A DESCRIPTIVE STUDY OF MUTATIONS IN THE LOW DENSITY LIPOPROTEIN RECEPTOR (*LDLR)* GENE AMONG PATIENTS WITH FAMILIAL HYPERCHOLESTEROLAEMIA IN SRI LANKA

BY

Dr. JAYASINGHEGE RASIKA DAMAYANTHI KUMARI RAJAPAKSE

HUMAN GENETICS UNIT

FACULTY OF MEDICINE

UNIVERSITY OF COLOMBO

DISSERTATION SUBMITTED TO THE UNIVERSITY OF COLOMBO, SRI LANKA FOR THE DEGREE OF MASTER OF SCIENCE IN CLINICAL GENETICS

JULY 2012

TABLE OF CONTENTS

ACKNOWLEDGEMENT 5

DECLARATION 7

ABSTRACT 8

1. INTRODUCTION AND BACKGROUND 9

1.1. Familial Hypercholesterolaemia phenotype 9

1.2. Clinical diagnosis of FH 11

1.3. Pathophysiology of FH 14

1.4. Genetic basis of FH 16

1.4.1. Evidence for genetic basis of FH 16

1.5. Candidate genes 18

1.5.1. *LDLR* gene 18

1.5.1.1. Introduction 18

1.5.1.2. Genomic structure and functional studies 18

1.5.1.3. Mutational hot spots in *LDLR* gene 20

1.5.1.4. Genotype - phenotype correlation 22

1.5.2. *Apolipoprotein B-100* gene 23

1.5.2.1. Introduction 23

1.5.2.2. Genomic structure and functional domains of *ApoB 100* gene 23

1.5.2.3. Genetic basis of *APO B 100* mutation 24

1.5.3. *PCSK* 9 gene 24

1.5.3.1. Introduction 24

1.5.3.2. Genomic structure 25

1.6. Justification 26

1.7. Objectives **28**

2. METHODS **29**

2.1. Ethical issues relevant to study **29**

2.2. Recruitment of patients **30**

2.2.1. Study population and place of study **30**

2.2.2. Inclusion criteria **30**

2.2.3. Exclusion criteria **31**

2.3. Clinical evaluation **31**

2.4. Biological samples and molecular genetic testing **32**

2.4.1. DNA extraction **32**

2.4.2. Polymerase chain reaction **33**

2.4.3. Agarose gel electrophoresis **35**

2.4.4. Cycle sequencing **35**

2.5. Data collection and software tools **36**

2.6. Statistical analysis **37**

3. RESULTS **38**

3.1. Familial Hypercholesterolaemia phenotype **38**

3.2. Demographic Characteristics **38**

3.3. Effects of FH **39**

3.4. Family history **39**

3.5. Biochemical analysis **40**

3.6. Molecular genetic testing **42**

3.6.1. Single nucleotide polymorphisms (SNPs) **42**

3.6.1.1. Exonic SNPs 42

3.6.1.2. Intronic SNPs 45

3.6.2. Large structural variants 45

4. DISCUSSION 46

4.1. Demographic Characteristics 46

4.2. Familial hypercholesterolaemia phenotype 47

4.3. Coronary heart disease and Familial hypercholesterolaemia 48

4.4. Biochemical and genetic testing 48

5. CONCLUSIONS 50

6. LIMITATIONS AND FUTURE RECOMMENDATIONS OF THE STUDY 51

REFERENCES 52

APPENDIX 1: LIST OF ABBREVIATIONS 59

APPENDIX 2: LIST OF SUPPLIERS 60

APPENDIX 3: DOCUMENTS USED FOR SUBJECT RECRUITMENT 61

ACKNOWLEDGEMENT

It's my pleasure to thank my supervisors Prof. Vajira H.W. Dissanayake, Professor in Anatomy and Medical Geneticist, Human Genetics Unit, Faculty of Medicine, University of Colombo, Prof. Rohan W. Jayasekara, Professor of Anatomy, Director of the Human Genetics Unit, and Dean, Faculty of Medicine, University of Colombo and Dr. Godwin Contantine, Department of Clinical Medicine, Faculty of Medicine, University of Colombo for their, guidance, supervision and encouragement during the study. This research was supported by the NOMA grant funded by NORAD in collaboration with the University of Colombo, Sri Lanka & the University of Oslo, Norway.

I would like to thank Dr. Prasad Katulanda, Consultant Endocrinologist, Department of Clinical Medicine, Faculty of Clinical Medicine, University of Colombo for the valuable support rendered in recruiting patients for the study. I would like to thank all the Consultant Cardiologists in the National Hospital of Sri Lanka for granting permission to recruit patients for the research. I wish to thank all the patients and their families for their participation in this study.

Biochemical analysis was performed in the Medical Laboratory of the Asiri Hospital, Colombo. DNA extraction and PCR analysis were performed at the Human Genetics Unit of the Faculty of Medicine, University of Colombo. Automated sequencing was carried out at the Genetic Laboratory of the Asiri Surgical Hospital, Colombo. I wish to thank Mr. A.A.G.S. Abeysekara, Laboratory Scientist, Human Genetics Unit, Faculty of Medicine, University of Colombo and the staff of the Medical and Genetic Laboratories of the Asiri Surgical Hospital, Colombo for their support.

I also wish to thank Miss P.K.D.S. Nisansala and Mrs. S.S.S.M. Bandaranayake of the Human Genetics Unit, Faculty of Medicine, University of Colombo for their support and assistance.

Without the support from my husband and parents I would not be able to complete this project. I wish to thank all of them for their understanding, encouragement and support.

DECLARATION

I declare that contents of this dissertation are my own work except for the genotyping of *LDLR* gene by Mr. A.A.G.S. Abeysekara, Laboratory Scientist, Human Genetics Unit, Faculty of Medicine, University of Colombo.

ABSTRACT

Introduction: Familial hypercholesterolemia (FH) is an inherited disorder of lipoprotein metabolism characterized by elevated Low density lipoprotein (LDL) cholesterol in serum, presence of tendon xanthoma and premature atherosclerosis resulting in cardiovascular disease. FH has primarily an autosomal dominant inheritance. This is commonly caused by mutations of low density receptor gene (*LDLR*), encoding low density lipoprotein receptor protein. This is the first study on molecular genetic analysis of FH in Sri Lanka.

Objectives: The aims of this study were to identify mutations in exons 4, 9, 10 and 14 of Low Density Lipoprotein Receptor (*LDLR*) gene among patients clinically diagnosed with FH and to correlate with the phenotype.

Methods: 25 clinically diagnosed patients with FH, according to Modified Simon Broom, US MedPed and Dutch Lipid Clinic Network criteria were recruited. Clinical history including family history and the physical examination findings were recorded. Eight millilitres (8ml) of blood was drawn for a lipid profile and for molecular genetic testing. DNA was extracted and exons 4,9,10 and 14 of *LDLR* were screened for mutation by sequencing.

Results: Participants were equal in sex distribution (Male- 48%, Female-52%). Only 6 (24%) patients had xanthelasma. None of the patients had tendon xanthoma or corneal arcus. Commonest complication was coronary vascular disease, n= 11 (44%) of which 9 (36%) have undergone surgical intervention. Eighteen patients (72%) had a family history of coronary vascular disease and hypercholesterolaemia, Mean cholesterol level was 366.28mg/dl (SD-60.47). Mean LDL level was 259.80mg/dl (SD-66.98).

One heterozygous pathological mutation was detected in a patient in exon 4 of *LDLR* gene. (NM_000527.2(*LDLR)*:c.682G>C;p.Glu228Gln).Synonymous single nucleotide

polymorphisms were detected in exon 4 (c.369 T>G), exon 9 (c.1431C>T) and in exon 10 at position 1413 (c.1413A>G). Fifteen (60%) patients had a single nucleotide polymorphism in the intervening sequence between exon 8 and 9 (IVS9-30C>T). An 11bp deletion was also detected in the intervening sequence between exon 4 and 5 (c.694+8_18delCGGGGCCAGGG).

Conclusion: Stigmata of FH were uncommon .There was a strong family history of coronary vascular disease and hypercholesterolaemia. Since, only one pathological mutation was found, genotype phenotype correlation was not possible. Further investigations are needed to detect mutations in other exons of *LDLR* and rest of the genes related to cholesterol metabolism. In addition, study should be extended to demonstrate the functional significance of the detected mutations.

1. INTRODUCTION AND BACKGROUND

Familial hypercholesterolemia (FH) is an inherited disorder of lipoprotein metabolism characterized by elevated Low density lipoprotein (LDL) cholesterol in serum, tendon xanthoma and increased risk of premature coronary heart disease. FH has mainly an autosomal dominant inheritance. The frequency of heterozygous familial hypercholesterolemia is about 1 in 500 in most populations (Soutar *et al.* 2007). Homozygous FH is rare ($<1/10^6$) in a population with a low rate of consanguineous marriage (Soutar *et al.* 2007). This dissertation describes the mutations of the LDL receptor gene (*LDLR*) among clinically diagnosed Sri Lankan Sinhalese patients with FH using molecular genetics methods and the correlation between the genotype and the phenotype.

1.1 Familial Hypercholesterolaemia phenotype

High cholesterol levels normally do not cause any symptoms. Excess cholesterol can get deposited in various places causing xanthelasma (around the eye lid), corneal arcus (outer margin of the iris) or tendon xanthomas (tendons of hands, elbows, knees and feet). FH is gene - dose dependant. Patients with homozygous or compound heterozygous FH are more severely affected and they develop severe heart disease in childhood needing early intervention than heterozygotes.

Fifty percent of men and 20% of women with heterozygous FH for LDL receptor gene mutation will have clinically suggestive coronary heart disease by age of 45(2008). Patients with homozygous or compound heterozygous FH for mutations of both alleles of *LDLR* gene usually have cholesterol levels four to ten times than normal. They develop tendon xanthoma in early childhood and coronary heart disease by 30 years. Phenotypic variation among different ethnic groups with same mutation of *LDLR* gene has been identified (Pimstone *et al.*

1998). Dietary factors, environmental factors, and life style factors may influence the variability in phenotypic expression.

Less commonly they develop transient ischaemic attacks or cerebro-vascular accidents. Patients with a history of smoking have a higher tendency to develop peripheral vascular disease.

1.2 Clinical diagnosis of FH

Different criteria are used to diagnose FH. Presence of tendon xanthoma, LDL cholesterol, mutations in LDL receptor gene or *apolipoprotein B* 100 are considered in Simon bloom criteria for definitive diagnosis. In the absence of facilities for genetic testing tendon xanthoma is a compulsory criterion to establish a definitive diagnosis of FH, using the Simon Bloom criteria.

To establish a probable diagnosis in accordance with the Simon Bloom criteria, elevated LDL cholesterol together with family history of coronary artery disease or hypercholesterolaemia should be present.

Simon Bloom criteria (Minhas *et al.* 2009)(Minhas *et al.* 2009)

Definite familial hypercholesterolaemia

a)Total cholesterol >6.7 mmol/l or LDL cholesterol above 4.0 mmol/l in a child <16 years or total cholesterol level >7.5 mmol/l or LDL cholesterol above 4.9 mmol/l in an adult(Level either pre-treatment or highest on treatment)

PLUS

b) Tendon xanthomas in patients, or in first degree relative (parent, sibling, child), or in second degree relative

c) DNA based evidence of LDL receptor mutation or familial defective apo B-100

OR

Possible familial hypercholesterolaemia

a) Above PLUS ONE of d) or e)

d) Family history of myocardial infarction: below age 50 in second degree relative or below age 60 in first degree relative

e) Family history of raised cholesterol: >7.5 mmol/l in adult first degree relative or >6.7 mmol/l in child or sibling under 16

Total cholesterol level of the patient and the relatives are used to diagnose FH in US Med Ped criteria (Table 1.1). In this method the clinical features are not considered.

Table 1.1: **Total cholesterol cut points (mmol/L) for diagnosis of FH**

	1st degree relative with FH	2nd degree relative with FH	3rd degree relative with FH	General population
Age (years)				
<20	5.7	5.9	6.2	7.0
20-29	6.2	6.5	6.7	7.5
30-39	7.0	7.2	7.5	8.8
> 40	7.5	7.8	8.0	9.3

Diagnosis: FH is diagnosed if total cholesterol level exceeds the cut point.

The Dutch Lipid Clinic Network (Table 1.2) uses a numeric score to diagnose FH. If a patient gets a higher score possibility of having FH is high. In contrary to Simon Bloom criteria, the

tendon xanthoma is not necessary for the definitive diagnosis, but the presence will increase the score substantially.

Table 1.2: Dutch Lipid Clinic Network criteria for making diagnosis of FH in adults (Walma *et al.* 2006).

	Score
Family History	
First degree relative with known premature coronary and vascular disease (Men<55years, Females <60 years) OR First degree relative with known LDL cholesterol above the 95th percentile for age and sex	1
First degree relative with tendinous xanthomata and/ or arcus cornealis OR Children aged less than 18 years with LDL cholesterol above the 95th percentile for age and sex	2
Clinical history	
Patient with premature coronary artery disease (Men<55years, Females <60 years)	2
Patient with premature cerebral or peripheral vascular disease (Men<55years, Females <60 years)	1
Physical examination	
Tendinous xanthomata	6
Arcus cornealis prior to age 45 years	4
LDL cholesterol (m mol/L)	

LDL-C ≥8.5	6
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, APOB* or *PCSK 9* gene	8
STRATIFICATION	**Total Score**
Definite FH	**≥8**
Probable FH	**6-7**
Possible FH	**3-5**
Unlikely FH	**<3**

Patient recruitment for the study was done using one of the above criteria depending on the clinical presentation.

1.3 Pathophysiology of FH

The LDL receptor is a cell surface glycoprotein synthesized in the endoplasmic reticulum and processed in the Golgi apparatus for maturation. These receptors specifically bind apolipoprotein B in LDL molecules. This LDL receptor complex is internalized by endocytosis via clatrin coated pits. (*LDLR*AP1/ARH). Dissociation of the ligand-receptor complex occurs in the endosomes due to its acidic environment. The LDL particles are degraded in lysosomes and the receptor particles are recycled to the cell surface (Figure 1.3). The proprotein convertase (*PCSK 9*) normally reduces LDL by post translational modification. Mutations in the *LDLR, Apolipoprotein B 100,* and *PCSK 9* genes can cause FH (Soutar *et al.* 2007).

Figure 1.3: Pathogenesis of FH

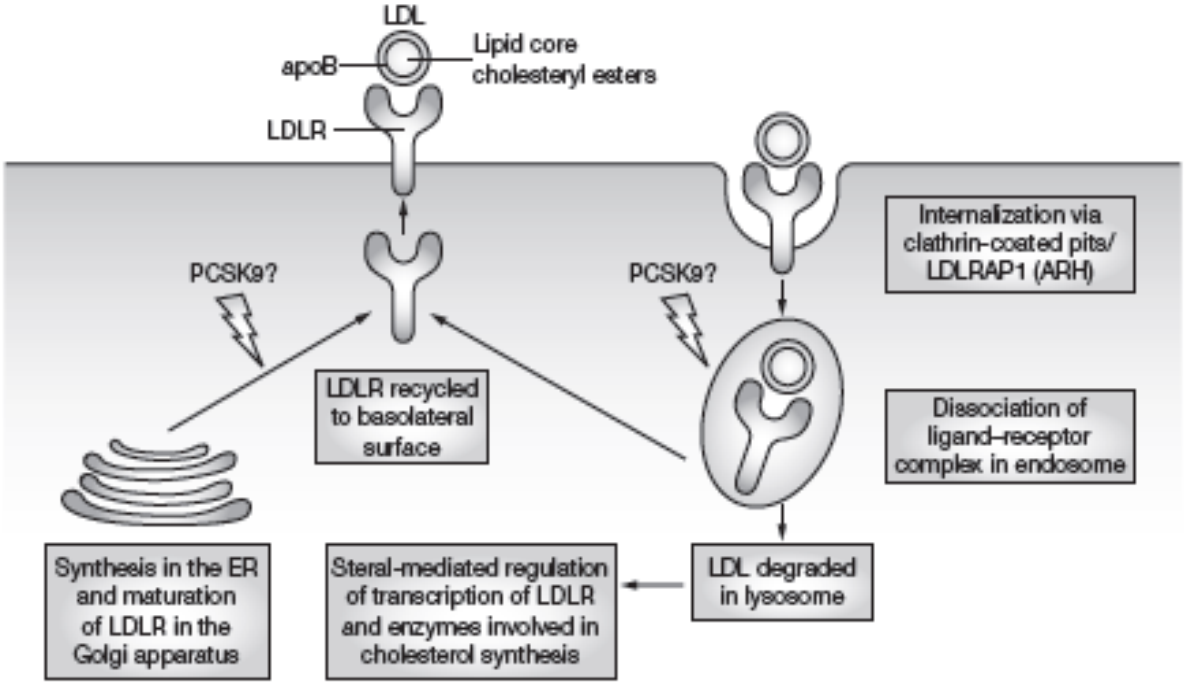

Figure 1.3.The LDL-receptor pathway for uptake and degradation of LDL.ER- Endoplasmic reticulum, LDLRAP1- LDL receptor adaptor protein Adapted from Anne T.H, R. S., Hans M.,Peter S,Borge G.N (1998). "association of mutations in the Apolipoprotein B gene with hypercholesterolemia and the risk of ischemic heart disease." N Engl J of Med **338**: 1577-1584.

Autosomal Recessive Hypercholesterolaemia (ARH) gene encodes LDL receptor adaptor protein (*LDLR*AP1/ARH) containing phosphotyrosine binding domain. These phosphothyrosine domains have been shown to bind FXNPXY motifs present in cytoplasmic end of the LDL proteins and involve in the process of internalization of the receptor- ligand complex via clathrin – coated pits. Therefore, defective ARH results in disturbed intracellular *LDLR* flux.

1.4 Genetic basis of FH

FH is mainly caused by mutations in the *LDLR* gene, encoding low density lipoprotein receptor protein (Hobbs *et al.* 1992). Less commonly mutations in *apolipoprotein B 100* and proprotein convertase subtilisin/kexin type 9 *(PCSK 9)* genes have been reported (Dedoussis *et al.* 2004). Genotype phenotype co-relation has been demonstrated in FH (Graham *et al.* 1999).

Autosomal recessive inheritance (ARH) has also been suggested by Khachandurian et al (Khachadurian 1964). Mutations in ARH gene lead to defective internalization of LDL receptor (*LDLR*) molecules in the liver, causing autosomal recessive FH (Guocheng He *et al.* 2002).

1.4.1Evidence for genetic basis of FH

Brown and Goldstein demonstrated that the phenotype of FH can be caused by mutations in the LDL receptor gene (*LDLR*) (Hobbs *et al.* 1992). Many different types of mutations have been identified in LDL receptor gene worldwide including large rearrangements, single amino acid substitutions, splice site mutations and mutations involving promoter region (Soutar *et al.* 2007). About 90% are point mutations (Mihaylov *et al.* 2004).

Although genetic heterogeneity has been reported in *LDLR* gene there are some notable exceptions. A study on molecular basis of FH in Netherland revealed four common mutations in the LDL receptor gene responsible for about 47.9% of patients. Nineteen percent of the patients had a combination of N543H and 2393del9 bp, present on the same allele which was the commonest mutation. A splice site mutation in intron 9, 1359-1(G>A), intron3 (313+1G>A and G>C,313 +2 T>C), W23X mutation accounted for about 29% of the patients with hypercholesterolaemia (Defesche *et al.* 2001).

