Preclinical Atherosclerosis in Monogenic Familial Hypercholesterolaemia and Polygenic Hypercholesterolaemia

Dr Mahtab Sharifi

University College London

Cardiovascular Genetic Laboratory

MD(Res) Degree

DECLARATION

I, Mahtab Sharifi confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Dr Małgorzata Walus-Miark provided the Polish cohort samples from the Jagiellonian University in Krakow in Poland. Dr Mourad Labib at the Dudley Group NHS Foundation Trust and Dr Jeanine Roeters van Lennep at the Erasmus Medical Centre in Rotterdam provided DNA samples and CT scan results of their patients. The patients at the Royal Free Hospital were recruited by me from Dr Devaki Nair clinic. Ms Lucy Jenkins facilitated the DNA samples collection and MLPA analysis of the samples at the Great Ormond Street Hospital Genetic Lab in London.

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Ms KaWah Li performed some of the genotyping for six LDL-C raising SNPs and Polish samples. Ms Maryam Zain performed some of the *LPA* genetic analysis. The statistical analysis in chapter three, four and five were carried out by me under supervision and help from Ms Jackie Cooper, the statistician at the Cardiovascular Genetic Lab.

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ABSTRACT

Familial Hypercholesterolaemia (FH) is an autosomal dominant disorder with a frequency of 1 in 250 to 500 in most European populations. It is characterised by a raised low density lipoprotein-cholesterol (LDL-C) and a high incidence of premature coronary heart disease (CHD).

There are three genes where mutations are known to cause FH: the low-density lipoprotein receptor (*LDLR*) gene, the apolipoprotein B (*APOB*) gene and the pro-protein convertase subtilisin/kexin type 9 (*PCSK9*) gene. An FH-causing mutation can be found in around 40% of patients with a possible diagnosis of FH. It has been suggested that the patients with a clinical diagnosis of FH where no mutation were found might have a polygenic cause for their raised LDL-C.

FH disorder is an under-diagnosed condition in many countries such as Poland. An analysis of a Polish FH cohort in this thesis, demonstrated the heterogeneous aetiology of FH. We found 39 different pathogenic mutations in the *LDLR* gene with 10 of them being novel and an overall detection rate of 43.4%.

The aim of this thesis was to compare preclinical atherosclerosis between patients with monogenic FH and subjects with polygenic hypercholesterolaemia by means of a neck ultrasound to measure carotid Intima Media Thickness and a cardiac CT scan to assess coronary artery calcification. This study showed that preclinical atherosclerosis was greater in patients with monogenic FH.

Lipoprotein(a) [Lp(a)] is a well-known biomarker for CHD risk prediction. The Lp(a) concentration and its association with two *LPA* single nucleotide polymorphisms (rs3798220 and rs6919346) were assessed in FH patients participating in the Simon Broome registry and a group of the general population participating in the Northwick Park Heart Study II. The results showed that the Lp(a) concentration and the frequency of rs3798220 was significantly higher in the FH patients compared to the general population.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ApoB	Apolipoprotein B
Bp	Base Pair
CAC	Coronary Artery Calcium
CHD	Coronary Heart Disease
CIMT	Carotid Intima Media Thickness
DNA	Deoxyribose Nucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FH	Familial Hypercholesterolaemia
GWAS	Genome Wide Association Study
HDL	High Density Lipoprotein
HRM	High Resolution Melting
LDL-C	Low Density lipoprotein-Cholesterol
LDLR	Low Density lipoprotein Receptor
LIPA	Lysosomal Acid Lipase
Lp(a)	Lipoprotein(a)
M	Mole
MADGE	Microplate-Array Diagonal Gel Electrophoresis
MLPA	Multiplex Ligation-dependent Probe Amplification
mM	Millimole
MRI	Magnetic Resonance imaging
mL	Milli Litre
nM	Nanomole
NPHSII	Northwick Park Heart Study II.
PCR	Polymerase Chain Reaction
PCSK9	Protein Convertase Subtilisin/kexin type 9
pmol	Picomole
PNPLA5	Patatin-like Phopspholipase-Domain-Containing Family
RFLP	Restriction Fragment Polymorphism
Rpm	Revolutions per Minute
SBFH	Simone Broome FH study
SD	Standard Deviation
SMR	Standardised Mortality Ratio
SNP	Single Nucleotide Polymorphism
STAP1	Signal Transducing Adaptor Protein Family 1
TC	Total Cholesterol
TG	Triglyceride
WHII	Whitehall Phase II study

1 CHAPTER ONE: INTRODUCTION

1.1 Familial Hypercholesterolaemia (FH)

Familial Hypercholesterolaemia (FH) is an autosomal dominant disorder of low density lipoprotein-cholesterol (LDL-C) metabolism (Nordestgaard *et al.* 2013). It is characterised by substantially raised concentrations of LDL-C and five to eight times higher than average risk of premature coronary heart disease (CHD) due to accelerated atherosclerosis from birth (Marks *et al.* 2004).

1.2 Genetic causes of monogenic heterozygous FH

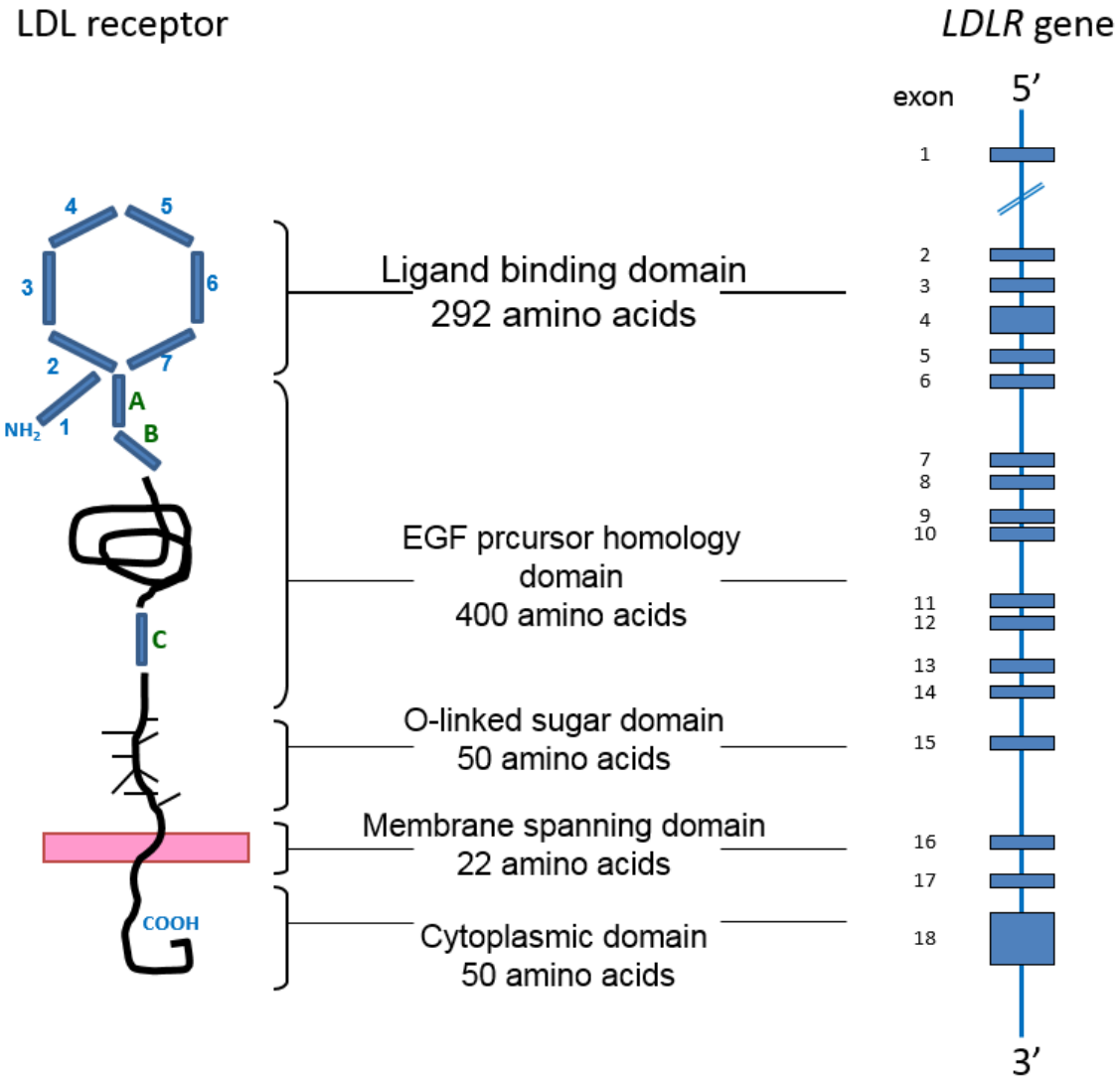
The most common genes causing FH are as follows:

1.2.1 Low-Density Lipoprotein Receptor (*LDLR*) gene

The *LDLR* gene was the first gene found to cause FH. It spans 45 kilobases (kb) on the short arm of chromosome 19 and comprises 18 exons that are transcribed and translated into five distinct domains which form the cell surface LDL-receptor (Figure 1) (Hobbs *et al.* 1992). Any defect in the *LDLR* gene can cause loss of function of LDL-receptor, resulting in reduced LDL-C uptake from blood.

There are more than 2900 different variants identified in the *LDLR* gene with majority of them being exonic substitutions and small (<100 bp) or large rearrangements (>100 bp) (Leigh *et al.* 2016). More than 90% of the reported variants are likely to be disease-causing (Usifo *et al.* 2012). Most FH cohort studies showed that among the variants found, a large proportion cluster in exon 4 (Grenkowitz *et al.* 2016). In contrast, the mutation frequency in exon 15 and 16 is extremely low (Leigh *et al.* 2016).

Figure 1: *LDLR* gene domains and related sections of the LDL-receptor



1.2.2 Apolipoprotein B (*APOB*) gene

Apolipoprotein B (apoB) is the major apolipoprotein on lipoprotein molecules, especially LDL-C, and functions as a ligand to the LDL-receptor. The *APOB* gene is located on chromosome 2p and spans more than 43kb. The gene comprises 29 exons and is transcribed and translated into a protein of 4563 amino acids (Chen *et al.* 1986).

Mutations of the *APOB* gene causing hypercholesterolaemia are missense mutations that result in ligand-defective apoB protein. The LDL-C particles made from this allele, therefore, have reduced apoB affinity to the LDL-receptor and accumulate in the blood (Myant 1993).

A single mutation occurs due to G to A transition at nucleotide 10580 in exon 26 of the *APOB* which leads to substitution of Glutamine to Arginine, p.(Arg3527Gln), accounts for approximately 6-10% of all FH cases in the European population (Myant *et al.* 1997). Other *APOB* mutations in other regions of the gene such as p.(Arg50Trp), p.(Arg1164Thr) and p.(Gln4494del) were also recently found to cause FH (Thomas *et al.* 2013, Fernandez-Higuero *et al.* 2015).

1.2.3 Pro-protein Convertase Subtilisin/Kexin type 9 (*PCSK9*) gene

The *PCSK9* gene (proprotein convertase subtilisin/kexin type 9) encodes an enzyme that is involved in regulating the degradation of the LDL-receptor protein in the lysosome of the cell, preventing it from being recycled to the cell surface. The gene is found on chromosome 1p and comprises 12 exons, covering 39kb (Humphries *et al.* 2009).

The PCSK9 molecule is synthesized as an inactive proprotein and undergoes cleavage in the endoplasmic reticulum to produce an enzyme with the prodomain noncovalently bound to the catalytic site, preventing further enzyme action. PCSK9 is secreted mostly from the liver and its binding to the LDL-receptor directs the receptor to the lysosome for degradation (Lagace *et al.* 2006).

Any ‘gain-of-function’ mutations in the *PCSK9* gene increase LDL-receptor degradation and consequently reduce the number of receptors on the cell surface. Although more than 20 such variants have been reported world-wide, the only common *PCSK9* variant in the

UK is p.(Asp374Tyr), which occurs in about 2% of the mutation-positive FH patients. This variant was found to increase the affinity of PCSK9 for the LDL-receptor. It is associated with a raised cholesterol level and a high risk of developing premature CHD, compared with a mutation in the *LDLR* gene (Naoumova *et al.* 2005).

The 'loss-of-function' mutations in the *PCSK9* gene that inactivate the PCSK9 protein are known to have a beneficial effect on LDL-C levels (Zhao *et al.* 2006). The most common of these variants, p.(Arg46Leu), enhances the clearance of LDL-C from the plasma and lowers cholesterol level in plasma. In the European populations, approximately 3% of individuals are carriers of this variant (Benn *et al.* 2010).

1.2.4 Other genetic causes of monogenic FH

Autosomal recessive hypercholesterolaemia is a very rare form of FH caused by mutations in the low-density lipoprotein receptor adaptor protein 1 gene (*LDLRAP1*) which encodes a cytosolic protein that interacts with the cytoplasmic tail of the LDL-receptor. This gene is located on the short arm of chromosome 1 (Michaely *et al.* 2004). Mutations in this gene, that usually cause premature truncations of the protein, lead to LDL-receptor malfunction and hypercholesterolaemia. The LDL-C level in these cases is typically intermediate between homozygote and heterozygote autosomal dominant FH patients (Austin *et al.* 2004).

A specific mutation, p.(Leu167del), in *APOE* gene has been reported to cause autosomal dominant FH (Marduel *et al.* 2013). This mutation has been previously reported to be associated with sea-blue histiocytosis and familial combined hyperlipidaemia (FCH) but the overlap between the FCH and FH phenotype have been shown before as hypertriglyceridaemia can be seen due to many common genetic and environmental factors (Talmud 2001, Civeira *et al.* 2008).

Other genes causing significantly elevated LDL-C and possibly the clinical phenotype of FH have been found, such as *STAP1* (signal transducing adaptor protein family 1), *LIPA* (lysosomal acid lipase) and *PNPLA5* (patatin-like phopspholipase-domain-containing family) (Stitziel *et al.* 2013, Fouchier *et al.* 2014, Lange *et al.* 2014). The *STAP1* and *PNPLA5* genes and their variants have yet to be independently confirmed as FH-causing.

1.3 Clinical diagnosis of FH

A clinical diagnosis of FH is based on having raised cholesterol levels, physical stigmata (e.g. tendon xanthomata and corneal arcus), or an evidence of these signs in first- or second-degree relatives and having a family history of premature CHD (DeMott K 2008). There are three available criteria that are used widely: the Simon Broome criteria from the UK, the Dutch Lipid Clinic Network criteria from the Netherlands and the MedPed criteria from the US (Marks *et al.* 2003).

In the UK, the National Institute for Health and Care Excellence (NICE) guideline recommends the use of the Simon Broome criteria (DeMott K 2008). A 'definite' diagnosis of FH is made if a patient has elevated cholesterol levels and tendon xanthomata or a mutation is found by sequencing a DNA sample from the patient. A 'possible' diagnosis of FH is made if the patient has high levels of cholesterol levels and a family history of hypercholesterolaemia or premature CHD.

Umans-Eckenhausen *et al.* defined the widely used Dutch Lipid Clinic Network criteria (DLCN) criteria for the diagnosis of heterozygous familial hypercholesterolaemia (Umans-Eckenhausen *et al.* 2001). The DLCN use a point system based on the patient's measured LDL-C levels, personal and family history of premature CHD, physical examination and carriage of an FH-causing mutation to give a possible, probable or definite diagnosis of FH (Table 1) (Austin *et al.* 2004).

Table 1: Simon Broome and Dutch Lipid Clinic Network diagnostic criteria for index FH individuals

Simon Brome Criteria		
Diagnosis of definite FH	Cholesterol concentration (Adults: TC >7.5 mmol/L and LDL-C >4.9 mmol/L; Children: TC >6.7 mmol/L and LDL-C >4.0 mmol/L); and tendon xantomata; or DNA-based evidence of a mutation in the *LDLR, APOB,* or *PCSK9* genes	
Diagnosis of possible FH	Cholesterol concentration (Adults: TC >7.5 mmol/L and LDL-C >4.9 mmol/L; Children: TC >6.7 mmol/L and LDL-C >4.0 mmol/L) And at least one of the below: Family history of myocardial infarction (aged younger than 50 years in 2nd degree relatives or aged younger than 60 years in 1st degree relatives) Family history of raised TC >7.5 mmol/L in 1st or 2nd degree adult relatives; or TC >6.7 mmol/L in 1st or 2nd degree child relatives	
Dutch Lipid Clinic Network Criteria		Points
Family history	1st degree relative (men <55 years and women < 60 years) with known premature coronary and vascular disease; or 1st degree relative with known LDL-C above the 95th percentile	1
	1st degree relative with tendon xanthomata; and/or arcus cornealis; or children aged less than 18 years with LDL-C above the 95th percentile	2
Clinical history	Patient with premature CHD	2
	Patient with premature cerebral or peripheral vascular disease	1
Physical examination	Tendon xanthomata	6
	Arcus cornealis prior to age 45 years	4
Cholesterol levels	LDL-C ≥ 8.5	8
	LDL-C : 6.5-8.4	5
	LDL-C : 5.0-6.4	3
	LDL-C : 4.0-4.9	1
DNA analysis	Functional mutation in the *LDLR* gene	8
Diagnosis	A 'definite' diagnosis requires more than 8 points A 'probable' diagnosis requires 6-8 points A 'possible' diagnosis requires 3-5 points	

TC: total cholesterol; CHD: coronary heart disease

A recent publication from 133,540 people from a large population-based prospective cohort conducted in the north of the Netherlands analysed percentiles of all lipid parameters in the general population (Balder *et al.* 2017). The Besseling *et al.* also studies the pre-treatment lipid profiles and data from 14,283 mutation carriers from the national FH screening programme in the Netherlands and produced the tables with age and gender specific percentiles of untreated LDL-C for FH patients (Besseling *et al.* 2014).

The DLCN criteria have been modified by clinicians in Wales to take into account that an elevated triglyceride (TG) level in a suspected FH patient makes it less likely that the patient has monogenic FH (Table 2) (Haralambos *et al.* 2015).

Table 2: Dutch Lipid Clinic Network criteria modified by Wales's clinicians (Haralambos *et al*)

Fasting triglyceride levels (mmol/L)	Points for Fasting triglyceride levels
2.5-3.4	-2
3.5-4.9	-3
>5.0	-4
Total points: Points from Dutch Lipid Clinic Network plus Points for Fasting triglyceride levels (genetic testing offered to anyone with total points > 6)	

The MedPed criteria are used for diagnosis of probable FH in the US and are mainly based on the total cholesterol (TC) and LDL-C cut offs stratified by age and family history. The cut offs are different in individuals with first, second and third degree relatives with FH (Williams *et al.* 1993).

1.4 Frequency of FH

Although the prevalence of FH has historically been estimated at 1 in 500, the likely true prevalence of FH-causing mutation carriage now appears to be between 1 in 250 and 1 in 300 in many European populations. In Denmark, 98,098 participants from the Copenhagen General Population Study were genotyped for three common *LDLR* mutations and the commonest *APOB* mutation p.(Arg3527Gln). The prevalence of the four FH mutations was 1 in 565, accounting for ~39% of pathogenic mutations in the country and equating to a total

prevalence of FH-mutation carriers of 1 in 217 (Benn *et al.* 2016). A prevalence of 1 in 270 was reported in Wald *et al* in a sample of ~10,000 UK children, (Wald *et al.* 2016) and a similar prevalence in subjects in the UK 10,000 genome project and in the US (Consortium *et al.* 2015, Do *et al.* 2015, Wald *et al.* 2016).

The frequency of heterozygous FH is also considerably higher in some populations due to a "founder" effect. This occurs when immigration of a small number of subjects to a geographical area is followed by a population expansion from those individuals. If, by chance, one (or more) of these "founder" individuals have FH, then genetic drift could lead to a high proportion of affected people in that population. Such founder effects have been reported in French Canadian, South-African Afrikaners, Jews and Finns (Austin *et al.* 2004).

In countries, such as Poland, the population remains highly understudied and the scale of FH under-diagnosis has been recently highlighted (Rynkiewicz *et al.* 2013). It is estimated that based on FH frequency of 1 in 500, more than 80,000 people in Poland might be affected by FH. Only about 20% of these appears to have been diagnosed to date, (Mysliwiec *et al.* 2014) with only few studies examining the spectrum of *LDLR* mutations in FH families in Poland (Bednarska-Makaruk *et al.* 2001, Chmara *et al.* 2010). A recent review in Poland also estimated the prevalence of FH at approximately 404 in 100,000, which equates to approximately 1 in 250 people (Pajak *et al.* 2016).

The information regarding molecular diagnosis of FH in other parts of the world such as Latin America and South Asia are also scant. In Brazil and Mexico, the countries with the largest cohorts in Latin America, only a few *LDLR* mutations have been reported, many of which have been encountered in the European population previously (Mehta *et al.* 2016).

1.5 Polygenic hypercholesterolaemia

Currently, an FH mutation can be found in 60-80% of patients with a clinical diagnosis of definite FH (Taylor *et al.* 2010). In those where a causative mutation cannot be found, there is a strong possibility that there may be a polygenic cause for FH. The Global Lipids Genetics Consortium meta-analysis identified over 100 loci where common variants influence LDL-C levels (Teslovich *et al.* 2010). In combination, such LDL-C-raising single nucleotide polymorphisms (SNPs) can have a substantial effect on LDL-C levels.

Talmud *et al* used a calculated gene score, derived from 12 common LDL-C-raising SNPs in 11 genes (Table 3) in a sample of UK FH patients with the European ancestry who had a clinical diagnosis of FH with or without an identified FH-causing mutation and compared the mean score with those of healthy men and women of the European ancestry from the UK Whitehall II (WHII) cohort study (Talmud *et al.* 2013).

Table 3: Twelve LDL-C raising SNPs used in Talmud *et al*

Global Lipid Genetic Consortium 12-SNP LDL-C gene score calculation					
	Chromosome number	Gene	Minor allele	Common allele	weight for score calculation
rs2479409	1	*PCSK9*	G[1]	A	0·052
rs629301	1	*CELSR2*	G	T[1]	0·15
rs1367117	2	*APOB*	A[1]	G	0·10
rs4299376	2	*ABCG8*	G[1]	T	0·071
rs1564348	6	*SLC22A1*	C	T[1]	0·014
rs1800562	6	*HFE*	A	G[1]	0·057
rs3757354	6	*MYLIP*	T	C[1]	0·037
rs11220462	11	*ST3GAL4*	A[1]	G	0·050
rs8017377	14	*NYNRIN*	A[1]	G	0·029
rs6511720	19	*LDLR*	T	G[1]	0·18
rs429358	19	*APOE*	C	T	..
rs7412	19	*APOE*	T	C	..
e2e2	19	*APOE*	..	..	−0·9
e2e3	19	*APOE*	..	..	−0·4
e2e4	19	*APOE*	..	..	0·2
e3e3	19	*APOE*	..	..	0
e3e4	19	*APOE*	..	..	0·1
e4e4	19	*APOE*	..	..	0·2

[1] Risk alleles (LDL-C raising)

Talmud *et al* showed that mean weighted LDL-C raising gene score in the mutation negative group was significantly higher compared to the mutation positive and control group. In the FH group with a known mutation, the LDL-C gene score was also significantly higher than the score in the healthy control group but was significantly lower than that of the mutation-negative group.

Using this score, it appears that at least 80% of FH mutation-negative patients are likely to have a polygenic (not a monogenic) explanation for their LDL-C level of over 4.9 mmol/L. In contrast, in individuals with a low LDL-C SNP score, there is a high likelihood that there is a yet unidentified monogenic cause (Talmud *et al.* 2013).

The additional polygenic contribution in the mutation positive group might explain the variation in the LDL-C concentrations among the family members of the FH patients. In these families, it might be possible that the FH phenotype is caused by the combination of a single mutation of large effect in *LDLR/APOB/PCSK9* and an accumulation of common small-effect LDL-C-raising alleles.

The LDL-C SNP score analysis in 7 independent European cohorts confirmed the findings reported by Talmud *et al*. (Futema *et al.* 2015). Futema *et al* reported that addition of 21 further LDL-C-raising SNPs did not significantly improve the ability of the SNP score to discriminate between the mutation negative and positive group compared with the 12-SNP score used in Talmud *et al*. From a diagnostic point of view, Futema *et al* showed that reducing the number of SNPs to the top 6 was as good at discriminating polygenic hypercholesterolaemia as the 12 SNP score, and using a smaller number of SNPs would clearly have cost benefits.

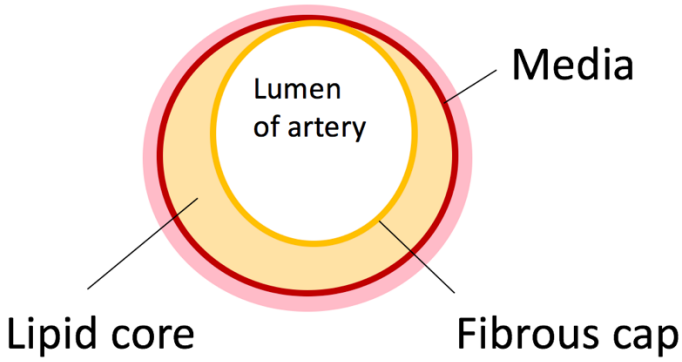
1.6 Atherosclerosis

The starting point for arterial atherosclerosis is artery endothelial dysfunction. The most important contributors to endothelial dysfunction are hemodynamic disturbances, hypercholesterolaemia and inflammation (Ross 1999).

