UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA

Molecular study of Familial Dyslipidaemias by Next Generation Sequencing

Beatriz de Carvalho Vieira Raposo Miranda

Mestrado em Bioquímica
Especialização em Bioquímica Médica

Dissertação orientada por:
Doutora Mafalda Bourbon
Doutora Ana Catarina Alves

2022

Acknowledgements

A realização desta dissertação de mestrado contou com o apoio e incentivo de diversos elementos aos quais estarei eternamente grata.

"If I have seen further it is by standing on the shoulders of Giants." (Issac Newton)

Primeiramente, quero agradecer à Doutora Mafalda Bourbon por me ter recebido prontamente no seu grupo de investigação (GIC) e por ter orientado a minha dissertação de mestrado. A sua preocupação, dedicação e entusiasmo pela investigação tornaram-me numa aluna mais capaz e mais apaixonada por ciência. O segundo agradecimento é dirigido à coorientadora deste trabalho, a Doutora Ana Catarina Alves, por me ter acompanhado (dentro e fora do laboratório) e motivado durante todas as fases deste projeto. Desta forma, quero agradecer a ambas as orientadoras por toda a compreensão demonstrada, pela confiança que depositaram em mim e no meu trabalho, e em último, por tudo o que me têm ensinado. Um grande Obrigada por terem (e me ensinarem a ter) "amor à camisola", e por terem sido verdadeiras mentoras.

"I am among those who think that science has great beauty." (Marie Curie)

Também a todos os outros elementos do GIC (Ana, Joana, Paulo, e Maria) devo um agradecimento por terem feito parte de uma etapa tão importante da minha vida, e por me terem ensinado o que realmente significa "trabalho de grupo". Um particular agradecimento à Ana Medeiros, pela sua alegria, e por estar sempre disposta a explicar-me tudo o que eu pergunto (teórico ou prático, com direito a esquema). De igual forma, gostaria de fazer uma agradecimento individualizado à Joana Chora por toda a ajuda, principalmente no que diz respeito a *guidelines*, e por todo o *brainstorming* durante as reuniões de grupo. Para além do GIC, gostaria de agradecer aos restantes grupos de investigação do Departamento de Promoção da Saúde e Doenças Não Transmissíveis do INSA pelo fantástico ambiente que se vive nas bancadas dos laboratórios do Edifico Lemes, e por se mostrarem sempre disponíveis para me ajudar. Uma palavra de agradecimento é também dirigida a todos os doentes (incluindo as respetivas famílias) e médicos que colaboram com o GIC ao fazerem parte do Estudo Português de Hipercolesterolemia Familiar (coordenado pela Doutora Mafalda Bourbon), pois sem eles, e sem o seu contributo, a realização deste trabalho não seria possível.

"Above all, don't fear difficult moments. The best comes from them." (Rita Levi-Montalcini)

À Márcia, (dedico aproximadamente cinco linhas, como combinado) por ser um apoio constante e uma verdadeira amiga nestes últimos cinco anos, por amparar lágrimas e partilhar risos, por ser minha cúmplice em tantos planos. Não tenho palavras para descrever o quão essencial foste nesta jornada, só posso agradecer por tudo até agora e por seres como um malmequer. Obrigada por seres uma excelente colega de casa, juntamente com a Bruna e a Margarida. Obrigada por me terem feito adorar Lisboa (a "nossa" casa), e por serem um verdadeiro algoritmo de felicidade.

Ao Vasco, que torna tudo mais leve e fácil, que é um dos meus maiores apoios, e para o qual 200-300 km não são nada, um grande Obrigada.

Deixo um agradecimento ao meus colegas de mestrado, particularmente à Filipa por me ter acolhido tão bem na FCUL e por me ter notado desde a passadeira! Obrigada por teres sido uma verdadeira companheira nestes últimos dois anos, e por representares uma amizade incrível, forjada em pouco tempo mas com tanto significado para mim.

À minha família académica, obrigada por todos os momentos que passámos juntos! Por me terem feito crescer e por ter encontrado em vocês verdadeiros amigos. Em especial, à minha Madrinha Ana, por ser morada e ser amparo tanto nas horas vagas como nas horas ocupadas; e, aos meus "irmãos", por toda a paciência e por estarem sempre cá para mim. Aos meus afilhados, obrigada por serem sempre Luz para mim, por me animarem, confiarem e acreditarem em mim.

Aos meus amigos Daniela, Joana e Leonardo, obrigada por (15 anos de) tudo. Já são amigos de uma vida inteira, e continuarão a sê-lo. Estou muito grata por vos ter na minha vida, obrigada por todo o carinho e apoio em (literalmente) todas as fases da minha vida.