A common mutation designated as FH-North Karelia has been found among the Finnish patients with FH. This mutation included deletion of seven nucleotides from exon 6 of the *LDLR* gene. Functional studies showed the association of receptor negative phenotype. This mutation has resulted in a frame shift producing a truncated receptor protein (Koivisto *et al.* 1992).

A large deletion involving the promoter region and the first exon of the *LDLR* gene has been detected in about 63% of French Canadian patients with heterozygous FH. The homozygotes were more severely affected than heterozygotes and had LDL levels over 6 times than normal (Hobbs *et al.* 1987). The common mutations in the Spanish population with familial hypercholesterolaemia were found in exon 2 (c114- 115insA, c.91G>T), exon 4 (c.346T > C) and exon 6 (c.829G>A)(Dedoussis *et al.* 2004).

About 80% South African Afrikaners with FH had two common mutations in the LDL receptor gene. They were FH Afrikaner -1 (FH1) mutation ($Asp_{206} \rightarrow Glu$) in exon 4 and FH Afrikaner – 2 (FH 2) mutations ($Val_{406} \rightarrow Met$) in exon 9. Those with mutations in FH1 had significantly lower cholesterol than those with mutations in FH2 (Kotze *et al.* 1989)' (Kotze *et al.* 1993).

Analysis of the spectrum of mutations among patients with heterozygous FH in United Kingdom showed that the mutations in the *LDLR* gene were widely scattered throughout the *LDLR* gene although some were clustered in specific sites (Sun *et al.* 1992).

Sobia et al .identified 2 SNPs within *LDLR* gene which were located in exon 12 (rs688) and exon 13, (rs5925) among Pakistan population (Sobia 2011). An association between SNPs and increased levels of cholesterol was noted. In this study polymorphism among APO B was not identified.

1.5 Candidate genes

Approximately 85% of the FH have mutations in the *LDLR* and in about 10% of cases mutations in the *Apolipoprotein B 100* gene were reported.

1.5.1. LDL receptor gene

1.5.1.1 Introduction

Mutations in the LDL receptor gene are responsible for the autosomal dominant FH.

1.5.1.2 Genomic structure and functional studies

The *LDLR* gene is located in chromosome 19 (19p 13.1-13.3), the chromosome with highest number of *Alu* repeats. *Alu* repeats represent 85% of *LDLR* intronic sequence outside exon intron junctions (Amsellem *et al.* 2002). It spans about 43kb with 18 exons and 17 intervening introns encoding 839 amino acids (Defesche *et al.* 2001). This encodes a protein involving 7 domains: promoter translation signal, signal sequence (exon 1), ligand binding domain (exons 2–6), epidermal growth factor precursor homology domain (exons 7–14), O-linked sugar domain (exon 15), membrane-spanning domain (exons 16 and 17) and the cytoplasmic domain (exons 17 and 18) (Figure 1.4). More than 1100 mutations have been identified in the gene (Dedoussis *et al.* 2004).

Five classes of mutations have been defined with functional significance. Null alleles result in complete absence of *LDLR* (class 1) due to disruption of the promoter sequence, nonsense, frame shift or splicing mutations. Both defective transport alleles (Class 2 mutations) and defective binding alleles (class 3 mutations) occur in the ligand-binding and epidermal growth factor precursor regions. Class 2 mutations disrupt transport of the *LDLR* from endoplasmic reticulum to the Golgi apparatus. Class 3 mutations interfere with cell surface binding of the receptor to LDL. Defective internalization alleles (Class 4) inhibit the clustering of LDL receptors on cell surface, causing defective internalization (Garcia-Garcia

et al. 2001). Class 5 mutations (defective recycling alleles) occur in the epidermal growth factor precursor region which prevents the release of LDL particles in the endosome leading to defective recycling of the LDL receptor (Austin *et al.* 2004). Class 1mutations are alternatively named as "null" or "receptor negative" mutations in the literature, whereas class 2-5 mutations are considered as "receptor defective" mutations (Austin *et al.* 2004).

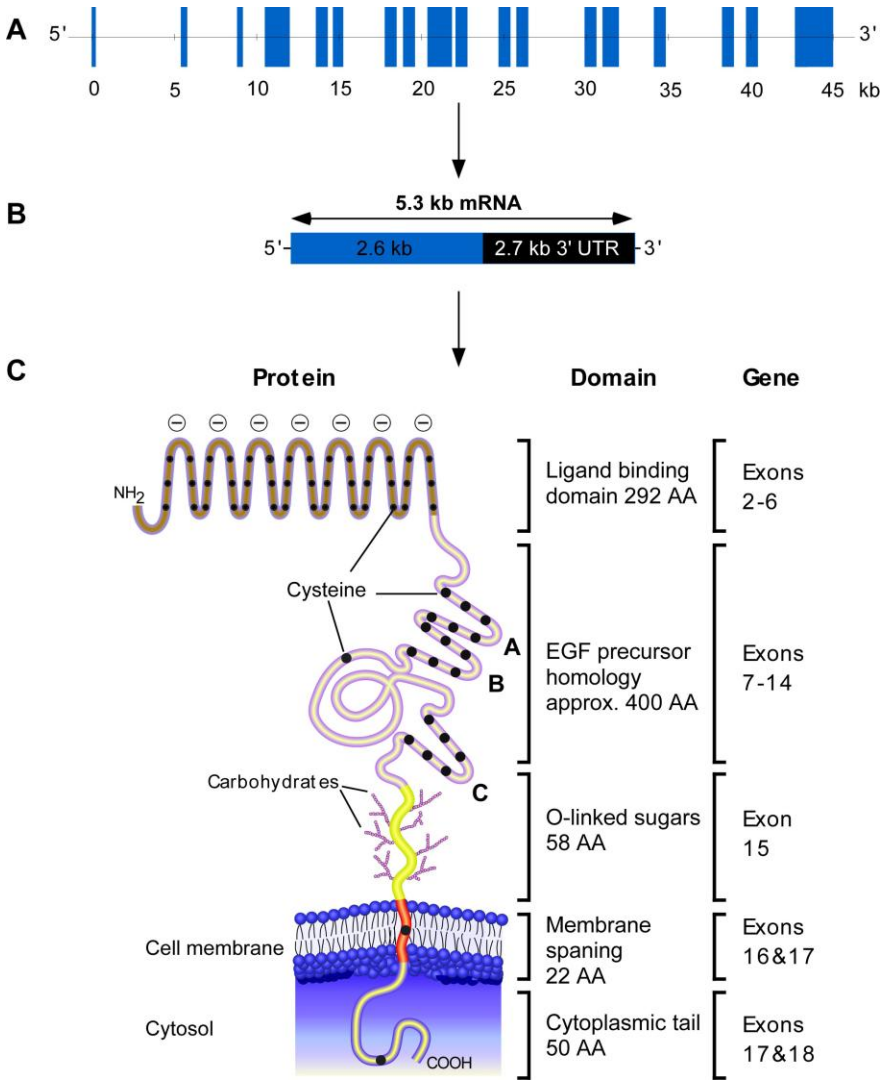

Figure 1.4: Schematic representation of human *LDLR* gene, mRNA and protein A, B and C respectively. UTR – Un translated region of the mRNA transcript. Al-Allaf, F.A.,C. Coutelle et al. "LDLR-Gene therapy for familial hypercholesterolaemia: problems,progress, and perspectives." Int Arch Med **3**: 36.By permission from the author Faisal Ahmed Allaf.

1.5.1.3 Mutational hot spots in *LDLR* gene

So far hot spots are not defined in *LDLR* gene. *LDLR* worldwide website in Familial Hypercholesteroleamia indicated the spectrum of the mutations (excluding major rearrangements) reported in each exon (Figure 1.1). This showed that the highest number of mutations reported in exon 4 which has been associated with severe phenotype.

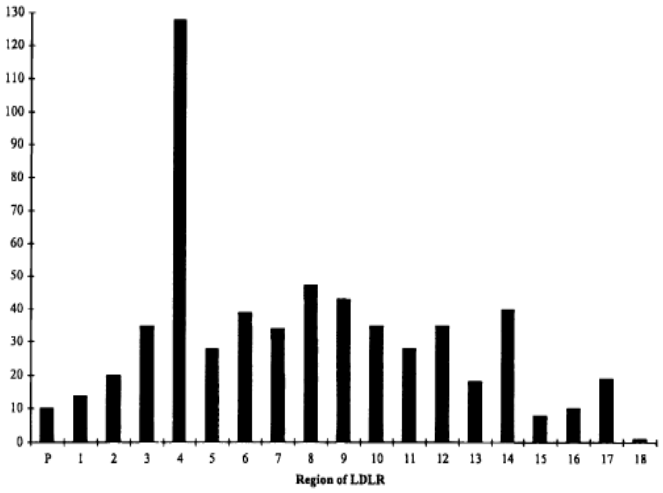

Figure 1.1: The spectrum of mutations across the promoter and 18 exons of *LDLR* found in FH mutation database. Adapted from Heath, K. E., M. Gahan, et al. (2001). "Low-density lipoprotein receptor gene (LDLR) world-wide website in familial hypercholesterolaemia: update, new features and mutation analysis." Atherosclerosis **154**(1): 243-246, by permission from the author Hamphries, Steve)

A recent study on mutation analysis of *LDLR* the gene in Indian families with FH reported 3 novel mutations unique to South Indian population. All the patients had a mutation in exon 10 (g.29372_29373insC) which had a functional effect on the *LDLR* gene. Another mutation has been found in the same exon at position 55, A>G (g.20209A) which is considered as a silent mutation.

According to the Huge prevalence review on monogenic heterizygous FH by Austin *et* al the largest numer of varients have been reported in exon 4 (Figure 1.2), partly explained by the

larger size of this exon (Austin *et al.* 2004). Exon 4 codes for repeat 5 which is required for LDL binding via apolipoprotein B.So mutations within this region are associated with a more severe phenotype than others.

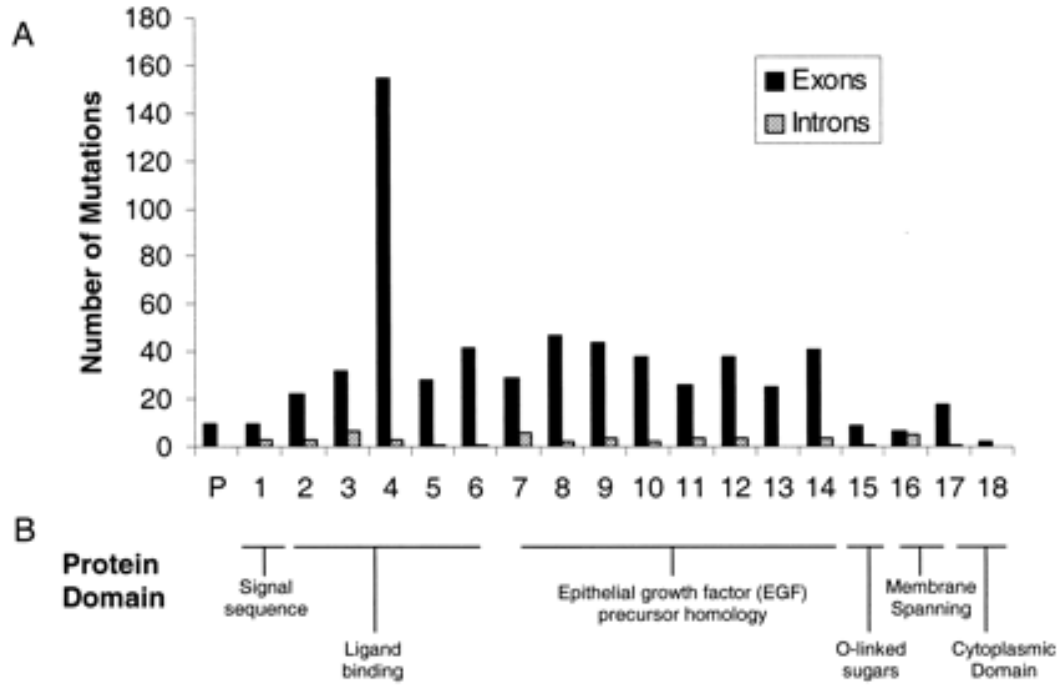

Figure 1.2: A,Location of 647 unique mutations (excluding major reaarngements)in the *LDLR* gene by promotor(P), exon and intron regions(data were extracted from www.ucl.ac.uk/fh on july 1,2003);B,correspondence between the LDLR gene organization and LDLR protein domain structure.O-linked sugars, sugars attached to a hydroxyl (-OH) group on the side chains of serine or threonine. Austin, M. A., C. M. Hutter, et al. (2004). "Genetic causes of monogenic heterozygous familial hypercholesterolemia: a HuGE prevalence review." Am J Epidemiol **160**(5): 407-420.By permission from the author Austin M.A)

Bulgarian patients with FH have been screened for the mutations in six exons, namely exon 3, 4, 6, 8, 9, and 14 as these exons constitute a region over which more than 60% of all

known point mutations in the *LDLR* were identified. (http://www.umd.necker.fr and http://www.ucl.ac.uk/fh).

1.5.1.4 Genotype - phenotype correlation

Due to marked genetic heterogeneity of the *LDLR* mutations it is difficult to collect sufficient number of patients with same mutation to determine the genotype phenotype co-relation. A study done in South India has found a genotype phenotype co-relation, with the presence of a mutation in exon 3 (g.18298 A>C) among patients who had tendon xanthomas (Anne T.H 1998). Graham *et al* have investigated genotype- phenotype correlation in *LDLR* mutation and *Apo B 100* mutation in patients with heterozygous FH. Families with *LDLR* mutations have been reported with higher total and LDL cholesterol levels and a higher incidence of tendon xanthoma than those with the common Apo B variant. The patients with frame shift mutations had the most severe presentation than those with missense mutations (Graham *et al.* 1999).

A study had been performed among patients with FH in UK to investigate the phenotypic expression for mutations in the 3' part of the exon 4 of *LDLR*. This area of the gene codes for repeat 5 of the binding domain of the LDL receptor. The mean LDL cholesterol levels were higher among patients with a mutation creating a null allele and also were similar to levels in those with a mutation affecting repeat 5 which causes defective protein production. In contrast patients with defective protein mutation outside repeat 5 had a lower LDL cholesterol level (Gudnason *et al.* 1994). Vohl *et al* compared the effect of two *LDLR* class mutations on coronary artery disease among French Canadian patients for heterozygous FH. The FH patients who were null allele carriers had the highest plasma total and LDL cholesterol levels and the highest cholesterol/HDL-cholesterol ratio than the patients who were defective allele carriers. The age at first coronary angiography and revascularization

were compared. They observed a trend for a younger age at first angiogram and first revascularization in FH heterozygotes bearing a null allele than in carriers of a defective allele (Vohl *et al.* 1997).

1.5.2 Apolipoprotein B-100 gene

1.5.2.1 Introduction

Up to 10% of FH occurs due to Familial ligand defective APO B 100 which is an autosomal dominant disorder leading to plasma cholesterol elevation and coronary vascular disease. *Apolipoprotein B-100* gene product is the main apolipoprotein of chylomicrons and low density lipoproteins. It occurs in plasma as two main isoforms, apoB-48 and apoB-100. Both isoforms are coded by a single gene from a single long m-RNA and they share a common N terminal sequence. Both are components of lipoproteins. Apo - B 48 (2152 amino acids) is required for chylomicron production in the intestine, whereas apo B – 100 (4536 amino acids) is required for VLDL production in the liver and also act as a ligand for *LDLR*-mediated endocytosis of LDL particles. Mutation of this gene results in abeta/hypobetalipoproteinemia, normotriglyceridemic hypobetalipoproteinemia, and hypercholesterolemia due to ligand-defective apoB.

1.5.2.2. Genomic structure and functional domains of *Apo B 100* gene

The Apo B gene is located in the short arm of chromosome 2 (2p24-p23) which is 43kb in length. It consists of 28 introns and 29 exons (Innerarity *et al.* 1990). Exon 26 is the longest exon detected in the human genome which is responsible for coding of more than half of Apo B 100 molecule. Apolipoprotein B 100 has several functions. This is required for the syndissertation, assembly and secretion of triglyceride rich lipoproteins. It is an essential structural component of very low density lipoproteins (VLDL) and LDL.

1.5.2.3 Genetic basis of *APO B 100* mutation

At least 5 mutations have been reported in *APO B 100* gene. Apolipoprotein B 100 interact with the Arg3500Gln (substitution of G to A at 10,699) which is designated as familial defective ApoB-100. This mutation is common to the western population and their descendents and account for 2-5 % patients with FH. This mutation has been demonstrated in other populations like Denmark, Poland, Finland, Scottland, Bulgaria and United Kingdom (Gorski *et al.* 1998)' (Aalto-Setala *et al.* 1988; Gaffney *et al.* 1995; Anne T.H 1998; Graham *et al.* 1999; Horvath *et al.* 2001). Both homozygotes and compound heterozygotes for Arg3500Gln and Thy3531Cys are at high risk of developing coronary artery disease. A second mutation Thy3531Cys in LDL receptor has been reported which is responsible for the clearance of LDL molecules from plasma. Another mutation, CAC (3543) TAC results in His (3543) Tyr substitution of Apo B-100 (H3543Y) has been found as the commonest mutation in Germany (Soufi *et al.* 2004). Mutation in the critical region of the *APO B-100* gene results in defective binding of the LDL molecules and increased cholesterol levels in the blood.

1.5.3. *PCSK* 9 gene

1.5.3.1. Introduction

PCSK 9 gene encodes a proprotein convertase belonging to the proteinase K subfamily of the secretory subtilase family. The encoded protein is synthesized as a soluble zymogen that undergoes autocatalytic intramolecular processing in the endoplasmic reticulum. This protein binds to epidermal growth factor like repeat A (EGF-A) domain of the LDL receptor which induces *LDLR* degradation. As it plays a role in cholesterol homeostasis and mutations in this gene have been associated with autosomal dominant FH. (Cenarro *et al.* 1996).

1.5.3.2. Genomic structure

This gene is located in chromosome 1 (1p32.3, NC_000001.10 (55505149..55530526) which is about 25kb in size, encodes a protein of 692 amino acids. This consists of 12 exons. Mutations in the *PCSK 9* gene have been reported as a cause of both hypercholesterolaemia and hypocholesterolaemia (Figure 1.5) (Berge *et al.* 2006).A study among the individuals with low cholesterol levels who are of African descent has reported 2 nonsense mutations in *PCSK 9* gene (Y142X and C679X).These were associated with a 40 % reduction in plasma levels of LDL cholesterol probably by loss of function mutations (Cohen *et al.* 2005).