Chronic endothelial injury in arteries results in endothelial dysfunction and increased permeability, and induces LDL oxidation and accumulation in the arterial wall (Flavahan

1992). This is followed by platelet aggregation, lymphocyte and monocyte adhesion and infiltration, thus initiating the inflammatory process (Cushing *et al.* 1990, Cybulsky *et al.* 1991). As monocytes are attracted into to the endothelium, they mature into macrophages and take up oxidized LDL transforming into 'foam' cells that eventually form the lipid core of the atherosclerotic plaque after apoptosis occurs. Atherosclerosis has a silent course for several decades before reaching clinical significance, when it typically presents as cardiovascular diseases (Ross 1999) Thus, CHD is thought to be primarily a problem of dysfunctional coronary endothelium which leads to inflammation, lipid accumulation and fibromuscular hyperplasia (Gallo *et al.* 2017). Figure 2 shows a typical atherosclerotic plaque comprises of the lipid core and the fibrous cap (Stary 2000, Doran *et al.* 2008).

Figure 2: A schematic picture of a plaque in an artery with a lipid core and a fibrous cap



1.7 Coronary heart disease (CHD) in FH

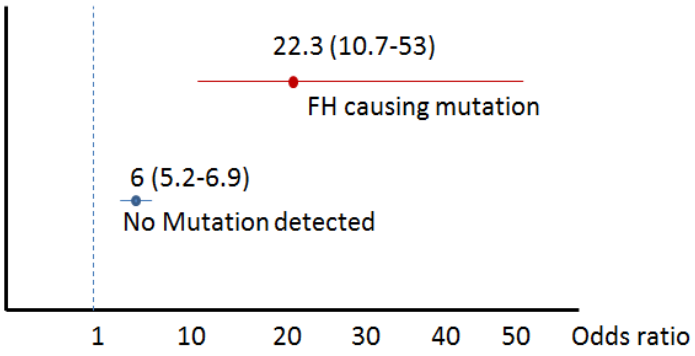
An earlier study of heterozygous FH patients, before statin therapy became a standard treatment, showed that the risk of fatal or nonfatal CHD by the age of 60 years was about 50% for male and 30% for females compared with 10% in the relatives without FH (Neil *et al.* 2003).

Premature CHD is an established phenomenon of FH, with the average age of onset of coronary symptoms shown to be significantly lower in men than women, with a mean age of 45 years compared to 55 years for women (Hill *et al.* 1991, Neil *et al.* 2004).

A major benefit of statin treatment appears to be in primary prevention of fatal coronary disease. Data from the Simon Broome Registry in the UK (1980 to 2006) showed 48% reduction in CHD mortality when statin was taken for primary prevention compared to 25% reduction when stains only used for secondary prevention.

This elevated risk for CHD in FH patients with a detected mutation has been convincingly confirmed by Khera *et al* in a population-based analysis (Khera *et al.* 2016). Using Next Generation Sequencing for the known FH genes among 20,485 CHD-free individuals, 1,386 (6.7%) had LDL-C >4.9 mmol/L, and of these, 24 (1.7%) carried a known FH mutation. Compared with individuals with LDL-C <3.7 mmol/L and no mutation, those with LDL-C >4.9 mmol/L and no FH mutation had a six-fold higher risk for CHD, but those with both LDL-C >4.9 mmol/L and an FH mutation had a 22-fold higher risk (Figure 3). This risk could be explained by the substantially higher accumulated 'LDL-C burden' since patients have had genetically-determined lifelong high LDL-C.

Figure 3: Odds ratios for people with LDL-C > 4.5 mmol/L with and without FH-causing mutations (data from Khera *et al*)



The Norwegian Cause of Death Registry data during 1992-2013 for 5518 patients with a genetically determined FH also showed that the cardiovascular disease mortality, including all cardiovascular disease deaths, was significantly higher in FH patients compared to the general Norwegian population under 70 years of age (standardised mortality ratio 2.29, 95% CI 1.65 to 3.19 in men and women combined; standardised mortality ratio 2.00, 95% CI 1.32 to 3.04 in men; standardized mortality ratio 3.03, 95% CI 1.76 to 5.21 in women) (Mundal *et al.* 2017).

1.7.1 CHD in monogenic FH versus polygenic hypercholesterolaemia

There are several lines of evidence to suggest that the extent of atherosclerosis is likely to be higher in monogenic compared to polygenic FH patients. There are many papers showing that the prevalence of CHD is higher in groups of FH patients where a mutation can be found compared to those with no mutation (Ten Kate *et al.* 2013, Vilades Medel *et al.* 2013).

The Simon Brome register work (Neil *et al.* 2003) showed that patients with a clinical diagnosis of definite FH had a higher Standardised Mortality Ratio (SMR) for CHD than those with a clinical diagnosis of possible FH (2.94 vs 2.05). Since Talmud *et al* (Talmud *et al.* 2013) suggested that a mutation can be found in ~80% of definite FH patients; this means that the majority of this group will have a monogenic cause. By contrast, a mutation can only be detected in 30% of possible FH patients, (Graham *et al.* 2005) meaning the majority of this group will have a polygenic cause of their elevated LDL-C.

The second line of evidence comes from the Simon Broome DNA study (Humphries *et al.* 2006) where 410 definite FH patients were examined. Compared to those where no mutation could be found, in those with a mutation, the odds ratio for having CHD was 1.84 (95% CI, 1.10 to 3.06) (Neil *et al.* 2008). This effect was maintained even after adjustment for pre-treatment lipid levels and other CHD risk factors. The definite FH no-mutation group had a ~30% lower prevalence of CHD than the *LDLR* mutation carriers, translating to an odds ratio of CHD of ~0.80 compared to those with a detected mutation. Talmud *et al* showed that this no-mutation group had higher LDL-C raising score i.e. they were polygenic (Talmud *et al.* 2013).

One reason that the CHD risk is higher in monogenic versus polygenic FH patients could be due to the fact that monogenic group have had severely elevated LDL-C since birth and thus have a greater cumulative "LDL-C burden", (Nordestgaard *et al.* 2013) while the polygenic group have developed elevated LDL-C only with increasing age or it might be due to other CHD risk factors.

1.8 CHD risk stratification in FH

Because the physical stigmata of the FH develop later in life, establishing the diagnosis in young asymptomatic individuals with no previous history of cardiovascular disease is often difficult. Since the age of onset, as well as, the severity of the CHD in FH patients varies, it is also difficult to decide when to initiate treatment to prevent the progress of atherosclerosis, and how best and how often to monitor CHD progression in those FH patients without any symptoms or signs of cardiovascular disease.

1.8.1 Traditional risk factors

Existing cardiovascular risk algorithms such as Framingham risk score (Anderson *et al.* 1991) and Joint British Societies (JBS) Guidelines on cardiovascular disease prevention in clinical practice are not recommended for use in FH patients (Nordestgaard *et al.* 2013). These risk scores are based on the general population data and using them in clinical practice significantly underestimates the lifetime CHD risk in FH patients.

As expected, the usual CHD risk factors such as age, male gender, smoking, hypertension, higher LDL-C and lower high density lipoprotein-cholesterol (HDL-C) (Hill *et al.* 1991, Neil *et al.* 2004) play a role in determining the risk of CHD in patients with FH. Not all individuals with FH develop atherosclerosis and CHD to the same extent.

The risk of CHD in FH is not solely due to elevated LDL-C levels and its severity and clinical expression are even variable within a family, where all relatives carry the same *LDLR* gene defect (Ferrieres *et al.* 1995, Jarauta *et al.* 2009, Besseling *et al.* 2014). A family history of an early cardiovascular event in first- or second-degree relatives generally puts the patient at higher risk (Taira *et al.* 2002, Goldberg *et al.* 2011).

The *LDLR* mutations that most severely impair the function of LDL-receptor (e.g. a null allele) are known to cause more advanced CHD with earlier onset of symptoms (Besseling *et al.* 2014). The *PCSK9* mutation p.(Asp374Tyr) (Naoumova *et al.* 2005, Alonso *et al.* 2008) and other common functional genetic variants such as apolipoprotein E (Eto *et al.* 1988) are also known to increase CHD risk, while some *PCSK9* variants with loss of

function (Scartezini *et al.* 2007) and *APOB* gene mutations (Vogt *et al.* 2014) are associated with lower risk. No other genetic risk variant for CHD in individuals with FH has been identified so far (Versmissen *et al.* 2014). Aortic valve and supra-valve calcification is also common among the FH patients with *LDLR*-negative mutations (Rafeiyian *et al.* 2007, Santos *et al.* 2008).

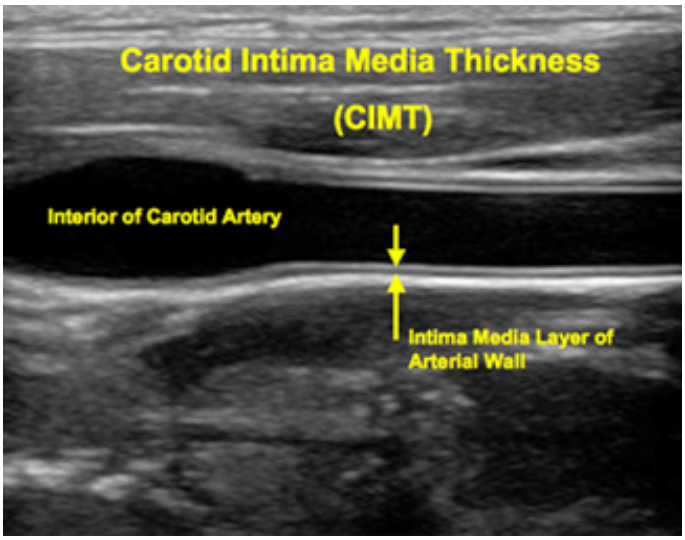
1.8.2 Imaging techniques

Non-invasive imaging modalities might be a useful approach for the identification of asymptomatic FH individuals with higher cardiovascular risk. Imaging techniques were recommended to detect the asymptomatic people at the intermediate and high risk of CHD in the 2012 European Society of Cardiology Guidelines for Cardiovascular Prevention (Perk *et al.* 2012).

1.8.3 Carotid Intima-Media Thickness (cIMT)

The cIMT is a measure of the thickness of the inner two layers of the carotid artery (Figure 4). The arterial segment e.g. the common carotid artery, the carotid bifurcation and the internal carotid artery are the common areas where the cIMT is measured. The far wall cIMT measurement accurately reflects the true thickness of the carotid wall and it is used more commonly in clinical practice.

Figure 4: Carotid Intima Media Thickness measurement



The cIMT measurement is a non-invasive procedure with a good sensitivity and reproducibility (Peters *et al.* 2013). It could detect atherosclerotic diseases in early and asymptomatic stages and allows for observation of plaques on the arterial wall. However, there is no standardised protocol for measurement of cIMT, which might lead to inaccurate estimation of the progression and regression of the cIMT in different studies by different performers. In general, the cIMT measurements only remain as an indirect assessment of the possible atherosclerotic burden in the coronary arteries (Kasliwal *et al.* 2014).

Over the last few years, many studies have reported on the relation of increased cIMT and the risk of cardiovascular disease in the general population. The IMPROVE study, a multicentre European study, showed that all cIMT measures (common, bifurcation, internal, mean and maximum, and aggregated cIMT estimates) have a value in relation to an increased risk of cardiovascular disease (Baldassarre *et al.* 2012). However, the addition of the cIMT measurements to traditional cardiovascular risk prediction models in the general population has not lead to a significant increase in the performance of these models (Den Ruijter *et al.* 2012, van den Oord *et al.* 2013).

The cIMT measurement has been used as a surrogate end-point marker for subclinical vascular disease in FH patients in several lipid-lowering clinical trials (Vergeer *et al.* 2010).

These studies showed that the changes in cIMT are highly sensitive to the changes in the LDL-C levels achieved on statin treatment; and, thus, changes in cIMT could be used in the monitoring of carotid atherosclerosis progression and risk stratification in FH patients (Vergeer *et al.* 2010).

It has been reported that individuals with FH have a higher cIMT compared to the people with normal cholesterol levels (Tonstad *et al.* 1998, Descamps *et al.* 2001) or other types of inherited hypercholesterolaemia such as Familial Combined Hypercholesterolaemia (Jarauta *et al.* 2012). Among FH patients, individuals carrying null alleles also have a higher cIMT than those with defective alleles (Junyent *et al.* 2010).

The changes in cIMT start from the young age. Measures of cIMT have been shown to be significantly higher among dyslipidaemic children compared to the children with normal lipid levels (Guardamagna *et al.* 2009). The difference in mean cIMT measurement between children with FH and their unaffected siblings was found to be statistically significant as early as the age of 8 years (Guardamagna *et al.* 2009, Fahed *et al.* 2014). As age increases, the cIMT measurements gradually increase (Dalmau Serra *et al.* 2009).

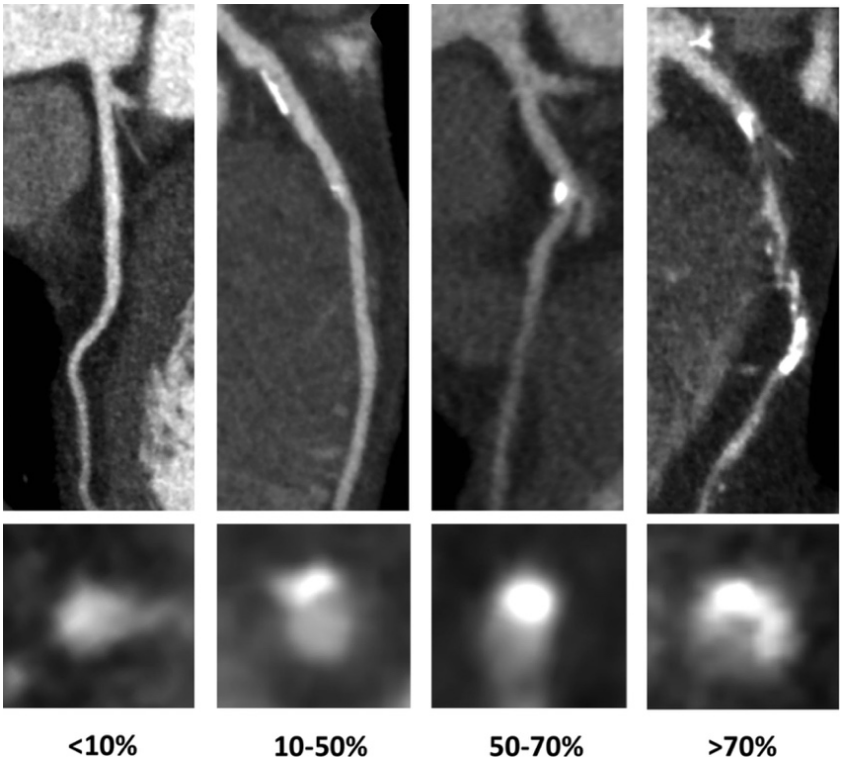
In summary, these studies support the view that the cIMT measurement can be used in the evaluation of the carotid atherosclerosis progression.

1.8.4 CT scan and MRI

There is very limited information available on the role of cardiac CT scan, Magnetic Resonance Imaging (MRI) or other imaging techniques in risk assessment of asymptomatic FH patients. In recent years, cardiac CT scan has emerged as a non-invasive imaging modality to assess coronary artery atherosclerosis in symptomatic (Budoff *et al.* 2007) and asymptomatic high risk patients (Choi *et al.* 2008). Direct examination of the vessel lumen using CT coronary angiogram (CTCA) has also shown a diagnostic capability comparable to that of invasive methods for visualisation of the anatomical details and degree of coronary lumen stenosis, and as a mean to assess plaque burden (Meijboom *et al.* 2008, Williams *et al.* 2015). It requires injection of contrast agents but with improved detector technology, it

can be optimised to reduce radiation exposure (Sun *et al.* 2012). Figure 5 shows the CT coronary angiography of lesions with different stenosis severity.

Figure 5: CT coronary angiography curved planar reformations and vessel cross sections showing lesions with different stenosis severity (none: <10%; mild: 10–49%; moderate: 50–70%; severe: >70%) (Pictures from Williams *et al)*



Myocardial perfusion imaging modalities such as stress echocardiography, nuclear myocardial perfusion tests and MR myocardial perfusion imaging, rely on the visualisation of myocardial perfusion or related cardiac wall motion abnormalities. Myocardial perfusion scans are only recommended for patients with a clinical symptom of chest pain (Jones *et al.* 2007).

Only few studies have reported calcium score and plaque burden by CT scan in FH patients (Ye *et al.* 2007, Miname *et al.* 2010, Neefjes *et al.* 2011, Neefjes *et al.* 2011, Ten Kate *et al.* 2013, ten Kate *et al.* 2013, Vilades Medel *et al.* 2013). Aortic wall calcification and lipid-

rich plaques assessments by MRI in asymptomatic FH patients were also reported in few studies (Schmitz *et al.* 2008, Soljanlahti *et al.* 2008, Caballero *et al.* 2012). Table 4 shows a list of these studies.

Due to lack of large scale data, neither a CT scan nor a cardiac MRI is currently recommended as a primary prevention investigation by either the American or European guidelines (Reiner *et al.* 2011, Perk *et al.* 2012, Goff *et al.* 2014).

Table 4: Case control studies using CT scan and MRI in FH individuals

Study	Journal	Asymptomatic FH subjects no.	Controls no.	Scans	Results
Ten Kate *et al*	Am J Cardiol. 2013	67	30 healthy subjects	CTCA	FH Patients had greater coronary calcium score
Viladés Medel *et al*	Am J Cardiol. 2013	50	70 healthy subjects	CTCA	FH patients had a greater prevalence, extension, and severity of subclinical CAD
Ten Kate *et al*	Athero. 2013	59 FH with null mutation	86 FH with reduced or normal *LDLR* function	CTCA	*LDLR*-negative patients had higher number of diseased coronary artery segments per patient
Neefjes *et al*	Athero.2011	140	All subjects had follow-ups	CTCA	Higher number of calcified plaques in FH patients.
Neefjes *et al*	Heart. 2011	101	126 non-FH patients with non-angina chest	CTCA	Total calcium score was significantly higher in patients with FH
Miname *et al*	Athero.2010	102	35 healthy subjects	CTCA	FH patients had a significantly higher number of plaques, stenosis, segments with plaques and calcium scores
Martinez *et al*	Athero.2008	89	31 healthy subjects	16 or 64 slice CT	Coronary artery calcification prevalence and severity were higher in FH
Ye ZX *et al*	Am J Cardiol. 2007	32	34 healthy subjects	electron-beam CT	Coronary artery calcification was higher in FH
Caballero *et al*	Atherosclerosis. 2012	36	19 healthy subjects	MRI of aorta	Atherosclerotic plaques in descending aorta were significantly higher in FH cases
Soljanlahti *et al*	Vasc Health Risk Manag. 2008	39	25 healthy subjects	MRI of aorta	No difference in any of the morphologic or functional aortic parameters between patients and controls
Schmitz *et al*	J Vasc Interv Radiol. 2008	11	26 subjects	MRI of aorta	The descending thoracic aorta wall area was significantly larger in patients with FH

CTCA: CT coronary angiogram

1.8.5 Coronary artery calcification (CAC)

Coronary artery calcification has been shown to be a surrogate marker for atherosclerosis with the calcium score being proportional to atherosclerosis plaque burden and cardiovascular risk (Perk *et al.* 2012). There is strong evidence that the calcium score represents a good marker of risk for the future cardiovascular event, and is a good tool for monitoring atherosclerosis progression.

The 'Agatston score', as measured by electron-beam computed tomography, remains the most commonly used method in the research settings due to a large set of data available from previous large-scale trials and cross-sectional studies; However, it has limitations in its diagnostic value in accurately evaluating the severity of CHD and plaque vulnerability (Sun *et al.* 2012).

The score is calculated using a weighted density score given the highest attenuation value (HU) multiplied by the area (sq mm) of the calcification speck (Agatston *et al.* 1990). Grading of coronary artery disease based on total calcium score is shown in Table 5.

Table 5: Grading of coronary artery disease based on calculated CAC score

Coronary artery calcium (CAC) score	Presence of coronary artery disease
0	No evidence
1 - 10	Minimal
11 - 100	Mild
101 - 400	Moderate
> 400	Severe

1.9 Biomarkers

One of the biomarkers that has been established as an indicator for CHD is Lipoprotein(a) (Nordestgaard *et al.* 2010).

1.9.1 Lipoprotein(a)

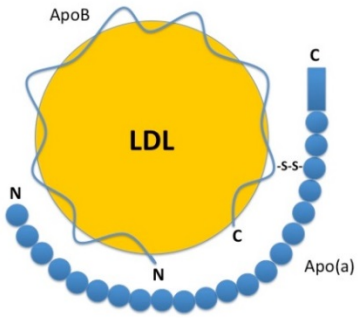
Lipoprotein(a) [Lp(a)] is an independent risk factor for cardiovascular diseases, irrespective of LDL-C levels (Holmes *et al.* 2005, Kamstrup *et al.* 2009, Nordestgaard *et al.* 2010). The Lp(a) association with myocardial infarction (MI) (Kamstrup *et al.* 2008), stroke (Emerging Risk Factors *et al.* 2009), peripheral vascular disease (Hopewell *et al.* 2011) and aortic valve stenosis (Thanassoulis *et al.* 2013) has been shown in several studies. In the study of the adult Danish general population, a stepwise increase in the risk of MI with increasing levels of Lp(a) in both genders was observed. Furthermore, Kampstrup *et al* showed an extremely high Lp(a) levels associated with a three to four-fold increase in the risk of MI and absolute 10-year risks of 20% and 35% in high-risk women and men (Kamstrup *et al.* 2008).

Several previous small studies have reported that the mean or median Lp(a) level was significantly higher in the FH patients compared to subjects having normal cholesterol level (Mbewu *et al.* 1991, Alonso *et al.* 2014). Also, it has been reported that FH patients with early CHD events had significantly higher Lp(a) levels compared to those with no or late CHD (Nenseter *et al.* 2011).

1.9.1.1 Lp(a) Structure

The Lp(a) consists of the LDL-C particle and a large glycoprotein, apolipoprotein(a) [apo(a)], which is covalently linked to apoprotein B-100 in the LDL-C particle by a single disulphate bond (Figure 6) (Marcovina *et al.* 1999). The protein and lipid composition of Lp(a) differs only slightly with LDL-C, with the protein:lipid ratio in Lp(a) and LDL-C of 1:2.2 and 1:3.5 respectively. The lipid content is 69% in Lp(a) and 79% in LDL-C but free cholesterol relative to total lipid is approximately the same (Boerwinkle *et al.* 1992).

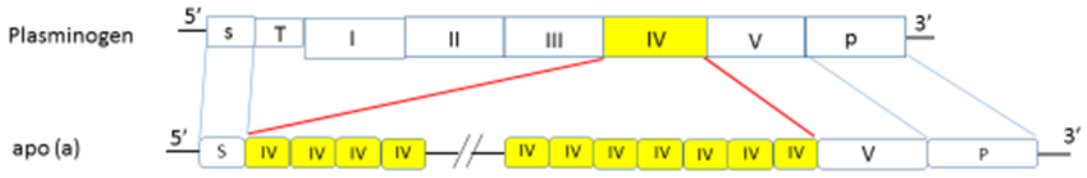
Figure 6: Lp(a) particle structure



The apo(a) protein is markedly similar to plasminogen, one of the proteins of the fibrinolytic system (Scanu *et al.* 1991). It is synthesized by the liver and attached to LDL-C on the surface of hepatocytes. Apo(a) has repeated motifs called kringles that are tri-looped structures containing 3 intramolecular disulfate bonds (Marcovina *et al.* 1999).

Plasminogen contains five kringle domains followed by a serine protease domain. Apo(a) in contrast contains multiple copies of a sequence that closely resembles plasminogen kringle IV and V (Marcovina *et al.* 1999). The kringle IV, which is present only once in the plasminogen structure, has 10 different types of apo(a) (Kringle IV types 1 to 10). Only Kringle IV type 2 occurs repeatedly in the apo(a) sequence, coinciding with about 84% of the amino acid sequence of Kringle IV in plasminogen. The number of Kringle IV repetitions is genetically determined, resulting in different apo(a) isoforms (Figure 7) (McLean *et al.* 1987, Maranhao *et al.* 2014).

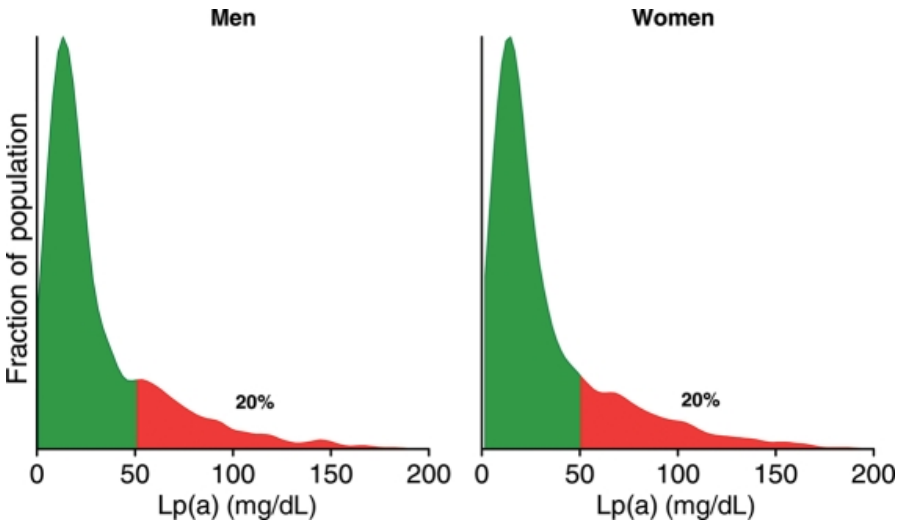
Figure 7: Sequence structure comparison of domains of plasminogen and apo(a) (derived from Scanu *et al*)



Connecting lines indicate regions of homology. S= signal peptide sequence, T=tail or preactivation peptide, I/II/III/IV/V= kringle domains, P=protease domain

The distribution of Lp(a) level is highly skewed toward low levels in most ethnic groups except sub-Saharan Africa (Figure 8) (Kronenberg *et al.* 2013, Nordestgaard *et al.* 2013). It has been estimated that the kringle IV type 2 repeat alone explains 61–69% of the variability observed in Lp(a) levels in the European population, between 19-44% in populations of African descent and 22–48% in Mexican Americans (Dumitrescu *et al.* 2011). Lp(a) levels associated with short kringle IV type 2 repeat alleles are much higher in Europeans than Africans (Kronenberg *et al.* 2013).

