the most affected gene in FH (24,49,51,52,60,101,115), all

of these eight patients had a mutation in the *LDLR* gene. The clusterplots of all detected mutations appeared robust.

When comparing these cases with those which considered definite FH when the clinical scoring system was used, the CARRENAL array showed a very high specificity with no fals-positives according to either clinical scoring system. The fact that no false-positive were detected makes it in fact impossible to calculate a numerical value for specificity, as the formula would contain a division by zero. Both clinical scoring systems, however, found a number of patients with definite or probable FH diagnoses where the CARRENAL array did not detect a mutation which led to relatively low NPV and sensitivity values. It is possible that in these cases a mutation is present that is not included in the array. Comparison with sequencing data showed that this is indeed the case for some of the patients. Other patients might suffer from a polygenetic type of FH (15,27).

Accordingly, there is only a partial match between the results of the Simon Broome criteria scoring, the DLCNS and the CARRENAL array, where some individuals fall into an area of overlap between two of the three diagnostic tools while only a few are considered FH according to all three systems (Figure 24). This could be due in part to imperfections in the clinical scores as highlighted by the discrepancies between Simon Broome Criteria and the DLCNS.

On the other hand, the presence of a mutations in a gene involved in the lipid metabolism does not necessarily lead to familial FH as several mutations have been noted in the *LDLR* and other related genes that seem to be benign, i.e. they do not seem to have an appreciable effect on LDL-C levels (23). While the CARRENAL assay was designed to include the most common mutations previously described to be associated with FH, the actual impact of these genes on LDL-C levels has not been thoroughly investigated for most of them. It is, therefore, possible that the CARRENAL array detects benign mutations and consequently overdiagnoses FH in some cases. When used as a screening tool in a population of patients with dyslipidemia this would, however, have few clinical consequences as these patients would most likely require pharmaceutical intervention to address the dyslipidemia whether FH is present or not. Interestingly, in this population none of the patients who were considered unlikely to suffer from FH according to either clinical criteria were CARRENAL positive, i.e. all eight CARRENAL positive patients were scored either “definite” or “probable FH” using both the Simon Broome and the DLCNS systems which highlights the diagnostic power of the CARRENAL array.

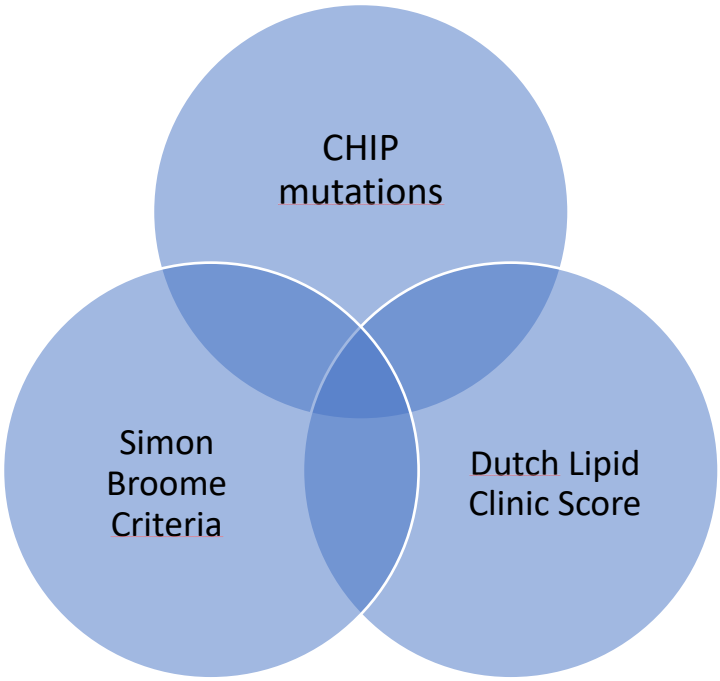

Figure 24: Schematic depiction of the overlap between the two clinical diagnostic systems and the CARRENAL array

Comparison of the CARRENAL array data with DNA-sequencing also showed discrepancies. In most cases these differences consisted of a mutation that was detected by DNA-sequencing that was not detected by the CARRENAL array. Every of these mutations were not included in the CARRENAL array due to their rarity or the fact that they had not previously been described as associated with FH. Additionally, every DNA sequencing method can introduce changes in the sequence which are called sequencing artifacts (118–121). Without replication of the variants detected by DNA sequencing, it is impossible to know whether these are actual mutations in the respective individuals or mere sequencing artifacts. In one case, the CARRENAL array found a mutation in the *LDLR* gene that was not detected by DNA sequencing. Instead, DNA sequencing detected a sequence variant near the one detected by the CARRENAL array. It is plausible that the existing mutation i.e. the one detected by sequencing that was not included in the CARRENAL array design altered the hybridization pattern of the CARRENAL probes in a way that gave the impression of the presence of the adjacent mutation that was included in the array design.