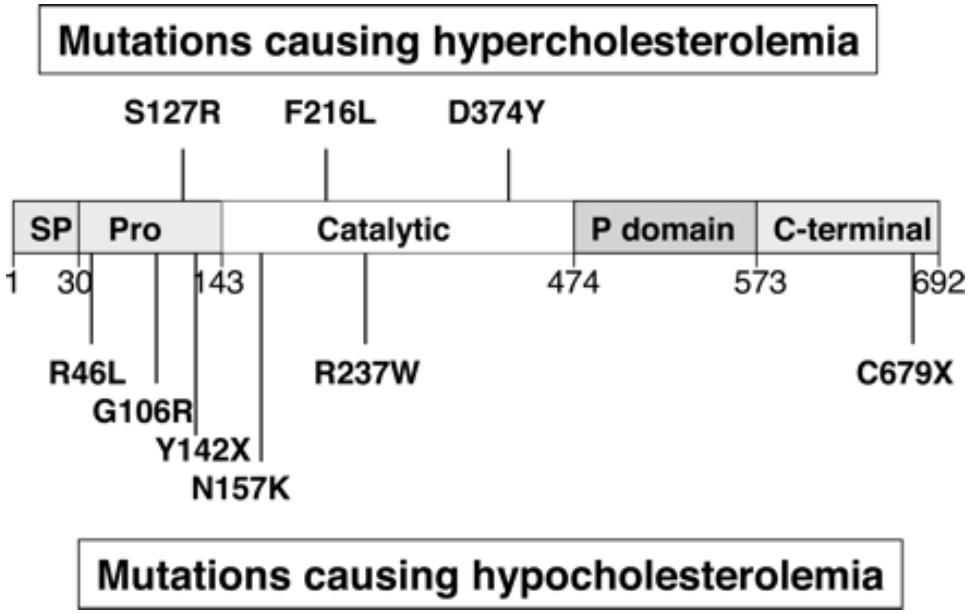

Figure 1.5: Functional domains and mutation in the *PCSK 9* gene. Functional domains of the *PCSK 9* gene and the mutations in the gene associated with hypercholesterolaemia or hypocholesterolaemia.The 30 amino acid signal peptide (SP) is followed by a prosegment (Pro) that is cleaved-off in ER. The catalytic domain is followed by a P domain and a cysteine-rich C-terminal domain. (Adapted from Berge, K. E., L. Ose, et al. (2006) "Missense mutations in the *PCSK 9* gene are associated with hypocholesterolemia and possiblyincreased response to statin therapy." Arterioscler Thromb Vasc Biol **26**(5): 1094-1100.By permission of the author Dr.Trond Paul Leren)

Several studies have reported the mutations in the *PCSK 9* gene causing FH. Allard *et al.* have described four novel heterozygous missense mutations among patients with (c.654>T, c.1070G>A, c.1405>T and c.1327G>A) which were absent in normolipidemic controls (Allard *et al.* 2005). Another study done in France detected the effect of the mutation S127R in *PCSK* on apolipoprotein B- 100 overproduction (Cameron *et al.* 2006). The patients with *PCSK* 9 mutations were reported to have an increased response to the cholesterol lowering effect of statins (Berge *et al.* 2006). Efforts are being made by certain pharmaceutical agencies to find a drug to treat FH by inhibition of *PCSK 9* gene. A recent clinical trial has shown positive results of ALN-PCS which acts by *PCSK* 9 inhibition through RNA interference (Steinberg *et al.* 2009).

1.6 JUSTIFICATION

FH is a risk factor for coronary heart disease which is a burden to the family as well as to the society, requiring intense medical and surgical management. The diagnosis of FH is by clinical signs and the parameters of the lipid profile. Most of the stigmata of hypercholesterolaemia appear later in life making the diagnosis of FH difficult in the young population. But detecting the mutations in the *LDLR* gene confirms early diagnosis. This leads to early intervention, reducing both morbidity and mortality.

Molecular diagnosis of FH helps in predicting response to specific lipid lowering drugs prescribed in hypercholesterolaemia. A study has been done to assess the influence of mutations in the *LDLR* gene, on treatment response to Simvastatin (HMG CoA reductase) in Spain (Chaves *et al.* 2001). This study showed that FH with null mutations (Class 1) responded poorly to Simvastatin compared to those with mutations causing defects in function of the *LDLR* receptor. Patients with null mutation had lower level of high density lipoprotein (HDL) cholesterol as well (Chaves *et al.* 2001).

The response to Simvasatin has also been assessed in FH in the South African population. Patients with mutations of FH2 (FH Afrikaners2) had a significant reduction in total plasma cholesterol than patients with FH1 (FH Afrikaners1) following treatment with Simvastatin (Jeenah *et al.* 1993). The reduction of LDL cholesterol level was also greater in patients with mutations in FH2. It also showed a difference in response to the drug between males and females with the same mutation (Jeenah *et al.* 1993). So the molecular approach helps in stratifying the patient's risk individually and possibly selecting the most effective treatment (Mihaylov *et al.* 2004).

There are more than 100 lipid clinics conducted in England where genetic testing is provided for patients with definite or possible diagnosis of FH. After the detection of a mutation cascade genetic screening is offered to the family members. When a mutation cannot be detected, cascade genetic screening is only recommended when there is strong clinical evidence of familial hypercholesterolemia(2007). Cascade genetic testing in Lipid clinics is feasible, desirable, cost effective and acceptable by patients as well as clinicians. Cascade screening is a systematic approach to testing of relatives of patients affected by genetic disorders.

To date there is no cure for FH. The primary aim of management is to control the cholesterol level by drugs and life style modifications. Several novel therapeutic options are now available which are still under clinical trial. These include squalene inhibitors, microsomal triglyceride transfer protein inhibitors, siRNA for *PCSK 9* or for apolipoprotein B-100 silencing, antisense *PCSK 9* and antisense apolipoprotein B-100 (Mipomersen sodium) (Belter *et al.* ; Gouni-Berthold *et al.* ; Akdim *et al.* 2007; Cuchel *et al.* 2007; Frank-Kamenetsky *et al.* 2008). Both *ex vivo* and *in vivo* approach of gene therapy has been experimented related to *LDLR (Al-Allaf et al. ; Grossman et al. 1994)*. The identification of the mutations will be beneficial in future in view of treating these patients.

Mutation analysis of Sri Lankan patients with FH has not been performed before. Very few studies have been carried out in Sri Lanka related to FH. This include detection of increased lipoprotein (a) level among a group of children with FH and determination of the efficacy as well as the safety of statin (HMG CoA reductase inhibitor) therapy in children and adolescents with heterozygous FH (Widhalm *et al.* 1988; Arambepola C 2009).

As a preliminary study, 25 Sri Lankan Sinhalese patients with clinically diagnosed FH were selected to detect mutations in selected exons in *LDLR* gene. Due to financial and time constrains, as an initial assessment only exons 4, 9, 10 and 14 were selected. These exons have been reported with high frequency of mutations in previous scientific literature and *LDLR* databases (Ashavaid *et al.* 2000)'(Kotze *et al.* 1996)'(Nissen *et al.* 2006)'(Chang *et al.* 2003; Mihaylov *et al.* 2004)'(Villeger *et al.* 2002),(Heath *et al.* 2001; Khan *et al.* 2011)

This will enable us to identify the spectrum of mutations among the Sri Lankan Sinhalese with FH, which will be of use to initiate a cascade screening programme for FH in Sri Lanka.

1.7 Objectives

The objectives of this study were:

1) To identify mutations in exons 4, 9, 10 and 14 of the *LDLR* gene among 25 clinically diagnosed patients with FH in the Sri Lankan population.

2) To correlate the genotype with the phenotype of the 25 patients with clinically diagnosed FH in Sri Lanka.

2. METHODS

2.1 Ethical issues relevant to study

This study was conducted according to the Declaration of Helsinki (2008). The study was done in collaboration with clinicians and cardiologists who were informed about the study. The study has social value because it contributed to improve knowledge about familial hypercholesterolaemia. The study was designed appropriately to ensure scientific validity. There was fare participant selection as any patient with FH was allowed to participate in the study. The patients, first and second degree relatives were interviewed privately in the medical/cardiology clinic. Informed written consent was obtained after providing the necessary information and giving them time to make a decision in private. The data collection booklet was designed to ensure confidentiality of information gathered. Soon after collecting the personal information, the identification page was removed and filled separately. The only identification number in the rest of the booklet was a coded subject study number which can not linked to an individual without the page containing the personal information which was kept by the principal investigator under lock and key. The electronic database containing the data only had the subject study number thus ensuring confidentiality. The database and the counter containing the database were password protected. These measures ensured that the main risk of this study, i.e. loss of confidentiality was minimized. The other risk to subjects participating in this study was the risk of pain, bruising, and infection at the venepuncture site. These were minimized by performing venepuncture under aseptic conditions by a trained phlebotomist. In contrast to these risks, the subjects benefited by participating in this study in the following ways: Firstly since they were tested for *LDLR* mutations, the genetic diagnosis was known, as genetic testing was not freely available in Sri Lanka this was valuable to these patients. Secondly this enabled initiating a cascade genetic screening programme for the relatives. Once results of this study are published, a written summary of the results, in lay

language will be posted to all subjects. In addition patients, first degree relatives and second degree relatives were provided genetic counselling about each subjects' test results.

This study has received the ethical clearance from the ethics committee of the Faculty of Medicine, Colombo and the National Hospital of Sri Lanka.

2.2 Recruitment of patients

2.2.1 Study population and place of study

The subjects comprised twenty five patients with clinically diagnosed FH according to modified Simon Bloom criteria (Minhas *et al.* 2009),US MedPed criteria (Katherine Herman 2009) and Dutch Lipid Clinic Network criteria (Walma *et al.* 2006) . The patients registered in medical and cardiology clinics in National Hospital of Sri Lanka were recruited after taking written informed consent.

2.1.2 Inclusion criteria

The patients were recruited according to following criteria.

- Clinically diagnosed patients with FH according to one of the following criteria.

  Modified Simon Bloom criteria

  Dutch Lipid Clinic Network criteria

  US Med Ped criteria
- Sex- males and females
- Country of origin and ethnicity –Sri Lankan Sinhalese
- Age >12 years

The above mentioned 3 criteria were used to diagnose FH clinically. DNA based evidence of an *LDLR* mutation or familial defective *apo B* 100 is not available in Sri Lanka. So modified Simon Broom criteria was used. Tendon xanthoma is not common among Sri

Lankan patients. For the clinical diagnosis it is difficult to confine to one diagnostic criterion. Depending on the clinical presentation the relevant criteria were selected. Screening of all the first degree and second degree relatives was not possible. So depending on the presentation a first degree or second degree relative was screened for hypercholesterolaemia.

Sex –Both males and females are affected with FH. Males are at a higher risk than females. So both sexes were selected.

Ethnicity- Studies have shown different mutations specific to certain ethnic groups. As a preliminary study of mutation analysis, Sri Lankan Sinhalese were selected as they are the major ethnic group in Sri Lanka. The study will be further extended to do the mutational analysis of the other ethnic groups in the future.

2.1.3 Exclusion criteria

- Non Sinhalese
- Age <12 years

2.3 Clinical evaluation

All the patients were clinically evaluated for stigmata of hypercholesterolaemia like tendon xanthomas, xanthelasma and corneal arcus. Body Mass Index (BMI) was calculated by using height and weight to detect patients with obesity (BMI = Weight (kg)/Height in meters^2). Detailed examination of the central nervous system and the cardiovascular system was performed. Family history of coronary vascular disease, peripheral vascular disease, cerebral vascular disease and hypercholesterolaemia were entered in a three generation pedigree chart.

All the findings were entered to the stigmata of hypercholesterolemia data collection booklet. The relevant data was obtained from the previous clinic records and entered accordingly.

2.4 Biological samples and molecular genetic testing

A sample of 8 ml of venous blood (EDTA) was drawn from the patient and 5ml from the first or second degree relative at recruitment. Blood samples were stored in a refrigerator at -80°C. Mutation analysis was performed after DNA extraction.

2.4.1 DNA extraction

Blood was collected into EDTA containing tubes and stored at -80°C prior to DNA extraction. DNA extraction was done using Promega Wizard® Genomic DNA purification kit according to the manufacturer's protocol. The Promega Wizard® Genomic DNA purification procedure was carried out as follows: 300 µl of whole blood was added to 900 µl of cell lysis solution. The mixture was incubated for 10 minutes at room temperature to lyse the red blood cells. Following incubation, the mixture was centrifuged briefly at 14,000 rpm for 20 seconds. Then the supernatant was discarded without disturbing the white pellet formed. This was followed by vigorous vortexing for about 10-15 seconds until the white blood cells were resuspended. Next 300 µl of nuclei lysis solution was added to the microcentrifuge tubes containing the resuspended cells. This solution was pipetted 5-6 times to lyse the white blood cells. The extraction process was continued by adding 1.5 µl of RNase solution to the nuclear lysate and incubating at 37°C for 15 minutes and by cooling back to room temperature. This was then followed by adding 100 µl of protein precipitation solution and vortexting vigorously for 10-20 seconds. Next the DNA extraction process was followed by centrifugation at 14,000 rpm for 3 minutes and the supernatant was transferred to 1.5µl microcentrifuge tubes containing 300 µl of room temperature isopropanol. This mixture was

then gently mixed until thread-like strands of DNA form a visible mass. This was again centrifuged at 14,000 rpm for 1 minute and the supernatant was discarded. Next 300 µl of room temperature 70% ethanol was added to the DNA and the tubes were gently inverted to wash the DNA pellet and the sides of the micro centrifuge tubes. Then the ethanol was carefully aspirated and the tubes were inverted on clean absorbent paper and the pellets were air-dried for 10-15 minutes. Lastly, 100 µl of DNA rehydration solution was added to the microcentrifuge tubes containing DNA and the DNA was rehydrated by incubating the solution overnight at 4°C. The eluted DNA samples were labelled and stored at -20°C. These samples were used for PCR experiments, which is described next. The left over blood samples were stored in a -80°C freezer under the supervision of the supervisors of this study indefinitely for future research into genetics of FH.

2.4.2 Polymerase chain reaction

Amplification of selected DNA segments of interest was done using the polymerase chain reaction (PCR). PCR amplification involves simultaneous primer extension on complementary strands of DNA with two oligonucleotide primers which are specific to each strand flanking the genomic region to be amplified. This is carried out using thermostable *Taq* DNA polymerase enzyme in the presence of deoxynucleotides and a reaction buffer containing Mg^{2+}. All PCR primers and buffers used in these investigations were from Avon Pharma.

The entire coding regions and flanking intronic sequences of exons 4, 9, 10, 14 of the *LDLR* gene were amplified by PCR using of primers (Xie *et al.* 2007) (Table:2.1). PCR was performed on isolated genomic DNA of all subjects according to the instructions for the HotStar Taq DNA PCR system (QIAGEN, Valencia, CA, USA). PCR was carried out on 30 ng DNA, 1 µl 10×buffer ,0.5 µl 25 mmol/L MgCl2,0.15 µl 10 mmol/L dNTPs, 0.6 µl each

primer (forward and reverse),and 0.12 µl HotStar Taq polymerase. The reactions were amplified under the following conditions: one circle of 95°C 15 minutes, 12 cycles of 94°C for 45 seconds, 63°C for 45 seconds, 72°C for 45 seconds, 25 cycles of 94°C for 45 seconds, 59°C for 45 seconds, 72°C for 45 seconds, and one cycle of 72°C for 10 minutes. PCR products were examined using electrophoresis in 6% polyacrylamide gel and treated with exonuclease and shrimp alkaline phosphatase to remove residual primers and remaining dNTPs. An analysis of the corresponding DNA fragment was performed using the ABI 3100 Genetic Analyzer.

A negative control was included in the PCR analysis to rule out carryover contamination. At the end of the process the presence of amplified PCR products were confirmed by subjecting an amplicon (10 µL) to gel electrophoresis which is explained below. This would reveal a single band if the PCR has worked as expected and has amplified a single segment of the target DNA. The remaining 40 µL were further purified using a DNA fragment purification kit B (BioDev Tech.) pending DNA sequencing. The DNA sequencing was carried out using an ABI 3730 xl DNA analyzer (Applied Biosystems)(Xie *et al.* 2007).

Primers	**Forward primer sequence**	**Reverse primer sequence**
***LDLR*-4**	CGTCCCCGGCTATAGAATG	AAATCACTGCATGTCCCACA
***LDLR*-9**	CTGCAGGATGACACAAGGGG	CATAGGAAGAGACGCCGTGG
***LDLR*-10**	TCTGACCTGTCCCAGAGAATG	CACTAACCAGTTCCTGAAGCTC
***LDLR*-14**	CCCAACCTTGAAACCTCCTTG	CGACCTTGAGGTACCCATTTGA

Table 2.1: The forward and reverse primers of exon 4, 9, 10 and 14 of *LDLR* gene

Adapted from Xie, L., Q. H. Gong, et al. (2007). "Two novel mutations of the LDL receptor gene associated with familial hypercholesterolemia in a Chinese family." Chin Med J (Engl) **120**(19): 1694-1699.

2.4.3 Agarose gel electrophoresis

The presence of PCR amplified products was confirmed by electrophoresing a fraction of the reaction mix on an agarose gel. Electrophoresis through agarose is the standard method used to separate and identify DNA fragments. This was performed by moving negatively charged DNA molecules towards the anode through an agarose gel with an electric field with smaller fragments having greater mobility. The location of DNA within the agarose gel was determined directly by staining with low concentrations of ethidium bromide dye which intercalates with DNA as it migrates and fluoresces when examined over ultra violet (UV) light on a UV transilluminator. Agarose gels were prepared by mixing an appropriate volume of 1x TBE buffer and an appropriate amount of molecular biology grade agarose. This helps to obtain the correct percentage gel (weight/volume). Ethidium bromide 0.5 μg/ml of was also added to each gel. Gels were poured into gel casting trays fixed with combs with the desired number of wells and allowed to set at room temperature. When the gels are set it was submerged in 1x TBE buffer and DNA samples which were mixed with gel loading dye were loaded into the wells of the agarose gel. Appropriate level of current was applied to move the negatively charged nucleic acid molecules. Subsequently the patterns created by different size DNA fragments were visualized by examining the agarose gel on a UV transilluminator.

2.4.4 Cycle sequencing

The DNA sequencing was performed with the PCR primers using the BigDye® Terminator Mix v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the sequencing reaction products were separated on an ABI-PRISM® 3100 Genetic Analyzer (ABI) after removal of unincorporated dye terminators. This system works on the basis of the Sanger dideoxy chain termination principle. It contains four ddNTPs with different fluorescence labels and AmpliTaq® DNA polymerase. At the end of the reaction PCR products of varying

sizes all terminating with a fluorescent-labelled dideoxy nucleotide are generated. The sequence was then read out automatically by capillary electrophoresis of cycle sequencing products through an automated sequencing machine. DNA sequencing analysis was done using Chromas Lite 2.01 software.

This was carried out using both forward and reverse primers separately in two different reactions and the readouts were aligned accordingly, aligned in order to overcome any errors or artefacts that may arise during the sequence reading reaction.

The aligned result was used as the base sequence of the each particular exon. This was then compared with the standard reference in NCBI data base and mutations were detected.