Figure 8: Distributions of Lp(a) in the general population based on non-fasting fresh serum samples from ~3000 men and ~3000 women from the Copenhagen General Population Study collected from 2003 to 2004 (picture from Nordestaard *et al*)



1.9.1.2 Lp(a) measurement in serum

The variable number of kringle IV type 2 repeats in different apo(a) isoforms and apo(a) structure similarity to plasminogen constitute a major challenge in its serum level measurement. A variety of methods such as enzyme-linked immunosorbent assay (ELISA), immunonephelometry and immunoturbidimetry have been used to measure Lp(a). In old immunochemical methods, the assays expressed Lp(a) values in mg/dL of the total lipoprotein particle. The measured Lp(a) mass does not reflect the number of Lp(a) particles due to the variable apo(a) isoforms. Lack of calibrators with an assigned target value also contributes to poor assay performance.

Immunoassays using antibodies directed to epitopes in the repeated apo(a) kringle IV type 2 tend to underestimate the Lp(a) concentration for smaller apo(a) isoforms and overestimate Lp(a) concentration for larger apo(a) isoforms. Using monoclonal antibodies directed against unique epitopes in apo(a) might improve the measurement, but limits the choice of the assay to the ELISA method.

To solve the problems of the apo(a) size variability, the units to express Lp(a) concentration were changed to nmol/L to reflect the number of Lp(a) particles in the solution. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and a Working Group on Lp(a) together with research institutions and several diagnostic companies, have developed primary and secondary international reference material for Lp(a) measurements to be used as calibrators by manufacturers (Tate *et al.* 1999, Dati *et al.* 2004).

In the assays from Denka company used by Randox, the lower level of apo(a) size variation impact observed is primarily due to the use of five independent calibrators, each containing a suitable distribution of apo(a) isoforms and accurately assigned values in ng/L. There are also other assays such as DiaSorin assays that are not influenced by the apo(a) isoform size, and they have increased the specificity of the Lp(a) measurement tests.

The principle of the Denka Seiken assay licensed under Randox company in the UK is agglutination, an antigen-antibody reaction between Lp(a) in a sample and anti Lp(a) antibody adsorbed to latex particles. The agglutination is detected as an absorbance change at 700 nm proportional to the concentration of Lp(a) in the sample. The assay is run on Randox Daytona or Randox imola. A multi-point calibration is used to calibrate the assay. A sample collected in Lithium heparin tube, sodium heparin tube, EDTA (Ethylenediaminetetraacetic acid) or citrate tubes could be used with this assay.

The assay range is between 2-79 mg/dL for all the sample types. If the sample concentration exceeds the assay upper limit, the sample would be diluted according to the manufacturers recommended procedure. The analytical coefficient variant (CV) of the assay has been estimated between 2% and 6% at high and low levels retrospectively.

The Marcovia *et al.* study suggested that the biological CV of Lp(a) for individuals with Lp(a) protein < 60 mg/L is about 26%, ranging from 3% to 51% and for individuals with

Lp(a) protein > 60 mg/L, the biological CV is considerably lower (11%), ranging from 1% to 16% (Marcovina *et al.* 1994). The biological CV difference of Lp(a) should be considered if the Lp(a) value are near or below the cut-off point; however, it is less likely to be a major contributor to the misclassification of a person's risk if the level is very high as the Lp(a) concentration might vary more than 1000-fold among low-risk and high-risk subjects.

The Statins, 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase inhibitors, while effective at lowering the LDL-C, do not reduce Lp(a) levels and may even increase them slightly (Hunninghake *et al.* 1993) . Reports have suggested that statins might raise Lp(a) by 10% to 20% (Tsimikas 2017). A possible explanation of this might be related to the function of the PCSK9 molecule. Statins upregulate both LDL-receptor expression on hepatocytes and PCSK9 production. The PCSK9 binding to the LDL-receptor might reduce the affinity of the large Lp(a) molecule binding to the LDL-receptor would have less of an effect on Lp(a) than otherwise predicted. PCSK9 inhibitors, on the other hand, have been shown to reduce Lp(a) concentration by ~30% (Kotani *et al.* 2017).

1.9.1.3 LPA gene

Lp(a) concentration in the blood is under strict genetic control and it is not influenced by diet, lifestyle or lipid-lowering agents. The glycoprotein, apo(a), is encoded by the *LPA* gene on chromosome 6q26 and is responsible for Lp(a) level in the blood (Thompson *et al.* 2013).

The gene is characterised by an extensive size polymorphism which is transcribed and translated into protein isoforms of different sizes. Two types of genetic variants in *LPA* have been associated with Lp(a) levels: variations in the number of copies of the kringle IV type 2 repeat and single nucleotide polymorphisms (SNPs) (Nordestgaard *et al.* 2010).

In a recent genome-wide association study, multiple SNPs in the *LPA* gene region were reported to be associated with an increased level of Lp(a) (Ober *et al.* 2009). Ober *et al*. GWAS study revealed a more complex genetic architecture of Lp(a) levels, with multiple contributing loci on 6q26-q27. The SNPs found in the *LPA* gene were shown to be associated with high Lp(a) concentration independent of the kringle IV number. The SNPs in other

genes such as *IGF2RA* and *PARK2* might also be associated with plasma Lp(a) levels but further studies are needed to confirm this association.

Several studies identified the association between SNPs in the *LPA* gene and cardiovascular risk (Luke *et al.* 2007, Clarke *et al.* 2009). A GWAS meta-analysis on Lp(a) concentration showed a directly proportional relationship of *LPA* variants with an increase in CHD risk for Lp(a)-increasing variants and a decrease in risk for Lp(a)-decreasing variants (Mack *et al.* 2017). Carriers of the minor alleles of two SNPs (rs10455872 and rs3798220) have been repeatedly reported and are already well-associated with higher Lp(a) concentration and consequently higher risk of developing coronary artery disease (Clarke *et al.* 2009).

The rs3798220 SNP in the region that encodes the protease-like domain of apo(a) (Luke *et al.* 2007) has been reported to have a strong association with Lp(a) level, a moderate association with LDL-C level and is a risk factor for CHD (Clarke *et al.* 2009). Another SNP in the *LPA* (rs6919346) has been identified independently of kringle IV type 2 copy number (Ober *et al.* 2009) and this variant is shown to be independently associated with an increased Lp(a) level. The mechanism through which this intronic SNP acts is unknown, but it suggests that rs6919346 could influence gene expression (Lanktree *et al.* 2010).

2 CHAPTER TWO: MATERIALS AND METHODS

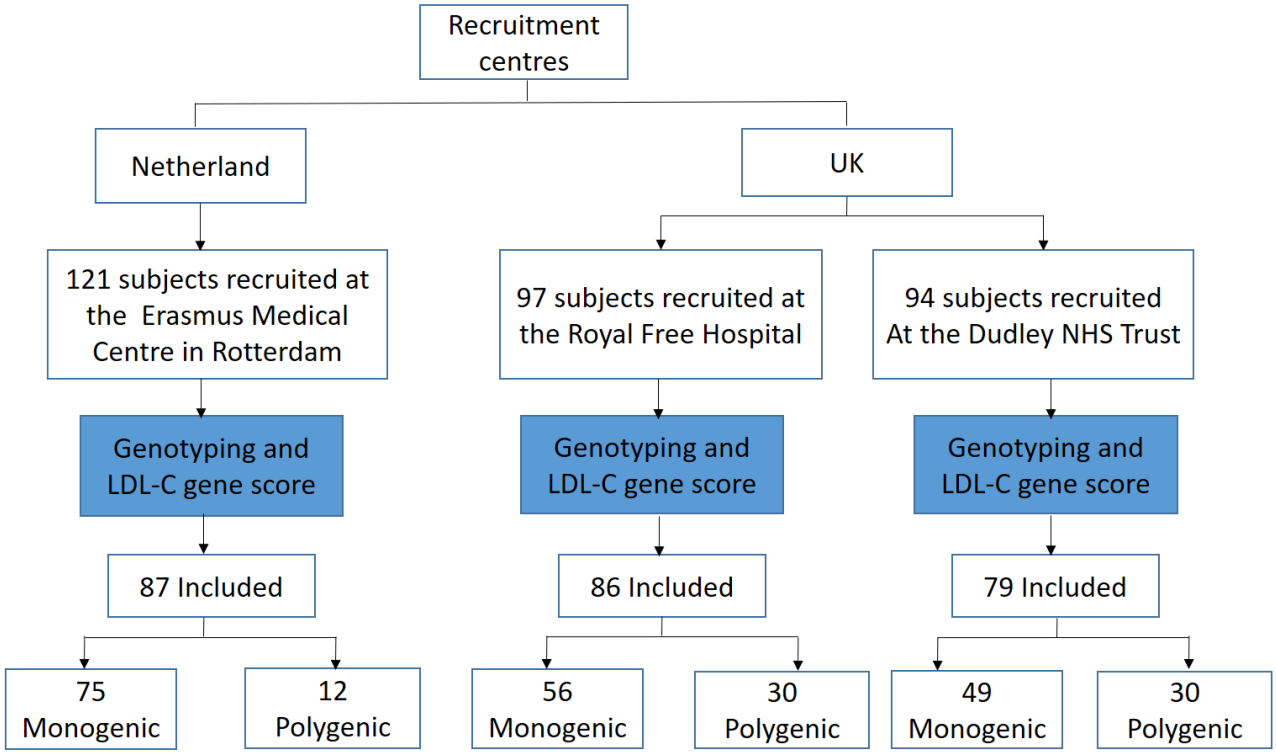
2.1 Patient selection criteria for cIMT measurement and CT scan imaging

Subjects recruited from two outpatient lipid clinics in the UK, the Royal Free Hospital in London and the Dudley NHS Trust, and an outpatient lipid clinic in the Netherlands, the Erasmus Medical Centre in Rotterdam over the period of 2014 to 2016. All patients had a clinical diagnosis of FH and patients with secondary causes of hypercholesterolaemia (such as renal disease, liver disease, thyroid disease) or a history of CHD were excluded from the study.

All patients underwent molecular genetic screening and SNP genotyping to confirm their monogenic or polygenic cause (please see below) and they all had a CT scan to measure coronary calcium score or a carotid ultrasound to measure carotid Intima Media Thickness. All patients were clinically asymptomatic, meaning they had no cardiac symptoms or any history of CHD. The inclusion age for the study was between 30-70 years for having a carotid ultrasound and 40-70 years for the CT scan. All patients gave written informed consent. Ethical approval was obtained from the relevant ethics committees (13/LLO/1334).

Data from all the monogenic patients and the patients with no mutation and a gene score in the top two quartiles of LDL-C gene scoring were included in the final analysis of this study. The data from the patients with no mutation in genotyping and a low gene score were excluded from the analysis. The patients at the Rotterdam and the Dudley NHS Trust in the UK only underwent a CT scan to measure the CAC score, while the patients at the Royal Free hospital had only a carotid IMT measurement. From a total number of 312 patients (94 patients at the Dudley Group NHS Foundation Trust and 97 at the Royal Free hospital in the UK and 121 patients in the Netherlands), data from 166 patients with CAC score and 86 patients with a carotid IMT measurement were included in the final analysis (Figure 9).

Figure 9: Recruitment flowchart



2.2 Sample collection criteria for lab analysis

2.2.1 Samples for FH genotyping

Blood samples of 169 unrelated Caucasians patients (56 males and 113 females) from the south-eastern part of Poland with a clinical diagnosis of FH based on the Simon Broome Criteria were recruited from the Department of Metabolic Diseases at Jagiellonian University in Krakow, Poland.

2.2.2 Samples for Lp(a) analysis

Samples from different studies were used for Lp(a) analysis. All studies received ethical approval from their respective Ethics Committees.

2.2.2.1 The Northwick Park Heart Study II (NPHSII)

The Northwick Park Heart Study (NPHSII) is a prospective study of healthy middle aged men (50-64 years) recruited from nine general practices in the UK, who have been followed for a period of ten years (Miller *et al.* 2008). From 3012 participants, 305 developed a CHD event during the study (Seed *et al.* 2001, Ken-Dror *et al.* 2012).

2.2.2.2 Simon Broome FH Study (SBFH)

The Simon Broome FH study (SBFH) is a registry of white patients aged 18 years or more with treated heterozygous definite FH with and without clinically documented CHD, who had been registered from 1980 onwards with the Simon Broome FH register in the UK. (Betteridge 1999) Of 410 participants, 159 people had a documented CHD having undergone coronary artery bypass grafting or percutaneous transluminal coronary angioplasty (Neil *et al.* 2004).

2.2.2.3 Rare Genetic Variants in Health and Disease (UK10K)

The UK10K is a large-scale whole exome sequencing project based on the collaboration between Welcome Trust Sanger Institute, and clinical experts in different genetic disorders (http://www.uk10k.org/). A cohort of 125 unrelated definite FH patients was sequenced as a part of the rare disease group of the project. The patients' characteristics and the sequencing methods have been reported previously (Futema *et al.* 2012).

2.2.2.4 Whitehall II study (WHII)

The WHII study recruited 10,308 British Civil Servants aged 20-64 (76% men) between 1985 and 1989 from 20 London-based civil service departments. Blood samples for DNA extraction were collected from 5066 individuals at a follow-up screening in 2003-2004 (Zabaneh *et al.* 2011). Data from a group of 3,020 participants of the WHII study (Marmot *et al.* 1991) was used as a representative of the general population for comparison in this thesis. Baseline characteristics of WHII are shown in Table 6.

Table 6: Baseline characteristics of the Whitehall II (WHII) study participants

Characteristics		WHII (n=3020)
Male	%	76 %
Age (years)	Mean ± SD	49.0 ± 6.0
Pre-treatment TC (mmol/L)	Mean ± SD	6.4 ± 1.1
Pre-treatment LDL-C (mmol/L)	Mean ± SD	4.4 ± 1.0

2.2.2.5 Belgian FH study

These samples came from a single lipid clinic in Belgium (Descamps *et al.* 2003). The genetic testing for FH-causing mutations in all Belgium samples were performed in the Centre de Recherche de Jolimont. All samples from the patients with clinical diagnosis of FH and a known mutation in LDL-C raising genes were included in our Lp(a) analysis study.

2.2.2.6 Additional subjects

Additional subjects with a clinical diagnosis of FH with a consent for genetic studies were obtained from the Great Ormond Street Diagnostic Laboratories archived material and their characteristics are as described previously (Talmud *et al.* 2013).

2.3 Molecular genetic analysis

2.3.1 Whole blood DNA extraction

The whole blood DNA extraction method was adapted from Miller *et al* 'salting out' method (Miller et al 1988).

Solutions:

- Reagent A: 0.32 M sucrose, 5 mM $MgCl_2$ and 10 mM Tris-HCL (pH 7.5) in deionised water
- Reagent B: 10 mM Tris-HCL (pH 8.2), 0.4 M NaCl and 2 mM EDTA (pH 8.0) in deionised water
- TE buffer: 10 mM Tris and 1mM EDTA in deionised water (PH 7.6)

Blood samples were collected into EDTA tubes and the DNA extraction was done in the following steps:

Cell lysis: 3-5 mL of blood was thoroughly mixed with 20 mL of iced-cold reagent A, and centrifuged at 10,000 rpm (13,416 x g) for 10 minutes at 4 °C. The supernatant was discarded into the waste tube without disturbing the pellet. The pellet was re-suspended and washed in 20 mL of reagent A and the centrifugation step repeated.

Nuclear Lysis: The pellet was re-suspended in 2 mL of reagent B.

Deproteinisation: 1mL of 5 M sodium perchlorate was added and mixed thoroughly and incubated on a shaker for 15 minutes.

Extraction: 2 mL of iced-cold chloroform was added, mixed and centrifuged at 10,000 rpm (13,416 x g) for 3 minutes to separate the DNA into upper aqueous part.

Precipitation: The aqueous part was transferred without disturbing the organic part to a fresh 30 mL polypropylene tube. 10 mL of ice-cold 100% ethanol was slowly added to the tube and incubated on a bench for 3 minutes. The tube was then inverted several times to precipitate the DNA.

Washing: Precipitated DNA was removed from the solution with a sterile Pasteur tip, washed briefly in 70% ethanol, and placed in a nuclease free tube containing 0.5 mL of TE buffer (pH=7.6). Samples were then incubated overnight at 37°C to dissolve.

Concentration and purity of each sample were measured using the Nanodrop ND8000 spectrometer, supplied by Labtech International. DNA was standardised to concentration of 15 ng/µL and stored at 4°C.

2.3.2 High Resolution Melt (HRM)

2.3.2.1 Primers

Oligonucleotide primers used for PCR-HRM were designed to cover intron-exon junctions and up to 40 bp of the intron, and the promoter and coding regions of *LDLR*. (Whittall *et al.* 2010) In addition, a fragment of exon 26 of *APOB* to cover the area for common mutation p.(Arg3527Gln) and exon 7 of *PCSK9* to cover p.(Asp374Tyr) were included for screening. Exon 10 of *LDLR* was screened by two overlapping PCR fragments and exon 4 by four overlapping fragments. The fragments and primer sequences and PCR conditions used for each fragment are shown in Table 7.

Table 7: Primers and PCR conditions used for the *LDLR*, *APOB* and *PCSK9* genes in HRM

Fragment	primer 5' - 3'	Region Covered	PCR		
			Denaturation (Temp (°C) / second)	Annealing (Temp (°C) / second)	Extension (Temp (°C) / second)
LDLR					
Promoter	CAGCTCTTCACCGGAGACCC ACCTGCTGTGTCCTAGCTGG	c.-298 - c.-62	95/5	60/10	70/20
Exon 1	AATCACCCCACTGCAAACTC GGGCTCCCTCTCAACCTATT	c.-139 - c.67+23	95/5	60/10	70/20
Exon 2	TTGAGAGACCCTTTCTCCTTTTCC GCATATCATGCCCAAAGGGG	c.68-10 - c.190+6	95/5	55/10	70/20
Exon 3	TCAGTGGGTCTTTCCTTTGAG CAGGACCCCGTAGAGACAAA	c.191-28 - c.313+58	95/5	60/10	70/20
Exon 4	TGGTGTTGGGAGACTTCACA CACTCATCCGAGCCATCTTC	c.314-35 - c.519	95/5	60/10	70/20
	AAGTGCATCTCTCGGCAGTT CCCCTTGGAACACGTAAAGA	c.377 - c.557	95/5	60/10	70/20
	AGCTTCCAGTGCAACAGCTC CATACCGCAGTTTTCCTCGT	c.474 - c.679	95/5	60/10	70/20
	TGTTCCAAGGGGACAGTAGC AAATCACTGCATGTCCCACA	c.586 - c.694+60	95/10	66/10	72/20
Exon 5	AGAAAATCAACACACTCTGTCCTG GGAAAACCAGATGGCCAGCG	c.695-8 - c.817+5	95/5	60/10	70/20
Exon 6	TCCTCCTTCCTCTCTCTGGC TCTGCAAGCCGCCTGCACCG	c.818-8 - c.940+8	95/5	60/10	70/20

Exon 7	GGCGAAGGGATGGGTAGGGG GTTGCCATGTCAGGAAGCGC	c.941-38 - c.1060+36	95/5	60/10	70/20
Exon 8	CTAGCCATTGGGGAAGAGCC TGCCTGCAAGGGGTGAGGC	c.1061-31 - c.1186+30	95/5	60/10	70/20
Exon 9	TCCATCGACGGGTCCCCTCTGACCC AGCCCTCATCTCACCTGCGGGCCAA	c.1187-26 - c.1358+25	95/5	60/10	70/20
Exon 10	AGATGAGGGCTCCTGGTGCGATGCC GCCCTTGGTATCCGCAACAGAGACA GATCCACAGCAACATCTACTGGACC AGCCCTCAGCGTCGTGGATA	c.1359-26 - c.1490 c.1475 - c.1586+5	95/5 95/5	60/10 60/10	70/20 70/20
Exon 11	TCCTCCCCCGCCCTCCAGCC GCTGGGACGGCTGTCCTGCG	c.1587-28 - c.1705+7	95/5	60/10	70/20
Exon 12	GCACGTGACCTCTCCTTATCCACTT CACCTAAGTGCTTCGATCTCGTACG	c.1706-10 - c.1845+10	95/5	56/20	70/30
Exon 13 Exon 14	AGAGGGTGGCCTGTGTCTC TCCACAAGGAGGTTTCAAGG CTGATGATCTCGTTCCTGCCC GCAGAGAGAGGCTCAGGAGG	c.1846-47 - c.1987+29 c.1988-23 - c.2140+46	95/5 95/5	58/10 60/10	70/20 70/20
Exon 15	GGCACGTGGCACTCAGAAGAC ACCCGTCTCTGGGTGAAGAGG	c.2141-18 - c.2311+25	95/5	60/10	70/20
Exon 16	CCTTCCTTTAGACCTGGGCC CATAGCGGGAGGCTGTGACC	c.2312-23 - c.2389+32	95/5	60/10	70/20
Exon 17	GGGTCTCTGGTCTCGGGCGC GGCTCTGGCTTTCTAGAGAGGG	c.2390-33 - c.2547+10	95/5	60/10	70/20
Exon 18	GCCTGTTTCCTGAGTGCTGG TCTCAGGAAGGGTTCTGGGC	c.2548-35 - c.2607	95/5	60/10	70/20

PCSK9					
Exon 7	CCCTCTCTTGGGCTCCTTTCT AAAGGGGCTGTTAGCATCACG	c.997-27 - c.1180+29	95/10	60/10	70/20
APOB					
Exon 26	TGTCAAGGGTTCGGTTCTTT GGGTGGCTTTGCTTGTATGT	c.10516 - c.10745	95/5	60/10	70/20

2.3.2.2 Reaction set up

The HRM reactions were performed using AccuMelt HRM SuperMix (Quanta BioSciences, USA). Each 10µL reaction contained:

- 5 µL of AccuMelt HRM SuperMix (containing the Syto9 saturating dye)
- 4 pmol of Forward Primer/4 pmol of Reverse Primer
- 5 µL DNA template at 1.5 ng/µL

2.3.2.3 Cycling conditions

PCR and subsequent HRM have performed on Rotor-Gene 6000 (Qiagen, UK) analyser. The PCR conditions are explained in Table 7. The number of PCR cycles and the HRM temperature gradient are presented in Table 8.

Table 8: Number of PCR cycles and HRM temperature gradient

Gene (exon)	No. of PCR cycles	HRM Temperature
LDLR (All exons)	40	80-94° C
APOB (exon 26)	40	80-95° C
PCSK9 (exon 7)	45	80-94° C

2.3.3 Restriction Fragment Length Polymorphism (RFLP)

The samples were genotyped for common polymorphisms in *LDLR* by RFLP and their genotypes compared with the HRM result as described previously (Whittall *et al.* 2010). Table 9 contains a list of all common *LDLR* SNPs and the restriction enzymes (NEB (UK) Ltd. Hitchin, Herts, UK) used for genotyping.

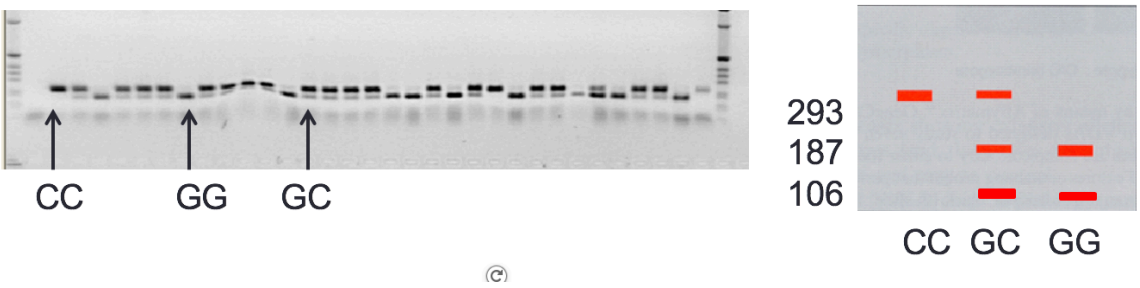
Table 9: List of restriction enzymes used for RFLP

***LDLR* exon**	**Enzyme**	**Polymorphism**
ex 2	Hhal	rs2228671
ex 7	SmaI	rs12710260
ex 8	StuI	rs11669576
ex 10	BsmA1	rs5930
ex 11	AciI	rs5929
ex 12	BSMAI& HincII	rs1799898 and rs688
ex 13	AvaII	rs5925
ex 14	MSII	rs72658867
ex 15	MspI	rs5927

The samples with shifts on the HRM suspected of polymorphism were not sequenced initially. Samples without the polymorphism but with an HRM shift were sequenced to find the cause of melt temperature shift.

The HRM-PCR products for each sample stored after HRM analysis were used for digestion with the appropriate restriction enzyme using 5 µl of HRM-PCR product and 3 units of appropriate enzyme in a total volume of 15 µl and run on a 1.5% agarose gel or Microplate-Array Diagonal Gel Electrophoresis (MADGE) gel in Tris-Boric ethylenediaminetetraacetic acid (TBE) buffer. An example of RLPF on agarose gel is shown in Figure 10.

Figure 10: RFLP with SmaI enzyme for polymorphism in exon 7 (rs12710260, c.1060+10 C>G) on agarose gel (SmaI enzyme cuts G allele and produce fragments of 187 bp and 106 bp for GG genotype and 293 bp, 187 bp and 106 bp for GC genotype; 293 bp fragment remains for CC genotype)



2.3.4 Electrophoresis

The DNA fragments produced by restriction enzyme digest were separated using electrophoresis o n a non-denaturing polyacrylamide gel, using Microtitre Array Diagonal Gel Electrophoresis (MADGE) or agarose gel.