DNA sequencing failed to detect five mutations detected by the CARRENAL array, since due to logistic and time constraints when this study was undertaken not all probes included in the CARRENAL array had been thoroughly investigated in terms of their hybridization behaviour. While it is, therefore, possible that these five patients represent false positives of the array, the clinical data suggests that this is not the case. It is, therefore, likely that these events represent

sequencing errors such as allele dropouts where during sequencing due to the preferential amplification of one allele in a heterozygous sample the results indicate the presence of a homozygous sample as it has been described in other research (118,122,123).

4.4 Conclusion

The diagnosis of FH using clinical scoring systems to data represents significant challenges with different systems consistently arriving at notably different results when comparing the same population of patients. Since underdiagnosis and consequently undertreatment can lead to severe clinical consequences such as premature ASCVD, myocardial infarction, and stroke, better options for fast and accurate diagnosis are vital (116,117,124).

The CARRENAL array was developed to fill this need. The results showed that all chip-positive patients scored high with both clinical scoring systems, however discrepancies between CARRENAL array data and DNA sequencing warrant further research into the cause of these discrepancies as well as into the clinical consequences of given mutations in genes involved in lipid metabolism.

Bibliography

1. Khera A v., Hegele RA. What Is Familial Hypercholesterolemia, and Why Does It Matter? Circulation. 2020 Jun 2;141(22):1760–3.

2. Kramer AI, Trinder M, Brunham LR. Estimating the Prevalence of Familial Hypercholesterolemia in Acute Coronary Syndrome: A Systematic Review and Meta-analysis. Canadian Journal of Cardiology. 2019 Oct 1;35(10):1322–31.

3. Santos RD, Gidding SS, Hegele RA, Cuchel MA, Barter PJ, Watts GF, et al. Defining severe familial hypercholesterolaemia and the implications for clinical management: a consensus statement from the International Atherosclerosis Society Severe Familial Hypercholesterolemia Panel. Lancet Diabetes Endocrinol. 2016 Oct 1;4(10):850–61.

4. Kaptoge S, Pennells L, De Bacquer D, Cooney MT, Kavousi M, Stevens G, et al. World Health Organization cardiovascular disease risk charts: revised models to estimate risk in 21 global regions. Lancet Glob Health. 2019 Oct 1;7(10):e1332–45.

5. Statistisches Bundesamt (Destatis). Destatis. 2021 [cited 2021 Apr 18]. p. 1 Die 10 häufigsten Todesfälle durch Herz-Kreislauf-Erkrankungen - Statistisches Bundesamt. Available from: https://www.destatis.de/DE/Themen/Gesellschaft-Umwelt/Gesundheit/Todesursachen/Tabellen/sterbefaelle-herz-kreislauf-erkrankungen-insgesamt.html

6. Statistisches Bundesamt (Destatis). Sterbefälle durch Herz-Kreislauf-Erkrankungen insgesamt 2018 [Internet]. 2018. Available from: https://www.destatis.de/DE/Themen/Gesellschaft-Umwelt/Gesundheit/Todesursachen/Tabellen/sterbefaelle-herz-kreislauf-erkrankungen-insgesamt.html

7. Statistisches Bundesamt (Destatis). Gesundheit - Todesursachen in Deutschland. Destatis [Internet]. 2017;12(4):Wiesbaden. Available from: https://www.destatis.de/DE/Publikationen/Thematisch/Gesundheit/Todesursachen/Todesursachen2120400157004.pdf?__blob=publicationFile

8. Chipayo-Gonzales D, Ramakrishna H, Nuñez-Gil IJ. Score2: A New Updated Algorithm to Predict Cardiovascular Disease Risk in Europe. J Cardiothorac Vasc Anesth. 2022 Jan 1;36(1):18–21.

9. Townsend N, Wilson L, Bhatnagar P, Wickramasinghe K, Rayner M, Nichols M. Cardiovascular disease in Europe: Epidemiological update 2016. Eur Heart J. 2016 Nov 7;37(42):3232–45.

10. Bolijn R, Kunst AE, Appelman Y, Galenkamp H, Moll van Charante EP, Stronks K, et al. Prospective analysis of gender-related characteristics in relation to cardiovascular disease. Heart. 2022 Jun 10;108(13):1030–8.

11. Kastelein JJP, Reeskamp LF, Hovingh GK. Familial Hypercholesterolemia: The Most Common Monogenic Disorder in Humans. J Am Coll Cardiol. 2020 May 26;75(20):2567–9.

12. Chacón-Camacho OF, Pozo-Molina G, Méndez-Catalá CF, Reyes-Reali J, Méndez-Cruz R, Zenteno JC. Familial Hypercholesterolemia: Update and Review. Endocr Metab Immune Disord Drug Targets. 2021 Feb 10;22(2):198–211.

13. Amerizadeh A, Javanmard SH, Sarrafzadegan N, Vaseghi G. Familial Hypercholesterolemia (FH) Registry Worldwide: A Systematic Review. Curr Probl Cardiol. 2022 Oct 1;47(10).

14. Bérard E, Bongard V, Haas B, Dallongeville J, Moitry M, Cottel D, et al. Prevalence and Treatment of Familial Hypercholesterolemia in France. Canadian Journal of Cardiology. 2019 Jun 1;35(6):744–52.