2.5 Data collection and software tools

At recruitment patients were interviewed and examined. Clinical features and the details of medical records and family history were entered into a computational database for statistical analysis. Gen Bank at the National Centre for Biotechnology Information (NCBI), USA was searched to obtain DNA sequences and gene mapping information. This is a free database that can be accessed online at URL: http://www.ncbi.nlm.nih.gov. The support from the already existing data bases related to *LDLR* mutation was taken to detect mutations. A database and a software tool had been designed by a research group in France which contains about 210 mutations which can be accessed through http://www.ncbi.nlm.nih.gov/pmc/articles/PMC146377/. There is a UMD-*LDLR* database contains 870 entries and a website (www.umd.necker.fr) containing information about polymorphisms, major rearrangements, and promoter mutations. These were accessed through http://www.ncbi.nlm.nih.gov/pubmed/12124988. About 1700 allelic variants had been included in University of College London (UCL) *LDLR* FH data base which was reached through http://www.ucl.ac.uk/*ldlr*/LOVDv.1.1.0/ (Leigh *et al.* 2008). The Human

Genome Mutation Data base was referred to detect previously reported mutations (http://www.hgmd.org/). Recommendations for the description of sequence variants by the Human Genome Variation Society were referred to interpret the results http://www.hgvs.org/mutnomen/recs.html.

2.6 Statistical analysis

Phenotypic data was entered to in to computational database. In the phenotype analysis, summary values are presented as mean (standard deviation (SD)) with the assumption that data were normally distributed. When data are not normally distributed chi-square test was used to analyse the association between 2 categorical variables within the group. One way Analysis of Variance (ANOVA) was used to find the association between continuous dependant variable and a categorical independent variable.

3. RESULTS

3.1 Familial Hypercholesterolaemia phenotype

In this section the phenotypic data of the 25 Sri Lankan Sinhalese patients with FH will be discussed. Most of the patients didn't have any stigmata of hypercholesterolaemia. None of them had tendon xanthoma. Only 6 (24 %) patients had xanthelasma. Corneal arcus was not detected among the recruited population.

3.2 Demographic Characteristics

The demographic characteristics of the patients with Familial hypercholesterolaemia are listed in Table 3.1.

	Sinhalese population (n=25)
Mean age (years) (SD)	52.48 (12.477)
Mean weight (Kg) (SD)	58.92 (12.227)
Mean height (cm) (SD)	161.88 (9.506)
Mean Body Mass Index (BMI) (SD)	22.38 (3.50)

Table 3.1: Demographic characteristics of patients with FH. SD: Standard Deviation

The participants were almost equal in sex distribution (Male - 48%, Female - 52%).

The age ranged from 24 to 74 years. The mode of the age group was 53. Majority had a BMI (BMI = Weight (kg)/Height in meters^2) < 24.5kg/m2 (n=19). Six patients had a BMI between 25- 29.9 kg/m2 (n=6).None of the patients had a BMI >30kg/m2 .There was no statistical significance between the BMI and the LDL cholesterol level (P= 0.204) or total cholesterol level (P=0.197).

3.3 Effects of FH

The commonest complication of FH was coronary vascular disease n=11 (44%). Chest pain was a complaint of n= 14 (56%) patients and 11 (44%) patients were detected as having ischaemic changes in electrocardiogram. Various structural and functional abnormalities were detected in echocardiogram of n=8 (32%) patients. Coronary angiogram demonstrated coronary vascular disease in n=9 patients (36%) with, 20% triple vessel disease and 16% with double vessel disease, needing surgical intervention. Past history of a cerebrovascular accident was found only in 2 patients. None of the patients had peripheral vascular disease.

3.4 Family history

A family history of coronary heart disease was noted in n=18 (72%) patients. Most of them had a first degree relative affected with a coronary heart disease at young age (Table 3.2) and some of them had already undergone a surgical intervention. Eighteen patients (72%) had first degree relatives affected with hypercholesterolaemia.

	Age groups			
	<40yrs	**41-50yrs**	**51-60yrs**	**>60yrs**
No members	96%	60%	60%	60%
Affected one member	4%	36%	32%	36%
Affected two members	None	4%	8%	4%

Table 3.2: Percentage of patients with a family history of FH

The commonest age group with a first degree relative with hypercholestraolemia was 41-50 years. History of cerebrovascular accident was found in first degree relatives of n=9 (36%) patients. None of them had a family history of peripheral vascular disease or transient ischemic attacks. Although the patients with a family history of coronary heart disease had high levels of total cholesterol (Figure 3.1) and LDL cholesterol, statistically significant (P=0.242, P=0.705) difference was not observed.

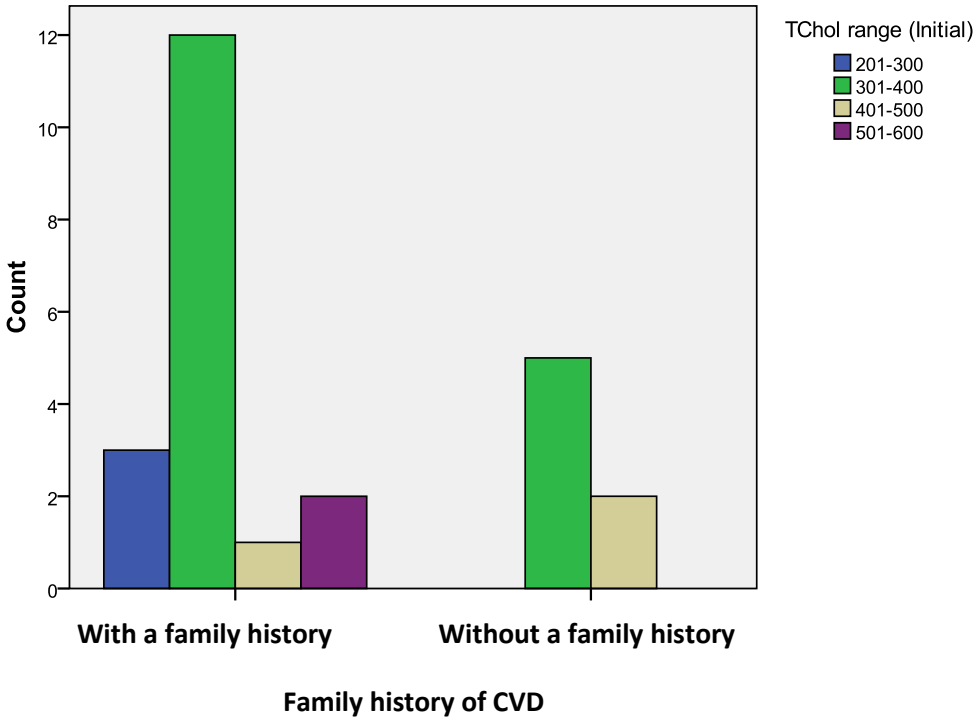

Figure 3.1: Comparison of initial (before treatment) total cholesterol levels among patients with or without family history of coronary vascular disease.

3.4 Biochemical analysis

The biochemical analysis results are shown in figure 3.2, indicating high levels of LDL, total cholesterol and triglyceride levels. Mean cholesterol level was 366.28mg/dl (SD-60.47). Mean LDL level was 259.80mg/dl (SD-66.98). Among the patients with FH the mean HDL

level was around 53.60mg/dl. Mean cholesterol/ HDL ratio was 7.01. (SD-1.60) where as mean LDL/HDL ratio was 4.69 (SD-1.376)

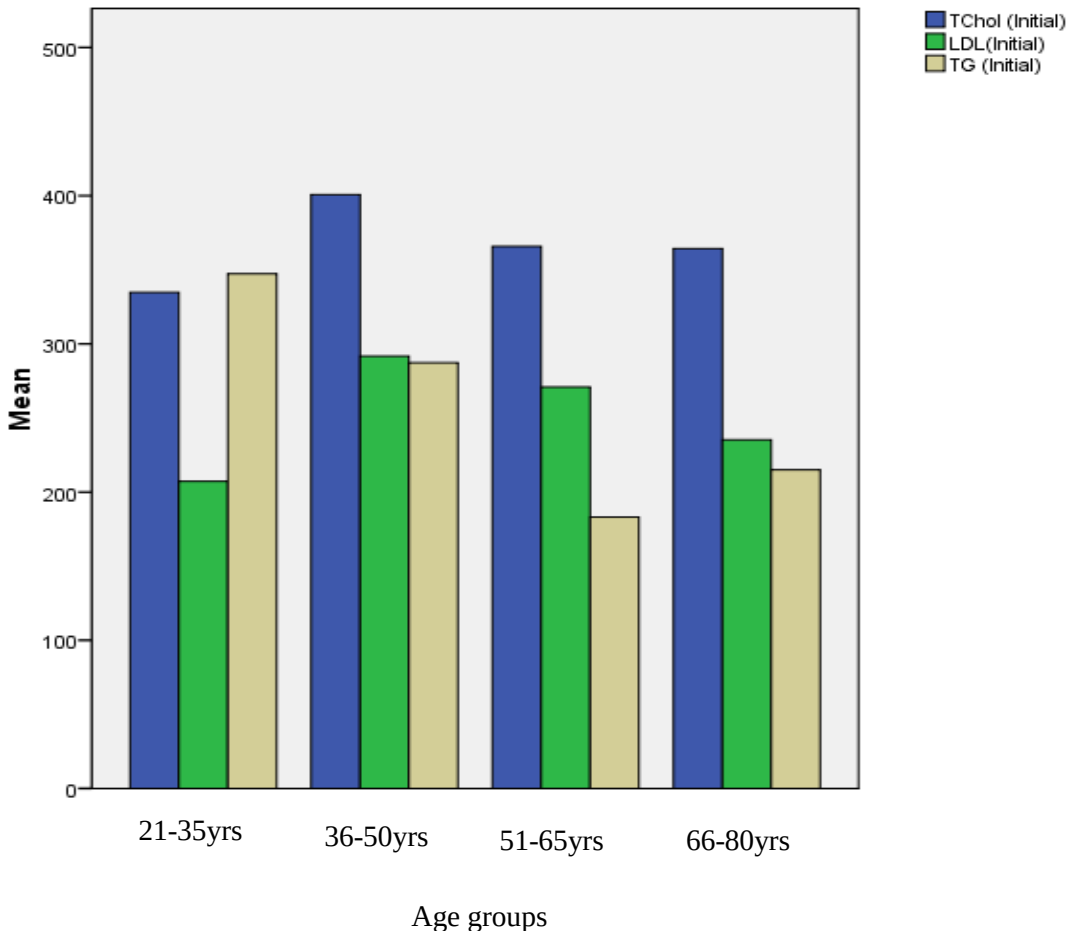

Figure 3.2: Comparison of mean total cholesterol, LDL and triglyceride levels in different age groups.

Most of the patients are currently on treatment for hypercholesterolaemia. The current values (following treatment) of total cholesterol, LDL cholesterol and triglycerides are shown in the figure 3.3 in different age groups. Most of the patients have responded to medical treatment and dietary modifications.

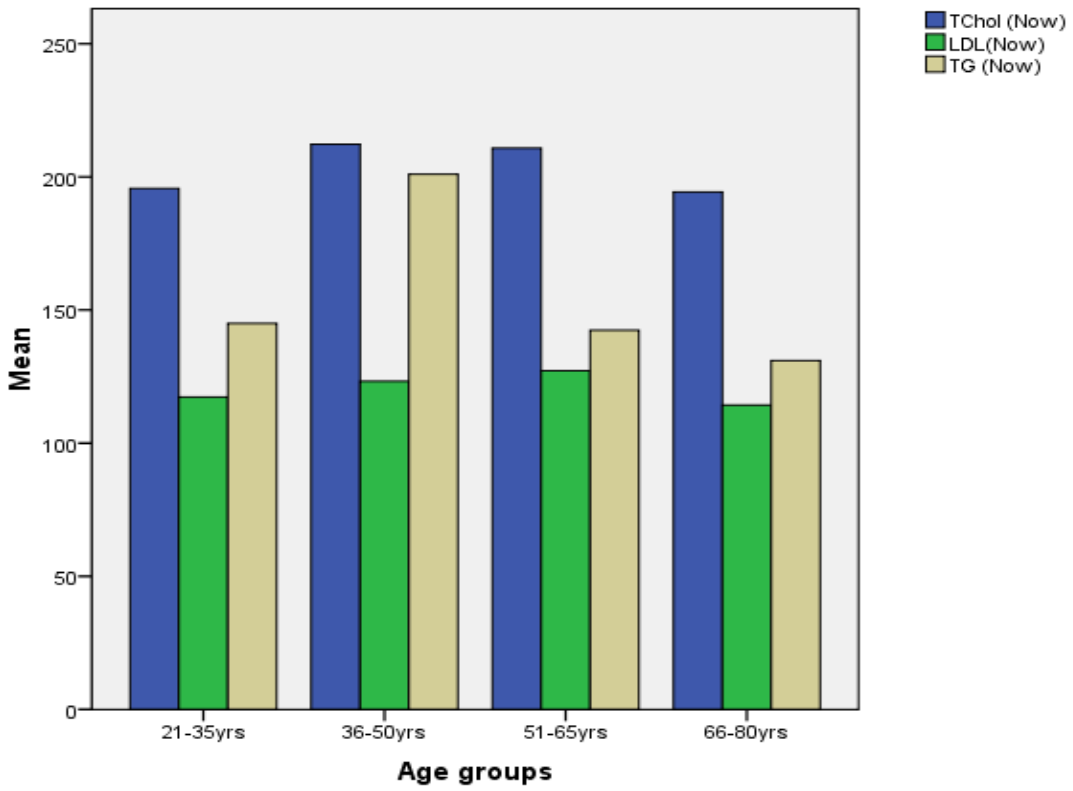

Figure 3.3: Comparison of current (following treatment) total cholesterol levels in different age groups

3.6 Molecular genetic testing

After sequencing of exons 4, 9, 10 and 14 of *LDLR* gene few mutations were detected.

3.6.1 Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms were found in both exonic and intronic regions.

3.6.1.1. Exonic SNPs

Non synonymous SNP was detected in a patient in exon 4 of *LDLR* gene which has been reported in literature, located at position 682 (Figure 3.4). It was a substitution of G by C causing substitution of Glutamine (Q) in to Glutamic acid (E) at amino acid position 228 (NM_000527.2(*LDLR)*:c.682G>C;p.Glu228Gln)

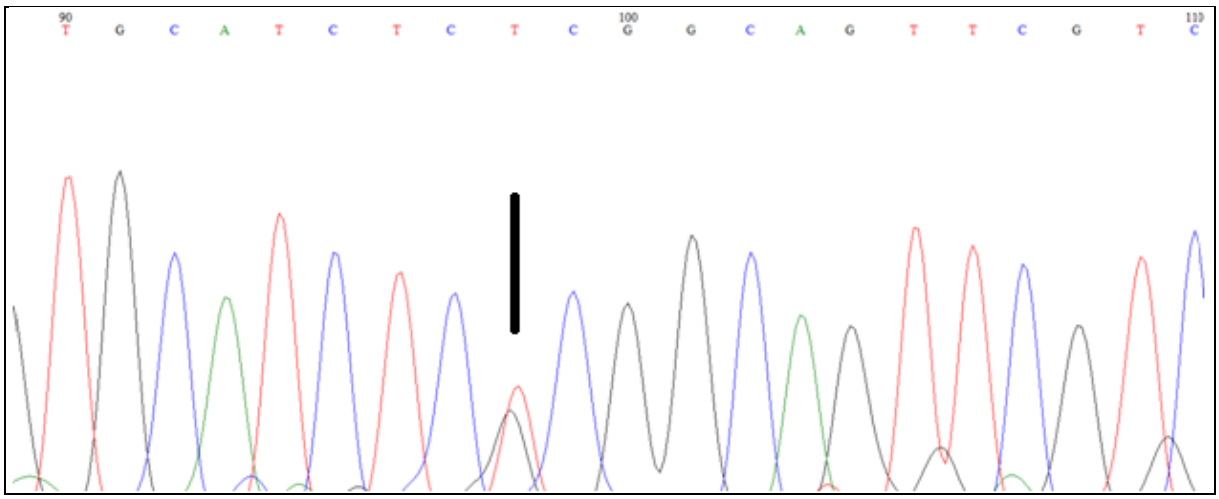

Figure 3.4: Electropherogram showing c.682G>C in exon 4 of *LDLR*

Synonymous mutations were detected in exons 4, 9, and 10.One patient had a heterozygous mutation in the exon 4 located at position 369 (Figure 3.5). This was a substitution of T by G. (c.369T>G; p. (=)).This has not been reported in literature.

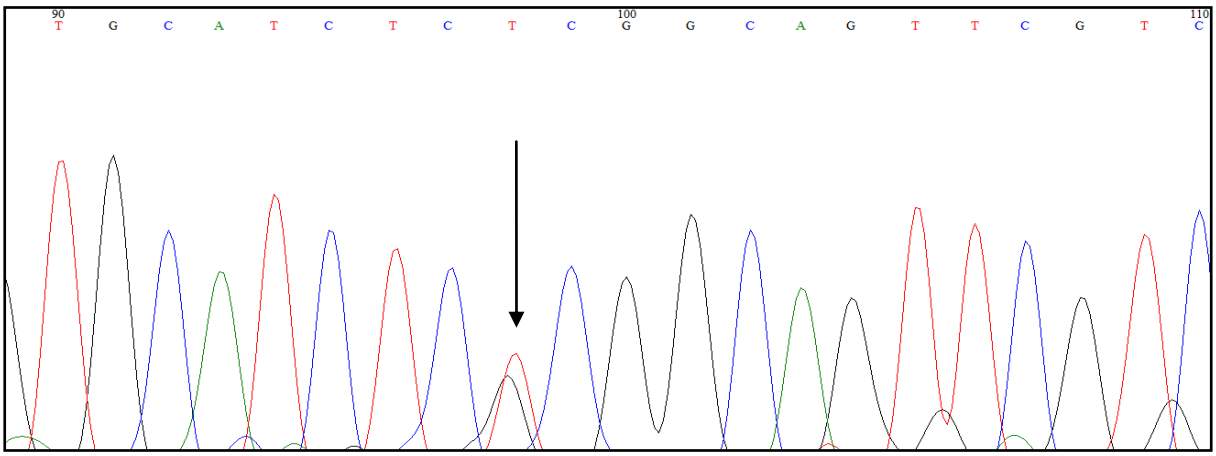

Figure 3.5: A patient's electrophoregram indicating c.369 from T>G in exon 4 of *LDLR*

Another patient had a homozygous substitution at nucleotide position 1431(Figure 3.6) in exon 9 which was a substitution of C by T without causing change in amino acid. (1431C>T; p. (=)).

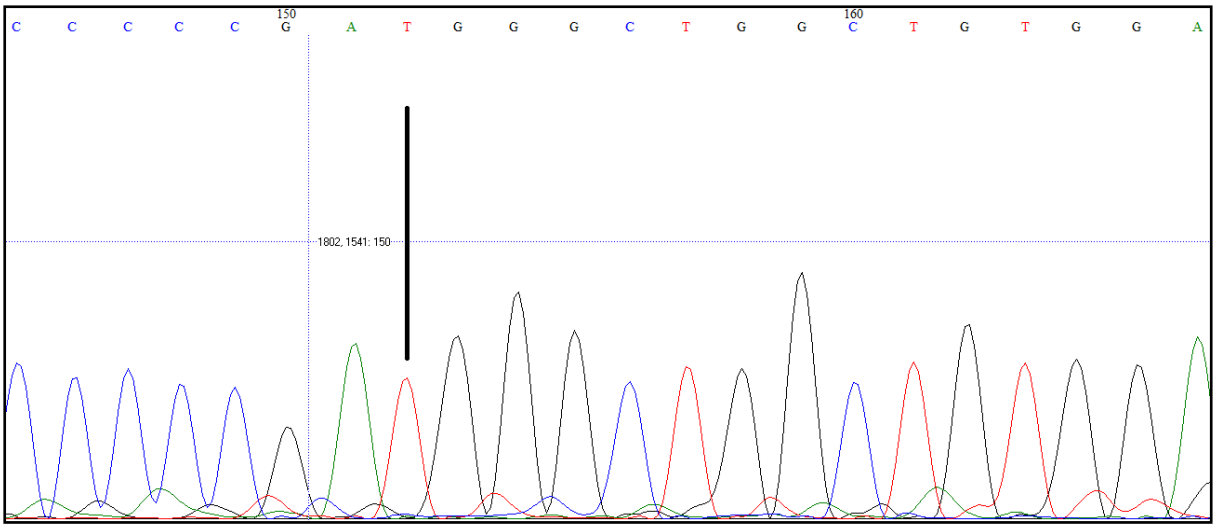

Figure 3.6: Electrophoregram showing mutation in position 1431C>T in exon9 of *LDLR*

Sequence analysis of exon 10 showed a single nucleotide polymorphism. Fifty six percent of patients (14) had a SNP at position 1413 (Figure 3.7) which was a substitution of A by G without causing any change in amino acid. (c.1413A>G; p. (=)).