2.3.4.1 Gel solution for MADGE gel

The MADGE gel consists of an open arrangement of 8x12 wells each 2 mm deep. The wells are arranged at an angel of 71.2 degree to the short axis of the array, but perpendicular to the long axis of the Perspex formers used. Thus, the maximum track length is 26.5 mm allowing sufficient travel for genotype resolution. Glass plate of appropriate size (160x100x2 mm) was rigorously cleaned and hand dried. 5 drops of gamma-methacryloxypropyltrimethoxysilane (silane) spread across the plates and left to air-dry. The silane was used to ensure that MADGE gel would adhere to the glass plates.

Gel solution for MADGE gel contained:

- 5 mL of TBE
- 12.2 mL of 30% acrylamide bisacrylamide (in a ratio of 19:1)
- 32.5 mL of distilled water
- 150 µL N-tetramethylethylenediamine (TEMED)
- 150 µL of 25% ammonium per sulphate (APS)

The solution was mixed and quickly poured into the three-dimensional plate. A glass plate was then gently placed over the mould (silanised side facing down) taking care not to trap the air bubbles. This was then left for 15 minutes to set, using small weight on top of the glass. The MADGE gels were stored in plastic Stuart box containing neat TBE and used within 7 days.

2.3.4.2 Gel solution for agarose gel

The gel mould was set up with comb in gel casting tray. Then 4.5 g (1%) agarose was added to 270 mL TBE in Erlenmeyer flask and was heat to boiling point in microwave for 2 minutes. When the agarose was dissolved completely, 30 mL of Ethidium Bromide was added to mix. Then the agarose solution was poured into gel mould with comb. The gel was allowed to solidify for half an hour before gently loosening the comb and removing it. Then the gel was placed in the electrophoresis tank.

2.3.4.3 Gel staining and loading

For staining, 2 µL of formamide dye (98% formamide, 10 mmol/L EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue) was added to each well of a new, round-bottomed, loading tray followed by 5 µL of each digested sample.

After placing the MADGE gel or agarose gel into an electrophoresis tank containing 1000 mL of TBE, a multi-channel pippete was used to transfer 5 µL of the digest/dye mixture to the wells of the gel. A ladder was used at the end of each row for comparison. The gel was electrophoresed at 120 volts for 40 minutes. Following the electrophoresis, the gel was viewed and photographed under ultra-violent light. The produced image was used for genotyping.

All genotyping was performed using both positive and negative controls. Two individuals rechecked the results at the time of MADGE imaging and during data entry into compute database.

2.3.5 Sequencing

2.3.5.1 PCR product purification

Prior to Sanger sequencing, the DNA purification was performed using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The HRM-PCR product was mixed with 500 µL of Capture Buffer and loaded onto the assembled GTX MicroSpin column and collection tube. The sample was then centrifuged at 11,000 rpm (16,233 x g) for 30 seconds. The flow through was discarded and the column was placed back inside the collection tube. Next, 500 µL of the Wash Buffer (type 1) containing 70% ethanol was added onto the column and the centrifuge at 11,000 rpm (16,233 x g) for 30 seconds was repeated. The PCR product was then eluted from the column with 30 µL of sterile nuclease-free water by another centrifugation step, 11,000 rpm (16,233 x g) for 1 minute. DNA concentration of the eluted product was measured on the Nanodrop ND8000 spectrophotometer. The aliquot of 1 ng/µL per 100 bp of in volume of 15µL was sent for Sanger sequencing.

2.3.5.2 Sanger sequencing

The HRM-PCR products, that showed a shift in the melt profile and melt temperature, were Sanger sequenced. The DNA purification was performed using Illustra GFX PCR DNA and Gel Band Purification Kit (from GE Healthcare). The same primers used for HRM were used for sequencing, which was performed by Source BioSciences, LifeSciences.

2.3.6 Multiplex Ligation-dependent Probe Amplification (MLPA)

To detect rearrangements within the coding sequence of *LDLR*, the multiplex ligation-dependent probe amplification (MLPA) was performed as per manufacturer's protocol (MRC-Holland, Amsterdam, the Netherlands). The protocol is enclosed in appendix 1.

2.3.7 LDL-C raising gene score

To assess the polygenic cause of hypercholesterolaemia, patients were genotyped for LDL-C-raising SNPs based on recent findings (Teslovich *et al.* 2010, Talmud *et al.* 2013). KASP

PCR technique (Kbiosciences, UK Hoddesdon, Herts, UK) or TaqMan assays (Life Technologies, Carlsbad, California, US) was carried out using an automated system, the results of which were checked manually by study personnel using SNPviewer software. One SNP (rs4299376) could not be genotyped and a proxy was used instead (rs6544731). The LDL-C gene score was calculated using weighted sums for six SNPs of the highest effect (Table 10).

Table 10: Six LDL-C raising SNPs used for gene score calculation

	Chromosome	Gene	Minor allele	Common allele	Weight for score calculation
rs629301	1	*CELSR2*	G	T[1]	0.15
rs1367117	2	*APOB*	A[1]	G	0.1
rs4299376	2	*ABCG8*	G[1]	T	0.071
rs6511720	19	*LDLR*	T	G[1]	0.18
rs429358	19	*APOE*	C	T	.
rs7412	19	*APOE*	T	C	.
e2e2	19	*APOE*	.	.	-0.9
e2e3	19	*APOE*	.	.	-0.4
e2e4	19	*APOE*	.	.	-0.2
e3e3	19	*APOE*	.	.	0
e3e4	19	*APOE*	.	.	0.1
e4e4	19	*APOE*	.	.	0.2

[1] Risk allele

2.3.7.1 KASP genotyping

The KASP (Kbioscience, UK Hoddeston, Herts, UK) genotyping was used for genotyping of all SNPs shown in above Table 10 except *APOE* variants.

The method utilises a form of competitive allele-specific PCR. The bi-allelic discrimination was achieved by the competitive binding of two allele-specific primers, each with a unique tail sequence that corresponded with two universal fluorescence resonant energy-transfer cassettes. Each allele-specific primer was labelled, one with FAM dye and the other with HEX dye, which enabled the discrimination between alleles. The reverse primer was universal for both forward allele-specific primers.

The genotyping reactions were carried out in 384-well plate format. 5 ng of DNA was transferred from a standardised array, using the Biomek 2000 Laboratory Automation Workstation, into a 384-well plate and centrifuged at 3,000 rpm (1,207 x g) for 1 minute before being air-dried. Each plate contained at least two no-template-control wells.

KASP Master Mix:

- 2.5 µL KASP Reaction Mix
- 0.07 µL KASP-by-Design assay
- 2.5 µL water

Cycling conditions on a standard thermocycler:

- 94°C for 15 minutes
- 10 cycles (dropping 0.8°C per each cycle)
    - 94°C for 20 seconds
    - 65-57°C for 60 seconds
- 26 cycles
    - 94°C for 20 seconds
    - 57°C for 60 seconds

2.3.7.2 TaqMan genotyping

The *APOE* e2/e3/e4 haplotype is made up of two SNPs (rs429358 and rs7412) and results in different isoforms of apoE protein. TaqMan probes (Life Technologies, Carlsbad, California, US) was used for *APOE* genotyping.

Each TaqMan genotyping assay contained two target SNP specific primers for the region of interest and two allele-specific TaqMan probes. Each allele-specific probe has three main elements: a receptor dye, a minor groove binder (MGB), and a non-fluorescent quencher (NFQ). Two commonly used receptor dyes, VIC and FAM, enabled to distinguish between each allele. The MGB was added to increase the melting temperature for a given probe length, which increases the efficiency of allele discrimination. The NFQ does not florescence and will quench the fluorescent signal in any receptor dye linked to the 5' end for as long as the probe remains intact.

TaqMan genotyping reaction were carried out in a 384-well plate format. 5 ng of DNA was transferred from a standardised array, using the Biomek 2000 Laboratory Automation Workstation, into a 384-well plate and centrifuged at 3,000 rpm (1,207 x g) for 1 minute before being air-dried. Each plate contained at least two no-template-control wells. The TaqMan genotyping reaction is carried out as per the TaqMan genotyping mastermix manufacturer's instruction. Each plate was centrifuged at 3,000 rpm (1,207 x g) for 1 minute and PCR reactions were carried out using the 7900HT Fast Real-Time PCR system.

Cycling conditions:

- 95°C for 10 minutes
- 40 cycles
    - 92°C for 15 seconds
    - 60°C for 60 seconds

2.3.8 In Silico mutation analysis

To predict the pathogenicity of the novel *LDLR* variants, *in silico* mutation prediction tools, including Polymorphism Phenotyping version2 (PolyPhen-2) (Adzhubei *et al.* 2010), Scale-invariant feature transform (SIFT) (Ng *et al.* 2001), Berkeley Drosophila Genome Project - Splice Site Prediction (BDGP) (Hoskins *et al.* 2007) and Mutation Taster (Schwarz *et al.* 2010) were used. The data from Exome Variant Server (http://evs.gs.washington.edu/EVS/) and UCL website (www.ucl.ac.uk/fh) were also used to check if any of the mutations were novel. Mutation nucleotide numbers were designated using the *LDLR* sequence reported in (www.ucl.ac.uk/fh) (Fokkema *et al.* 2005). Mutations were designated according to recommendations from Human Genome Variation Society (www.hgvs.org). Information from the novel mutations was added to the UCL *LDLR* database (www.ucl.ac.uk/fh).

2.3.8.1 Polyphen 2

Polymorphism Phenotyping 2 (PolyPhen 2) is a web-based tool (http://genetics.bwh.harvard.edu/pph2/) to predict possible impact of an amino acid substitution on the structure and function of a human protein. The mutation prediction

algorithm classifies variants into three groups: benign, possibly damaging and probably damaging (Adzhubei *et al.* 2010).

2.3.8.2 SIFT

Sorting Intolerant Form Tolerant (SIFT) is a web-based amino-acid change prediction tool (http://sift.jcvi.org/). It examines the amino acid change in the protein family it belongs to and summarises the outcomes in tolerated and non-tolerated forms (Ng *et al.* 2001).

2.3.8.3 Mutation taster

Mutation Taster is a free web-based tool designed to assess the effect of a SNP or deletion/insertion in a human gene (http://www.mutationtaster.org/). A studied variant is evaluated according to its evolutionary conservation, its ability to cause splice-site alteration and the protein feature that it may affect. The results are shown as disease-causing or polymorphism (Schwarz *et al.* 2010).

2.3.8.4 Human splicing finder

Human Splicing Finder was used for assessment of single base changes that occur in the region in or around intronic/exonic boundaries and ambiguous effect on amino acid composition of a give protein (http://www.umd.be/HSF3/) (Desmet *et al.* 2009).

2.4 Biomarkers

2.4.1 Lipids

TC and TG were measured by an enzymatic colorimetric test and HDL-C by a homogenous enzymatic colorimetric test. An automated Roche cobas® and Vitros® Fusion 5.1 analyser (Ortho-Clinical Diagnostics, Rochester, NY, U.S.A.) were used at the Royal Free Hospital and the Dudley Group NHS Foundation Trust respectively. The calculation of the LDL-C concentration was done according to Friedewald's formula [TC(mmol/L) =LDL-C(mmol/L) + HDL-C(mmol/L) + TG(mmol/L)/2.2]. The internal QC material was run every day in the

labs and all the QC values were within the acceptable limits designated by the manufacturer. The labs were registered with the External Quality Assurance Scheme and the performances of the analytes were within the acceptable range. All the laboratory methods had a between run coefficient variance (CV) of < 4%. Lipid and lipoprotein measures were performed using standard assays as previously reported in the samples from the Netherland (Bos *et al.* 2015).

2.4.2 Lp(a)

2.4.2.1 Serum concentration

A subset analysis of samples from the NPHSII and the SBFH study was performed to compare the Lp(a) concentration measured in these studies and a most recent developed assay by the Randox company.

In NPHSII study, serum samples (stored at −80°C) were assayed for Lp(a) concentration by ELISA method (Biopool AB, Umea, Sweden), with a coefficient of variance (CV) of repeat measurement of 6%. This method was validated by the Centres for Disease Control, Atlanta, Georgia, and in an international survey of Lp(a) measurement methods (Seed *et al.* 2001).

In the SBFH study, venous blood specimens were collected into EDTA, fluoride, and citrate vacutainers and centrifuged immediately to separate plasma for the measurement of lipids, lipoproteins, apolipoproteins, and Lp(a) by the Department of Chemical Pathology, University College Hospital, London. Lp(a) was measured by immunoturbidimetry on a Cobas-Bioanalyser (Roche Diagnostics), with kits obtained from DiaSorin using SPQ II test systems calibrators (DiaSorin Ltd, Wokingham, Berks, UK) (Neil *et al.* 2004).

To validate Lp(a) measures in the two above studies, 83 citrate samples from the NPHSII and 80 serum samples from the SBFH study were available. These samples were used for Lp(a) measurement by the immuno-turbidimetry method on a Randox RX Daytona analyser at the Randox Lab in London. The kits has a CV of repeat measurements of 2.3%.

2.4.2.2 LPA genotyping

The *LPA* genotyping was done in DNA samples available from 6 different studies as mentioned in sample collection criteria. Two *LPA* SNPs (rs3798220 and rs6919346) were genotyped using TaqMan technology (Applied Bioscience, ABI, and Warrington, UK) as described above.

2.5 Imaging

Two imaging techniques were used for cardiovascular risk stratification in asymptomatic FH patients.

2.5.1 Carotid Intima Media Thickness (cIMT)

The carotid IMT was measured in B-mode by a Philips CX50 machine equipped with a 5-10 MHz linear array probe at Royal Free Hospital in the UK. Measurements were done in the far wall of common carotid artery (in the second centimetre proximal to the bifurcation), the bifurcation and the internal carotid artery on both right and left arteries. Three scan angles of lateral, posterior and anterior during diastole were used and each segment was measured in at least four different frames. IMT analysis was performed by Philips QLAB® software after completed examination. In the case of plaque presence, the IMT was measured away from the plaque.

2.5.2 CAC score

The CAC was measured using Symbia TruePoint T6 SPECT/CT scanner (Siemens Medical Solutions, Forchheim, Germany) and dual-source CT scanner (Siemens Medical Solutions, Forchheim, Germany) (Ten Kate *et al.* 2013) in the UK and in the Netherlands respectively. The CAC score measurement was done using the same standard Agatston calcium scoring algorithm (Agatston *et al.* 1990).

CT scans of the heart (from the carina to the apex of the heart) were acquired during one inspiratory breath-hold without the use of contrast medium. CAC was quantified using calcium scoring software (Syngo CaScore, Siemens) and measurements were performed using the standard Agatston calcium scoring algorithm, (Agatston *et al.* 1990) which has

been validated in several large studies. It has been shown previously that in asymptomatic individuals with a CAC score <100, the prevalence of cardiac ischemia is generally very low (<10%) (He *et al.* 2000, Wiegman *et al.* 2004). Therefore, in our study, the participants were divided in two groups for comparison with the calcium score above and below 100 Agatston units.

2.6 Statistical analysis

The lipid traits in the Polish cohort data were not normally distributed and log-transformed data were used for the analysis. One-way ANOVA was used to compare the lipid parameters and gene score between the mutation positive and negative groups (SPSS® version 21). A p value < 0.05 was used to denote significance.

For cIMT and CAC score results, demographic and biochemical data are presented as mean with standard deviation (SD) or number (percent). The cIMT and CAC score data were not normally distributed so log-transformed data were used to compare the groups after adjustment for age and gender (SPSS® version 21) and they were transformed back to the original scale and presented as geometric means and 95% confidence intervals.

For cIMT, a linear regression model was used. For CAC score, a tobit model was used due to the high frequency of zero scores. Values were recoded to CACS+1 to allow a censored threshold of zero in the tobit model. In addition, CAC score was analysed as two groups using a cut-point of 100. Logistic regression was used to adjust for age and gender for this analysis. The characteristics between the patient groups were compared using unpaired t-tests for continuous variables and chi-squared tests for categorical data.

Linear regression and tobit regression models were also fitted using age, mutation and an age*mutation interaction term to determine differences between the increase in cIMT and CAC sore with age in monogenic and polygenic groups respectively. Based on the carotid IMT data in Jarauta et al, (Jarauta *et al.* 2012) a sample of 50 in each monogenic and polygenic group, would give 80% power at the 5% significance level to detect an 11% difference in carotid IMT.

For Lp(a) analysis, allele frequencies between the groups were compared using the chi-squared test, and tested for Hardy-Weinberg equilibrium. The baseline characteristics of the study participants were given as mean ± SD and study differences were tested by t-test. Lp(a) differences were also presented as median with interquartile range and tested using the Mann-Whitney U test as the distribution differed from normality. Differences between samples measured by the two different methods were assessed using the Bland-Altman method. Genetic associations with Lp(a) were tested using the Kruskal-Wallis test and interactions with study were assessed using an ordinal logistic regression model based on quintiles of the Lp(a) distribution.

3 CHAPTER THREE: THE GENETIC SPECTRUM OF FAMILIAL HYPERCHOLESTEROLAEMIA IN SOUTH-EASTERN POLAND

161 patients with a clinical diagnosis of FH were recruited. Using the HRM-melt method, all patients were screened for mutations in all 18 exons of *LDLR* gene, plus a fragment of exon 26 of *APOB* to cover p.(Arg3527Gln) and plus exon 7 of *PCSK9* to cover p.(Asp374Tyr).

3.1 Patient characteristics

161 patients were recruited in this cohort. The characteristics of these patients are shown in table 11.

Table 11: Characteristics of the patients in the Polish cohort

Variable	**N (%)**
Male	55 (34.1)
Tendon xanthomata	92 (57.1)
Family history of premature CHD	79 (49.1)
Personal history of premature CHD	21 (13.0)
On lipid-lowering medication	110 (68.3)
	mean (SD)
Age (years)	42 (17.6)
Maximum TC reported (mmol/L)*	9.9 (2.6)
Lipid levels:**	
TC (mmol/L)	7.0 (1.8)
LDL-C (mmol/L)	4.8 (1.8)
HDL-C (mmol/L)	1.5 (0.3)
TG (mmol/L)	1.5 (0.8)

*Highest pre-treatment total cholesterol level reported for the patient;
**lipid levels at the start of this study; TC: Total cholesterol;
TG: Triglyceride

3.2 Mutation spectrum

Overall, a mutation was detected in 70 out of 161 (43.4%) patients. The most frequent mutation was in *APOB* gene p.(Arg3527Gln). Mutations in the *LDLR* gene were identified in 57 patients and accounted for the majority (81.4%) of all the mutations found in this cohort. No patient carried the *PCSK9* p.(Asp374Tyr) mutation.

12 patients (17.1%) of all FH causes in this cohort had a major rearrangement in the *LDLR* gene. Among the intronic variants found, all were previously reported as splice-site-modifying mutations and therefore considered to be pathogenic (www.ucl.ac.uk/ldlr) except c.2390-16G>A which is not near to the splice site; thus, based on prediction tools it was designated as non-pathogenic. 13 *LDLR* variants were considered non-pathogenic. Seven of these variants were present in patients already identified with a pathogenic mutation. All mutations are shown in Table 12.

Table 12: *LDLR* and *APOB* variants identified in the Polish cohort study

DNA level	Protein level	N	exon	Prediction			
				PolyPhen	SIFT	Mutation Taster	splice site effect
LDLR gene							
Major rearrangement							
c.-187-?_67+?dup	p.(?)	1	Promoter-ex1 dup	n/a	n/a	n/a	n/a
c.-187-?_67+?del	p.(?)	1	>30kb upstream of the promoter-ex1 del	n/a	n/a	n/a	n/a
c.-187-?_190+?del	p.(?)	1	Promoter-ex2 del	n/a	n/a	n/a	n/a
c.941-?_1060+?del	p.(Gly314_Glu353del)	1	Ex7del	n/a	n/a	n/a	no
c.314-?_1186+?dup	p.(Gly396Ala;Pro106_Val395dup)	2	Ex4-ex8 dup	n/a	n/a	n/a	no
c.695-?_1586+?del	p.(Val233Serfs*18)	6	Ex5-10 del	n/a	n/a	n/a	no
Probably pathogenic							
c.100T>G	p.(Cys34Gly)	3	2	Probably damaging	Not tolerated	Disease causing	no
c.185C>T	p.(Thr62Met)	1	2	Probably damaging	Tolerated	Disease causing	no
c.380T>A	p.(Val127Asp)	1	4[1]	Possibly Damaging	Not tolerated	Disease causing	no
c.501C>A	p.(Cys167*)	1	4	n/a	n/a	n/a	no
c.530C>T	p.(Ser177Leu)	1	4	Benign	Not tolerated	Disease causing	no
c.654_656delTGG	p.(Gly219del)	1	4	n/a	n/a	Disease causing	no
c.666C>A	p.(Cys222*)	1	4	n/a	n/a	n/a	no

c.681C>G	p.(Asp227Glu)	1	4	Probably damaging	Not tolerated	Disease causing	no
c.986G>T	p.(Cys329Phe)	4	7[1]	Probably damaging	Not tolerated	Disease causing	no
c.1048C>T	p.(Arg350*)	1	7	n/a	n/a	n/a	no
c.1085delA	p.(Asp362Alafs*8)	1	8	n/a	n/a	Disease causing	no
c.1246C>T	p.(Arg416Trp)	2	9	Probably damaging	Not tolerated	Disease causing	no
c.1449G>T	p.(Trp483Cys)	1	10[1]	Possibly damaging	Not tolerated	Disease causing	no
c.1567G>A	p.(Val523Met)	1	10	Benign	Not tolerated	Disease causing	no
c.1720C>T	p.(Arg574Cys)	1	12	Probably damaging	Not tolerated	Disease causing	no
c.1737C>G	p.(Asp579Glu)	3	12[1]	probably damaging	Not tolerated	Disease causing	no
c.1775G>A	p.(Gly592Glu)	4	12	probably damaging	Not tolerated	Disease causing	no
c.1834G>T	p.(Ala612Ser)	2	12[1]	Possibly damaging	Not tolerated	Disease causing	no
c.1862C>G	p.(Thr621Arg)	1	13[1]	Probably damaging	Not tolerated	Disease causing	no
c.1975_1987+16del	p.(?)	1	13[1]	n/a	n/a	n/a	yes
c.2026G>C	p.(Gly676Arg)	1	14	Probably damaging	Not tolerated	Disease causing	no
c.2032C>T	p.(Gln678*)	2	14	n/a	n/a	n/a	no
c.2054C>T	p.(Pro685Leu)	1	14	Probably damaging	Not tolerated	Disease causing	no
c.2096C>T	p.(Pro699Leu)	2	14	Probably damaging	Not tolerated	Disease causing	no
c.2096delC	p.(Pro699Argfs*10)	1	14[1]	n/a	n/a	Disease causing	no
c.2546C>A	p.(Ser849*)	1	17[1]	n/a	n/a	n/a	no
Intronic pathogenic							
c.313+1G>A	p.(Leu64_Pro105delins Ser)	1	intron 3	n/a	n/a	n/a	yes
c.2389+5G>A		1	Intron 16	n/a	n/a	n/a	yes

Non-pathogenic							
c.1171G>A	p.(Ala391Thr)	3	8	Benign	Tolerated	Polymorphism	no
c.1545C>T	p.(Asn515Asn)	1	10	n/a	Tolerated	Polymorphism	no
c.1920C>T	p.(Asn640Asn)	1	13	n/a	n/a	Polymorphism	no
c.1959C>T	p.(Val653Val)	1	13[1]	n/a	n/a	n/a	no
c.2025C>T	p.(Gly675Gly)	1	14[1]	n/a	n/a	Disease causing	no
c.2177C>T	p.(Thr726Ile)	2	15	Benign	Tolerated	polymorphism	no
c.2231G>A	p.(Arg744Gln)	1	15	Benign	Tolerated	polymorphism	no
c.2390-16G>A	Intronic	3	intron17[1]	n/a	n/a	n/a	no
APOB gene							
c.10580G>A	p.(Arg3527Gln)	13	*APOB* ex26	Probably damaging	Not tolerated	n/a	n/a

[1] Novel; n/a: not applicable

3.2.1 Novel mutations

In total, 10 novel mutations were found in the *LDLR* gene (Table 13). The mutation c.1975_1987+16del, is predicted to delete the last four amino acids of exon 13 and the consensus splice site, and is predicted to result in a frame shift. The mutation c.2096delC will also result in a frame shift in exon 14 (p.Pro699Argfs*10) and would be pathogenic.

The mutations p.(Cys255Tyr) and p.(Cys329Phe), would cause loss of cysteine in the ligand binding domain of the LDL-receptor and cause aberrant protein folding. The mutation p.(Ser849*) causes a premature stop codon at position 849 in the cytoplasmic tail of LDL-receptor, known to be important for the localisation of the receptor in coated pits on the cell surface.

The other novel mutations, p.(Ala612Ser), p.(Asp579Gly), p.(Trp483Cys), p.(Val127Asp) were also predicted to be pathogenic; however, family members of these patients were not available for segregation analysis.

The novel mutation p.(The621Arg) is predicted to cause aberrant recycling of the LDL-receptor protein to the cell surface and is thus pathogenic. Analysis of the proband's family members (see below) showed that this mutation segregated with the disease.