15. Brandts J, Dharmayat KI, Ray KK, Vallejo-Vaz AJ. Familial hypercholesterolemia: Is it time to separate monogenic from polygenic familial hypercholesterolemia? Curr Opin Lipidol. 2020 Jun 1;31(3):111–8.

16. Goldberg AC, Gidding SS. Knowing the prevalence of familial hypercholesterolemia matters. Circulation. 2016;133(11):1054–7.

17. Brunham LR, Hegele RA. What Is the Prevalence of Familial Hypercholesterolemia? Arterioscler Thromb Vasc Biol. 2021;2629–31.

18. Polychronopoulos G, Tzavelas M, Tziomalos K. Heterozygous familial hypercholesterolemia: prevalence and control rates. Expert Rev Endocrinol Metab. 2021;16(4):175–9.

19. Cui Y, Li S, Zhang F, Song J, Lee C, Wu M, et al. Prevalence of familial hypercholesterolemia in patients with premature myocardial infarction. Clin Cardiol. 2019 Mar 1;42(3):385–90.

20. Beheshti SO, Madsen CM, Varbo A, Nordestgaard BG. Worldwide Prevalence of Familial Hypercholesterolemia: Meta-Analyses of 11 Million Subjects. J Am Coll Cardiol. 2020 May 26;75(20):2553–66.

21. Hu P, Dharmayat KI, Stevens CAT, Sharabiani MTA, Jones RS, Watts GF, et al. Prevalence of Familial Hypercholesterolemia among the General Population and Patients with Atherosclerotic Cardiovascular Disease: A Systematic Review and Meta-Analysis. Circulation. 2020 Jun 2;141(22):1742–59.

22. Sánchez-Ramos A, Fernández-Labandera C, Vallejo-Vaz A, Bonacho EC, Quevedo-Aguado L, Catalina-Romero C, et al. Prevalence of familial hypercholesterolemia phenotype and ten-year risk of cardiovascular events in a working population in primary prevention: The ICARIA study. Atherosclerosis. 2021 Dec 1;338:39–45.

23. Warden BA, Fazio S, Shapiro MD. Familial Hypercholesterolemia: Genes and Beyond. Endotext [Internet]. 2021 Oct 23 [cited 2023 Aug 3]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK343488/

24. Berberich AJ, Hegele RA. The complex molecular genetics of familial hypercholesterolaemia. Nat Rev Cardiol [Internet]. 2019 Jan 1 [cited 2023 Aug 3];16(1):9–20. Available from: https://pubmed.ncbi.nlm.nih.gov/29973710/

25. Harada-Shiba M, Ako J, Arai H, Hirayama A, Murakami Y, Nohara A, et al. Prevalence of familial hypercholesterolemia in patients with acute coronary syndrome in Japan: Results of the EXPLORE-J study. Atherosclerosis [Internet]. 2018 Oct 1 [cited 2023 Feb 7];277:362–8. Available from: https://pubmed.ncbi.nlm.nih.gov/30270072/

26. Bobrowska B, Zasada W, Rajtar-Salwa R, Dziewierz A, Dudek D. Prevalence of familial hypercholesterolemia in patients with acute coronary syndromes. Kardiol Pol. 2019;77(4):475–7.

27. Narayanaswamy M, Sharma S. Polygenic Hypercholesterolemia. StatPearls [Internet]. 2022 Jun 27 [cited 2023 Mar 22]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK559143/

28. Talmud PJ, Shah S, Whittall R, Futema M, Howard P, Cooper JA, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: A case-control study. The Lancet [Internet].

2013 Apr 13 [cited 2023 Mar 28];381(9874):1293–301. Available from: http://www.thelancet.com/article/S0140673612621278/fulltext

29. Gratton J, Finan C, Hingorani AD, Humphries SE, Futema M. LDL-C Concentrations and the 12-SNP LDL-C Score for Polygenic Hypercholesterolaemia in Self-Reported South Asian, Black and Caribbean Participants of the UK Biobank. Front Genet [Internet]. 2022 Mar 31 [cited 2023 Mar 23];13:1. Available from: /pmc/articles/PMC9010053/

30. Futema M, Bourbon M, Williams M, Humphries SE. Clinical utility of the polygenic LDL-C SNP score in familial hypercholesterolemia. Atherosclerosis. 2018 Oct 1;277:457–63.

31. Katzmann JL, Lehmann M, Tünnemann-Tarr A, an Haack I, Dressel A, März W, et al. Cutaneous manifestations in familial hypercholesterolaemia. Atherosclerosis. 2021 Sep 1;333:116–23.

32. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999 May 1;340(2):115–26.

33. Steinberg D. Hypercholesterolemia and inflammation in atherogenesis: Two sides of the same coin. Mol Nutr Food Res. 2005 Nov;49(11):995–8.

34. Stokes KY, Cooper D, Tailor A, Granger DN. Hypercholesterolemia promotes inflammation and microvascular dysfunction: Role of nitric oxide and superoxide. Free Radic Biol Med. 2002 Oct 15;33(8):1026–36.