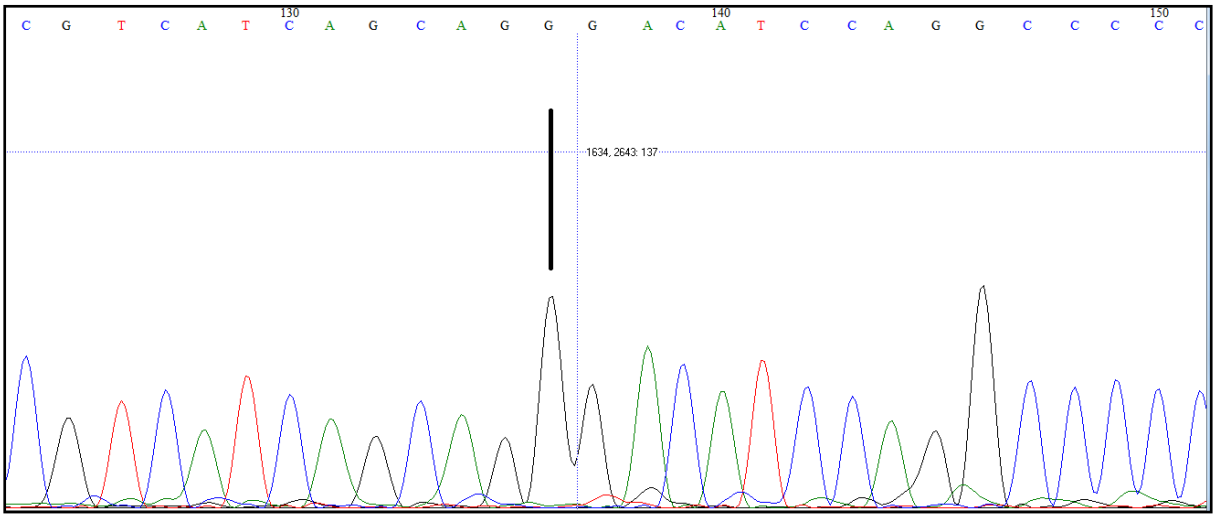

Figure 3.7: Electropherogram of a patients showing c.1413A>G in exon 10 of *LDLR*

3.6.1.2 Intronic SNPs

A SNP was detected in the intervening sequence between exon 8 and 9 (IVS9-30C>T) in 15 patients (60%) (Figure 3.8).

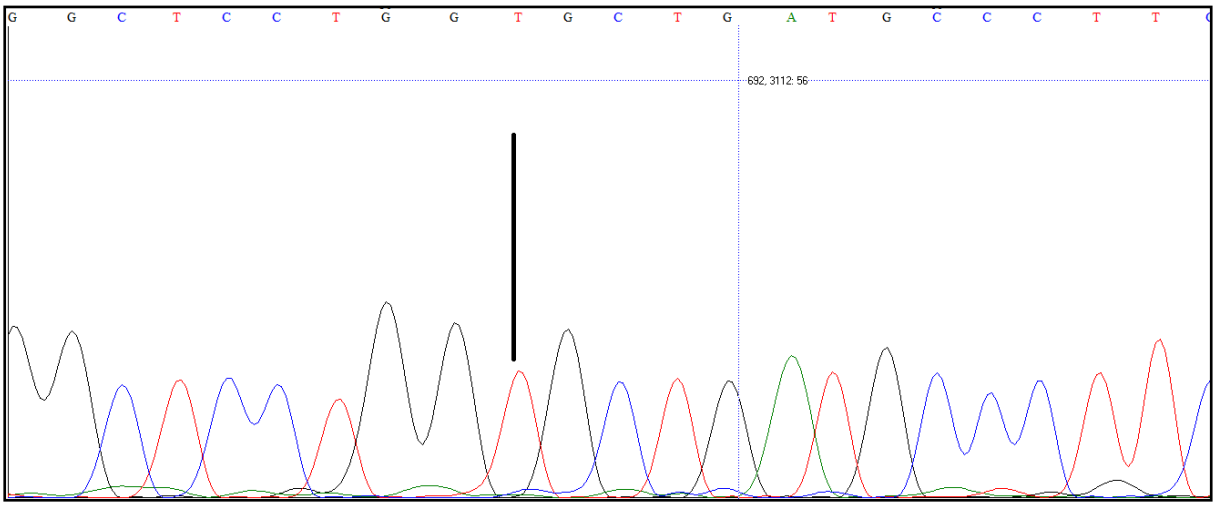

Figure 3.8: Electropherogram of a patients intervening sequence showing IVS9-30C>T located between exon 8 and 9 of *LDLR*

3.6.2. Large structural variants

An 11bp deletion (c.694+8_18delCGGGGCCAGGG) was reported in the intervening sequence between exon 4 and 5 (Figure 3.9) along with c.1413A>G in exon 10 and IVS9-30C>T in a patient.

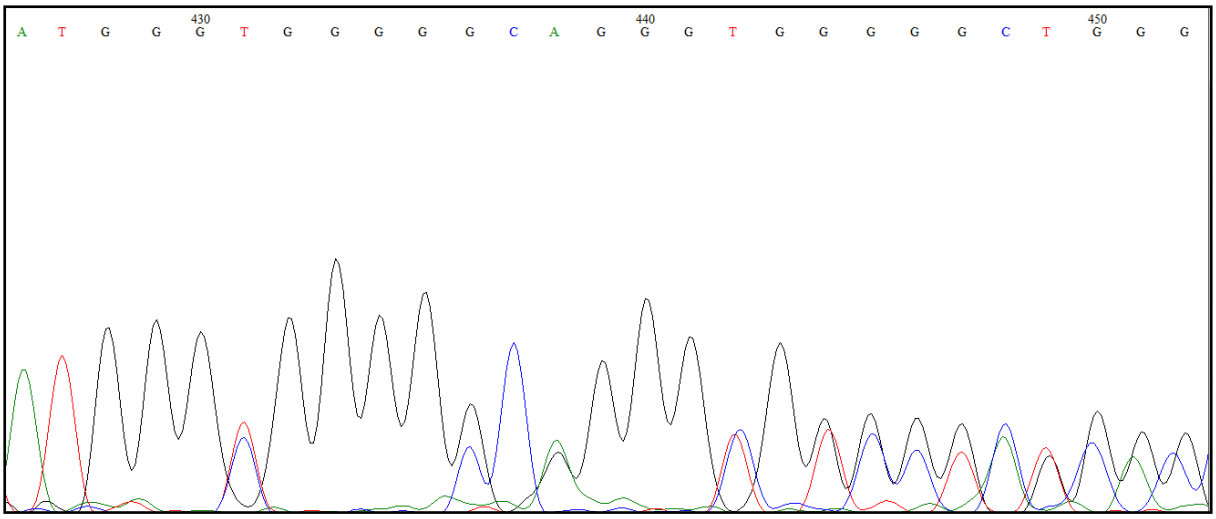

Figure 3.9: Electrophoregram showing the deletion (c.694+8_18delCGGGGCCAGGG) in the intervening sequence between exon 4 and 5 of *LDLR*.

4. DISCUSSION

4.1 Demographic Characteristics

This study is the first to report the mutations and polymorphic variants in *LDLR* gene in Sri Lankan Sinhalese population. The mean age of presentation was 52 years which is closer to the mean age found in other populations. A study done in Japan had a population with age ranged from 35-74 years with a mean of 54 years (Kawasuji *et al.* 1995). Nevertheless much earlier presentations were reported in literature such as, the report of coronary revascularization performed in a 13 year old boy with homozygous FH in Iran (Nemati 2010). Sex distribution among study population was approximately equal. However, increasing age and male gender are associated with increased risk of coronary heart disease, with men typically developing clinically significant disease 10-15 years before women (Austin *et al.* 2004). This occurs as females are generally protected until menopause (Austin *et al.* 2004).

Lack of exercise and associated adiposity is a known risk factor for hypercholesterolaemia (Austin *et al.* 2004). But in this study population obesity was not found as a risk factor for FH as expected. This may be partly due to low prevalence of obesity in Sri Lanka compared to developed countries. The prevalence of obesity was estimated to be 20.3% in men and 36.5% in women in four provinces in Sri Lanka (Wijesinghe 2006). Miltiadous *et al* also failed to demonstrate significant relationship between obesity and heterozygous FH in a case control study done in Greece (Miltiadous *et al.* 2004). This is consistent with the finding that other chronic illnesses also have shown different associated features in different demographic populations.

4.2 Familial hypercholesterolaemia phenotype

The presence of tendon xanthoma is generally considered as diagnostic of FH. But this can be due to other rare conditions namely type III hyperlipoptoteinemia, phytosterolemia or cerebrotendinous xanthomatosis (Bourbon *et al.* 2006). About 30-50% of genetically diagnosed patients with heterozygous Familial hypercholesterolaemia were reported to be having tendon xanthoma (Civeira *et al.* 2005).Unlike in western populations, the patients with clinically evident tendon xanthoma were not present in the study population (Kotze *et al.* 1993). Since, sonography of the achilles tendon was not performed, there can be patients with undiagnosed sub clinical tendon xanthoma among the population (Bureau *et al.* 1998). Xanthelasma were found in a few patients of this study group which is not specific for FH. On average only half of the patients with xanthelasma are hyperlipidemic (Bergman 1994).This is not uncommon in elderly with normal cholesterol levels.

4.3 Coronary heart disease and Familial hypercholesterolaemia

FH causes excess deposition of cholesterol in tissues, leading to accelerated atherosclerosis and increased risk of premature coronary heart disease, the same trend was evident in our population with high percentage of patients with ischemic changes on electrocardoigram, needing surgical intervention. Statistically significant associations between FH and coronary vascular disease have been demonstrated in studies done in both Asian and European countries (Austin *et al.* 2004).

Hawe *et al* have found a 1.7 fold increased risk of developing coronary vascular disease with the presence of a family history (Hawe *et al.* 2003). Higher proportion of patients gave a family history of premature coronary heart disease and hypercholesterolaemia in a first degree relative as expected.

4.4 Biochemical and genetic testing

This population had high levels of mean cholesterol and LDL cholesterol which indicates increased risk of coronary heart disease in these patients. The total cholesterol/HDL ratio is more indicative of coronary heart disease than total cholesterol level (Haq *et al.* 1999). Higher Cholesterol/ HDL ratio was observed among the study population, which may account for the increased risk of coronary heart disease. The goal is to keep the value of total cholesterol/ HDL ratio below 5:1; the optimum ratio is under 3.5: 1(Lichtenstein *et al.* 2006).

The most significant finding was the heterozygous non synonymous SNP found in exon 4 of *LDLR* is known as FH Tulsa-2.This has been reported previously (Hobbs *et al.* 1992). This affects the repeat 5 of the ligand binding domain of the LDL receptor. Ligand binding domain of the LDL receptor consists of seven repeats (~50% identical) each 40 amino acids long, with 6 cysteine repeats. Repeat 5 is unique among these repeats as its structural integrity is

required for binding of both Apo B 100 and Apo E receptor ligands (Russell *et al.* 1989; Theart *et al.* 1995). Binding of ApoB requires repeats 2-7 while binding ApoE requires only repeat 5 of the ligand binding domain of *LDLR*. Substitution of 2 amino acids causes marked reduction in LDL binding. The presence of heterozygous FH with Tulsa 2 results in reduction of *LDLR* activity to a very low level (2-5 %) (*LDLR* @ www.ucl.ac.uk).

The patient with the Tulsa 2 mutation had a first degree relative died of cerebrovascular accident and her son also had hypercholesterolaemia diagnosed at 32 years of age. There was no family history of coronary heart disease. Her total cholesterol level was 441mg/dl with a LDL level of 344mg/dl. She was on atorvastatin and dietary modifications and now her cholesterol levels are within the normal range.

The single nucleotide polymorphisms found in the exon 4 and 9 were not previously reported in scientific literature. The single nucleotide polymorphism which was found in exon 10 is a common polymorphic variant. This has been reported in other populations as well. Tejedor *et al* has reported this SNP in a Spanish population whereas Tatishcheva *et al* has described this in the Russian population (Tatishcheva Iu *et al.* 2001; Tejedor *et al.* 2011). According to the UCL *LDLR* database (*LDLR* @ www.ucl.ac.uk/*ldlr*/LOVDv.1.1.00) this single nucleotide polymorphism (SNP) doesn't have a noticeable phenotypic effect.

The SNP which was found in the intervening sequence between exon 8 and 9 is a common polymorphic variant. This didn't affect mRNA splicing in vitro and also not associated with significant difference in mean plasma cholesterol (Webb *et al.* 1996). A population base case control study in Shanghai, China had reported that there was a 1.5 fold increased risk of bile duct cancer among males and female carriers of this IVS9-30C>T polymorphism (Andreotti *et al.* 2008).

An 11bp deletion which was reported in the intervening sequence between exon 4 and 5 has failed to demonstrate any functional significance through biological sequence analysis using NetGene2 World Wide Web server (http://www.cbs.dtu.dk/services/NetGene2/). Further investigations are needed to detect the functional significance of this mutation.

A comparison was done between Chinese patients living in China and Canada with the same or similar mutations in the LDLR gene. This revealed that the individuals with heterozygous FH residing in China exhibit a milder phenotype despite having deleterious mutations in the *LDLR* gene. This study has shown the significant role of dietary and environmental factors in modulating the phenotype of heterozygous FH (Pimstone *et al.* 1998). In our study population details of the diet or environmental factors such as smoking and life style were not collected. Out of 25 patients with FH only one patient was detected with a pathological mutation, as only 4 exons were screened for mutations. Due to these reasons correlation of genotype with phenotype was not possible.

Only 2 patients had hypothyroidism and were on thyroxine and they didn't have a mutation of exon 4,9,10 and 14 of *LDLR* gene. They may either have hypecholesterolaemia due to hypothyroidism or may have mutations in other exons or other genes related to FH which we have not tested.

5.0 CONCLUSIONS

Stigmata of FH were uncommon .There was a strong family history of coronary vascular disease and hypercholesterolaemia. A pathological mutation was found in the exon 4 of LDLR. There were 2 common polymorphic variants without known functional significance. Since, only one pathological mutation was found, genotype phenotype correlation was not possible. Further investigations are needed to detect mutations in other exons of *LDLR* and rest of the genes related to cholesterol metabolism. In addition, study should be extended to

screen the first and second degree relatives and to demonstrate the functional significance of the detected mutations.

7. LIMITATIONS AND FUTURE RECOMMENDATIONS OF THE STUDY

In the present study most of the patients were recruited from the cardiology wards and clinics at a tertiary care hospital hence, there was a selection bias for patients with severe coronary heart disease. Further extension of this research to include FH patients diagnosed at primary health care facilities as well as from different provinces in the country is needed in future.

Other minor ethnic groups also needed to be included to detect the spectrum of mutations in Sri Lanka.

Due to financial and time constrains only 4 exons were screened. Screening of other exons in LDLR gene, Apo B 100 gene and *PCSK 9* gene is recommended in future. Lack of details of dietary and environmental factors is a limitation for analysis of genotype phenotype correlation. Further extension of this research to collect relevant data is recommended in future. Due to heterogeneity of this mutation a larger sample size is also needed.

Family members, of the patients detected with mutations in *LDLR* should be screened to initiate a cascade screening program in Sri Lanka. This enables early diagnosis, leading to prompt management. This invariably helps to reduce morbidity and mortality related to hypercholesterolaemia.

Several novel ex vivo and in vivo therapeutic options related to LDLR are now available which are still under clinical trials *(Al-Allaf et al. ; Grossman et al. 1994).* The identification of the mutations in this population will be beneficial in future in view of treating these patients.

REFERENCES

"HEART UK FH GIT TOOLKIT,Diagnosis of Familial Hypercholesterolaemia."(2007). Department of Health Familial Hypercholesterolemia Cascade Testing Audit Project. (2008).

Aalto-Setala, K., M. J. Tikkanen, et al. (1988). "XbaI and c/g polymorphisms of the apolipoprotein B gene locus are associated with serum cholesterol and LDL-cholesterol levels in Finland." Atherosclerosis **74**(1-2): 47-54.

Akdim, F., E. S. Stroes, et al. (2007). "Antisense apolipoprotein B therapy: where do we stand?" Curr Opin Lipidol **18**(4): 397-400.

Al-Allaf, F. A., C. Coutelle, et al. "LDLR-Gene therapy for familial hypercholesterolaemia: problems, progress, and perspectives." Int Arch Med **3**: 36.

Allard, D., S. Amsellem, et al. (2005). "Novel mutations of the PCSK9 gene cause variable phenotype of autosomal dominant hypercholesterolemia." Hum Mutat **26**(5): 497.

Amsellem, S., D. Briffaut, et al. (2002). "Intronic mutations outside of Alu-repeat-rich domains of the LDL receptor gene are a cause of familial hypercholesterolemia." Hum Genet **111**(6): 501-510.

Andreotti, G., J. Chen, et al. (2008). "Polymorphisms of genes in the lipid metabolism pathway and risk of biliary tract cancers and stones: a population-based case-control study in Shanghai, China." Cancer Epidemiol Biomarkers Prev **17**(3): 525-534.

Anne T.H, R. S., Hans M.,Peter S,Borge G.N (1998). "ASSOCIATION OF MUTATIONS IN THE APOLIPOPROTEIN B GENE WITH

HYPERCHOLESTEROLEMIA AND THE RISK OF ISCHEMIC HEART DISEASE." N Engl J of Med **338**: 1577-1584.

Arambepola C, F. A. J., Perera R, Neil H A (2009). "Statin treatment for children and adolescents with heterozygous familial

hypercholesterolaemia: a systematic review and meta-analysis." Atherosclerosis(195): 339-347.

Ashavaid, T. F., A. A. Kondkar, et al. (2000). "Identification of two LDL receptor mutations causing familial hypercholesterolemia in Indian subjects." J Clin Lab Anal **14**(6): 293-298.

Austin, M. A., C. M. Hutter, et al. (2004). "Familial hypercholesterolemia and coronary heart disease: a HuGE association review." Am J Epidemiol **160**(5): 421-429.

Austin, M. A., C. M. Hutter, et al. (2004). "Genetic causes of monogenic heterozygous familial hypercholesterolemia: a HuGE prevalence review." Am J Epidemiol **160**(5): 407-420.