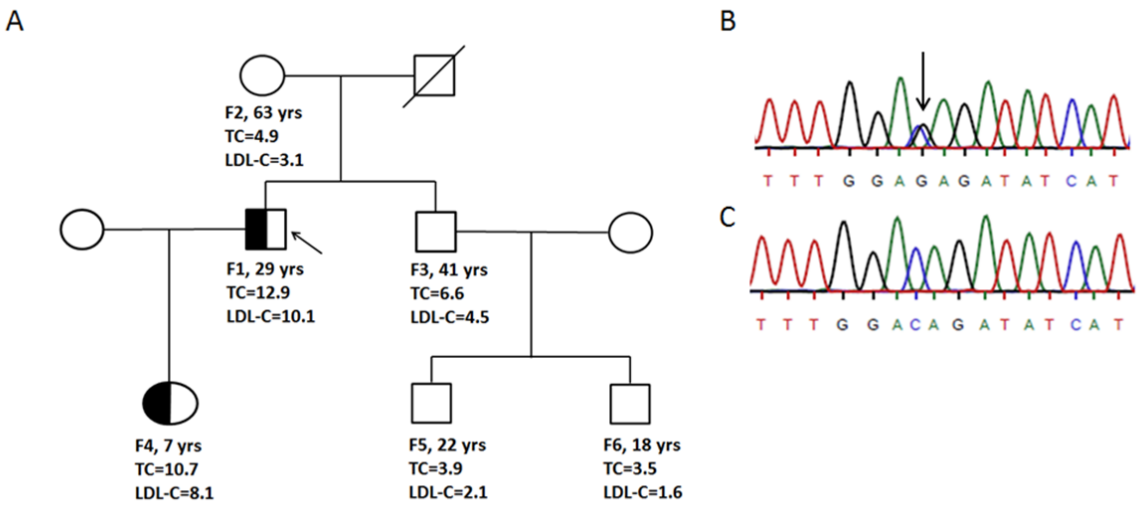
Table 13: Novel mutations found and their pathogenic prediction

DNA level	Protein level	N	exon	Prediction			
				PolyPhen	SIFT	Mutation Taster	splice site effect
c.380T>A	p.(Val127Asp)	1	4	Possibly damaging	Not tolerated	Disease causing	no
c.764G>A	p.(Cys255Tyr)	1	5	Probably damaging	Not tolerated	Disease causing	no
c.986G>T	p.(Cys329Phe0	4	7	Probably damaging	Not tolerated	Disease causing	no
c.1449G>T	p.(Trp483Cys)	1	10	Possibly damaging	Not tolerated	Disease causing	no
c.1737C>G	p.(Asp579Gly)	3	12	Probably damaging	Not tolerated	Disease causing	no
c.1834G>T	p.(Ala612Ser)	2	12	Possibly damaging	Not tolerated	Disease causing	no
c.1862C>G	p.(Thr621Arg)	1	13	Probably damaging	Not tolerated	Disease causing	no
c.1975_1987+16del	p.(?)	1	13	n/a	n/a	n/a	yes
c.2096delC	p.(Pro699Argfs*10)	1	14	n/a	n/a	Disease causing	no
c.2546C>A	p.(Ser849*)	1	17	n/a	n/a	n/a	no

3.2.2 Proband study

From five family members of the patient with novel mutation p.(The621Arg), the daughter was found to have a raised total cholesterol level (10.7 mmol/L) and LDL-C level (8.1 mmol/L) and inherited the p.(Thr621Arg) mutation. The index father, who had raised serum cholesterol levels, died of myocardial infarction at the age of 46 (Figure 11).

Figure 11: Family co-segregation of the novel c.1862C>G [p.(Thr621Arg)] *LDLR* mutation. A-A family pedigree of the index patient (F1) with the novel mutation including age (years), TC level (mmol/L) and LDL-C level (mmol/L). Five members of the family (F2, F3, F4, F5 and F6) were screened and sequenced for the mutation. Only F4 was found to carry the novel variant as the index, which co-segregated with FH phenotype. B- *LDLR* exon 13 sequencing for the index patient (appropriate base arrowed), C- Wild type exon 13 sequence

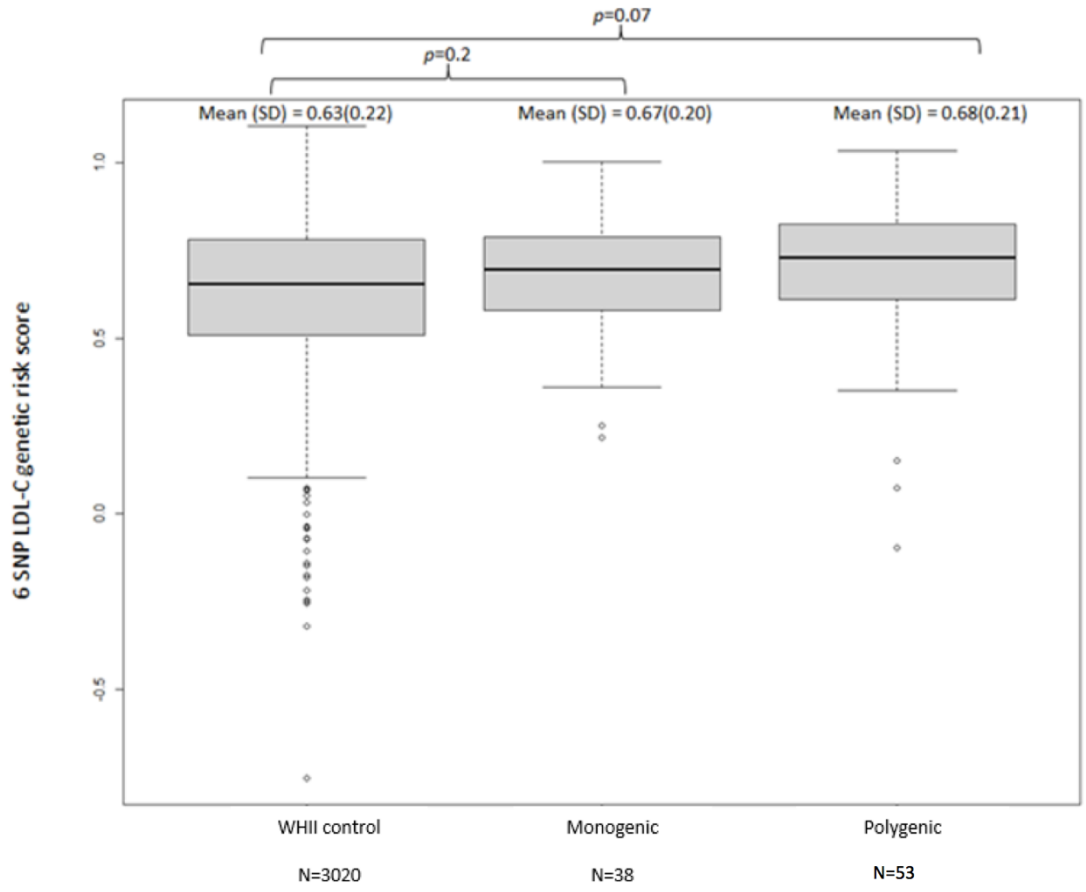


3.2.3 LDL-C raising gene score

Genotypes for all six SNPs were obtained for 101 patients. Using the control cohort (WHII) and SNP score quartiles published in Talmud *et al* 2013, (Talmud *et al.* 2013) out of the 63-genotyped mutation negative FH patients, 53 (84.1%) had an SNP score in the top three quintiles of gene score (>0.51) and therefore the cause of high LDL-C in these patients is likely to be polygenic.

The highest mean score (mean ± SD) was observed in individuals with polygenic hypercholesterolaemia (0.68 ± 0.21). Individuals from the control cohort (WHII) had the lowest mean score (0.63 ± 0.22), whereas those with a confirmed FH mutation had intermediate score (0.67 ± 0.20). The differences between the polygenic group and the control group was borderline statistically significant (p=0.07) (Figure 12).

Figure 12: LDL-C gene score analysis based on six-SNP scores in different groups



3.2.4 Characteristics of the mutation positive and mutation negative patients

Based on the genotyping results and the gene score findings, the characteristics of the patients with and without a mutation were compared in Table 14.

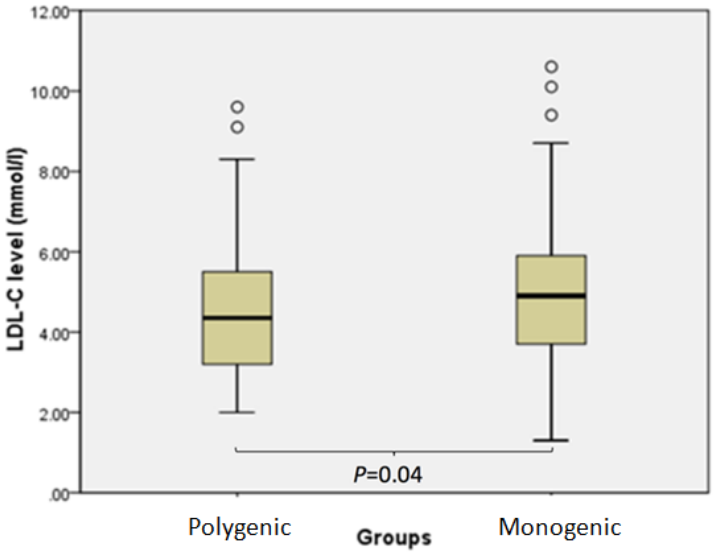
Table 14: Baseline characteristics of the patients where a mutation found and without a mutation

Variable	Mutation positive N=70	Mutation negative N= 91	*p*
	N (%)	N (%)	
Male	26 (37.1)	29 (31.8)	0.48
Tendon xanthomata	43 (61.4)	49 (53.8)	0.53
Family history of premature CHD	38 (54.2)	41 (45.0)	0.38
Personal history of premature CHD	10 (14.2)	11 (12.0)	0.77
On lipid-lowering medication	40 (57.1)	70 (76.9)	0.007
	mean (SD)	mean (SD)	
Age (years)	38 (17.9)	46 (16.6)	0.004
Maximum TC reported (mmol/L)*	10.5 (3.2)	9.5 (2.1)	0.039
Lipid levels:**			
TC (mmol/L)	7.3 (2.2)	6.8 (0.5)	0.31
LDL-C (mmol/L)	5.2 (2.0)	4.5 (1.5)	0.17
HDL-C (mmol/L)	1.5 (0.3)	1.5 (0.3)	0.22
TG (mmol/L)	1.3 (0.7)	1.7 (0.9)	0.001

*Highest pre-treatment total cholesterol level reported for the patient; ** lipid levels at the start of this study; TC: Total cholesterol; TG: Triglyceride

The monogenic group had significantly higher pre-treatment total cholesterol and lower triglyceride level (Figure 14). There was no significant difference in other cardiovascular risk factors. There was also significantly higher number of mutation negative patients on lipid-lowering treatment compared to individuals with monogenic FH.

Figure 13: Pre-treatment LDL-C level in mutation positive and mutation negative patients



3.3 Discussion

This study showed a broad spectrum of mutations and high heterogeneity of FH-causing mutations in this cohort of Polish population with 39 different mutations in 161 FH patients. Overall, FH mutation detection rate was 43.4%. This finding is in agreement with previous studies of European populations, (Fouchier *et al.* 2005) and similar to what was reported in the UK (Taylor *et al.* 2010, Futema *et al.* 2012).

The spectrum of FH mutations varies between countries; from Greece where relatively small numbers of mutations account for the majority of the FH cases, to the Netherlands where the mutation spectrum was found to be extensive (Dedoussis *et al.* 2004, Glynou *et al.* 2008). The cause of FH in the UK is highly heterogeneous with over 200 different mutations reported (Futema *et al.* 2012). The information regarding molecular diagnosis of FH in some parts of the world such as Latin America and South Asia are scant.

The most common *APOB* mutation in European populations, p.(Arg3527Gln), usually accounts for 5-7% of FH patients in European countries. (Liyanage *et al.* 2011) 8% of the patients in this cohort had this mutation, which is similar to that reported in previous studies from Poland (Bednarska-Makaruk *et al.* 2001, Chmara *et al.* 2010). The penetrance of *APOB* mutation has been shown to be <100% and patients with *APOB* mutations usually have a less severe phenotype than FH patients due to *LDLR* mutations (Myant 1993, Vrablik *et al.* 2001).

The frequency of large insertion/deletion was also higher in our study compared with a recently reported UK study (Futema *et al.* 2013) (16.7% vs. 10%). Interestingly, in an FH cohort from northern Poland, nearly 11 out of 13 studied probands had the same mutation (c.662A>G) and they were all originated from a same region in the north. This finding indicates that despite the high heterogeneity of the FH in the country, there might also be a founder effect present in Poland (Mickiewicz *et al.* 2016). This mutation was not found in our study of southern Poland.

We also found ten novel pathogenic mutations in the *LDLR* gene based on multiple prediction algorithms and demonstrated co-segregation of the novel mutation p.Thr621Arg with the FH phenotype in a family in Poland.

This study emphasises the importance of FH screening in the less studied population such as Polish people. The broad variety of FH mutations in Poland requires health care strategies to include a comprehensive diagnostic test to cover the entire coding region of the *LDLR* gene and *APOB* mutations in the screening of the FH patients in this country.

In this cohort of patients, there were a significantly lower number of monogenic patients on lipid-lowering treatment than patients where no mutation found. This could be explained by the fact that majority of the patients with monogenic FH were young women during child-bearing ages who might have been spared from a lipid-lowering medication for family planning. The other reason could also be the intolerance to statins.

In patients where no mutation was found, 84.1% had a gene score in the top three quintiles of the score based on the healthy comparison group, suggesting they might have a polygenic cause for their high cholesterol levels. Comparing the mean weighted LDL-C raising SNPs gene score in the monogenic and polygenic patients showed that the gene score was higher in the polygenic group as was shown in the previous studies in Europe (Talmud *et al.* 2013, Futema *et al.* 2015). The six LDL-C SNPs score analysis in seven independent European cohorts in Futema *et al* consistently confirmed the findings reported by this study.

The limitation of this study was the small number of the samples and a possibility that few novel mutations might have been missed as only the regions of *APOB* and *PCSK9* where the most common FH-causing mutations occur were examined. Also, due to lack of consent and unavailability of family members, the co-segregation in all patients with novel variants was not possible. The results of this chapter have been published in the metabolism journal (appendix 2).

4 CHAPTER FOUR: PRE-CLINICAL ATHEROSCLEROSIS IN MONOGENIC FAMILIAL HYPERCHOLESTEROLAEMIA VERSUS POLYGENIC HYPERCHOLESTEROLAEMIA

In total, 86 patients were recruited to have a cIMT measurement in the UK and 166 patients to have a CT scan and a CAC score in the UK and in the Netherlands.

4.1 Molecular analysis

54 samples from the Dudley Group NHS Foundation Trust in the UK were screened for mutations in all 18 exons of *LDLR* gene, a fragment of exon 26 of *APOB* to cover p.(Arg3527Gln) and exon 7 of *PCSK9* to cover p.(Asp374Tyr) by HRM-melt. MLPA was performed to detect gross deletions and insertions in the *LDLR* as described previously.

Among these 54 patients, 7 patients found to have a mutation detected by the HRM-melt method. The list of all mutations is provided in Table 15.

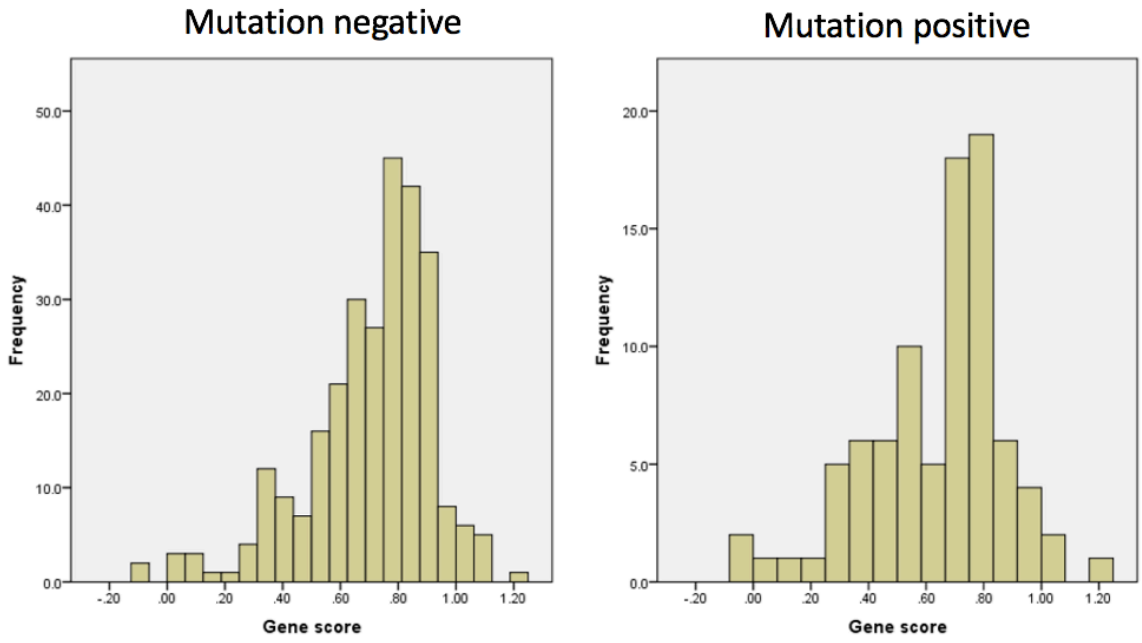
Table 15: Mutations detected in a subset study of the UK samples

Mutation detected	**No**	**Protein**	**Gene/ exon**	**Prediction**		
				Polyphen	**SIFT**	**Refined SIFT**
c.1247-1260del	1	p.(Arg416Glnfs*20)	*LDLR*/ ex9	Benign	Not tolerated	Not tolerated
c.10580G>A	2	p.(Arg3527Gln)	*APOB* ex26	-	-	-
c.2054C>T	3	p.(Pro685Leu)	*LDLR*/ ex14	Not-tolerated	-	-
c.938-939delinsAT	1	p.(Cys313Tyr)	*LDLR*/ ex6	Probably damaging	Not tolerated	Not tolerated

4.1.1 Gene score analysis

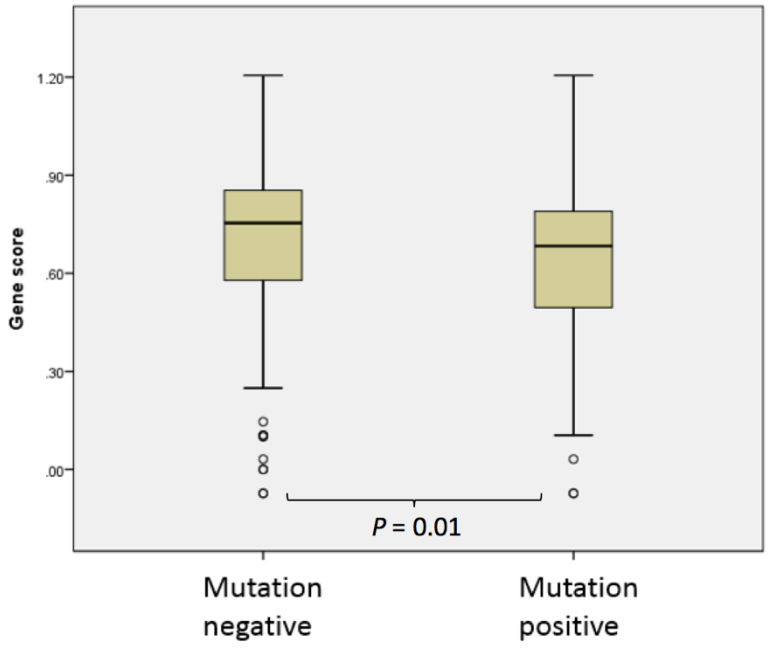
All patients where no mutation was detected in their FH-causing genes had a gene score for six LDL-C raising SNPs. This includes 61 patients at the Dudley NHS Trust, 144 patients at the Royal Free Hospital and 80 patients in the Netherlands. The distribution of gene score found in mutation negative and positive groups were shown in Figure 14.

Figure 14: Gene score distribution in mutation negative and mutation positive group



The mean (SD) of the gene score in mutation negative group was significantly higher than the mutation positive group (0.70 (0.21) vs 0.63 (0.24), p=0.01). The boxplot graph of the data is shown in Figure 15.

Figure 15: Gene score in mutation negative and mutation positive group



4.2 Pre-clinical atherosclerosis assessed by carotid Intima Media Thickness (cIMT)

At the Royal Free Hospital, 56 patients with a known FH-causing mutation were recruited. From 140 patients with a clinical diagnosis of FH where no mutation was detected in their FH-causing genes, 30 patients who had a calculated LDL-C raising gene score in the top two quartiles of the LDL-C raising gene scores were also recruited. This cut off is based on the cut-off reported in Futema *et al* which was designated as having a polygenic cause of FH phenotype. (Futema *et al.* 2015)

4.2.1 Patient characteristics

The demographics of 86 patients (56 monogenic and 30 polygenic) with a cIMT measurement are shown in Table 16.

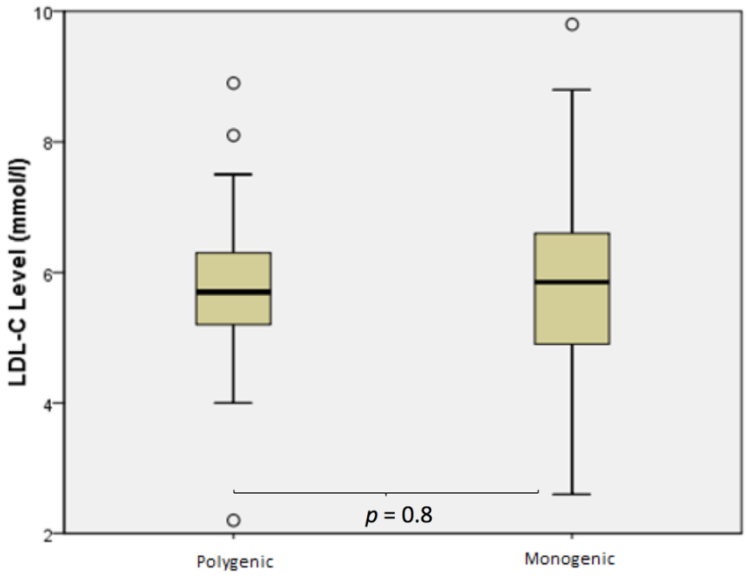
Table 16: Characteristics of the subjects with a cIMT measurement

Characteristics		**Monogenic (N = 56)**	**Polygenic (N = 30)**	***p***
Male	N (%)	22 (39.2)	14 (46.6)	0.3
Age (years)	Mean (SD)	50 (14)	57 (12)	0.03
Pre-treatment lipid levels				
TC (mmol/L)	Mean (SD)	8.1 (1.5)	8.2 (1.0)	0.5
LDL-C (mmol/L)**	Mean (SD)	5.8 (1.6)	5.9 (0.9)	0.8
HDL-C (mmol/L)**	Mean (SD)	1.5 (0.4)	1.9 (1.1)	0.1
TG (mmol/L)**	Mean (SD)	1.2 (0.5)	1.6 (0.7)	0.01
Tendon xanthoma	N (%)	29 (51.7)	10 (33.3)	0.1
Family History of premature CHD*	N (%)	30 (53.5)	20 (66.6)	0.24
BMI (kg/m²)	Mean (SD)	26.1 (4.6)	26.4 (4.4)	0.1
Patients with Hypertension	N (%)	4 (7.1)	1 (3.3)	0.1
Patients with Diabetes	N (%)	0 (0)	1 (3.3)	0.3
Smoker	N (%)	0 (0)	0 (0)	
Post-treatment lipid levels				
TC (mmol/L)	Mean (SD)	4.8 (0.8)	5.0 (0.9)	0.3
LDL-C (mmol/L)	Mean (SD)	2.9 (0.8)	2.8 (0.8)	0.8
HDL-C (mmol/L)	Mean (SD)	1.5 (0.3)	1.6 (0.4)	0.2
TG (mmol/L)	Mean (SD)	0.8 (0.2)	1.3 (0.6)	0.001
Patients on lipid lowering medication	N (%)	42 (75.0)	25 (83.3)	0.7
Years treated with statin	Mean (SD)	10 (5)	8 (3)	0.2

[1] In 1st degree relative (<60 years old) or in 2nd degree relatives (<50 years old); **Data was available for 40 subjects in monogenic group and 25 patients in polygenic group

There was no significant difference in pre-treatment LDL-C level (Figure 16) and other conventional cardiovascular risk factors such as smoking, hypertension, diabetes or body mass index between the groups. The triglyceride level was significantly higher in the polygenic group compared to the monogenic patients (p=0.01).

Figure 16: Pre-treatment LDL-C level in the monogenic and polygenic group with a cIMT measurement



4.2.2 Carotid IMT results

As shown in Table 17, mean of all cIMT readings (mean cIMT) was 12% higher in the monogenic than the polygenic patients after adjustment for age and gender [0.74mm (0.7-0.79) vs 0.66mm (0.61-0.72), p=0.038]. Similar differences were seen in the different segments analysed, with those of mean bifurcation IMT and mean internal carotid artery IMT being statistically significant.