35. Linton MF, Yancey PG, Davies SS, Jerome WG, Linton EF, Song WL, et al. The Role of Lipids and Lipoproteins in Atherosclerosis. Science (1979) [Internet]. 2019 Jan 3 [cited 2023 Feb 26];111(2877):166–86. Available from: https://www.ncbi.nlm.nih.gov/books/NBK343489/

36. Gaggini M, Gorini F, Vassalle C. Lipids in Atherosclerosis: Pathophysiology and the Role of Calculated Lipid Indices in Assessing Cardiovascular Risk in Patients with Hyperlipidemia. Int J Mol Sci. 2023 Jan 1;24(1).

37. Libby P, Buring JE, Badimon L, Hansson GK, Deanfield J, Bittencourt MS, et al. Atherosclerosis. Nat Rev Dis Primers. 2019 Dec 1;5(1).

38. Collado A, Marques P, Domingo E, Perello E, González-Navarro H, Martinez-Hervás S, et al. Novel Immune Features of the Systemic Inflammation Associated with Primary

Hypercholesterolemia: Changes in Cytokine/Chemokine Profile, Increased Platelet and Leukocyte Activation. J Clin Med [Internet]. 2018 Dec 22 [cited 2023 Feb 21];8(1). Available from: http://www.ncbi.nlm.nih.gov/pubmed/30583563

39. Collado A, Domingo E, Piqueras L, Sanz MJ. Primary hypercholesterolemia and development of cardiovascular disorders: Cellular and molecular mechanisms involved in low-grade systemic inflammation and endothelial dysfunction. Int J Biochem Cell Biol [Internet]. 2021 Oct 1 [cited 2023 Feb 21];139. Available from: https://pubmed.ncbi.nlm.nih.gov/34438057/

40. Miao J, Zang X, Cui X, Zhang J. Autophagy, hyperlipidemia, and atherosclerosis. Adv Exp Med Biol. 2020;1207:237–64.

41. Dhawan UK, Bhattacharya P, Narayanan S, Manickam V, Aggarwal A, Subramanian M. Hypercholesterolemia Impairs Clearance of Neutrophil Extracellular Traps and Promotes Inflammation and Atherosclerotic Plaque Progression. Arterioscler Thromb Vasc Biol. 2021;2598–615.

42. Wang Q, Wang Y, Xu D. Research progress on Th17 and T regulatory cells and their cytokines in regulating atherosclerosis. Front Cardiovasc Med. 2022 Sep 21;9:2516.

43. Beverly JK, Budoff MJ. Atherosclerosis: Pathophysiology of insulin resistance, hyperglycemia, hyperlipidemia, and inflammation. J Diabetes. 2020 Feb 1;12(2):102–4.

44. Fan J, Watanabe T. Atherosclerosis: Known and unknown. Pathol Int [Internet]. 2022 Mar 1 [cited 2023 Feb 22];72(3):151–60. Available from: https://pubmed.ncbi.nlm.nih.gov/35076127/

45. McGowan MP, Hosseini Dehkordi SH, Moriarty PM, Duell PB. Diagnosis and Treatment of Heterozygous Familial Hypercholesterolemia. Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease [Internet]. 2019 Dec 12 [cited 2023 Feb 26];8(24). Available from: /pmc/articles/PMC6951065/

46. Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, et al. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular riskThe Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS). Eur Heart J [Internet]. 2020 Jan 1 [cited 2023 Aug 4];41(1):111–88. Available from: https://dx.doi.org/10.1093/eurheartj/ehz455

47. Athero.org.au. DLCN questionnaire. 2021 [cited 2023 Feb 26]. Dutch Lipid Clinic Network Score (DLCNS) for FH. Available from: http://www.athero.org.au/fh/wp-content/uploads/Dutch-Lipid-Clinic-Network-Score2.pdf

48. Tada H, Okada H, Nomura A, Usui S, Sakata K, Nohara A, et al. Clinical Diagnostic Criteria of Familial Hypercholesterolemia - A Comparison of the Japan Atherosclerosis Society and Dutch Lipid Clinic Network Criteria. Circ J [Internet]. 2021 [cited 2023 Feb 26];85(6):891–7. Available from: https://pubmed.ncbi.nlm.nih.gov/33268660/

49. Sturm AC, Knowles JW, Gidding SS, Ahmad ZS, Ahmed CD, Ballantyne CM, et al. Clinical Genetic Testing for Familial Hypercholesterolemia: JACC Scientific Expert Panel. J Am Coll Cardiol. 2018 Aug 7;72(6):662–80.

50. Klose G, Laufs U, März W, Windler E. Familial hypercholesterolemia: Developments in diagnosis and treatment. Dtsch Arztebl Int [Internet]. 2014 Aug 4 [cited 2023 Jun 18];111(31–32). Available from: https://pubmed.ncbi.nlm.nih.gov/25145510/

51. Genetic Testing for Familial Hypercholesterolemia: Health Technology Assessment. Ont Health Technol Assess Ser. 2022;22(3):1–155.