Belter, A., M. Skupinska, et al. "Squalene monooxygenase - a target for hypercholesterolemic therapy." Biol Chem **392**(12): 1053-1075.

Berge, K. E., L. Ose, et al. (2006). "Missense mutations in the PCSK9 gene are associated with hypocholesterolemia and possibly increased response to statin therapy." Arterioscler Thromb Vasc Biol **26**(5): 1094-1100.

Bergman, R. (1994). "The pathogenesis and clinical significance of xanthelasma palpebrarum." J Am Acad Dermatol **30**(2 Pt 1): 236-242.

Bourbon, M. and Q. Rato (2006). "Portuguese Familial Hypercholesterolemia Study: presentation of the study and preliminary results." Rev Port Cardiol **25**(11): 999-1013.

Bureau, N. J. and G. Roederer (1998). "Sonography of Achilles tendon xanthomas in patients with heterozygous familial hypercholesterolemia." AJR Am J Roentgenol **171**(3): 745-749.

Cameron, J., O. L. Holla, et al. (2006). "Effect of mutations in the PCSK9 gene on the cell surface LDL receptors." Hum Mol Genet **15**(9): 1551-1558.

Cenarro, A., H. K. Jensen, et al. (1996). "Two novel mutations in the LDL receptor gene: common causes of familial hypercholesterolemia in a Spanish population." Clin Genet **49**(4): 180-185.

Chang, J. H., J. P. Pan, et al. (2003). "Identification and characterization of LDL receptor gene mutations in hyperlipidemic Chinese." J Lipid Res **44**(10): 1850-1858.

Chaves, F. J., J. T. Real, et al. (2001). "Genetic diagnosis of familial hypercholesterolemia in a South European outbreed population: influence of low-density lipoprotein (LDL) receptor gene mutations on treatment response to simvastatin in total, LDL, and high-density lipoprotein cholesterol." J Clin Endocrinol Metab **86**(10): 4926-4932.

Civeira, F., S. Castillo, et al. (2005). "Tendon xanthomas in familial hypercholesterolemia are associated with cardiovascular risk independently of the low-density lipoprotein receptor gene mutation." Arterioscler Thromb Vasc Biol **25**(9): 1960-1965.

Cohen, J., A. Pertsemlidis, et al. (2005). "Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9." Nat Genet **37**(2): 161-165.

Cuchel, M., L. T. Bloedon, et al. (2007). "Inhibition of microsomal triglyceride transfer protein in familial hypercholesterolemia." N Engl J Med **356**(2): 148-156.

Dedoussis, G. V., H. Schmidt, et al. (2004). "LDL-receptor mutations in Europe." Hum Mutat **24**(6): 443-459.

Defesche, S. W. F. J. C., M. A. W. Umans-Eckenhausen, et al. (2001). "The molecular basis of familial hypercholesterolemia in the Netherlands." Humen Genetics **109**: 602-615.

Diakou, M., G. Miltiadous, et al. (2011). "Spectrum of LDLR gene mutations, including a novel mutation causing familial hypercholesterolaemia, in North-western Greece." Eur J Intern Med **22**(5): e55-59.

Frank-Kamenetsky, M., A. Grefhorst, et al. (2008). "Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates." Proc Natl Acad Sci U S A **105**(33): 11915-11920.

Gaffney, D., J. M. Reid, et al. (1995). "Independent mutations at codon 3500 of the apolipoprotein B gene are associated with hyperlipidemia." Arterioscler Thromb Vasc Biol **15**(8): 1025-1029.

Garcia-Garcia, A. B., J. T. Real, et al. (2001). "Molecular genetics of familial hypercholesterolemia in Spain: Ten novel LDLR mutations and population analysis." Hum Mutat **18**(5): 458-459.

Gorski, B., J. Kubalska, et al. (1998). "LDL-R and Apo-B-100 gene mutations in Polish familial hypercholesterolemias." Hum Genet **102**(5): 562-565.

Gouni-Berthold, I. and H. K. Berthold "Antisense oligonucleotides for the treatment of dyslipidemia." Curr Pharm Des **17**(9): 950-960.

Graham, C. A., E. McClean, et al. (1999). "Mutation screening and genotype:phenotype correlation in familial hypercholesterolaemia." Atherosclerosis **147**(2): 309-316.

Grossman, M., S. E. Raper, et al. (1994). "Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia." Nat Genet **6**(4): 335-341.

Gudnason, V., I. N. Day, et al. (1994). "Effect on plasma lipid levels of different classes of mutations in the low-density lipoprotein receptor gene in patients with familial hypercholesterolemia." Arterioscler Thromb **14**(11): 1717-1722.

Guocheng He, S. G., Ming Yi, Peter Michaely, Helen H. Hobbs and a. J. C. Cohen (2002). "ARH Is a Modular Adaptor Protein That Interacts with the LDL

Receptor, Clathrin, and AP-2*." The journal of biological chemistry **Vol. 277** (November 15): 44044–44049.

Hawe, E., P. J. Talmud, et al. (2003). "Family history is a coronary heart disease risk factor in the Second Northwick Park Heart Study." Ann Hum Genet **67**(Pt 2): 97-106.

Heath, K. E., M. Gahan, et al. (2001). "Low-density lipoprotein receptor gene (LDLR) world-wide website in familial hypercholesterolaemia: update, new features and mutation analysis." Atherosclerosis **154**(1): 243-246.

Hobbs, H. H., M. S. Brown, et al. (1992). "Molecular genetics of the LDL receptor gene in familial hypercholesterolemia." Hum Mutat **1**(6): 445-466.

Hobbs, H. H., M. S. Brown, et al. (1987). "Deletion in the gene for the low-density-lipoprotein receptor in a majority of French Canadians with familial hypercholesterolemia." N Engl J Med **317**(12): 734-737.

Horvath, A., A. Savov, et al. (2001). "High frequency of the ApoB-100 R3500Q mutation in Bulgarian hypercholesterolaemic subjects." J Med Genet **38**(8): 536-540.

Innerarity, T. L., R. W. Mahley, et al. (1990). "Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia." J Lipid Res **31**(8): 1337-1349.

Jeenah, M., W. September, et al. (1993). "Influence of specific mutations at the LDL-receptor gene locus on the response to simvastatin therapy in Afrikaner patients with heterozygous familial hypercholesterolaemia." Atherosclerosis **98**(1): 51-58.

Katherine Herman, C. V. H. a. D. W. (2009). "Cascade screening for familial hypercholesterolaemia and its effectiveness in the prevention of vascular disease." British Journal of Diabetes & Vascular Disease **9**(2009).

Kawasuji, M., N. Sakakibara, et al. (1995). "Coronary artery bypass grafting in familial hypercholesterolemia." J Thorac Cardiovasc Surg **109**(2): 364-369.

Khachadurian, A. K. (1964). "The Inheritance of Essential Familial Hypercholesterolemia." Am J Med **37**: 402-407.

Khan, S. P., R. Ghani, et al. (2011). "Two novel mutations in exon 3 and 4 of low density lipoprotein (LDL) receptor gene in patients with heterozygous familial hypercholesterolemia." J Coll Physicians Surg Pak **21**(7): 403-406.

Koivisto, U. M., H. Turtola, et al. (1992). "The familial hypercholesterolemia (FH)-North Karelia mutation of the low density lipoprotein receptor gene deletes seven nucleotides of exon 6 and is a common cause of FH in Finland." J Clin Invest **90**(1): 219-228.

Kotze, M. J., W. J. De Villiers, et al. (1993). "Phenotypic variation among familial hypercholesterolemics heterozygous for either one of two Afrikaner founder LDL receptor mutations." Arterioscler Thromb **13**(10): 1460-1468.

Kotze, M. J., E. Langenhoven, et al. (1989). "The identification of two low-density lipoprotein receptor gene mutations in South African familial hypercholesterolaemia." S Afr Med J **76**(8): 399-401.

Kotze, M. J., R. Thiart, et al. (1996). "Mutation analysis reveals an insertional hotspot in exon 4 of the LDL receptor gene." Hum Genet **98**(4): 476-478.

Leigh, S. E., A. H. Foster, et al. (2008). "Update and analysis of the University College London low density lipoprotein receptor familial hypercholesterolemia database." Ann Hum Genet **72**(Pt 4): 485-498.

Lichtenstein, A. H., L. J. Appel, et al. (2006). "Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee." Circulation **114**(1): 82-96.

Mihaylov, V. A., A. D. Horvath, et al. (2004). "Screening for point mutations in the LDL receptor gene in Bulgarian patients with severe hypercholesterolemia." J Hum Genet **49**(4): 173-176.

Miltiadous, Stavroulla Xenophontos, et al. (2004). "Familial Hypercholesterolaemia in NorthWestern Greece." Hellenic J Cardiol **45**: 299-304.

Minhas, R., S. E. Humphries, et al. (2009). "Controversies in familial hypercholesterolaemia: recommendations of the NICE Guideline Development Group for the identification and management of familial hypercholesterolaemia." Heart **95**(7): 584-587; discussion 587-591.

Nemati, M. H. (2010). "Coronary revascularization in a child with homozygous familial hypercholesterolemia." Interact Cardiovasc Thorac Surg **10**(1): 131-132.

Nissen, P. H., D. Damgaard, et al. (2006). "Genomic characterization of five deletions in the LDL receptor gene in Danish Familial Hypercholesterolemic subjects." BMC Med Genet **7**: 55.

Pimstone, S. N., X. M. Sun, et al. (1998). "Phenotypic variation in heterozygous familial hypercholesterolemia: a comparison of Chinese patients with the same or similar mutations in the LDL receptor gene in China or Canada." Arterioscler Thromb Vasc Biol **18**(2): 309-315.

Russell, D. W., M. S. Brown, et al. (1989). "Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins." J Biol Chem **264**(36): 21682-21688.

Sobia, R., Nuzhat, A., Anne, S., Raheel, Q. (2011). "The genetic characterization of Familial Hypercholesterolemia in Parkistan." Journal of Basic and Applied Sciences **7**(1): 21-25.

Soufi, M., A. M. Sattler, et al. (2004). "A new but frequent mutation of apoB-100-apoB His3543Tyr." Atherosclerosis **174**(1): 11-16.

Soutar, A. K. and R. P. Naoumova (2007). "Mechanisms of disease: genetic causes of familial hypercholesterolemia." Nat Clin Pract Cardiovasc Med **4**(4): 214-225.

Steinberg, D. and J. L. Witztum (2009). "Inhibition of PCSK9: a powerful weapon for achieving ideal LDL cholesterol levels." Proc Natl Acad Sci U S A **106**(24): 9546-9547.

Sun, X. M., J. C. Webb, et al. (1992). "Characterization of deletions in the LDL receptor gene in patients with familial hypercholesterolemia in the United Kingdom." Arterioscler Thromb **12**(7): 762-770.

Tatishcheva Iu, A., M. Mandel'shtam, et al. (2001). "[Four new mutations and polymorphic variants of the low density lipoprotein receptor in patients with familial hypercholesterolemia in Saint Petersburg]." Genetika **37**(9): 1290-1295.

Tejedor, M. T., A. Cenarro, et al. (2011). "New contributions to the study of common double mutants in the human LDL receptor gene." Naturwissenschaften **98**(11): 943-949.

Theart, L., M. J. Kotze, et al. (1995). "Screening for mutations in exon 4 of the LDL receptor gene: identification of a new deletion mutation." J Med Genet **32**(5): 379-382.

Villeger, L., M. Abifadel, et al. (2002). "The UMD-LDLR database: additions to the software and 490 new entries to the database." Hum Mutat **20**(2): 81-87.

Vohl, M. C., D. Gaudet, et al. (1997). "Comparison of the effect of two low-density lipoprotein receptor class mutations on coronary heart disease among French-Canadian patients heterozygous for familial hypercholesterolaemia." Eur J Clin Invest **27**(5): 366-373.

Walma, E. P., F. L. Visseren, et al. (2006). "[The practice guideline 'Diagnosis and treatment of familial hypercholesterolaemia' of the Dutch Health Care Insurance Board]." Ned Tijdschr Geneeskd **150**(1): 18-23.

Webb, J. C., D. D. Patel, et al. (1996). "Genetic variation at a splicing branch point in intron 9 of the low density lipoprotein (LDL)-receptor gene: a rare mutation that disrupts

mRNA splicing in a patient with familial hypercholesterolaemia and a common polymorphism." Hum Mol Genet **5**(9): 1325-1331.

Widhalm, K. and D. Genser (1988). "Increased lipoprotein(a) levels in children with familial hypercholesterolaemia." Lancet **2**(8622): 1262.

Wijesinghe, P. R. (2006). "Obesity is a wake up call for developing world too." BMJ **333**(7572): 809.

Xie, L., Q. H. Gong, et al. (2007). "Two novel mutations of the LDL receptor gene associated with familial hypercholesterolemia in a Chinese family." Chin Med J (Engl) **120**(19): 1694-1699.

APPENDIX 1: LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APO B	Apolipoprotein B
BMI	Body Mass Index
EGF A	Epidermal Growth Factor A
ER	Endoplasmic reticulum
FH	Familial Hypercholesterolaemia
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
PCSK 9	Proprotein convertase subtilisin/kexin type 9
PCR	Polymerase Chain Reaction
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
VLDL	Very Low Density Lipoprotein

APPENDIX 2: LIST OF SUPPLIERS

Applied Biosystems	7 Kingsland Grange, Woolston, Warrington, Cheshire, WA1 7SR, UK.
Promega UK Ltd.	Delta House, Chilworth Research Centre, Southampton SO16 7NS, UK.
Integrated DNA Technology (IDT) – USA.	

APPENDIX 3: DOCUMENTS USED FOR SUBJECT RECRUITMENT

The English and sinhala translation of the documents which were used for the recruitment of patients and first degree relatives for this study included in this appendix.

Data collection booklet (patient)

Information sheet- English (Patient)

Information sheet- Sinhala (Patient)

Consent form- English (Patient)

Consent form- Sinhala (Patient)

Data collection booklet (First degree relative)

Information sheet- English (First degree relative)

Information sheet- Sinhala (First degree relative)

Consent form- English (First degree relative)

Consent form- Sinhala (First degree relative)

A descriptive study of the mutations in the Low Density Lipoprotein Receptor *(LDLR*) gene among patients with familial hypercholesterolaemia in Sri Lanka

Phenotypic data

Subject study number | | | |

Name of the subject .....

.....

Date of Birth ...../...../.....

Sex **Male** ☐ **Female** ☐

Consanguinity **Yes** ☐ **No** ☐

Address .....

.....

.....

Telephone number (Home) ..... **(Mobile)**.....

E mail address .....

Clinic/Clinic number .....

Referring consultant .....

Date of Referral .....

Hospital .....**Ward:** .....

Clinic No/ BHT No .....

Data Protection and Confidentiality After completion of this page ensure that the subject study number is entered on all pages of this booklet. Then detach this page and store separately from the remainder of the booklet.

Subject study number

Date of entry

Date of Birth

CLINICAL HISTORY

Sex Male

Female

Symptoms

Chest pain	
Shortness of breath	
Calf pain while walking	
Temporary loss of vision	
dizziness	
Headache	
Impairment of balance	
Aphasia	
Parasthesia	
other	

Past medical history

Coronary vascular disease	
Transient ischemic attacks	
Peripheral vascular disease	
Diabetes mellitus	
Hypertension	
Other	

Subject study number

Family history and pedigree

(Indicating family history of coronary vascular disease, peripheral vascular disease, cerebral vascular diseases, hypercholesterolaemia)

Consanguinity Yes/No I
II
III
IV
V

Subject study number

Additional pedigree information

Location in pedigree	**Clinical or other information**

Clinical examination

Weight

Height

BMI (Kg/m2)

Features of hypercholesterolaemia

Xanthomas- Elbows

Hands

Knees

Buttocks

Tendons

Subject study number

Corneal arcus

Xanthelasma

Cardiovascular system

Pulse rate- /min Rhythm-

Pulse volume-

Blood pressure- mmHg

Heart-

Central Nervous System

Speech

Gait

Mental state

Involuntary movements

Cranial nerve examination

Motor System

	R/upper limb	L/upper limb	R/Lower limb	L/Lower limb
Inspection				
Tone				
Power				
Reflexes				

Cerebellar signs

Sensory system

Subject study number

Investigations

A) Patients

1. Lipid profile

	Total cholesterol (mg/dl)	LDL cholesterol (mg/dl)	HDL Cholesterol (mg/dl)	Triglycerides (mg/dl)	VLDL Cholesterol (mg/dl)	HDL/LDL ratio
Patient						

2. ECG changes (if present)

T inversions
ST depression
ST elevation
Arrhythmias

3. Echocardioghraphy (if available)

Valvular heart diseases
Left ventricular hypertrophy
Ejection fraction

Subject study number | | | |

4. Coronary angiogram (Date - - -)

Coronary arteries Stenosis of coronary arteries

5. Duplex scan (Date - - -)

Arterial blood flow Thrombosis

6. CT scan (Date - - -)

Cerebral edema Cerebral ischemia Cerebral infarction

7. MRI scan (Date - - -)

Cerebral ischaemia Cerebral infarction

8. MRA (Date - - -)

Cerebral blood flow Arterial thrombosis

Subject study number

Drug treatment

Current medication for hypercholesterolaemia

Sampling record

a) Patient

Date of collection | | | / | | | / | | | | |

	Label	volume	storage	comments
K/EDTA				
Plain tube				

Comments

Record reasons for any missing data and any additional relevant comments. **ENSURE THAT ANONYMITY IS PRESERVED.**

The booklet should be signed when ALL available data have been entered and cross checked with relevant data recorded elsewhere in this booklet .

Signed……. **Date……**

Investigator/Research Assistant

Information sheet

A descriptive study of mutations in the Low Density Lipoprotein receptor gene (*LDLR*) among patients with familial hypercholesterolaemia in Sri Lanka.

This is a descriptive study conducted by Dr.J. R. D. K. Rajapakse, an MSc student in Clinical Genetics at the faculty of Medicine, University of Colombo, Sri Lanka. I am the principal investigator of this study, currently attached to Human Genetic Unit, Faculty of Medicine, University of Colombo. I would like to invite to participate in this study titled "Detection of mutations of low density lipoprotein receptor gene among patients with Familial hypercholesterolaemia in Sri Lanka". This study is conducted under the supervision of Prof. Vajira H. W. Dissanayake, Human Genetics Unit, Faculty of Medicine, University of Colombo and Dr. Godwin Constantine, Deparment of Clinical Medicine, Faculty of Medicine, University of Colombo.

1. **Purpose of the study**

   Familial Hypercholesterolaemia is an inherited condition associated with mutations of LDL receptor gene. In this study we will analyse the mutations of the LDL receptor gene among patients with Familial hypercholesterolaemia which will be helpful in further management and family screening.

2. **Voluntary participation**

   Your consent is needed to participate in this study. If you are unwilling to participate in this study you are free to say no without any influence to the care that you are currently receiving. You are free to not to participate or withdraw the consent at any time during the study by informing us. But this is only possible if you inform us before the results of the study are published.