Table 17: Mean and max cIMT in each carotid segment in the monogenic and polygenic group after adjustment for age and gender

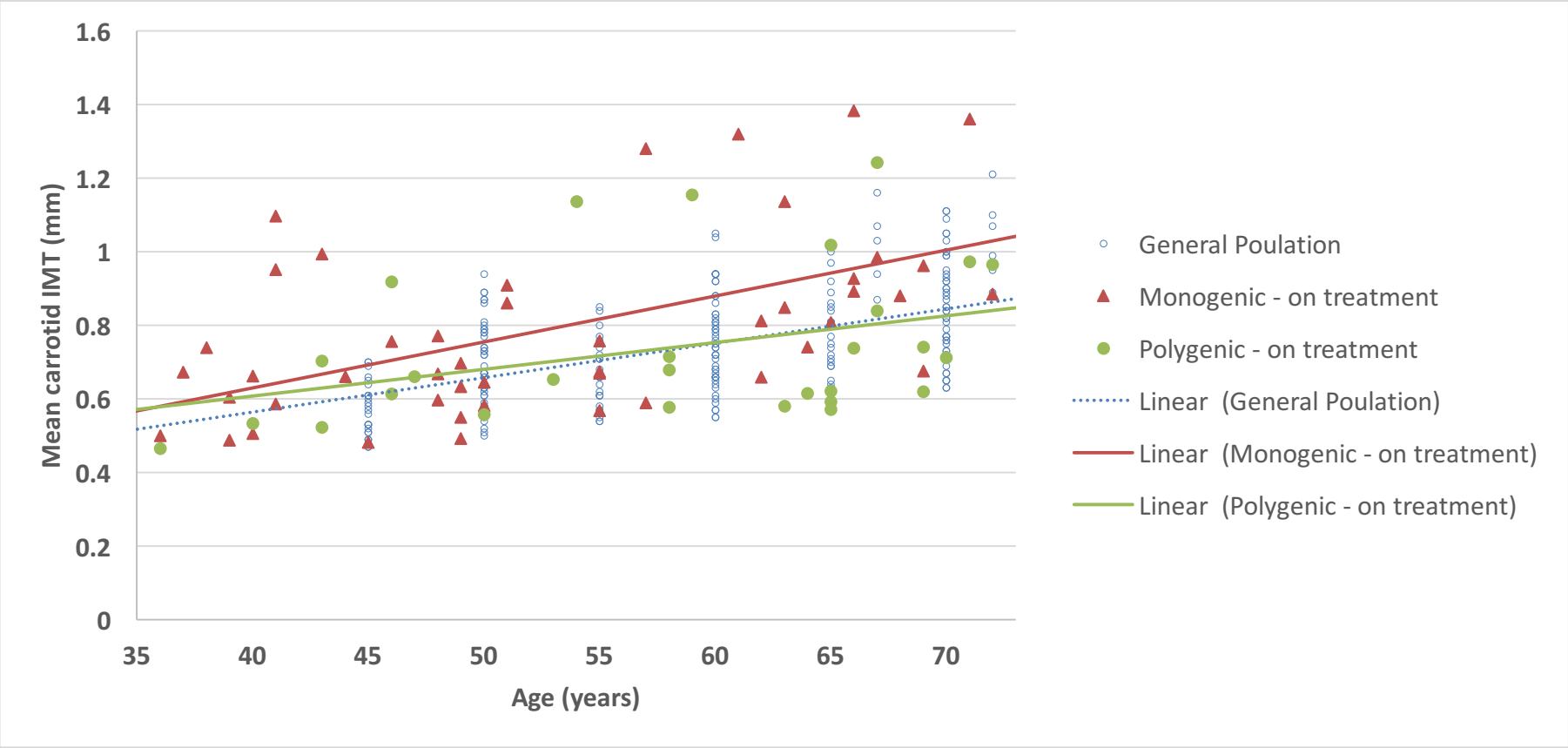
The cIMT results	Monogenic (N = 56)	Polygenic (N = 30)	*p*
	mean (95% CI)	mean (95% CI)	
Mean cIMT[1] (mm)	0.74 (0.70-0.79)	0.66 (0.61-0.72)	0.03
Mean CCA[2] IMT (mm)	0.65 (0.61-0.68)	0.62 (0.58-0.66)	0.3
Max CCA[2] IMT (mm)	0.72 (0.68-0.77)	0.70 (0.64-0.76)	0.5
Mean bifurcation IMT (mm)	0.81 (0.74-0.89)	0.70 (0.62-0.79)	0.05
Max bifurcation IMT (mm)	0.96 (0.85-1.07)	0.80 (0.69-0.93)	0.08
Mean ICA[3] IMT (mm)	0.74 (0.66-0.83)	0.60 (0.52-0.7)	0.04
Max ICA[3] IMT (mm)	0.82 (0.69-0.96)	0.65 (0.52-0.81)	0.1
N(%) patients with carotid plaque	12 (21%)	4 (13%)	0.4

[1] Mean cIMT: mean of all carotid IMT readings; [2] CCA: common carotid artery;
[3] ICA: internal carotid artery; CI: confidence interval.

As expected, and as shown in Figure 17, the cIMT increases with age in both the monogenic and polygenic groups compared to the general population. Using the median age (51 years) and comparing the cIMT in patients ≤ 51 versus > 51 years old showed mean(SD) cIMT of 0.63mm (0.15) vs 0.88mm (0.24) in the monogenic group (p=0.0005) and 0.60mm (0.12) vs 0.75mm (0.19) in the polygenic group (p=0.01).

The increase in mean cIMT with age was greater in the monogenic group with 1.4% increase per annum, compared to the polygenic group, a 1.0% increase per annum; however, this difference was not statistically significant (p=0.35).

Figure 17: Mean carotid IMT measurements against age in treated monogenic FH, polygenic hypercholesterolaemia and the general population[1] based on cIMT readings mean in each patient measured on a single occasion during the study



[1] General population data obtained from Stein *et al*. The coefficient of determination (R^2) between age and IMT values is the proportion of the variance in carotid IMT that can be explained by differences in age (R^2 for mutation positive=0.27, R^2 for mutation negative=0.08 and R^2 for general population=0.22). The value of 0.27 for mutation positive indicates that 27% of the variability in IMT can be explained by age variations, with 73% of the variability unexplained.

4.3 Pre-clinical atherosclerosis assessed by Coronary Artery Calcium (CAC) score

In total, a CT scan and a CAC score calculation performed for 49 monogenic and 30 polygenic in the UK and 75 monogenic and 12 polygenic patients in the Netherlands.

4.3.1 Patient characteristics

The characteristics of 166 patients (124 monogenic and 42 polygenic) with a CT scan and a CAC score are shown in Table 18.

Table 18: Characteristics of the subjects with a CAC score

UK patients				
		Monogenic (N=49)	**Polygenic (N=30)**	***p***
Male	N (%)	22 (44.8)	12 (40.0)	0.67
Age (years)	Mean (SD)	43.6 (9.8)	59.6 (8.1)	0.001
Pre-treatment lipid levels				
TC (mmol/L)	Mean (SD)	8.6 (0.8)	8.8 (1.3)	0.76
LDL-C (mmol/L)	Mean (SD)	6.3 (0.7)	6.1 (1.1)	0.66
HDL-C (mmol/L)	Mean (SD)	1.5 (0.4)	1.7 (0.5)	0.3
TG (mmol/L)	Mean (SD)	1.8 (0.8)	2.3 (1.2)	0.32
Tendon xanthoma	N (%)	30 (61.2)	3 (10.0)	0.001
Family History of premature CHD[1]	N (%)	30 (61.2)	15 (50.0)	0.03
BMI (kg/cm²)	Mean (SD)	27.7 (4.4)	29.7 (9.2)	0.58
Patients with Hypertension	N (%)	1 (2.0)	12 (40.0)	0.3
Patients with Diabetes	N (%)	0 (0)	3 (10.0)	0.9
Smoker	N (%)	1 (2.0)	4 (13.3)	1
Patients on lipid lowering medication	N (%)	40 (81.6)	23 (76.6)	0.1
Years treated with statin	Mean (SD)	9.0 (7.5)	3.0 (3.0)	0.006
Netherlands patients				
		Monogenic (N=75)	**Polygenic (N=12)**	***p***
Male	N (%)	52 (69.3)	8 (66.6)	0.85
Age (years)	Mean (SD)	51.4 (7.7)	55.8 (8.6)	0.07
Pre-treatment lipid levels				
TC (mmol/L)	Mean (SD)	9.9 (2.2)	8.7 (2.0)	0.09
LDL-C (mmol/L)	Mean (SD)	7.6 (2.1)	6.2 (1.7)	0.03
Tendon xanthoma	N (%)	28 (37.3)	0 (0)	-
Family History of premature CHD[1]	N (%)	36 (48.0)	7 (58.3)	0.5
BMI (kg/cm²)	Mean (SD)	26.7 (3.8)	24.6 (3.2)	0.07
Patients with Hypertension	N (%)	15 (20.0)	5 (41.6)	0.1
Patients with Diabetes	N (%)	2 (2.6)	1 (8.3)	0.36
Smoker	N (%)	14 (18.6)	3 (25.0)	0.7
Post-treatment lipid levels				
TC (mmol/L)	Mean (SD)	5.5 (1.4)	5.2 (1.7)	0.62
LDL-C (mmol/L)	Mean (SD)	3.5 (1.3)	2.8 (1.3)	0.11
HDL-C (mmol/L)	Mean (SD)	1.3 (0.3)	1.3 (0.3)	0.7
TG (mmol/L)	Mean (SD)	1.2 (0.8)	2.1 (3.5)	0.06
Patients on lipid lowering medication	N (%)	75 (100)	11 (91.6)	0.14
Years treated with statin	Mean (SD)	10.8 (7.6)	5.8 (7.6)	0.04

[1] In 1st degree relative (<60 years old) or in 2nd degree relatives (<50 years old)

In the UK group, there was no significant difference between the monogenic and polygenic patients in their LDL-C levels (p=0.66). In the Netherland group, the LDL-C level was significantly higher in monogenic patients (p=0.03).

Tendon xanthoma was present mainly in the monogenic patients in both the UK and the Netherland group. Only 10% of the polygenic patients in the UK were reported to have tendon xanthoma and no polygenic patient in the Netherlands.

4.3.2 CAC score results

The estimated mean (95% CI) CAC score in all centres was 24.5 (14.4 to 41.8) for the monogenic group which was significantly higher than 2.65 (0.94-7.44) for the polygenic group. The CAC score was estimated to be 9.27 (95% CI: 2.74 to 31.4) times higher in the monogenic compared to the polygenic group after adjustment for centre, age and gender (p=0.0004) (Table 19).

The CAC score did not differ, overall, between two centres (UK vs Netherlands: 14.2 vs 19.1, p=0.50) and both centres showed a significantly higher CAC score in the monogenic patients.

Table 19: CAC score in the monogenic and polygenic group after adjustment for age and gender

CAC score Results	Monogenic	Polygenic	*p*
	mean (95% CI), N	mean (95% CI), N	
UK centre	33.4 (13.9-81.5), N=49	1.0 (0.3-3.4), N=30	
Netherlands centre	22.9 (12.1-43.4), N=75	11.1 (2.3–54.0), N=12	
Total	24.5 (14.4-41.8), N=124	2.6 (0.9-7.4), N= 42	0.0004

Table 20 shows the number of monogenic and polygenic patients in each CAC score category: zero, 1-99, 100-399 and ≥400. A CAC score above 100 occurred in 51 (41%) of monogenic patients compared to 12 (28%) polygenic patients (p=0.43). A CAC score of zero was reported in 33 monogenic patients compared with 16 polygenic individuals (age and gender adjusted, p=0.01) and a CAC score >400 was found in 26 (21%) monogenic people in comparison to 5 (12%) polygenic individuals (age and gender adjusted, p=0.03).

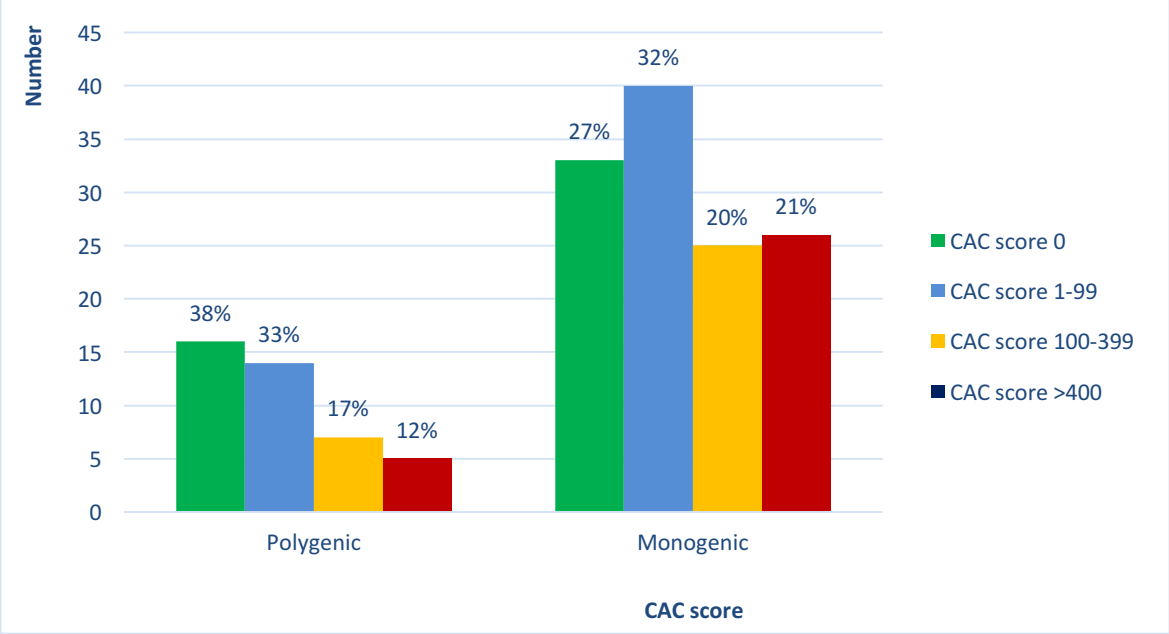
The data was also shown for 49 excluded subjects with a LDL-C SNP score below our recruitment cut-off.

Table 20: Number of patients with different CAC score in the monogenic group and the polygenic group

Groups	CAC score			
	0	**1 - 99**	**100 – 399**	**> 400**
Polygenic (n = 42)	16	14	7	5
Monogenic (n = 124)	33	40	25	26

After adjustment for centre, age and gender, the odds ratio for having a CAC score >100 in monogenic group was 4.7 (95% CI: 1.6-13.7, p=0.004).

Figure 18: Percentage of patients with different CAC score in different groups



4.4 Discussion

This study showed that preclinical atherosclerosis, as measured in carotid and coronary arteries, was greater in treated asymptomatic monogenic FH patients compared to individuals with a polygenic hypercholesterolaemia.

There was no significant difference in cardiovascular risk factors among the monogenic and polygenic groups except the longer lipid-lowering therapy for primary prevention in the monogenic group. Despite the longer lipid-lowering therapy, the CAC score was significantly raised in the monogenic group. In addition, a significantly higher number of monogenic patients had severe CAC score (≥400).

While there is some evidence that statin treatment itself may be associated with high levels of coronary calcium (possibly as the plaque becomes lipid-depleted and more stable), (Rodriguez-Granillo *et al.* 2016) it is unlikely that differences in lipid-lowering treatment in the two groups is the explanation for the differences seen here.

Zero calcium score was found in 38.1% of polygenic patients and was significantly higher than the monogenic FH patients (26.6%). The CAC score of zero has been reported in a range of 40-60% in the general population in previous population-based studies (Hoff *et al.* 2001, Schmermund *et al.* 2006). The MESA study, a prospective cohort, designed to investigate the prevalence, risk factors, and progression of subclinical cardiovascular disease, following about 6.000 asymptomatic subjects aged 45-84 years of age in the United States, demonstrated a score of zero was observed in nearly 62% of the women and in 40% of the men (McClelland *et al.* 2006).

Notably the zero CAC score does not exclude the presence of atherosclerosis but a previous study of FH patients showed the presence of non-calcified plaque only in 4% of FH patients with zero CAC score and they all had less than 50% luminal obstruction (Neefjes *et al.* 2011).

Coronary Artery Calcium Scoring is a robust and reproducible way of detecting coronary atherosclerosis to estimate future risk of cardiac events. It has the incremental benefit beyond traditional risk prediction biomarkers. It has a great benefit when applied to asymptomatic

individuals between ages 45 to 75 years old who are at intermediate risk as determined by Framingham Risk Score or similar tool calculators. It has the ability of re-classify many into either lower risk, with potential cost- savings in minimizing therapy or into higher risk group where appropriate therapies may improve outcomes.

Asymptomatic patients with monogenic FH might benefit from noninvasive imaging techniques to identify preclinical atherosclerosis and to initiate additional preventive measures and therapy at an earlier stage. This has been suggested in the 2013 risk assessment guideline by the American College of Cardiology (ACC) and the American Heart Association (AHA) to enhance atherosclerotic cardiovascular disease (ASCVD) risk estimation (Goff *et al.* 2014).

Although CAC scores are a reliable surrogate marker of atherosclerosis, their correlation with results of invasive coronary angiography, the current reference standard for diagnosing obstructive coronary artery disease, is limited in terms of detecting significant stenosis (Scholte *et al.* 2008). CT coronary angiography has been proposed as an alternative diagnostic modality for assessing coronary artery disease in patients with high risk of CHD (Meijboom *et al.* 2007). The coronary CT angiography provides comprehensive information regarding the location and severity of atherosclerotic plaque in the coronary arteries. Several studies have taken advantage of this feature of coronary CT angiography and demonstrated the independent prognostic utility of coronary CT angiography in patients suspected of having CHD (Ostrom *et al.* 2008, van Werkhoven *et al.* 2009).

FH patients are at increased risk of cardiovascular events at a relatively younger age. Whether accumulation of LDL-C raising SNPs which causes the high cholesterol levels in patients with polygenic hypercholesterolaemia can cause the same atherosclerosis severity as the monogenic FH patients who have raised LDL-C since birth would need further studies.

The aetiology for raised LDL-C in these patients may play a role in determining the degree of preclinical atherosclerosis. The substantially higher accumulated ‘LDL-C burden’ in monogenic patients since birth might explain the higher risk compared to patients with polygenic hypercholesterolaemia who may only reach the LDL-C threshold of monogenic

FH patients in later life after exposure to environmental factors (Humphries and Futema et al unpublished).

Carotid IMT has been shown to be thicker in children with FH than in their unaffected siblings, (Guardamagna *et al.* 2009) and in adults with a known FH causing-mutation compared to the general population, (Descamps *et al.* 2001) or patients with familial combined hypercholesterolaemia (Masoura *et al.* 2011). Our results show that in treated monogenic patients carotid IMT remains raised compared to treated polygenic patients and the general population throughout adulthood. This result should be viewed with caution since it is based on cross-sectional data, and validation of this using multiple measures is required. A total sample size of around 600 would be required to detect a significant (80% power at p= 0.05) interaction effect between the age and the carotid IMT thickness comparing monogenic with polygenic subjects.

Clearly, the differences in carotid IMT and coronary calcification seen here should be confirmed in a larger sample, and further studies of the coronary atherosclerosis burden would strengthen the inference.

The principal limitation of this study is the relatively small sample size. However, a strength of our study is that, in patients with a clinical diagnosis of FH, two different measures of atherosclerosis burden (carotid IMT and CAC score), in three completely independent centres, consistently found the lower burden in patients with a polygenic compared to a monogenic aetiology.

Although all subjects fulfilled the clinical diagnosis of FH, it is possible that a small proportion of those designated as “polygenic” may carry an FH-causing mutation in the *LDLR/APOB/PCSK9* genes that have been missed because of technical reasons in the methods used for mutation detection, although this is unlikely to be more than 1-2 for each of the study groups. The inclusion of a few monogenic FH patients in the polygenic group would mean that the measured mean levels of carotid IMT or CAC score would be higher than that in a “pure” polygenic group, and as such could not be a confounder of the differences seen here. Conversely, since all the identified mutations in this group have been previously reported as FH-causing, there is no inclusion of any “false-positive” cases in the

monogenic group. The results of this chapter have been published in the atherosclerosis journal (appendix 3).

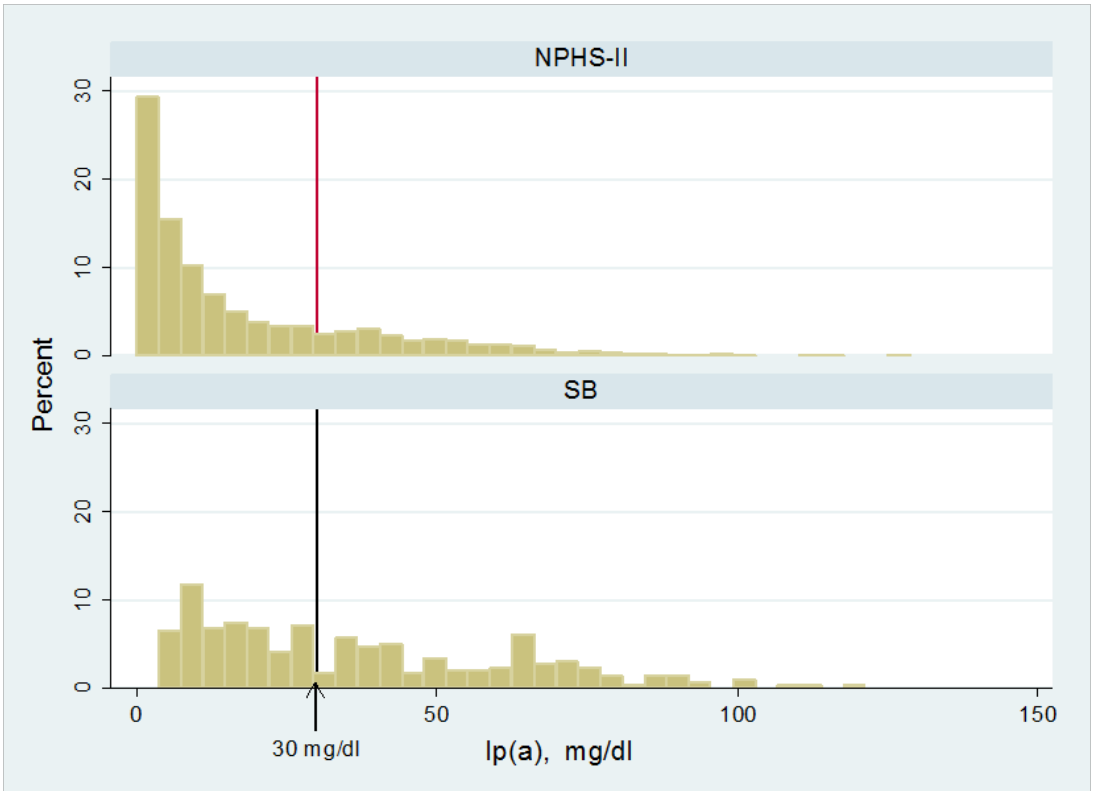
5 CHAPTER FIVE: LIPOPROTEIN(a)

5.1 Biochemical analysis

5.1.1 Lp(a) concentration in NPHSII and SBFH studies

The distribution of Lp(a) concentration measured in both original NPHSII and SBFH studies are presented in Figure 19. The Lp(a) concentration had a markedly skewed distribution in the healthy NPHSII subjects, with most people (88%) having the Lp(a) levels below 30mg/dL. In FH patients, the Lp(a) levels showed a less skewed distribution.

Figure 19: Distribution of Lp(a) concentration in the NPHSII and SBFH studies



30 mg/dL is the cut off for high risk patients

The patients with FH had significantly higher levels of Lp(a) compared to the healthy men recruited in the NPHSII (median [IQR]: 29 [15-56] vs 8.85 [2.8-26.], p <0.001) and this difference was maintained after adjustment for LDL-C and total cholesterol level (p<0.001). Overall, 35% of the SBFH subjects had an Lp(a) level above 30mg/dL compared to 12% in the NPHSII subjects (p=0.001).

5.1.2 Lp(a) concentration measurement by original assays and Randox assay

The Lp(a) concentration [mean (SD) and median (IQR)] measured by the original assays and the Randox assay in each study are shown in Table 21.

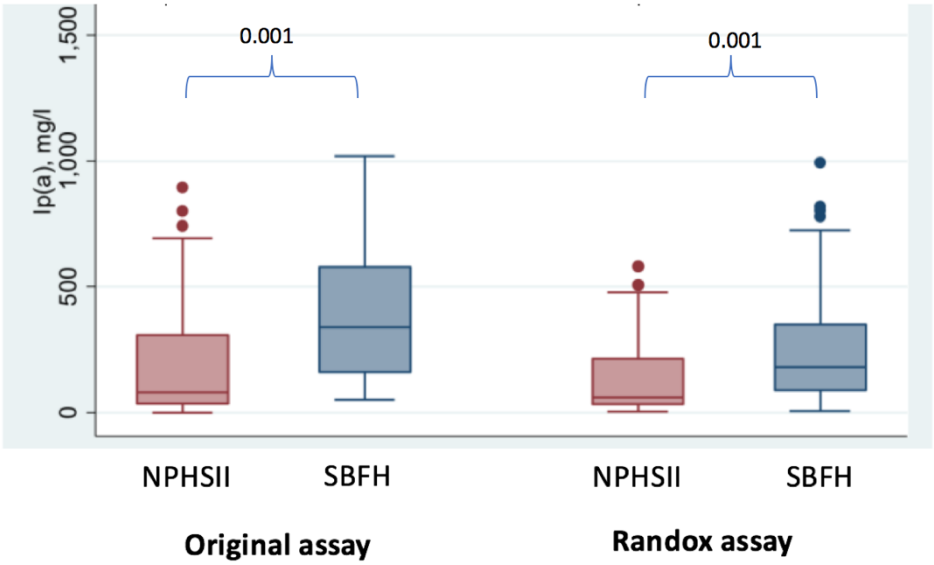
Table 21: Lp(a) concentration measured by the original assay and the Randox assay in the NPHSII and SBFH studies

Assay		**NPHSII N=83**	**SBFH N=80**	***P***
Original assay[1]				
Lp(a), mg/L	Mean (SD)	186.8 (219.0)	368.8 (248.4)	<0.001
Lp(a), mg/L	Median (IQR)	80 (34.0 - 309.2)	340 (160.0 -580.0)	< 0.001
Randox assay[2]				
Lp(a), mg/L	Mean (SD)	127.0 (136.1)	287.3 (287.1)	< 0.001
Lp(a), mg/L	Median (IQR)	60.8 (30.3 - 213.4)	181.5 (89.0 - 351.6)	<0.001

[1] ELISA assay in NPHSII and immune-turbidimetry with DiaSorin SPQ II assay on Cobas-Bioanalyser in SBFH; [2] Immuno-turbidimetry Daytona analyser with Denka Seiken reagents

Figure 20 shows a box plot graph of Lp(a) concentration distribution measured by the original assays and the Randox assay.