52. Futema M, Taylor-Beadling A, Williams M, Humphries SE. Genetic testing for familial hypercholesterolemia—past, present, and future. J Lipid Res. 2021 Jan 1;62.

53. Sturm AC, Truty R, Callis TE, Aguilar S, Esplin ED, Garcia S, et al. Limited-Variant Screening vs Comprehensive Genetic Testing for Familial Hypercholesterolemia Diagnosis. JAMA Cardiol. 2021 Aug 1;6(8):902–9.

54. John G, Sahajpal NS, Mondal AK, Ananth S, Williams C, Chaubey A, et al. Next-Generation Sequencing (NGS) in COVID-19: A Tool for SARS-CoV-2 Diagnosis, Monitoring New Strains and Phylodynamic Modeling in Molecular Epidemiology. Curr Issues Mol Biol [Internet]. 2021 Sep 1 [cited 2022 Nov 23];43(2):845. Available from: /pmc/articles/PMC8929009/

55. Metzker ML. Sequencing technologies — the next generation. Nature Reviews Genetics 2010 11:1 [Internet]. 2009 Dec 8 [cited 2023 Mar 28];11(1):31–46. Available from: https://www.nature.com/articles/nrg2626

56. Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nature Reviews Genetics 2016 17:6 [Internet].

2016 May 17 [cited 2023 Mar 28];17(6):333–51. Available from: https://www.nature.com/articles/nrg.2016.49

57. Illumina. Technology Spotlight: Illumina ® Sequencing. Illumina Technology Spotlight [Internet]. 2010 [cited 2022 Nov 23];1–5. Available from: https://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf

58. Rusk N. Torrents of sequence. Nature Methods 2011 8:1 [Internet]. 2010 Dec 20 [cited 2022 Nov 27];8(1):44–44. Available from: https://www.nature.com/articles/nmeth.f.330

59. Wang M, Cai J, Chen J, Liu J, Geng X, Yu X, et al. PCR Techniques and Their Clinical Applications. 2023 Feb 14 [cited 2023 Apr 23]; Available from: https://www.intechopen.com/online-first/86086

60. Leren TP, Manshaus T, Skovholt U, Skodje T, Nossen IE, Teie C, et al. Application of Molecular Genetics for Diagnosing Familial Hypercholesterolemia in Norway: Results from a Family-Based Screening Program. Semin Vasc Med [Internet]. 2004 Feb [cited 2023 Apr 23];4(1):75–85. Available from: http://www.thieme-connect.com/products/ejournals/html/10.1055/s-2004-822989

61. Reiman A, Pandey S, Lloyd KL, Dyer N, Khan M, Crockard M, et al. Molecular testing for familial hypercholesterolaemia-associated mutations in a UK-based cohort: development of an NGS-based method and comparison with multiplex polymerase chain reaction and oligonucleotide arrays. Ann Clin Biochem [Internet]. 2016 Nov 1 [cited 2023 Apr 23];53(6):654–62. Available from: https://pubmed.ncbi.nlm.nih.gov/26748104/

62. National Human Genome Research Institute. Fact Sheets about Genomics. 2020 [cited 2023 Aug 7]. DNA Microarray Technology Fact Sheet. Available from: https://www.genome.gov/about-genomics/fact-sheets/DNA-Microarray-Technology

63. Hernán MA. The C-Word: Scientific Euphemisms Do Not Improve Causal Inference From Observational Data. Am J Public Health [Internet]. 2018 May 1 [cited 2023 Mar 30];108(5):616. Available from: /pmc/articles/PMC5888052/

64. Lee DH, Rezende LFM, Ferrari G, Aune D, Keum NN, Tabung FK, et al. Physical activity and all-cause and cause-specific mortality: assessing the impact of reverse causation and measurement error in two large prospective cohorts. Eur J Epidemiol

[Internet]. 2021 Mar 1 [cited 2023 Apr 23];36(3):275–85. Available from: https://pubmed.ncbi.nlm.nih.gov/33428024/

65. Aggio D, Papachristou E, Papacosta O, Lennon LT, Ash S, Whincup P, et al. Trajectories of physical activity from midlife to old age and associations with subsequent cardiovascular disease and all-cause mortality. J Epidemiol Community Health (1978) [Internet]. 2020 [cited 2023 Apr 23];74(2). Available from: https://pubmed.ncbi.nlm.nih.gov/31704805/

66. Kaufman BG, Van Houtven CH, Greiner MA, Hammill BG, Harker M, Anderson D, et al. Selection Bias in Observational Studies of Palliative Care: Lessons Learned. J Pain Symptom Manage. 2021 May 1;61(5):1002-1011.e2.

67. Glesby MJ, Hoover DR. Survivor treatment selection bias in observational studies: Examples from the AIDS literature. Ann Intern Med. 1996;124(11):999–1005.

68. Beck CA. Selection bias in observational studies: Out of control? Neurology. 2009 Jan 13;72(2):108–9.

69. Pandis N. Bias in observational studies. American Journal of Orthodontics and Dentofacial Orthopedics. 2014;145(4):542–3.