3. Duration, Procedures of study and participant's responsibilities

This study will be conducted over a one year. We need your permission to acquire clinical history including family history, to examine you and to get the details from your medical records. As this condition has a familial inheritance you are requested to recruit two immediate relatives (parents/children/siblings) with hypercholesterolaemia to this study. For the study we need 10 ml of venous blood. We also need your permission to publish the data and the results of the study in scientific journals in an unidentifiable manner. Only one visit during the research will be enough to get the relevant details and the blood sample.

4. Potential Benefits

By participating in this study you will be able to know the genetic mutations causing familial hypercholesterolaemia. This will be helpful in further management. After obtaining consent, screening the family members will be possible if a mutation is detected. This also helpful in identifying mutations among Sri Lankan Sinhalese population which can be further extended

5. Risks, Hazards and discomforts

Blood will be drawn to detect the genetic defect causing familial hypercholesterolaemia. Approximately 10ml of blood will be taken for testing from you. The risk to you by participating in the study is the risk of pain, bruising and infection at the needle prick site. These will be minimized by performing blood drawing under aseptic conditions by trained phlebotomist

6. Reimbursement

There will be no payment for the participation in this study. You will be given the report of the genetic test and a post test counselling will be arranged in the Human Genetics Unit, Faculty of Medicine, University of Colombo.

7. Confidentiality

Confidentiality of all records is guaranteed and no information by which you can be identified will be released or published. The data collection booklet is designed to ensure confidentiality of information gathered. The electronic database containing the data will have only the subject study number and the database and the computer containing the database would be password protected. These data will never be used in such a way that you could be identified in any way in any public presentation or publication without your express permission.

8. Termination of study participation

You can withdraw your consent of participating in this study at any time before publication, without any effect on the medical care or benefits. But it will not possible for you to leave the study once the results are published or sent for publication.

9. Clarification

If you have any questions regarding the procedures/ tests and information feel free to ask any of person listed below by calling 0112689545

Prof. Vajira H.W.Dissanayake

Prof. Rohan Jayasekara

Dr. Godwin Constantine

Dr. J. R. D. K. Rajapakse

තොරතුරු පත්‍රිකාව

ශ්‍රී ලංකාවේ පරම්පරාගත අධික කොලෙස්ටරෝල් භාවය පවතින රෝගීන්ගේ ලෝඩෙන්සිටි රීසෙප්ටර් ජානයේ ජානමය විවිධත්වය පිළිබඳ අධ්‍යයනය

අපගේ පර්යේෂණ කණ්ඩායම මගින් සිදු කරනු ලබන ඉහත සඳහන් පර්යේෂණයට සහභාගි වීම සඳහා ඔබහට ආරාධනය කිරීමට අප කැමැත්තෙමු. අපගේ පර්යේෂණ කණ්ඩායමේ සාමාජිකයන් වනුයේ

- මහාචාර්ය වජිර එච්. ඩබ්ලිව්.දිසානායක (අධීක්ෂකවරයා) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීඨයේ මානව ප්‍රවේණි විද්‍යා ඒකකයේ මහාචාර්යයවරයෙකි.
- මහාචාර්ය රොහාන් ජයසේකර (අධීක්ෂකවරයා) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීඨයේ මානව ප්‍රවේණි විද්‍යා ඒකකයේ මහාචාර්යයවරයෙකි.
- වෛද්‍ය ගොඩිවින් කොන්ස්ටන්ටයින් (අධීක්ෂකවරයා) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීඨයේ විද්‍යා ඒකකයේ මහාචාර්යයවරයෙකි.
- වෛද්‍ය රසිකා රාජපක්ෂ (ප්‍රධාන පර්යේෂකවරිය) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීඨයේ මානව ප්‍රවේණි විද්‍යා ඒකකයට සම්බන්ධ පශ්චාත් උපාධි අපේක්ෂිකාවකි.

1) මෙම අධ්‍යනයේ අරමුණ

පරම්පරාගත අධික කොලෙස්ටරෝල් භාවය ආවේණිගත තත්වයකි. මෙය ලෝඩෙන්සිටි ලිපෝප්‍රෝටීන් ලයිපේස් රීසෙප්ටර් ජානයේ විකෘති තාවයන් මත සිදුවන බව පර්යේෂණ මගින් තහවුරු කර තිබේ. මෙම අධ්‍යනයේදී ශ්‍රී ලංකික රෝගීන්ගේ ජානමය විවිධත්වය සහ එහි රෝග ලක්ෂණ වලට තිබෙන සම්බන්ධය පිළිබඳ පර්යේෂණය සිදු කරනු ලැබේ.

මෙම ජාන විවිධත්වය හඳුනා ගැනීමෙන් එම රෝගී තත්වය සඳහා ප්‍රතිකාර කිරීමටත් පවුලේ අනිත් සාමාජිකයන්ගේ මෙම රෝගී තත්වය හට ගැනීමට හැකියාවක් තිබේද යන්නත් පරීක්ෂා කර ගත හැකිවේ.

2) ස්වේච්ඡා සහභාගිත්වය

මෙම අධ්‍යනය සඳහා ඔබගේ සහභාගිත්වය ඔබගේ කැමැත්තෙන්ම සිදු කරන්නෙකි.

මෙම අධ්‍යනය සඳහා ඔබට සහභාගි නොවීමට පූර්ණ අයිතිය ඇති අතර එමගින් දැනට ඔබට ලැබෙන ප්‍රතිකාර හෝ අනිකුත් වෛද්‍ය පහසුකම් කෙරෙහි කිසිදු බලපෑමක් ඇත නොවේ.මෙම පර්යේෂණට සහභාගි වීමට ඔබ කලින් කැමැත්ත ලබා දී තිබුණද ඔබට අවශ්‍ය අවස්ථාවක ඉන්

ඉවත් වීමට පූර්ණ අයිතිය ඇත. නමුත් එය කළ හැක්කේ පර්යේෂණ ප්‍රතිඵල ප්‍රසිද්ධියට පත් කිරීමට පෙර පමණි.

3) පර්යේෂණ කාල සීමාව ක්‍රියා පිළිවෙල හා සහභාගිවන්නන්ගේ වගකීම්

මෙම පර්යේෂණය අවුරුද්දක් තුළ සිදුකෙරේ. ඔබගේ සායනික වාර්තා ලබා ගැනීමට පවුලේ සාමාජිකයන්ගේ රෝගී තත්වයන් පිළිබඳ විස්තර ලබා ගැනීමට ඔබව සහ නෝරාගත් පවුලේ සාමාජිකයන් පරීක්ෂා කිරීමට ඔබගේ අවසරය අපට ලබාදිය යුතුවේ. මෙය පරම්පරාගත රෝගී තත්වයක් බැවින් ඔබගේ ළඟම ඥාතීන් දෙදෙනෙකු මෙම පර්යේෂණයට සහභාගී කරවීම අවශ්‍ය වේ. (දෙමව්පියන් / දරුවන් / සහෝදර සහෝදරියන්)

පර්යේෂණය සඳහා රුධිර සාම්පලයක් (මිලි ලීටර් 10) ලබා ගනු ලැබේ. රුධිර සාම්පලයේ කොලෙස්ටරෝල් මට්ටම සහ පරම්පරාගත අධික කොලෙස්ටරෝල් භාවයට බලපාන ජාන විකෘතිතාවයන් හඳුනා ගැනීමට භාවිතා කෙරේ. තවද එකතු කරගත් දත්ත සහ ප්‍රතිඵලයන් ප්‍රකාශයට පත් කිරීමට ඔබගේ අවසරය අවශ්‍ය වේ. මෙහිදී ඔබගේ අනන්‍යතාවය හෙළි නොවන ලෙස ඒවා ප්‍රකාශයට පත් කෙරේ. එක් වරක් සහභාගි වීමෙන් අවශ්‍ය දත්ත සහ රුධිර සාම්පලය අපට ලබා දිය හැක.

4) ලද හැකි ප්‍රතිලාභ

මෙම අධ්‍යනයට සහභාගි වීමෙන් ඔබට ඔබගේ අධික කොලෙස්ටරෝල් භාවයට හේතුවන ජාන විකෘති තාවය හඳුනා ගතහැකි වන අතර මෙය ඔබගේ පවුලේ සාමාජිකයන්ටද වැදගත් වනු ඇත. ඔබගේ අනුදැනුම ඇතිව ඔබගේ පවුලේ සාමාජිකයන්ද මෙම පර්යේෂණයට යොමුකරගත හැක. ශ්‍රී ලංකාවේ මෙම රෝගය පවතින අයගේ ජාන විකෘති තාවයන් හඳුනා ගැනීමටද මෙය උපකාර වේ.

5) අවදානම් අනතුරු සහ අපහසුතා

ලෝඩෙන්සිටි ලිපෝප්‍රෝටීන් ලයිපේස් රිසෙප්ටර් ජානයේ විකෘති තාවය පරීක්ෂා කිරීමට මිලි ලීටර් 10 පමණ රුධිර සාම්පලයක් ලබා ගත යුතු අතර මෙම සාම්පලය

ලබා ගැනීමේද යම් අපහසුතාවයක් ඇතිවිය හැකිය. එන්නත් කටුව නිසා එම ස්ථානයේ වේදනාවක් තැළීමක් හෝ කලාතුරකින් ආසාදනයක් හටගත හැක. මෙය අවම කිරීමට පුහුණු හෙදියක් විසින් සාම්පලය ලබා ගැනේ.

6) දිමනා

ඔබ මෙම අධ්‍යනයට සහභාගි වීම නිසා ඔබට දිමනාවක් නොලැබේ. එහෙත් ඔබට

පරම්පරාගත අධික කොලෙස්ටරෝල් භාවයට හේතු වූ ජාන විකෘති තාවය පිළිබඳ වාර්තාවක් සහ ඒ පිළිබඳ දැනුවත් කිරීමක් මානව ප්‍රවේණි විද්‍යා ඒකකය ලබා දෙනු ලැබේ.

7) රහසිගත භාවය

සියලුම තොරතුරු සහිත වාර්තාවන් සහ අධ්‍යයනය මගින් ලබාගන්නා දත්තයන්ගේ රහසිගතතාවය තහවුරු කරන අතර ඔබගේ අනන්‍යතාවය හෙළිවන ආකාරයේ කිසිවක් ඔබගේ කැමැත්තෙන් තොරව හෙළිකිරීමක් ඉදිරිපත් කිරීමක් හෝ ප්‍රකාශයට පත් කිරීමක් සිදු කරනු නොලැබේ.

8) අධ්‍යයනයට සහභාගි වීම අහෝසි කිරීම

අධ්‍යයනයට සහභාගි වීමට ඔබ මුලදී කැමැත්ත ප්‍රකාශ කළද ඔබට අවශ්‍ය ඕනෑම අවස්ථාවක ලබා දුන් කැමැත්ත ඉවත් කර ගැනීමට අවසර ඇත. එසේ සිදු කිරීමට අවශ්‍ය වූ විගස එය පර්යේෂකයන්ට දැනුම් දීම අවශ්‍ය වේ. එමගින් දැනට ඔබට ලැබෙන ප්‍රතිකාර හෝ අනිකුත් වෛද්‍ය පහසුකම් කෙරෙහි කිසිදු බලපෑමක් ඇති නොවේ. නමුත් එසේ කල හැක්කේ එකතු කරගත් දත්ත හෝ ප්‍රතිඵල ප්‍රකාශයට පත් කිරීමට පෙර පමණි.

9) වැඩිදුර තොරතුරු

ඔබට මෙම අධ්‍යනයට හෝ එහි ක්‍රියාපටිපාටිය පිළිබඳ කිසියම් ප්‍රශ්නයක් ඇතිනම් හෝ වැඩිදුර තොරතුරු අවශ්‍ය නම් කරුණාකර පහත සඳහන් වෛද්‍යවරුන් අමතන්න.

මහාචාර්ය වජිර දිසානායක

මහාචාර්ය රොහාන් ජයසේකර

වෛද්‍ය ගොඩිවින් කොන්ස්ටන්ටයින්

වෛද්‍ය රසිකා රාජපක්ෂ

දුරකථන 0112689545

CONSENT FORM

A descriptive study of mutations in the Low Density Lipoprotein Receptor (*LDLR*) gene among patients with familial hypercholesterolaemia in Sri Lanka

To be completed by the participant

The participant should complete the whole of this sheet by himself/ herself

1. Have you read the information sheet? (Please keep a copy for yourself) YES/NO

2. Have you had an opportunity to discuss this study and ask any questions? YES/NO

3. Have you had satisfactory answers to all your questions? YES/NO

4. Have you received enough information about the study? YES/NO

5. Who explained the study to you?

……

6. Do you understand that you are free to withdraw from the study at any time, without having to give a reason and without affecting your future medical care? YES/NO

7. Sections of your medical notes, including those held by the investigators relating to your participation in this study may be examined by other research assistants. All personal details will be treated as STRICTLY CONFIDENTIAL. Do you give your permission for these individuals to have access to your records? YES/NO

8. Have you had sufficient time to come to your decision? YES/NO

9. Do you agree to take part in this study? YES/NO

10. Do you agree for the samples to be sent abroad? YES/NO

11. Do you agree to let us take photographs of you? YES/NO

12. Do you give consent to the publication of one or more photographs in a scientific journal? YES/NO

13. Do you agree to keep the leftover sample for future studies relevant to Familial hypercholesterolaemia under the supervision of the supervisors? YES/NO

PARTICIPANT' NAME (BLOCK CAPITALS)

……

Participant's signature……..……

Date…….

INVESTIGATOR

I have explained the study to the above volunteer and he/ she has indicated her willingness to take part in the study.

Signature of investigator……...…….....

Date…….

Name (BLOCK CAPITALS)

…….....

කැමැත්ත ප්‍රකාශ කිරීමේ පත්‍රය

(a) සහභාගී වන්නන් විසින් පිරවීම සඳහාය.

මෙම පත්‍රය සහභාගී වන්නන් /භාරකරුවන් විසින් සම්පූර්ණයෙන් පිරවිය යුතුය.

1. අධ්‍යයනය සම්බන්ධයෙන් තොරතුරු පත්‍රිකාවෙ පැහැදිලි කරන ලද කරුණු ඔබට තේරුනාද? (කරුණාකර තොරතුරු පත්‍රිකාවේ පිටපතක් ඔබ ලබාගන්න)

ඔව්/නැහැ

2. මෙම අධ්‍යයනය සම්බන්ධව සාකච්ඡා කිරීමට හා ඒ පිළිබඳව ප්‍රශ්න ඇසීමට ඔබට අවස්ථාවක් ලැබුණා ද?

ඔව්/නැහැ

3. ඔබ ඇසූ ප්‍රශ්න සියල්ලටම සෑහීමකට පත්විය හැකි පිළිතුරු ලැබුණාද?

ඔව්/නැහැ

4. මෙම අධ්‍යයනය සම්බන්ධයෙන් ප්‍රමාණවත් තොරතුරු ලැබුණාද? **ඔව්/නැහැ**

5. මෙම අධ්‍යයනය සම්බන්ධයෙන් ඔබට පැහැදිලි කරන ලද්දේ කවුරුන් විසින්ද?

.....

6. කිසිදු කරුණු දැක්වීමකින් තොරව, මෙම අධ්‍යයනයෙන් ඉවත් වීමට ඔබ හට ඕනෑම අවස්ථාවක හැකියාව ඇති බව පැහැදිලි වූවාද?

ඔව්/නැහැ

7. ඔබෙ වෛද්‍ය වාර්තා සහ පර්යේෂණ දත්ත මෙම අධ්‍යයනය සම්බන්ධ සාමාජිකයින් විසින් අධ්‍යයනය කෙරෙ සියළු වාර්තා සහ දත්තවල රහස්‍යභාවය තහවුරු කෙරෙ මෙම අධ්‍යයනය සම්බන්ධ සාමාජිකයින්ට තොරතුරු ලබා දීමට එකඟ වෙනවාද?

ඔව්/නැහැ

8. මෙම අධ්‍යයනයෙන් පසුව ඉතිරි වන රුධිර සාම්පලයක් ඇතොත් ඉදිරියෙ පරම්පරාගත අධික කොලෙස්ටරෝල් භාවය රෝගය සම්බන්ධ පර්යේෂණ සඳහා භාවිතා කිරීමට ඔබ එකඟ වෙනවාද?

ඔව්/නැහැ

9. රුධිර සාම්පල පිටරට යැවීමට එකඟ වෙනවාද?

ඔව්/නැහැ

10. මෙම අධ්‍යයනයට සහභාගී වීම සම්බන්ධයෙන් තීරණයකට එළඹීමට ඔබට ඇති තරම් කාලය ලැබුණා ද?

ඔව්/නැහැ

11. ඔබ මෙම අධ්‍යයනයට සහභාගී වීමට එකඟ වෙනවාද? **ඔව්/නැහැ**

සහභාගී වන්නන්ගෙ /භාරකරුවන්

අත්සන:..... දිනය:.....

නම:.....

(b) පර්යේෂක විසින් පිරවීම සඳහාය.

මෙම අධ්‍යයනය සම්බන්ධ කරුණු, මා විසින් අධ්‍යයනයට ස්වේච්ඡාවෙන් සහභාගී වන්නන් හට පැහැදිලි කරන ලදී. ඔහු/ඇ විසින් මෙම අධ්‍යයනයට සහභාගී වීමට කැමැත්ත ප්‍රකාශ කරන ලදී.

පර්යේෂකගේ අත්සන:..... දිනය:.....

නම:.....

A descriptive study of the mutations in the Low Density Lipoprotein Receptor *(LDLR*) gene among patients with familial hypercholesterolaemia in Sri Lanka

Phenotypic data- First Degree relative

Subject study number | | | |

Name of the subject .....

.....

Date of Birth ...../...../.....

Sex **Male** ☐ **Female** ☐

Consanguinity **Yes** ☐ **No** ☐

Address .....

.....

.....

Telephone number(Home) ..... **(Mobile)**.....

E mail address .....

Clinic/Clinic number .....

Referring consultant .....

Date of Referral .....

Hospital .....**Ward:** .....

Clinic No/ BHT No .....

Relationship to the participant .....

Data Protection and ConfidentialityAfter completion of this page ensure that the subject study number is entered on all pages of this booklet. Then detach this page and store separately from the remainder of the booklet.