Figure 20: Boxplot graph of Lp(a) concentration in samples from the NPHSII and SBFH studies[1]

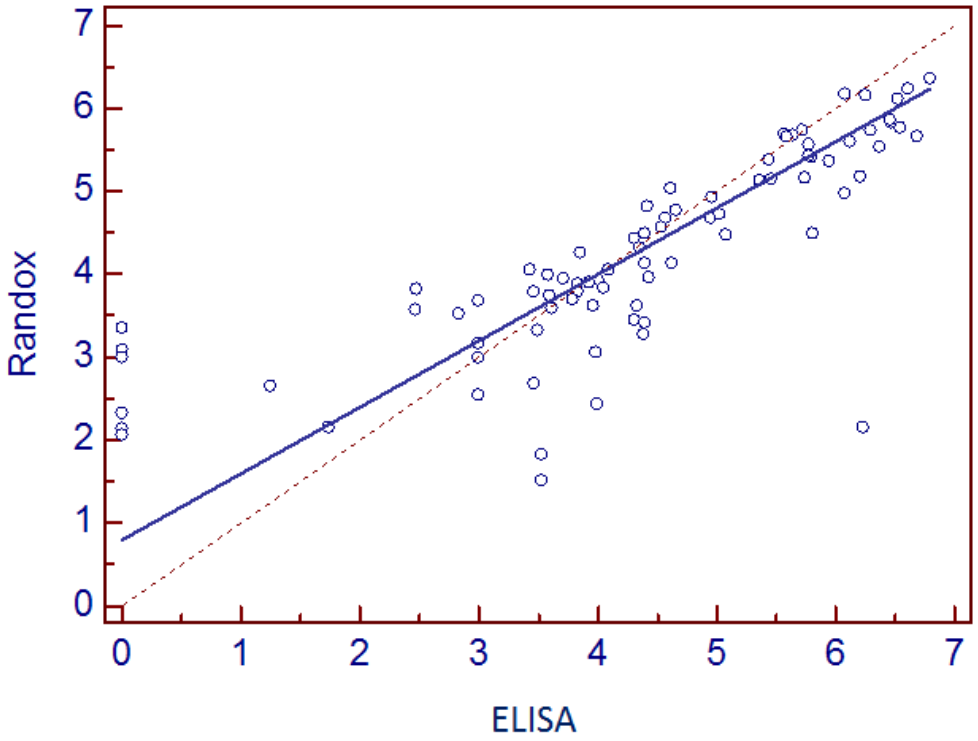


[1]Original assay in NPHSII: ELISA; Original assay in SBFH: Immune-turbidimetry with DiaSorin SPQ II assay on Cobas-Bioanalyser; Randox assay: immuno-turbidimetry on a Randox RX Daytona analyser with Denka Seiken reagents.

5.1.3 Original assay and Randox assay comparison in NPHSII

The scatter plot of the results by the original assay (ELISA) and the Randox assay in NPHSII samples is shown in Figure 21 using the log-transformed data. There was a strong correlation between the Lp(a) concentrations in both assays (r^2= 0.84, p<0.001).

Figure 21: Scatter plot of Lp(a) concentration measured by the original assay (ELISA) and the Randox assay in the NPHSII (log-transformed data)

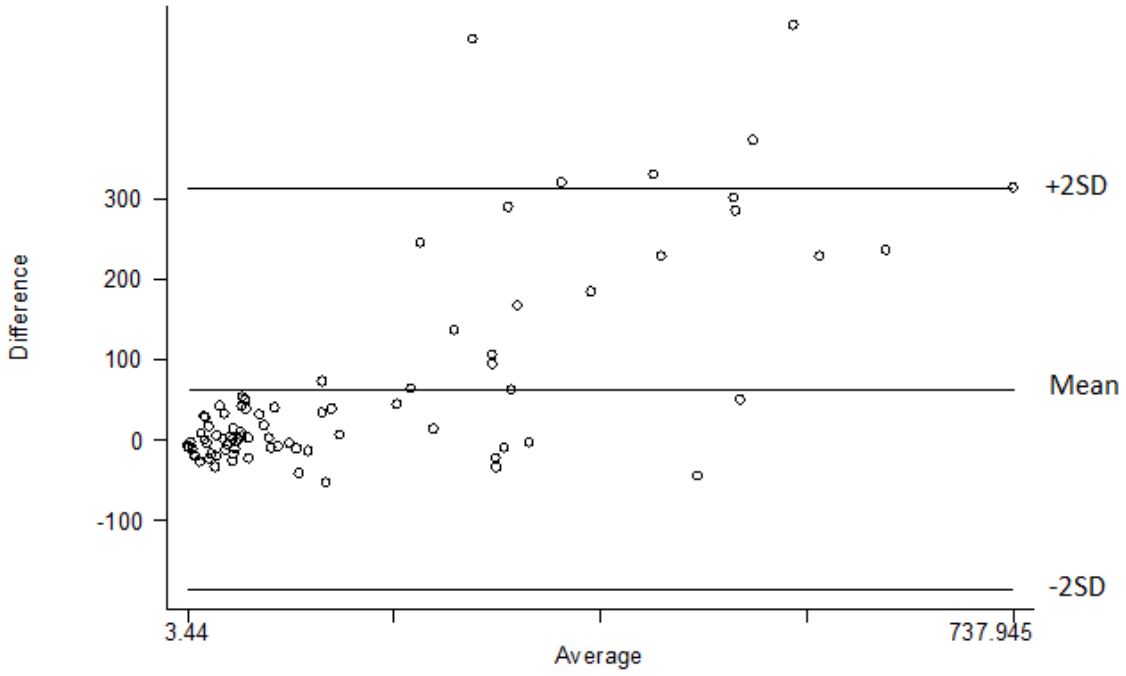


Sample size: 83 **Regression Equation:** y = 0.7903 + 0.8010 x			
Axis	**Mean**	**CV (%)**	
X axis= ELISA Y axis= Randox	4.3073 4.2404	6 2.3	
Parameter	**Coefficient**	**Standard Error**	**95% CI**
Intercept Slope	0.7903 0.801	0.4951 0.09535	0.1945 to 1.7752 0.6113 to 0.9907

Plotting the differences between the Lp(a) measurements by the original and the Randox assay on y-axis against the average concentration of Lp(a) measured by the original and the Randox assay for each sample on x-axis indicated that the levels measured by the original assay tend the to be higher than the concentrations measured by the Randox assay, especially for those with higher average levels (Figure 22). The limits of agreement (mean of difference ± 2SD) were very wide (-186.8 to 311.6), and the variability of the differences increased as

the Lp(a) levels raised. After using log-transformed data, the Lp(a) concentration was on average 6.9% higher when measured by the original assay.

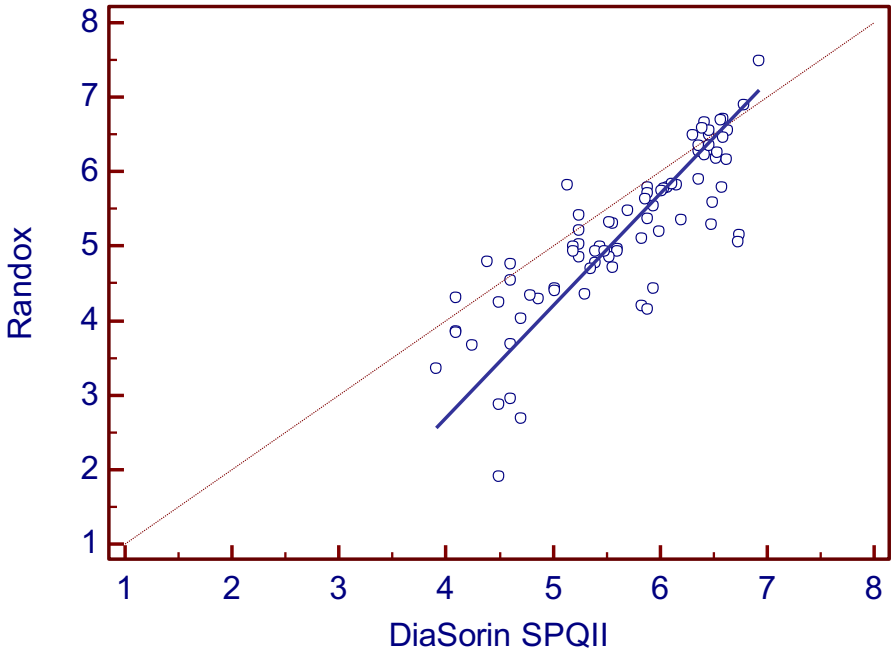
Figure 22: Bland Altman plot of Lp(a) concentration measured by the original and the Randox assay in the NPHSII



5.1.4 Original assay and Randox assay comparison in SBFH

Figure 23 shows the scatter plot of the Lp(a) concentration measured by the original assay (DiaSorin SPQII) and the Randox assay using log-transformed data. There was a strong correlation between the Lp(a) measurements (r^2= 0.86, p<0.001).

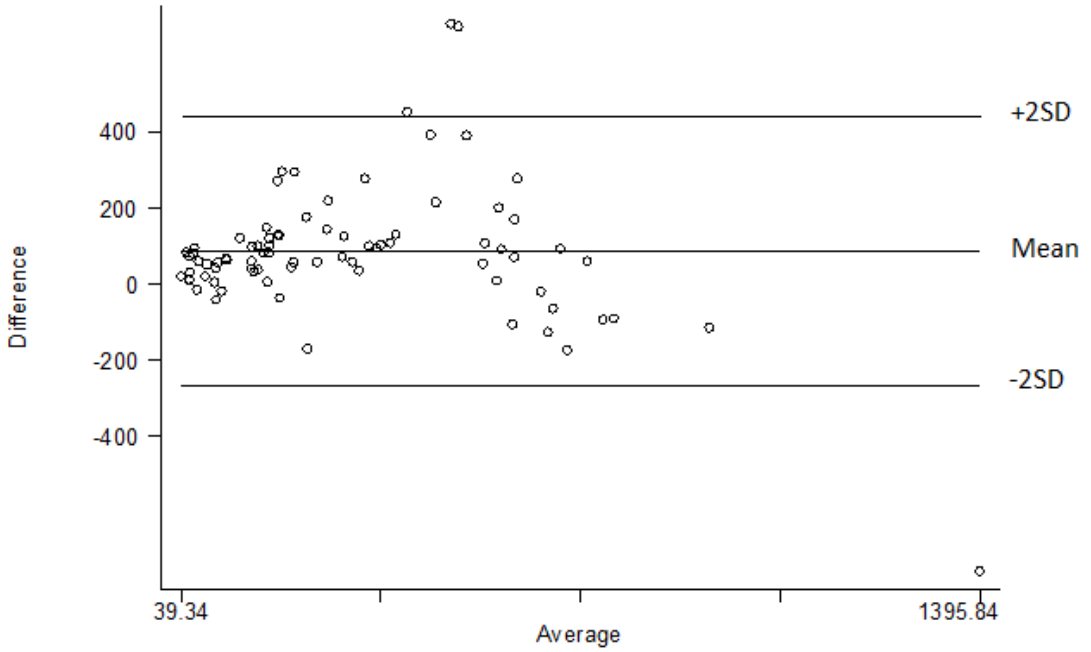
Figure 23: Scatter plot of Lp(a) concentration measured by the original assay (DiaSorin SPQII) and the Randox assay in the SBFH study (log transformed data)



Sample size: 80 **Regression Equation:** y = -3.3165 + 1.5036 x			
Axis	**Mean**	**CV (%)**	
X axis: DiaSorin SPQII	5.6564	3.1	
Y axis: Randox	5.1886	2.3	
Parameter	**Coefficient**	**Standard Error**	**95% CI**
Intercept	-3.3165	0.8146	4.9378 to 1.6951
Slope	1.5036	0.1365	1.2320 to 1.7752

The difference plot (Bland-Altman plot) of the Lp(a) concentration by the original assay (DiaSorin SPQII) and the Randox assay showed that the original levels were higher than the levels measured by the Randox assay (Figure 24). Plotting the difference between the original and the Randox assay against the average level of both assays for each sample indicated that limits of agreement were very wide between -267 and 441. No Systematic bias was detected. After using log-transformed data, the original measurements were on average 60% higher.

Figure 24: Bland Altman plot of Lp(a) concentration measured by the original and the Randox assay in the SBFH study



5.2 Genotype analysis

5.2.1 Association between SNPs (rs3798220 and rs6919346) genotype and Lp(a) concentration measured by the Randox assay

The Lp(a) concentration is measured in 79 samples from the NPHSII and 68 samples from the SBFH study with a known rs3798220 genotype. The Lp(a) concentration was also measured in 79 samples from each NPHSII and SBFH studies with a known rs6919346 genotype. The association of SNPs (rs3798220 and rs6919346) genotype with Lp(a) concentration is shown in Table 22.

Table 22: Association of *LPA* genotype with Lp(a) concentration in each study

SNP ID	Genotype	SBFH		NPHSII	
		Lp(a) concentration (mg/dL)		Lp(a) concentration (mg/dL)	
		Median [IQR]	N	Median [IQR]	N
rs3798220	**AA**	167.2 [77.7-339.8]	66	59.2 [30.3-213.4]	78
	AG	957.0 [142.4-1771.7]	2	580.9 [580.9-580.9]	1
rs6919346	**CC**	211.6 [138.3-519.0]	53	82.4 [41.4-214.7]	55
	CT	110.2 [39.4-239.9]	15	35.1 [18.9-87.5]	21
	TT	57.8 [46.1-69.6]	2	270.0 [30.3-320.2]	3

Subjects carrying the rare G allele of rs3798220 had significantly higher Lp(a) levels compared with AA genotype in FH patients (p=0.002). The association was not significant with rs6919346 (p=0.09).

5.2.2 Association between SNPs (rs3798220 and rs6919346) genotype and Lp(a) concentration measured by the original assays

The genotype distribution and minor allele frequency (MAF) for the two *LPA* SNPs (rs3798220 and rs6919346) of the original data in the NPHSII and SBFH studies are presented in Table 23.

Table 23: Genotype and allele frequency of rs3798220 and rs6919346 in each study

SNP ID	Genotype	SBFH	NPHSII	*p*
		N (%)	N (%)	
rs3798220	**AA**	344 (94.5)	2636 (96.7)	
	AG	20 (5.5)	90 (3.3)	
	MAF (G Allele)	*0.027*	*0.017*	*0.02*
rs6919346	**CC**	260 (70.5)	1875 (69.9)	
	CT	100 (27.1)	729 (27.2)	
	TT	9 (2.4)	80 (3.0)	
	MAF (T Allele)	*0.166*	*0.16*	*0.27*

MAF: minor allele frequency

Distribution of Lp(a) concentration measured by the original assays in the NPHSII and SBFH studies by rs3798220 and rs6919346 genotypes is shown in Table 24.

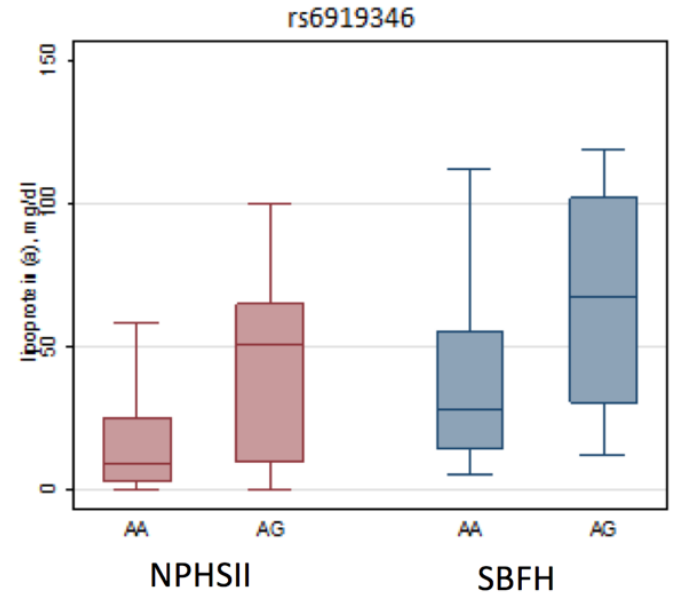
Table 24: Association of rs3798220 and rs6919346 with Lp(a) concentration in each study

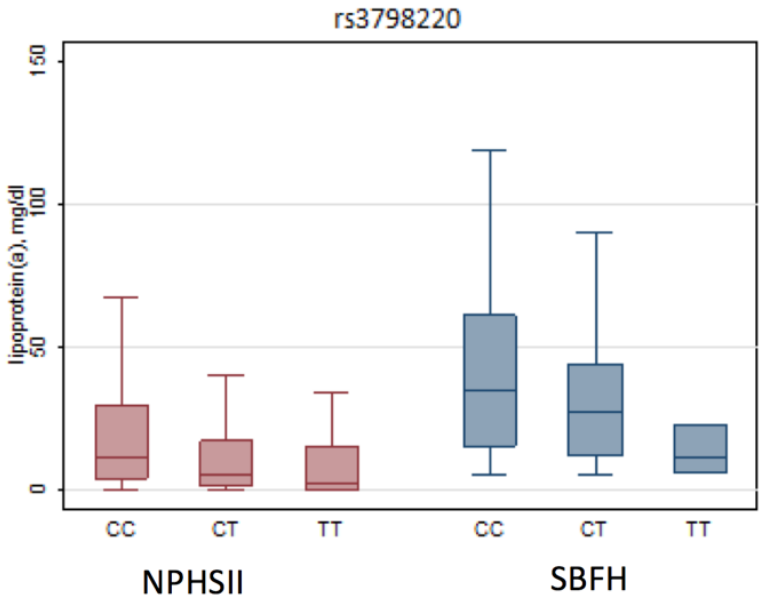
SNP ID	Genotype	SBFH		NPHSII		Interaction *p*
		Lp(a) concentration (mg/dL)		Lp(a) concentration (mg/dL)		
		median [IQR]	N	median [IQR]	N	
rs3798220	**AA**	28 [14-55]	255	8.6 [3-25]	2422	
	AG	68 [31-102]	8	51.1 [9-64]	84	
	p	*0.02*		*0.0001*		*022*
rs6919346	**CC**	35 [15-61]	195	11.4 [4-29]	1723	
	CT	27 [12-44]	67	5.3 [1.3-16]	669	
	TT	12 [6-23]	6	2.35 [0-14]	76	
	p	*0.05*		*0.0001*		*0.21*

Subjects carrying the rare G allele of rs3798220 had significantly higher Lp(a) levels compared with AA genotype in both FH patients and normal subjects. By contrast, for SNP rs6919346, carriers of the rare T allele had significantly lower Lp(a) levels than subjects carrying one or no T allele, in both FH and normal subjects. For both SNPs, the raising or lowering effects was similar in the FH and healthy subjects (interaction *p* value 0.21 and 0.22 respectively).

Figure 25 shows a box plot graph of Lp(a) concentration distribution in the NPHSII and SBFH studies by rs3798220 and rs6919346 genotypes.

Figure 25: Distribution of Lp(a) concentration measured in the NPHSII and SBFH studies by rs3798220 and rs6919346 genotypes





5.2.3 Rs3798220 genotype and allele frequency in different FH cohorts and two general population studies

When the rs3798220 genotype of FH patients in the Belgian cohort were added to the data available from the SBFH, the UK10K and other cohorts, rs3798220 G allele frequency was significantly higher in patients with a diagnosis of FH compared to the general population (p=0.001) (Table 25).

Table 25: Rs3798220 genotype and allele frequency in different FH cohort studies and groups of the UK general population

	FH cohorts					**The general population groups**		
rs3798220	**SBFH**	**UK10K**	**Additional UK FH cases**	**Belgian FH**	**All FH**	**NPHSII**	**WHII**	**All (NPHSII plus WHII)**
Genotype	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
AA	344 (94.5)	69 (89.6)	161 (92.0)	251 (96.1)	**825 (94.1)**	2636 (96.7)	2923 (96.8)	**5559 (96.7)**
AG	20 (5.5)	8 (10.4)	14 (8.0)	10 (3.9)	**52 (5.9)**	90 (3.3)	96 (3.2)	**186 (3.3)**
MAF(G allele)*	*0.027*	*0.052*	*0.04*	*0.019*	***0.03***	*0.017*	*0.016*	***0.016***
P****	*0.021*	*0.0005*	*0.0006*	*0.6*	***0.001***			

* MAF: minor allele frequency; ** *p* value of FH cohort versus combined NPHSII and WHII

5.3 Discussion

In patients with a clinical diagnosis of definite FH, Lp(a) levels were significantly higher in comparison with the general population, with 35% of the FH patients having an Lp(a) level above the value of 30 mg/dL compared to 12% in the general population. Previous studies also reported raised Lp(a) levels above 30 mg/dL in 50% of the FH patients compared to 27% in the normal population (Bowden *et al.* 1994), and levels above 50 mg/dL in 20% of the general population (Nordestgaard *et al.* 2010).

Lp(a) mass assays have an inherent limitation due to the heterogeneity of Lp(a) particle sizes, making it difficult to standardise assays with appropriate calibrators. In addition, because most antibodies used in the assays are polyclonal and cross-react with multiple Kingle IV type 2 repeats, these assays may overestimate Lp(a) levels in patients with large isoforms and underestimate levels in patients with small isoforms. Most clinical laboratories have overcome this limitation by using appropriate calibrators, along with linking the results to the World Health Organization/International Federation of Clinical Chemistry and Laboratory Medicine International Reference Reagent, making the assays relatively isoform independent.

Since the original methods used for the Lp(a) concentration measurements in the SBFH and NPSHII studies were different, we compared the original results with the measurements by the Randox assay. The Marcovina *et al.* paper (Marcovina, Albers et al. 2000) showed that Denka Seiken reagent achieved the best concordance with the reference method for Lp(a) measurement. This reagent is marketed under license by the Randox Laboratories. It is one of the few assays which is not affected by the varying size of apo(a) and has a good specificity.

The results showed similar Lp(a) concentration distribution by the original and the Randox method. This confirms the reports of other studies such as Lingenhel *et al.* (Lingenhel *et al.* 1998) and Jansen *et al.* (Jansen *et al.* 2004) that there may not be a major bias in Lp(a) measurements in methods affected by apo(a) sizes.

Appropriate selection, sampling and proper storage of analytes, in combination with analytes stability, can profoundly impact the interpretation of lab results. A common problem in clinical laboratories is maintaining the stability of the analytes during sample storage. The temperature at which the samples are stored constitutes an important pre-analytical variable that may affect analytical results in the clinical biochemistry laboratory setting.

The Lp(a) samples used in the NPHSII study were citrate samples stored at -80 degrees for 20 years. According to the Randox manufacturer, citrate samples could be used for analysis by the Randox assay. However, considering that these samples were stored at -80 for a long time, there would be an uncertainty about the stability of these samples. Our study showed that despite using old samples, the correlation between measurements by the Randox assay and the previous assays was strong, supporting the validity of the measurements.

In this study, the Lp(a) readings measured by Randox assay were overall 6.9% lower than the original readings. The Simo *et al.* study of Lp(a) measurements in samples stored for 5 years showed the same results with a significant correlation with the initial values (Simo *et al.* 2001). This was expected as long-term storage is known to cause more deterioration in Lp(a) particles of low molecular weight which is associated with high Lp(a) concentrations than high molecular weight particles. (Kronenberg *et al.* 1996)

Clark *et al.* showed that two common variants, rs10455872 and rs3798220, together explained 36% of the total variation in the Lp(a) level and were independently associated with an increased risk of coronary artery disease (Clarke *et al.* 2009). They also found that the effects of the *LPA* variants on the risk of coronary disease correlated with the effects on the Lp(a) concentration. The linear dose–response relationship of the *LPA* variants with both the Lp(a) concentration and the risk of coronary disease supports the role of an elevated Lp(a) concentration in the risk of coronary artery disease.

In both FH patients and healthy subjects, the *LPA* rs3798220 rare allele carriers had increased Lp(a) levels while the rare allele carriers of rs6919346 had lower levels, as reported previously (Clarke *et al.* 2009, Lanktree *et al.* 2010). The raising and lowering effect of the rare alleles were of similar magnitude in the FH patients and the healthy subjects, but the frequency of the raising allele for the missense SNP rs3798220 (Ile4399Met) was almost twice as high in the FH patients as in the healthy subjects.

This would suggest that, in part, the higher Lp(a) levels seen in the FH patients could have a genetic basis. Since the genes where mutations cause FH and the *LPA* gene, are on different chromosomes, they will segregate independently in families; which may also explain the variable age of onset of CHD in FH relatives, i.e. earlier if they have also inherited Lp(a) raising variants and later if they have not.

While it is clear that Lp(a) plays a role in causing atherosclerosis, the exact mechanism is unknown (Kronenberg *et al.* 2013). FH patients are at high risk of atherosclerosis due to raised LDL-C levels from birth. The increased frequency of the rs3798220 variant may also contribute to CHD in these patients. However, the size of the sample of FH patients available to us was insufficient to have the power to address this issue and larger studies are needed to investigate this further.

6 OVERALL DISCUSSION

The findings of Chapter 3 show that the spectrum of FH mutations is markedly heterogeneous in Poland and novel *LDLR* variants are discovered. The data produced by this thesis and similar studies in different parts of the world would contribute to a more comprehensive genetic database for FH disorder. Large databases such as the UK 100,000 genome project will also give us a better insight into frequency of the variants found and help to establish the pathogenicity of those variants.