70. Cohen AB, Mathews SC. The Digital Outcome Measure. Digit Biomark [Internet]. 2018 Sep 1 [cited 2023 Apr 23];2(3):94–105. Available from: http://www.ncbi.nlm.nih.gov/pubmed/32095761

71. Jefford M, Stockler MR, Tattersall MHN. Outcomes research: What is it and why does it matter? Intern Med J. 2003 Mar 1;33(3):110–8.

72. Tung A. Challenges in Outcome Reporting. Anesthesiol Clin. 2018 Jun 1;36(2):191–9.

73. Kozlov N, Benzon HT. Role of Gender and Race in Patient-Reported Outcomes and Satisfaction. Anesthesiol Clin. 2020 Jun 1;38(2):417–31.

74. Moltaji S, Gallo M, Wong C, Murphy J, Gallo L, Waltho D, et al. Reporting Outcomes and Outcome Measures in Digital Replantation: A Systematic Review. J Hand Microsurg [Internet]. 2020 Aug 1 [cited 2023 Apr 23];12(2):85–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33335363

75. Lee J, Little TD. A practical guide to propensity score analysis for applied clinical research. Behaviour Research and Therapy. 2017 Nov 1;98:76–90.

76. Dahabreh IJ, Sheldrick RC, Paulus JK, Chung M, Varvarigou V, Jafri H, et al. Do observational studies using propensity score methods agree with randomized trials? A systematic comparison of studies on acute coronary syndromes. Eur Heart J. 2012 Aug;33(15):1893–901.

77. Davis ML, Neelon B, Nietert PJ, Burgette LF, Hunt KJ, Lawson AB, et al. Propensity score matching for multilevel spatial data: accounting for geographic confounding in health disparity studies. Int J Health Geogr. 2021 Dec 1;20(1).

78. Kim DH, Pieper CF, Ahmed A, Colón-Emeric CS. Use and Interpretation of Propensity Scores in Aging Research: A Guide for Clinical Researchers. J Am Geriatr Soc. 2016 Oct 1;64(10):2065–73.

79. Wilkinson JD, Mamas MA, Kontopantelis E. Logistic regression frequently outperformed propensity score methods, especially for large datasets: a simulation study. J Clin Epidemiol. 2022 Dec 1;152:176–84.

80. Sekula P, Del Greco FM, Pattaro C, Köttgen A. Mendelian randomization as an approach to assess causality using observational data. Journal of the American Society of Nephrology. 2016;27(11):3253–65.

81. Bowden J, Holmes M V. Meta-analysis and Mendelian randomization: A review. Res Synth Methods. 2019 Dec 1;10(4):486–96.

82. Skrivankova VW, Richmond RC, Woolf BAR, Yarmolinsky J, Davies NM, Swanson SA, et al. Strengthening the Reporting of Observational Studies in Epidemiology Using Mendelian Randomization: The STROBE-MR Statement. JAMA - Journal of the American Medical Association. 2021 Oct 26;326(16):1614–21.

83. Mohammadi-Shemirani P, Chong M, Narula S, Perrot N, Conen D, Roberts JD, et al. Elevated Lipoprotein(a) and Risk of Atrial Fibrillation: An Observational and Mendelian Randomization Study. J Am Coll Cardiol. 2022 Apr 26;79(16):1579–90.

84. Smith GD, Hemani G. Mendelian randomization: genetic anchors for causal inference in epidemiological studies. Hum Mol Genet [Internet]. 2014 [cited 2023 Apr 23];23(R1). Available from: https://pubmed.ncbi.nlm.nih.gov/25064373/

85. Burgess S, Thompson SG. Avoiding bias from weak instruments in Mendelian randomization studies. Int J Epidemiol [Internet]. 2011 Jun [cited 2023 Apr 23];40(3):755–64. Available from: https://pubmed.ncbi.nlm.nih.gov/21414999/

86. Hemani G, Tilling K, Davey Smith G. Orienting the causal relationship between imprecisely measured traits using GWAS summary data. PLoS Genet [Internet]. 2017 Nov 1 [cited 2023 Apr 23];13(11). Available from: https://pubmed.ncbi.nlm.nih.gov/29149188/

87. Huang J, Zuber V, Matthews PM, Elliott P, Tzoulaki J, Dehghan A. Sleep, major depressive disorder, and Alzheimer disease: A Mendelian randomization study. Neurology. 2020 Oct 6;95(14):E1963–70.

88. Lu T, Forgetta V, Greenwood CMT, Zhou S, Richards JB. Circulating Proteins Influencing Psychiatric Disease: A Mendelian Randomization Study. Biol Psychiatry. 2023 Jan 1;93(1):82–91.

89. Hartwig FP, Borges MC, Horta BL, Bowden J, Davey Smith G. Inflammatory biomarkers and risk of schizophrenia: A 2-sample mendelian randomization study. JAMA Psychiatry. 2017 Dec 1;74(12):1226–33.