Subject study number

Date of entry

Date of Birth

CLINICAL HISTORY

Sex

Male

Female

Symptoms

Chest pain	
Shortness of breath	
Calf pain while walking	
Temporary loss of vision	
dizziness	
Headache	
Impairment of balance	
Aphasia	
Parasthesia	
other	

Past medical history

Coronary vascular disease	
Transient ischemic attacks	
Peripheral vascular disease	
Diabetes mellitus	
Hypertension	
Other	

Subject study number | | | |

Family history and pedigree

(Indicating family history of coronary vascular disease, peripheral vascular disease, cerebral vascular diseases, hypercholesterolaemia)

Consanguinity Yes/No I
II
III
IV
V

Subject study number | | | |

Additional pedigree information

Location in pedigree	**Clinical or other information**

Clinical examination

Weight

Height

BMI (Kg/m2)

Features of hypercholesterolaemia

Xanthomas- Elbows

Hands

Knees

Buttocks

Tendons

Subject study number

Corneal arcus

Xanthelasma

Cardiovascular system

Pulse rate- /min Rhythm-

Pulse volume-

Blood pressure- mmHg

Heart-

Central Nervous System

Speech

Gait

Mental state

Involuntary movements

Cranial nerve examination

Motor System

	R/upper limb	L/upper limb	R/Lower limb	L/Lower limb
Inspection				
Tone				
Power				
Reflexes				

Cerebellar signs

Sensory system

Subject study number | | | |

Investigations

A) Patients

9. Lipid profile

	Total cholesterol (mg/dl)	LDL cholesterol (mg/dl)	**HDL Cholesterol (mg/dl)**	Triglycerides (mg/dl)	VLDL Cholesterol (mg/dl)	HDL/LDL ratio
Patient						

10. ECG changes (if present)

T inversions
ST depression
ST elevation
Arrhythmias

11. Echocardioghraphy (if available)

Valvular heart diseases
Left ventricular hypertrophy
Ejection fraction

Subject study number | | | |

12. Coronary angiogram (Date - - -)

Coronary arteries Stenosis of coronary arteries

13. Duplex scan (Date - - -)

Arterial blood flow Thrombosis

14. CT scan (Date - - -)

Cerebral edema Cerebral ischemia Cerebral infarction

15. MRI scan (Date - - -)

Cerebral ischaemia Cerebral infarction

16. MRA (Date - - -)

Cerebral blood flow Arterial thrombosis

Subject study number

Drug treatment

Current medication for hypercholesterolaemia

Sampling record

b) Patient

Date of collection | | | / | | | / | | | | |

	Label	volume	storage	comments
K/EDTA				
Plain tube				

Comments

Record reasons for any missing data and any additional relevant comments. **ENSURE THAT ANONYMITY IS PRESERVED.**

The booklet should be signed when ALL available data have been entered and cross checked with relevant data recorded elsewhere in this booklet .

Signed……. **Date……**

Investigator/Research Assistant

Information sheet –(First degree relative)

A descriptive study of mutations in the Low Density Lipoprotein receptor gene (*LDLR*) among patients with familial hypercholesterolaemia in Sri Lanka.

This is a descriptive study conducted by Dr.J. R. D. K. Rajapakse, an MSc student in Clinical Genetics at the faculty of Medicine, University of Colombo, Sri Lanka. I am the principal investigator of this study, currently attached to Human Genetic Unit, Faculty of Medicine, University of Colombo. I would like to invite to participate in this study titled "Detection of mutations of the Low Density Lipoprotein receptor gene among patients with familial hypercholesterolaemia in Sri Lanka". This study is conducted under the supervision of Prof. Vajira H. W. Dissanayake, Human Genetics Unit, Faculty of Medicine, University of Colombo and Dr. Godwin Constantine, Deparment of Clinical Medicine, Faculty of Medicine, University of Colombo.

1. **Purpose of the study**

   Familial Hypercholesterolaemia is an inherited condition associated with mutations of LDL receptor gene. In this study we will analyse the mutations of the LDL receptor gene among patients with Familial hypercholesterolaemia which will be helpful in further management and family screening.

2. **Voluntary participation**

   Your consent is needed to participate in this study. If you are unwilling to participate in this study you are free to say no without any influence to the care that you are currently receiving. You are free to not to participate or withdraw the consent at any time during the study by informing us. But this is only possible if you inform us before the results of the study are published.

3. Duration, Procedures of study and participant's responsibilities

This study will be conducted over a one year. We need your permission to acquire clinical history including family history, to examine you and to get the details from your medical records. For the study we need 5 ml of venous blood to do the lipid profile. We also need your permission to publish the data and the results of the study in scientific journals in an unidentifiable manner. Only one visit during the research will be enough to get the relevant details and the blood sample.

4. **Potential Benefits**

By participating in this study you will help us to diagnose patients with familial hypercholesterolaemia to do the mutation analysis. If a mutation is detected in your close relative it is possible to get the screening done in other family members later.

5. Risks, Hazards and discomforts

Blood will be drawn to perform a lipid profile. Approximately 5ml of blood will be taken for testing from you. The risk to you by participating in the study is the risk of pain, bruising and infection at the needle prick site. These will be minimized by performing blood drawing under aseptic conditions by trained phlebotomist.

6. Reimbursement

There will be no payment for the participation in this study. You will be given the report of lipid profile.

7. Confidentiality

Confidentiality of all records is guaranteed and no information by which you can be identified will be released or published. The data collection booklet is designed to ensure confidentiality of information gathered. The electronic database containing the data will have only the subject study number and the database and the computer containing the database would be password protected. These data will never be used in such a way that

you could be identified in any way in any public presentation or publication without your express permission.

8. Termination of study participation

You can withdraw your consent of participating in this study at any time before publication, without any effect on the medical care or benefits. But it will not possible for you to leave the study once the results are published or sent for publication.

9. Clarification

If you have any questions regarding the procedures/ tests and information feel free to ask any of person listed below by calling 0112689545

Prof. Vajira H.W.Dissanayake

Prof. Rohan Jayasekara

Dr. Godwin Constantine

Dr. J. R. D. K. Rajapakse

තාරතුරු පත්‍රිකාව(පළමු පරම්පරාවේ ඥාතීන් සඳහා)

ශ්‍රී ලංකාවේ පරම්පරාගත අධික කොලෙස්ටරෝල් භාවය පවතින රෝගීන්ගේ ලෝඩෙන්සිටි රීසෙප්ටර් ජානයේ ජානමය විවිධත්වය පිළිබඳ අධ්‍යයනය

අපගේ පර්යේෂණ කණ්ඩායම මගින් සිදු කරනු ලබන ඉහත සඳහන් පර්යේෂණයට සහභාගි වීම සඳහා ඔබහට ආරාධනය කිරීමට අප කැමැත්තෙමු. අපගේ පර්යේෂණ කණ්ඩායමේ සාමාජිකයන් වනුයේ

- මහාචාර්ය වජිර එච්. ඩබ්ලිව්.දිසානායක (අධීක්ෂකවරයා) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීඨයේ මානව ප්‍රවේණි විද්‍යා ඒකකයේ මහාචාර්යයවරයෙකි.
- මහාචාර්ය රොහාන් ජයසේකර (අධීක්ෂකවරයා) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීඨයේ මානව ප්‍රවේණි විද්‍යා ඒකකයේ මහාචාර්යයවරයෙකි.
- වෛද්‍ය ගොඩ්වින් කොන්ස්ටන්ටයින් (අධීක්ෂකවරයා) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීඨයේ විද්‍යා ඒකකයේ මහාචාර්යයවරයෙකි.
- වෛද්‍ය රසිකා රාජපක්ෂ (ප්‍රධාන පර්යේෂකවරිය) - කොළඹ විශ්ව විද්‍යාලයේ වෛද්‍ය පීඨයේ මානව ප්‍රවේණි විද්‍යා ඒකකයට සම්බන්ධ පශ්චාත් උපාධි අපේක්ෂිකාවකි.

1) මෙම අධ්‍යනයේ අරමුණු

පරම්පරාගත අධික කොලෙස්ටරෝල් භාවය ආවේණිගත තත්වයකි. මෙය ලෝඩෙන්සිටි ලිපෝප්‍රෝටීන් ලයිපේස් රීසෙප්ටර් ජානයේ විකෘති තාවයන් මත සිදුවන බව පර්යේෂණ මගින් තහවුරු කර තිබේ. මෙම අධ්‍යනයේදී ශ්‍රී ලංකික රෝගීන්ගේ ජානමය විවිධත්වය සහ එහි රෝග ලක්ෂණ වලට තිබෙන සම්බන්ධය පිළිබඳ පර්යේෂණය සිදු කරනු ලැබේ.මෙම ජාන විවිධත්වය හඳුනා ගැනීමෙන් එම රෝගී තත්වය සඳහා ප්‍රතිකාර කිරීමටත් පවුලේ අනිත් සාමාජිකයන්ගේ මෙම රෝගී තත්වය හට ගැනීමට හැකියාවක් තිබේද යන්නත් පරීක්ෂා කර ගත හැකිවේ.

2) ස්වේච්ඡා සහභාගිත්වය

මෙම අධ්‍යයනය සඳහා ඔබගේ සහභාගිත්වය ඔබගේ කැමැත්තෙන්ම සිදු කරන්නෙකි.

මෙම අධ්‍යයනය සඳහා ඔබට සහභාගි නොවීමට පූර්ණ අයිතිය ඇති අතර එමගින් දැනට ඔබට ලැබෙන ප්‍රතිකාර හෝ අනිකුත් වෛද්‍ය පහසුකම් කෙරෙහි කිසිදු බලපෑමක් ඇත නොවේ.මෙම පර්යේෂණට සහභාගි වීමට ඔබ කලින් කැමැත්ත ලබා දී තිබුණද ඔබට අවශ්‍ය අවස්ථාවක ඉන්

ඉවත් වීමට පූර්ණ අයිතිය ඇත. නමුත් එය කළ හැක්කේ පර්යේෂණ ප්‍රතිඵල ප්‍රසිද්ධියට පත් කිරීමට පෙර පමණි.

3) පර්යේෂණ කාල සීමාව ක්‍රියා පිලිවෙල හා සහභාගිවන්නන්ගේ වගකීම්

මෙම පර්යේෂණය අවුරුද්දක් තුළ සිදුකෙරේ. ඔබගේ සායනික වාර්තා ලබා ගැනීමට පවුලේ සාමාජිකයන්ගේ රෝගී තත්වයන් පිළිබඳ විස්තර ලබා ගැනීමට ඔබව සහ නෝරාගත් පවුලේ සාමාජිකයන් පරීක්ෂා කිරීමට ඔබගේ අවසරය අපට ලබාදිය යුතුවේ. රුධිර කොලෙස්ටරෝල් මට්ටම පරීක්ෂා කිරීම සඳහා රුධිර සාම්පලයක් (මිලි ලීටර් **5**) ලබා ගනු ලැබේ. තවද එකතු කරගත් දත්ත සහ ප්‍රතිඵලයන් ප්‍රකාශයට පත් කිරීමට ඔබගේ අවසරය අවශ්‍ය වේ. මෙහිදී ඔබගේ අනන්‍යතාවය හෙළි නොවන ලෙස ඒවා ප්‍රකාශයට පත් කෙරේ. එක් වරක් සහභාගි වීමෙන් අවශ්‍ය දත්ත සහ රුධිර සාම්පලය අපට ලබා දිය හැක.

4) ලද හැකි ප්‍රතිලාභ

මෙම අධ්‍යනයට සහභාගි වීමෙන් පරම්පරාගත අධික කොලෙස්ටරෝල් භාවය පවතින රෝගීන් හඳුනාගැනීමට උපකාරීවේ.මෙමගින් අධික කොලෙස්ටරෝල් භාවයට හේතුවන ජාන විකෘති තාවය හඳුනා ගතහැකි වන අතර මෙය ඔබගේ පවුලේ සාමාජිකයන්ටද වැදගත් වනු ඇත.

5) අවදානම් අනතුරු සහ අපහසුතා

රුධිර කොලෙස්ටරෝල් මට්ටම පරීක්ෂා කිරීම සඳහා මිලිලීටර් **5** ක පමණ රුධිර සාම්පලයක් ලබා ගත යුතු අතර මෙම සාම්පලය ලබා ගැනීමේද යම් අපහසුතාවයක් ඇතිවිය හැකිය. එන්නත් කටුව නිසා එම ස්ථානයේ වේදනාවක් තැල්මක් හෝ කලාතුරකින් ආසාදනයක් හටගත හැක. මෙය අවම කිරීමට පුහුණු හෙදියක් විසින් සාම්පලය ලබා ගැනේ.

6) දීමනා

ඔබ මෙම අධ්‍යනයට සහභාගි වීම නිසා ඔබට දීමනාවක් නොලැබේ. එහෙත් ඔබට රුධිර කොලෙස්ටරෝල් වාර්තාවක් සහ ඒ පිළිබඳ දැනුවත් කිරීමක් මානව ප්‍රවේණි විද්‍යා ඒකකය ලබා දෙනු ලැබේ.

7) රහසිගත භාවය

සියලුම තොරතුරු සහිත වාර්තාවන් සහ අධ්‍යයනය මගින් ලබාගන්නා දත්තයන්ගේ රහසිගතභාවය තහවුරු කරන අතර ඔබගේ අනන්‍යතාවය හෙළිවන ආකාරයේ කිසිවක් ඔබගේ කැමැත්තෙන් තොරව හෙළිකිරීමක් ඉදිරිපත් කිරීමක් හෝ ප්‍රකාශයට පත් කිරීමක් සිදු කරනු නොලැබේ.

8) අධ්‍යයනයට සහභාගි වීම අහෝසි කිරීම

අධ්‍යයනයට සහභාගි වීමට ඔබ මුලදී කැමැත්ත ප්‍රකාශ කළද ඔබට අවශ්‍ය ඕනෑම අවස්ථාවක ලබා දුන් කැමැත්ත ඉවත් කර ගැනීමට අවසර ඇත. එසේ සිදු කිරීමට අවශ්‍ය වූ විගස එය පර්යේපකයන්ට දැනුම් දීම අවශ්‍ය වේ. එමගින් දැනට ඔබට ලැබෙන ප්‍රතිකාර හෝ අනිකුත් වෛද්‍ය පහසුකම් කෙරෙහි කිසිදු බලපෑමක් ඇති නොවේ. නමුත් එසේ කල හැක්කේ එකතු කරගත් දත්ත හෝ ප්‍රතිඵල ප්‍රකාශයට පත් කිරීමට පෙර පමණි.

9) වැඩිදුර තොරතුරු

ඔබට මෙම අධ්‍යනයට හෝ එහි ක්‍රියාපටිපාටිය පිළිබඳ කිසියම් ප්‍රශ්නයක් ඇතිනම් හෝ වැඩිදුර තොරතුරු අවශ්‍ය නම් කරුණාකර පහත සඳහන් වෛද්‍යවරුන් අමතන්න.

මහාචාර්ය වජිර දිසානායක

මහාචාර්ය රොහාන් ජයසේකර

වෛද්‍ය ගොඩිවින් කොන්ස්ටන්ටයින්

වෛද්‍ය රසිකා රාජපක්ප

දුරකථන 0112689545

CONSENT FORM FOR FIRST DEGREE RELATIVE

A descriptive study of mutations in the Low Density Lipoprotein receptor gene (*LDLR*) among patients with familial hypercholesterolaemia in Sri Lanka

To be completed by the participant

The participant should complete the whole of this sheet by himself/ herself

1. Have you read the information sheet? (Please keep a copy for yourself) YES/NO

2. Have you had an opportunity to discuss this study and ask any questions? YES/NO

3. Have you had satisfactory answers to all your questions? YES/NO

4. Have you received enough information about the study? YES/NO

5. Who explained the study to you?

……

6. Do you understand that you are free to withdraw from the study at any time, without having to give a reason and without affecting your future medical care? YES/NO

7. Sections of your medical notes, including those held by the investigators relating to your participation in this study may be examined by other research assistants. All personal details will be treated as STRICTLY CONFIDENTIAL. Do you give your permission for these individuals to have access to your records? YES/NO

8. Have you had sufficient time to come to your decision? YES/NO

9. Do you agree to take part in this study? YES/NO

10. Do you agree to let us take photographs of you? YES/NO

11. Do you give consent to the publication of one or more photographs in a scientific journal? YES/NO

13. Do you agree to keep the leftover sample for future studies relevant to Familial hypercholesterolaemia under the supervision of the supervisors ? YES/NO

PARTICIPANT' NAME (BLOCK CAPITALS)

…….

Participant's signature……..……

Date…….

INVESTIGATOR

I have explained the study to the above volunteer and he/ she has indicated her willingness to take part in the study.

Signature of investigator……...…….....

Date…….

Name (BLOCK CAPITALS)

…….....

කැමැත්ත ප්‍රකාශ කිරීමේ පත්‍රය

(a) සහභාගී වන්නන් විසින් පිරවීම සඳහාය.

මෙම පත්‍රය සහභාගී වන්නන් /භාරකරුවන් විසින් සම්පූර්ණයෙන් පිරවිය යුතුය.

1. අධ්‍යයනය සම්බන්ධයෙන් තොරතුරු පත්‍රිකාවෙ පැහැදිලි කරන ලද කරුණු ඔබට තේරුනාද? (කරුණාකර තොරතුරු පත්‍රිකාවේ පිටපතක් ඔබ ලබාගන්න)

ඔව්/නැහැ

2. මෙම අධ්‍යයනය සම්බන්ධව සාකච්ඡා කිරීමට හා ඒ පිළිබඳව ප්‍රශ්න ඇසීමට ඔබට අවස්ථාවක් ලැබුණා ද?

ඔව්/නැහැ

3. ඔබ ඇසූ ප්‍රශ්න සියල්ලටම සෑහීමකට පත්විය හැකි පිළිතුරු ලැබුණාද?

ඔව්/නැහැ

4. මෙම අධ්‍යයනය සම්බන්ධයෙන් ප්‍රමාණවත් තොරතුරු ලැබුණාද? **ඔව්/නැහැ**

5. මෙම අධ්‍යයනය සම්බන්ධයෙන් ඔබට පැහැදිලි කරන ලද්දේ කවුරුන් විසින්ද?

.....

6. කිසිඳු කරුණු දැක්වීමකින් තොරව, මෙම අධ්‍යයනයෙන් ඉවත් වීමට ඔබ හට ඕනෑම අවස්ථාවක හැකියාව ඇති බව පැහැදිලි වූවාද?

ඔව්/නැහැ

7. ඔබෙ වෛද්‍ය වාර්තා සහ පර්යේෂණ දත්ත මෙම අධ්‍යයනය සම්බන්ධ සාමාජිකයින් විසින් අධ්‍යයනය කෙරෙ සියළු වාර්තා සහ දත්තවල රහස්‍යභාවය තහවුරු කෙරෙ මෙම අධ්‍යයනය සම්බන්ධ සාමාජිකයින්ට තොරතුරු ලබා දීමට එකඟ වෙනවාද?

ඔව්/නැහැ

8. මෙම අධ්‍යයනයෙන් පසුව ඉතිරි වන රුධිර සාම්පලයක් ඇතොත් ඉදිරියෙ පරම්පරාගත අධික කොලෙස්ටරෝල් භාවය රෝගය සම්බන්ධ පර්යේෂණ සඳහා භාවිතා කිරීමට ඔබ එකඟ වෙනවාද?

ඔව්/නැහැ

9. රුධිර සාම්පල පිටරට යැවීමට එකඟ වෙනවාද?

ඔව්/නැහැ

10. මෙම අධ්‍යයනයට සහභාගී වීම සම්බන්ධයෙන් තීරණයකට එළඹීමට ඔබට ඇති තරම් කාලය ලැබුණා ද?

ඔව්/නැහැ

11. ඔබ මෙම අධ්‍යයනයට සහභාගී වීමට එකඟ වෙනවාද? **ඔව්/නැහැ**

සහභාගී වන්නන්ගෙ /භාරකරුවන්

අත්සන:..... දිනය:.....

නම:.....

(b) පර්යේෂක විසින් පිරවීම සඳහාය.

මෙම අධ්‍යයනය සම්බන්ධ කරුණු, මා විසින් අධ්‍යයනයට ස්වේච්ඡාවෙන් සහභාගී වන්නන් හට පැහැදිලි කරන ලදී. ඔහු/ඇ විසින් මෙම අධ්‍යයනයට සහභාගී වීමට කැමැත්ත ප්‍රකාශ කරන ලදී.

පර්යේෂකගේ අත්සන:..... දිනය:.....

නම:.....