Advances in technology and using next generation sequencing has led to finding large numbers of variants, though this also results in identifying variants of uncertain significance which result in an unclear diagnosis. Predicting pathogenicity of novel variants in *LDLR* gene is not always straightforward. We should be able to separate genuine disease-causing or disease-associated genetic variants reliably from the broader background of variants present in all human genomes that are rare, potentially functional, but not pathogenic. It is clearly of great importance for clinicians to be able to assess whether variants identified in the clinical settings or as incidental findings in genomics projects are pathogenic or not.

These results highlight the need for more advanced tools to enable a quick and decisive assessment of the functional effect of a variant. The already existing bioinformatics tools such as PolyPhen, SIFT and Mutation Taster provide an evaluation of the variant's effects. However, these tools are mainly designed to assess the effect of non-synonymous changes. It is hoped that as more information becomes available from *in vitro* functional studies, the development of additional *in silico* tools and from the various genomics studies, it will be possible to determine the pathogenicity of the variants.

One way to confirm the pathogenicity is to carry out a co-segregation study which was done in a family in the Polish cohort and the results were presented in Chapter 3. While co-inheritance of the variant with elevated LDL-C level was seen in a relative, the relatives without the inherited variant had normal levels of LDL-C. The interpretation of family data may be complicated by the overlay of environmental factors that influence lipid levels and by the presence of other genetic variants that raise or lower LDL-C. Also, the accessibility of the family members and the costs involved in this type of study would remain as major obstacles.

The findings of this thesis also emphasise better health strategies to identify FH patients in different countries such as Poland. Considering that *APOB* mutation p.(Arg3527Gln) and large rearrangements in *LDLR* gene are common in Poland, the screening programme to identify new probands in this country should include the diagnostic genetic testing to cover the entire coding region of the *LDLR* gene and *APOB* gene as the first step.

As the results presented in Chapter 4 show, a monogenic defect is a marker of a greater presence and severity of preclinical atherosclerosis in patients with monogenic FH. This would suggest that genetic testing will not only be important for confirming the diagnosis of FH and cascade screening of the family members of the patients with FH, but also could have prognostic implications. It is now accepted that subjects with an FH-causing mutation have a higher future risk of CHD than those with similar LDL-C levels but with no detected mutation, presumably because of the greater "LDL-C burden" they have experienced, with high LDL-C from birth.

The imaging techniques that are currently not considered for cardiovascular risk stratification in the general population might benefit monogenic FH patients. While there is a strong association between the CAC score and the cardiovascular risk, newer imaging techniques such as CT coronary angiogram are now available that not only provide a lower radiation dose but also better information on coronary plaque burden. A coronary atherosclerotic plaque burden assessment by CT angiography would provide us with better data on extent of atherosclerosis in patients with similar raised LDL-C levels but a polygenic versus a monogenic cause.

In addition, repeat measurements of the cIMT in these patients in a future study would help us to assess the rate of carotid atherosclerosis progression in monogenic and polygenic patients and whether any difference in carotid atherosclerosis could be seen between the groups.

Widespread use of imaging testing has the potential to increase health care expenditures but may also be cost saving through preventing the major cardiac events such as myocardial infarction in these high-risk asymptomatic young patients.

Treatment criteria remain the same in patients with severe hypercholesterolaemia with a monogenic or polygenic cause due to the undisputable causal role of LDL-C in atherosclerosis. Whether there would be any benefit in use of the potent and expensive monoclonal PCSK9 inhibitors in the monogenic FH patients whose cholesterol levels are not on target but the levels are below the recommended levels for treatment by the NICE guideline remains unclear.

This work suggests that, in future, integrated models of care, which could bring genetics into daily clinical practice would be needed. If it is confirmed by the larger studies that the risk of atherosclerosis is less severe in polygenic patients, then these patients could be managed under GP surgeries in primary care and could be discharged from the secondary lipid clinics in hospitals. This would provide more cost-effective management of FH patients in the health services.

One of the cardiovascular biomarkers which was studied in this thesis was Lp(a). The Lp(a) has been known as a cardiovascular risk factor for many years but is not measured in the clinical practices as a routine blood test. In the last few years, prospective studies have been published that highlighted the increased risk for cardiovascular disease in patients with elevated Lp(a). Its measurement in those at intermediate or high cardiovascular risk such as FH patients could provide another reliable screening test. Uncertainties about Lp(a) measurement and population-specific reference values mean that it cannot be used for any purpose except baseline cardiovascular risk assessment.

Lp(a) mass assays have an inherent limitation due to the heterogeneity of Lp(a) particle sizes, making it difficult to standardise assays for routine clinical biochemistry platforms. The most important issue with the Lp(a) methods without doubt is the selection of the reference material and the traceability of the calibrators. Findings of this thesis showed that there might not be a major bias in the Lp(a) measurements by different methods. However, for widespread use of Lp(a) concentration in clinical settings, fully standardised assays across all platforms would be needed.

The technologies for detecting genetic variation have advanced at a rapid pace in the last decade allowing the delineation of most of the genetic variation within a single individual

including both the common genetic variation described in GWAS and rare familial variations. Newer genotyping techniques such as next generation sequencing are now providing a cheaper, quicker and more accurate DNA test in patients.

The results of Chapter 5 in this thesis and other previous studies showed that some *LPA* variants are associated very well with raised Lp(a) concentration. These findings would suggest that, in future, genotyping of the *LPA* SNPs associated with raised Lp(a) concentration could become a part of routine clinical practices .

Since standardising the Lp(a) assays in the clinical biochemistry platforms would be costly and timely, the focus could be shifted to the *LPA* genotyping for the SNPs associated with high concentration of Lp(a). A future project to assemble large sample sizes from the pooling of different cohorts to confirm the association between the *LPA* genotype and the raised Lp(a) level would be essential.

In conclusion, advances in genotyping techniques and a better understanding of the clinical implications of genetic defects would enable us to implement genetic testing in routine clinical care of patients more often in near future. The availability of clinical genetic diagnostics depends on the level of evidence for clinical utility, the impact of such services on clinical decision making and the cost-effectiveness of genetic testing for a diagnosis. Newer technologies carry the additional costs of validation of novel platforms for clinical use but in near future, with the availability of better databases, integration of genetic diagnostics into clinical practice would be possible.

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8 APPENDIX

8.1 Appendix 1

MRC-Holland MLPA®

MLPA® General Protocol

Instructions For Use

General MLPA protocol for the detection and quantification of nucleic acid sequences.
To be used in combination with the appropriate MLPA probemix-specific product description.

Certain MLPA products from MRC-Holland are registered for In Vitro Diagnostic (IVD) use, but only in specific countries. **In all other cases, MLPA products are for Research Use Only (RUO).**
Information on IVD registration can be found in the appropriate probemix-specific product description.

An alternative "two-tube" protocol is available which is only recommended for use on low quality DNA samples (RUO).
Separate protocols exist for the detection of DNA methylation status (MS-MLPA®) and RNA expression (RT-MLPA®)
These protocols are available on www.mlpa.com

Manufacturer: MRC-Holland B.V. Willem Schoutenstraat 1, 1057 DL Amsterdam, the Netherlands
Website: www.mlpa.com Phone: +31 888 657 200; E-mail: info@mlpa.com (information & technical questions); order@mlpa.com (for orders); coffalyser@mlpa.com (for Coffalyser-related questions).

1. INTENDED USE

Copy Number Variations (CNVs) are a prominent source of genetic variation in human DNA and play a role in a wide range of disorders. Multiplex Ligation-dependent Probe Amplification (MLPA) is a semi-quantitative technique that is used to determine the relative copy number of up to 60 DNA sequences in a single multiplex PCR-based reaction. MRC-Holland manufactures and sells MLPA reagents and a wide range of MLPA probemixes. Together, these can be used to detect deletions and duplications in a DNA sample. Details on the intended use are specified in the MLPA probemix-specific Product Description.

2. MLPA ASSAY PRINCIPLE

As outlined in Figure 1, the principle of MLPA is based on the amplification (by use of a single PCR primer pair) of up to 60 probes, each of which detecting a specific DNA sequence of approximately 60 nt in length. After denaturation of the sample DNA, a mixture of MLPA probes is added to the sample. Each MLPA probe consists of two oligonucleotides that must hybridise to immediately adjacent target sequences in order to be ligated into a single probe. Each probe in an MLPA probemix has a unique amplicon length, typically ranging between 130-500 nt. During the subsequent PCR reaction, all ligated probes are amplified simultaneously using the same PCR primer pair. One PCR primer is fluorescently labelled, enabling the amplification products to be visualised during fragment separation. This is done on a capillary electrophoresis instrument, yielding a specific electropherogram (Figure 2, left). The relative height of each individual probe peak, as compared to the relative probe peak height in various reference DNA samples, reflects the relative copy number of the corresponding target sequence in the sample. A deletion of one or more target sequence thus becomes apparent as a relative decrease in peak height (Figure 2, right), while an increase in relative peak height reflects an amplification.

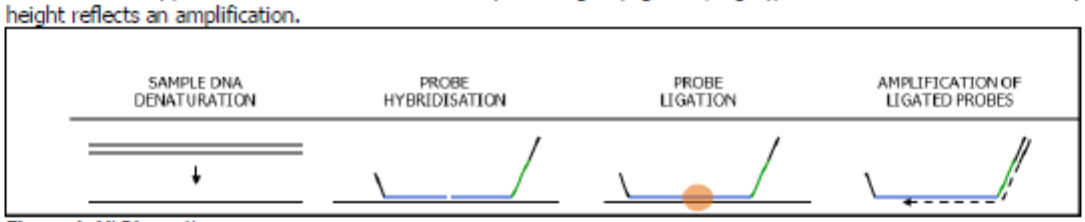

Figure 1. MLPA reaction.

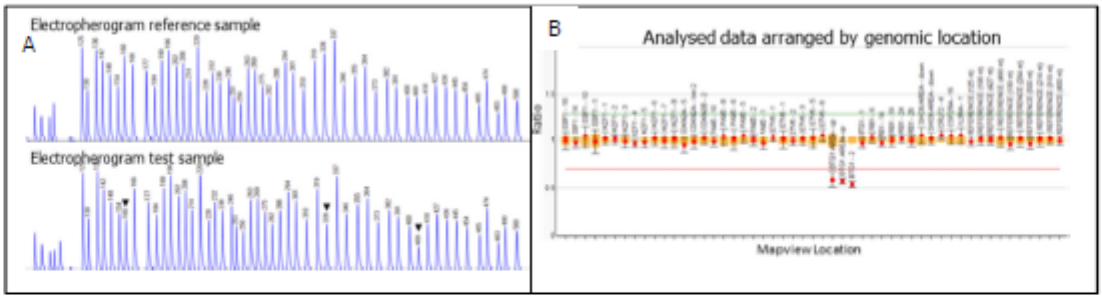

Figure 2. A: Electropherogram of a test sample (bottom) is compared to that of a reference sample (top) showing a relative decrease of three probes in the test sample (arrows). **B:** Calculated probe ratios of the same test sample (as displayed by Coffalyser.Net software) after analysis of these two samples: arranging probes by chromosomal location shows a reduced copy number for these three adjacent probes in the test sample.

3. SALSA® MLPA® ASSAY COMPONENTS & STORAGE CONDITIONS

3.1. REAGENT KIT ITEM NUMBERS

Item no.	Description
EK1-FAM	SALSA MLPA EK1 reagent kit – 100 rxn - FAM-labelled PCR primer
EK1-Cy5	SALSA MLPA EK1 reagent kit – 100 rxn - Cy5-labelled PCR primer
EK5-FAM	SALSA MLPA EK5 reagent kit – 500 rxn - FAM-labelled PCR primer
EK5-Cy5	SALSA MLPA EK5 reagent kit – 500 rxn - Cy5-labelled PCR primer

3.6. LIMITATIONS OF THE PROCEDURE

- For most MLPA applications, the major cause of most genetic defects are small (point) mutations, most of which will not be detected by MLPA probemixes. MLPA will not detect most inversions, balanced translocations, nor copy number changes that lie outside (or only partially inside) the sequence detected by an MLPA probe.
- False positive or negative results can be caused by various factors, including:
  a. Impurities in the test or reference DNA samples, including NaCl or KCl (>40 mM) and other salts, phenol, ethanol, heparin, EDTA (>1.5 mM) and Fe. The concentration of impurities will increase due to evaporation of sample DNA or by SpeedVac concentration of sample DNA– do not use this!
  b. Use of low quality plastics, as these may leak impurities such as releasing agents into the MLPA reaction
  c. Depurination of sample DNA during the initial 98°C heat treatment. This can occur when the sample has insufficient buffering capacity (sample dissolved in dH_2O instead of TE). A minimum of 5 mM Tris pH 8.2 in the sample DNA solution is required
  d. Sample DNA denaturation problems causing (part of) the DNA template to be unavailable for the MLPA probes. Certain DNA purification methods, e.g. Qiagen EZ1, result in a high salt concentration in DNA samples. Extremely GC-rich regions are not denatured at 98°C when more than 40 mM NaCl or KCl is present
  e. Use of whole genome amplification (WGA) of sample DNA, due to its amplification bias
  f. Use of incorrect DNA quantities
  g. Use of insufficient or unsuitable reference samples
  h. Improper mixing of enzyme solutions, e.g. by mixing insufficiently or too vigorously
  i. Deviating probe ratios that are due to (harmless) SNPs, mutations and small indels within the sequence detected by a probe
  j. Evaporation during o/n hybridisation, causing increased salt concentration and strong sample DNA secondary structure. This may prevent certain probes from binding to their target sequences
  k. Contamination during the MLPA reaction or electrophoresis with other PCR products
  l. Problems during capillary electrophoresis, including saturation of the device causing peaks to be off-scale
  m. Problems during normalisation, including the use of incorrect normalisation algorithms or software
  n. An incorrect interpretation of results due to insufficient knowledge of the application or gene(s) in question
  o. Insufficient knowledge of the clinical effect of a found genetic aberration. Not all deletions and duplications detected by MLPA may be pathogenic.

4. ASSAY SETUP INSTRUCTIONS

4.1. SAMPLE TREATMENT

1. Use a total quantity of 50-250 ng (preferably 50-100 ng) of human DNA in a 5 µl volume for each MLPA reaction. If necessary, DNA samples can be concentrated by ethanol precipitation. Glycogen (Roche 901393) can be used as carrier in ethanol precipitations. More info on www.mlpa.com.
2. Dissolve and dilute sample DNA in **TE0.1** (10 mM Tris-HCl pH 8.2 + **0.1 mM EDTA**). DNA preparations should contain 5-10 mM Tris buffer with a pH of 8.0-8.5 to prevent depurination during initial heat treatment at 98°C. If unknown whether sufficient buffer is present, add Tris-HCl: 4µl sample DNA + 1µl 50mM Tris-HCl pH 8.5.
3. In case of doubts about DNA quality: a) use only 50 ng of sample DNA; b) clean contaminated samples by ethanol precipitation or silica based clean-up kits; c) use the alternative two-tube PCR protocol which uses only part of the ligation reaction for the PCR (see section 5.1).
4. MLPA is more sensitive to contaminants than simple monoplex PCR assays. Contaminants left after DNA extraction (listed in 3.6) may influence MLPA performance. To minimise their effect, ensure the extraction method, tissue type, DNA concentration and treatment are as identical as possible in test and reference samples.
5. EDTA concentration of the samples should not be higher than 1.5 mM. Sample DNA should not be concentrated by evaporation or SpeedVac as this leads to a very high EDTA concentration.
6. Heparinised blood is not preferred for DNA extraction. Heparin is difficult to remove from DNA preparations and can distort the MLPA PCR reaction. Certain DNA purification methods (e.g. Nucleospin gDNA Clean-up XS) are capable of removing heparin contamination.
7. Do not use Qiagen M6, M48 and M96 systems for DNA purification (high salt concentrations). For Qiagen EZ1, only use the QIAGEN Supplementary Protocol for use in Third Wave Invader® assays (see www.mlpa.com).
8. DNA from whole genome amplification reactions (WGA) is not suitable for MLPA because of its amplification bias.
9. MRC-Holland has tested and can recommend the following extraction methods:
   - Qiagen Autopure LS (automated) and QIAamp DNA mini/midi/maxi kit (manual)
   - Promega DNA extraction Wizard (manual)
   - Salting out (manual)

4.2. SELECTING REFERENCE & OTHER CONTROL SAMPLES

1. REFERENCE SAMPLES. Reference samples should be included in each MLPA experiment. Minor differences in experimental execution may affect the MLPA peak pattern. Only compare samples that are a) included in the same MLPA experiment, b) tested with the same probemix lot.
2. MULTIPLE REFERENCE SAMPLES are needed to estimate the reproducibility of each probe within each MLPA run. Minimum is 3 different reference samples. When testing >21 samples: add 1 additional reference per 7 additional test samples. Reference samples should be distributed randomly over the sample plate to minimise variation.
3. SELECTING REFERENCE SAMPLES. Reference samples are DNA samples in which target and reference probe sequences are expected to have a normal copy number, obtained from healthy individuals/tissues. They should be as similar as possible to test samples in all other aspects (see 4.1). For formalin-fixed paraffin-embedded tissue, use reference samples derived from similarly treated healthy tissue.
4. COMMERCIAL DNA. In case of doubts about sample quality, include one or more commercial DNA samples for comparison (recommended: Promega Cat. Nr G1471 male, G1521 female DNA).
5. NO DNA CONTROL. Per MLPA run, include a No DNA Control reaction: replace 5 µl DNA by TE (10 mM Tris-HCl pH 8.2 + 0.1 mM EDTA) to check for contamination of TE, MLPA reagents, electrophoresis reagents or capillaries.
6. POSITIVE CONTROL SAMPLES. Including positive controls is recommended when possible. When using cell line DNA, note that this may have acquired additional copy numbers, incl. complete chromosomal gains/losses.
7. ALIQUOT PRECIOUS REFERENCE/CONTROL SAMPLES and store these at -20°C. Contamination with microorganisms or moulds can deteriorate samples that are stored at 4°C for an extended period.

5. MLPA REACTION - DNA DETECTION/QUANTIFICATION

5.1. NOTES TO READ BEFORE YOU START (DAY 1)

- Use a calibrated thermocycler with heated lid (99-105°C).
- Always vortex thawed buffers and probemix before use. MLPA buffer is typically frozen at -20°C but may remain liquid due to its high salt concentration.
- Centrifuge all MLPA reagent tubes for a few seconds before use, as drops may have adhered to the lid.
- Enzyme solutions contain 50% glycerol and remain fluid at the recommended storage temperature. **Never vortex enzyme solutions**. Master mixes containing enzymes should be mixed by gently pipetting up and down. When the viscous enzyme solution is not mixed properly with the buffers, unreliable results will occur! If the enzyme solution is mixed too vigorously however, enzyme inactivation occurs. When preparing master mixes, always add enzymes last.
- To minimise sample variation, prepare sufficiently large volumes of master mix solutions. Include a 5-10% volume surplus to allow for pipetting errors. Prepare Ligase-65 master mix and Polymerase master mix <1 hour before use and store on ice.
- When running a large number of samples, use multi-channel pipettes to avoid excessive evaporation.
- In this General MLPA protocol, the complete (40 µl) ligation reaction is used for the PCR. The alternative two-tube MLPA protocol, which uses only 10 µl of the ligation reaction for the PCR, is available on www.mlpa.com. The two-tube protocol may have advantages when using DNA samples containing impurities such as high EDTA concentrations (>1.5 mM) or PCR inhibitors. A vial of PCR buffer, required for this alternative protocol, can be ordered for free. The two-tube protocol is for research use only (RUO), not for in vitro diagnostic use (IVD).

5.2. THERMOCYCLER PROGRAM FOR THE MLPA REACTION

1. DNA denaturation			
1.	98°C	*5 minutes*	
2.	25°C	*pause*	
2. Hybridisation reaction			
3.	95°C	*1 minute*	
4.	60°C	*pause*	
3. Ligation reaction			
5.	54°C	*Pause*	
6.	54°C	*15 minutes*	
7.	98°C	*5 minutes*	
8.	20°C	*pause*	
4. PCR reaction			
9.	35 cycles:	• 95°C	*30 seconds*
		• 60°C	*30 seconds*
		• 72°C	*60 seconds*
10.	72°C	*20 minutes*	
11.	15°C	*pause*	

5.3. DNA DENATURATION (DAY 1)

- Label 0.2 ml tubes, strips or plates.
- Add 5 µl DNA sample (50-250 ng; 50-100 ng is optimal) to each tube. Use TE for 'no DNA control'.
- Place tubes in thermocycler; start MLPA thermocycler program (see above). Denature sample DNA for 5 min at 98°C; cool samples to 25°C before removing tubes from thermocycler.

5.4. HYBRIDISATION REACTION (DAY 1)

- Vortex MLPA buffer and MLPA probemix before use.
- Prepare hybridisation master mix containing, for each reaction: 1.5 µl MLPA buffer (yellow cap) + 1.5 µl probemix (black cap). Mix hybridisation master mix well by pipetting or vortexing.
- After DNA denaturation, add 3 µl hybridisation master mix to each sample tube. Mix well by pipetting up and down.
- Continue thermocycler program: incubate for 1 min at 95°C, then 16–20 hours at 60°C.

5.5. LIGATION REACTION (DAY 2)

- Vortex the two Ligase Buffers before use.
- Prepare a Ligase-65 master mix. For each reaction, mix: 25 µl dH_2O + 3 µl Ligase Buffer A (transparent cap) + 3 µl Ligase Buffer B (white cap). Then add 1 µl Ligase-65 enzyme (green cap). Mix well by pipetting gently up and down. Never vortex enzyme solutions.
- Continue thermocycler program: pause at 54°C.
- When the samples are at 54°C, add 32 µl ligase master mix to each tube. Mix by gently pipetting up and down. Add the ligase master mix while the samples are IN the thermocycler!
- Continue thermocycler program: 15 min incubation at 54°C (for ligation); 5 min at 98°C for heat inactivation of Ligase-65 enzyme. **Pause at 20°C**. At this point, tubes can be removed from the thermocycler.

5.6. PCR REACTION (DAY 2)

- Vortex SALSA PCR primer mix. Warm polymerase for 10 sec in your hand to reduce viscosity.
- Prepare polymerase master mix. For each reaction, mix: 7.5 µl dH_2O + 2 µl SALSA PCR primer mix (brown cap) + 0.5 µl SALSA Polymerase (orange cap). Mix well by pipetting up and down; do not vortex. Store on ice until use.
- **At room temperature,** add 10 µl polymerase master mix to each tube. Mix by pipetting gently up and down. Place the tubes in the thermocycler and continue the thermocycler program; 35 cycles of 30 seconds 95°C; 30 seconds 60°C; 60 seconds 72°C. End with 20 min incubation at 72°C; pause at 15°C.
- After the PCR reaction, do not open tubes in the room with the thermocycler. To avoid contamination, use different micropipettes for performing MLPA reactions and handling MLPA PCR products.
- PCR product can be stored at 4°C for 1 week. For longer periods, store between -25°C /-15°C. As fluorescent dyes are light-sensitive, store PCR products in dark box or wrapped in aluminium foil.

6. FRAGMENT SEPARATION BY CAPILLARY ELECTROPHORESIS

6.1. NOTES TO READ BEFORE YOU START

- Size standard, run conditions, polymer, fluorescent dye and volume of MLPA PCR reaction used depend on instrument type. Settings below are standard settings. Instrument settings may require optimisation for optimal MLPA fragment separation; follow instructions of the manufacturer of the capillary electrophoresis apparatus.
- Using old capillaries or polymer has a detrimental effect on MLPA results. Change capillaries and polymer regularly. Polymer quickly deteriorates after prolonged exposure to >25°C. In case size standard peaks are low and broad, the capillaries or polymer are usually deteriorated.
- Formamide can become acidic. Use high quality formamide and store it in aliquots at -20°C.
- In case all MLPA peaks are low, do not add more MLPA PCR product to injection mixture! Adding more PCR product increases the salt concentration in the injection mixture which competes with DNA for injection. When an increase in peak heights is desired, increase injection time/voltage. None of the peaks should be off-scale!
- Reset the bin settings in the fragment analysis software whenever using a different a) MLPA probemix lot; b) size standard; c) capillary electrophoresis apparatus or d) run settings.

6.2. ELECTROPHORESIS SPECIFICATIONS

Instrument	Primer Dye	Capillaries	Injection mixture	Initial Settings
Beckman CEQ-2000 CEQ-8000 CEQ-8800 GeXP	Cy5	33 cm	0.7 µl PCR reaction 0.2 µl CEQ - size standard 600 32 µl HiDi formamide / Beckman SLS Add one drop of high quality mineral oil.	run method: Frag capillary temperature: 50°C denaturation: 90°C for 120 sec injection voltage: 1.6 kV injection time: 30 sec run time: 60 min run voltage: 4.8 kV
ABI-Prism 3100 (Avant) ABI-3130 (xL) * ABI-3500 (xL)* ABI-3730 (xL)*	FAM	36, 50 cm **ABI3500: 50 cm!**	0.7 µl PCR reaction 0.3 µl (ROX) or 0.2 µl (LIZ) GS 500 size standard 9 µl HiDi formamide Seal the injection plate, **heat 3 min at 86°C, cool for 2 min at 4°C.‡**	run module: FragmentAnalysis injection voltage: 1.6 kV injection time: 15 sec run voltage: 15 kV run time: 1800 s oven temperature: 60°C polymer: POP4 or POP7
ABI-Prism 310	FAM	47 cm	0.75 µl PCR reaction 0.75 ul dH-O 0.5 µl GS 500 ROX/LIZ size standard 13.5 µl HiDi formamide **Heat 3 min at 86 °C, cool for 2 min at 4°C. ‡**	injection voltage: 1.6 kV injection time: 15 sec filter set: D polymer: POP4

* ABI-3130, 3500 & 3730 have been validated and found suitable to be used with IVD-registered MLPA products.
‡ Briefly heating the injection mixture before capillary electrophoresis is essential.