90. Gill D, Cameron AC, Burgess S, Li X, Doherty DJ, Karhunen V, et al. Urate, Blood Pressure, and Cardiovascular Disease: Evidence From Mendelian Randomization and Meta-Analysis of Clinical Trials. Hypertension. 2021 Feb 1;77(2):383–92.

91. Chen D, Zhang Y, Yidilisi A, Xu Y, Dong Q, Jiang J. Causal Associations Between Circulating Adipokines and Cardiovascular Disease: A Mendelian Randomization Study. Journal of Clinical Endocrinology and Metabolism. 2022 Jun 1;107(6):E2572–80.

92. Rosoff DB, Smith GD, Mehta N, Clarke TK, Lohoff FW. Evaluating the relationship between alcohol consumption, tobacco use, and cardiovascular disease: A multivariable Mendelian randomization study. PLoS Med. 2020 Dec 4;17(12).

93. Zhou W, Liu G, Hung RJ, Haycock PC, Aldrich MC, Andrew AS, et al. Causal relationships between body mass index, smoking and lung cancer: Univariable and multivariable Mendelian randomization. Int J Cancer. 2021 Mar 1;148(5):1077–86.

94. Larsson SC, Burgess S, Mason AM, Michaëlsson K. Alcohol Consumption and Cardiovascular Disease: A Mendelian Randomization Study. Circ Genom Precis Med. 2020 Jun 1;13(3):E002814.

95. Zhu M, Ma Z, Zhang X, Hang D, Yin R, Feng J, et al. C-reactive protein and cancer risk: a pan-cancer study of prospective cohort and Mendelian randomization analysis. BMC Med. 2022 Dec 1;20(1).

96. Brice P, Burton H, Edwards CW, Humphries SE, Aitman TJ. Familial hypercholesterolaemia: A pressing issue for European health care. Vol. 231, Atherosclerosis. Atherosclerosis; 2013. p. 223–6.

97. Alonso R, Defesche JC, Tejedor D, Castillo S, Stef M, Mata N, et al. Genetic diagnosis of familial hypercholesterolemia using a DNA-array based platform. Clin Biochem. 2009 Jun;42(9):899–903.

98. Damgaard D, Larsen ML, Nissen PH, Jensen JM, Jensen HK, Soerensen VR, et al. The relationship of molecular genetic to clinical diagnosis of familial hypercholesterolemia in a Danish population. Atherosclerosis. 2005;180(1):155–60.

99. Qiagen. QIAamp ® DNA Mini and Blood Mini Handbook [Internet]. 2016. Available from: www.qiagen.com.

100. Thermo Fisher Scientific. Product Overview. 2023 [cited 2023 Mar 15]. p. 1 Axiom™ myDesign™ 384HT Custom Array. Available from: https://www.thermofisher.com/order/catalog/product/000870

101. Bogsrud MP, Øyri LKL, Halvorsen S, Atar D, Leren TP, Holven KB. Prevalence of genetically verified familial hypercholesterolemia among young (<45 years) Norwegian patients hospitalized with acute myocardial infarction. J Clin Lipidol. 2020 May 1;14(3):339–45.

102. All genes - Global Variome shared LOVD [Internet]. 2023 [cited 2023 Apr 23]. Available from: https://databases.lovd.nl/shared/genes

103. Willer CJ, Schmidt EM, Sengupta S, Peloso GM, Gustafsson S, Kanoni S, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet [Internet]. 2013 Nov [cited 2023 Apr 23];45(11):1274–85. Available from: https://pubmed.ncbi.nlm.nih.gov/24097068/

104. Talmud PJ, Shah S, Whittall R, Futema M, Howard P, Cooper JA, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. Lancet [Internet]. 2013

[cited 2023 Apr 23];381(9874):1293–301. Available from: https://pubmed.ncbi.nlm.nih.gov/23433573/

105. Alves AC, Etxebarria A, Soutar AK, Martin C, Bourbon M. Novel functional APOB mutations outside LDL-binding region causing familial hypercholesterolaemia. Hum Mol Genet [Internet]. 2014 [cited 2023 Apr 23];23(7):1817–28. Available from: https://pubmed.ncbi.nlm.nih.gov/24234650/

106. Gangloff A, Bergeron J, Couture P, Martins R, Hegele RA, Gagné C. A novel mutation of apolipoprotein B in a French Canadian family with homozygous hypobetalipoproteinemia. J Clin Lipidol [Internet]. 2011 Sep [cited 2023 Apr 23];5(5):414–7. Available from: https://pubmed.ncbi.nlm.nih.gov/21981844/

107. Grenkowitz T, Kassner U, Wühle-Demuth M, Salewsky B, Rosada A, Zemojtel T, et al. Clinical characterization and mutation spectrum of German patients with familial hypercholesterolemia. Atherosclerosis [Internet]. 2016 Oct 1 [cited 2023 Apr 23];253:88–93. Available from: https://pubmed.ncbi.nlm.nih.gov/27596133/

108. Nikpay M, Goel A, Won HH, Hall LM, Willenborg C, Kanoni S, et al. A comprehensive 1,000 Genomes-based genome-wide association meta-analysis of coronary artery disease. Nat Genet [Internet]. 2015 Sep 29 [cited 2023 Apr 23];47(10):1121–30. Available from: https://pubmed.ncbi.nlm.nih.gov/26343387/

109. Mahajan A, Go MJ, Zhang W, Below JE, Gaulton KJ, Ferreira T, et al. Genome-wide trans-ancestry meta-analysis provides insight into the genetic architecture of type 2 diabetes susceptibility. Nat Genet [Internet]. 2014 [cited 2023 Apr 23];46(3):234–44. Available from: https://pubmed.ncbi.nlm.nih.gov/24509480/

110. Dehghan A, Dupuis J, Barbalic M, Bis JC, Eiriksdottir G, Lu C, et al. Meta-analysis of genome-wide association studies in >80 000 subjects identifies multiple loci for C-reactive protein levels. Circulation [Internet]. 2011 Feb 22 [cited 2023 Apr 23];123(7):731–8. Available from: https://pubmed.ncbi.nlm.nih.gov/21300955/

111. Köttgen A, Albrecht E, Teumer A, Vitart V, Krumsiek J, Hundertmark C, et al. Genome-wide association analyses identify 18 new loci associated with serum urate concentrations. Nat Genet [Internet]. 2013 Feb 1 [cited 2023 Apr 23];45(2):145–54. Available from: https://pubmed.ncbi.nlm.nih.gov/23263486/

112. Pattaro C, Teumer A, Gorski M, Chu AY, Li M, Mijatovic V, et al. Genetic associations at 53 loci highlight cell types and biological pathways relevant for kidney function. Nat Commun [Internet]. 2016 Jan 21 [cited 2023 Apr 23];7. Available from: https://pubmed.ncbi.nlm.nih.gov/26831199/

113. Nordestgaard BG, Chapman MJ, Humphries SE, Ginsberg HN, Masana L, Descamps OS, et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease Consensus Statement of the European Atherosclerosis Society. Eur Heart J [Internet]. 2013 Dec 1 [cited 2023 Jun 11];34(45):3478–90. Available from: https://dx.doi.org/10.1093/eurheartj/eht273

114. Austin MA, Hutter CM, Zimmern RL, Humpries SE. Familial hypercholesterolemia and coronary heart disease: A HuGE association review. Am J Epidemiol. 2004 Sep 1;160(5):421–9.

115. Austin MA, Hutter CM, Zimmern RL, Humphries SE. Genetic causes of monogenic heterozygous familial hypercholesterolemia: a HuGE prevalence review. Am J Epidemiol [Internet]. 2004 Sep 1 [cited 2023 Jun 11];160(5):407–20. Available from: https://pubmed.ncbi.nlm.nih.gov/15321837/

116. Schmidt N, Dressel A, Grammer TB, Gouni-Berthold I, Julius U, Kassner U, et al. Lipid-modifying therapy and low-density lipoprotein cholesterol goal attainment in patients with familial hypercholesterolemia in Germany: The CaReHigh Registry. Atherosclerosis. 2018 Oct 1;277:314–22.

117. Gitt AK, Laufs U, März W, Paar WD, Bramlage P, Marx N, et al. Hypercholesterolemia Diagnosis, Treatment Patterns, and 12-Month Target Achievement in Clinical Practice in Germany in Patients with Familial Hypercholesterolemia. J Clin Med [Internet]. 2022 Jun 30 [cited 2023 Aug 24];11(13). Available from: http://www.ncbi.nlm.nih.gov/pubmed/35807095

118. Chen L, Liu P, Evans TC, Ettwiller LM. DNA damage is a pervasive cause of sequencing errors, directly confounding variant identification. Science (1979). 2017 Feb 17;355(6326):752–6.

119. Kircher M. Analysis of high-throughput ancient DNA sequencing data. Methods in Molecular Biology. 2012;840:197–228.

120. Star B, Nederbragt AJ, Hansen MHS, Skage M, Gilfillan GD, Bradbury IR, et al. Palindromic sequence artifacts generated during next generation sequencing library preparation from historic and ancient DNA. PLoS One. 2014 Mar 7;9(3).

121. Tanaka N, Takahara A, Hagio T, Nishiko R, Kanayama J, Gotoh O, et al. Sequencing artifacts derived from a library preparation method using enzymatic fragmentation. PLoS One. 2020 Jan 1;15(1).

122. Blais J, Lavoie SB, Giroux S, Bussières J, Lindsay C, Dionne J, et al. Risk of misdiagnosis due to allele dropout and false-positive PCR artifacts in molecular diagnostics analysis of 30,769 genotypes. Journal of Molecular Diagnostics. 2015;17(5):505–14.

123. Høy Hansen M, Steensboe Lang C, Abildgaard N, Nyvold CG. Comparative evaluation of the heterozygous variant standard deviation as a quality measure for next-generation sequencing. J Biomed Inform. 2022 Nov 1;135.

124. Laufs U, Parhofer KG. Simplified algorithm to facilitate communication of familial hypercholesterolaemia. Eur Heart J. 2015;36(43):3004–6.

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Lebenslauf

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht

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