O ultimo agradecimento, e um dos maiores, é dirigido à minha família. Aos meus pais, Carla e Carlos, pois sem eles nenhuma das minhas conquistas seria possível. Obrigada por nunca me terem "cortado as asas", e por me terem proporcionado todas as condições para estudar em duas cidades diferentes sem nunca me pedirem algo em troca. Obrigada por acreditarem sempre em mim e por valorizarem a minha felicidade. À minha irmã, Matilde, um gigantesco obrigada por me apoiar sempre, por ser o melhor presente que os meus pais me poderiam ter dado, e por ser uma amiga ainda melhor do que eu poderia ter pedido.

Abstract

Dyslipidemia, a clinical condition defined by an abnormal concentration of lipids in the blood, can have a genetic etiology. Familial dyslipidemias constitute a group of genetically determined conditions, the majority being rare. In addition, these are often associated to serious conditions that can be prevented by the early identification of patients. Hypercholesterolemia promotes atherosclerosis, increasing patients' cardiovascular risk. Neurological manifestations and poor weight progression are frequent complications of hypocholesterolemia, and an increased risk of pancreatitis is often verified in cases of hypertriglyceridemia.

This project aimed to identify the genetic cause of dyslipidemia in 96 Portuguese individuals, referred to the Cardiovascular Research Group at the National Health Institute, with a clinical diagnosis of several genetic dyslipidemias associated to different traits: hypercholesterolaemia, hypocholesterolaemia, and hypertriglyceridaemia.

The lipid profile of the 96 index cases was determined for biochemical characterization. The molecular study of individuals was performed by Next Generation Sequencing with a customised target panel of 57 genes involved in lipid metabolism. Molecular diagnosis was provided after analysis of 18 genes strongly associated with several familial dyslipidemias. Rare variants detected were confirmed by PCR and Sanger Sequencing.

A definite cause of monogenic dyslipidemia was established in 35 cases: 22 individuals were diagnosed with familial hypercholesterolaemia, 3 with familial hypobetalipoproteinemia, 2 with familial chylomicronemia syndrome, 7 with multifactorial chylomicronemia, and one with autosomal recessive hypertriglyceridemia. Moreover, several variants with uncertain significance were found by NGS during this project, the majority lacking functional data. As these variants can constitute the cause of disease in some cases, functional studies will be essential to assess variant’s pathogenicity.

This work allowed an early and correct identification of Portuguese patients with different familial dyslipidemias, thus providing guidance for pharmacological treatment and lifestyle adaptations to reduce the chance of suffering disease complications, improving this way patients’ prognosis.

Key words: familial dyslipidemia, Next Generation Sequencing, molecular diagnosis, variants.

Resumo

A dislipidemia constitui uma condição caracterizada por alteração do perfil lipídico, variando entre valores baixos ou elevados de várias partículas lipídicas, nomeadamente colesterol LDL (LDL-c) e/ou colesterol HDL (HDL-c) e/ou triglicéridos. Esta condição pode ser determinada geneticamente, sendo designada de dislipidemia primária ou familiar. As dislipidemias familiares são um grupo de doenças do metabolismo lipídico, maioritariamente raras, com exceção da hipercolesterolemia familiar (FH) cuja prevalência estimada é de 1/300 indivíduos.

As dislipidemias familiares encontram-se relacionadas com diversas condições clínicas graves. Uma elevação anormal dos níveis plasmáticos de colesterol LDL(LDL-c) e/ou colesterol total traduz-se clinicamente em hipercolesterolemia. Uma vez que a acumulação destas partículas lipídicas promove aterosclerose, e sendo este um processo que pode resultar em eventos cardiovasculares e morte em idade precoce, a hipercolesterolemia está associada ao aumento do risco cardiovascular dos doentes. No caso de hipocolesterolemia, defeitos genéticos levam à diminuição drástica dos níveis plasmáticos de LDL-c. As principais complicações associadas a esta condição são problemas neurológicos, e/ou atrasos do crescimento. Em doentes cujo perfil lipídico é caracterizado por uma elevada concentração plasmática de triglicéridos (hipertrigliceridemia), complicações como dor abdominal, pancreatites recorrentes ou outros problemas hepáticos (como esteatose e hepatomegalia) são normalmente observados. As complicações que advêm como consequência da dislipidemia familiar (independentemente do tipo de partícula lipídica aumentada ou diminuída) podem ser prevenidas se existir uma identificação molecular precoce destes doentes.

O principal objetivo deste estudo foi a identificação molecular da causa de dislipidemia familiar em 96 indivíduos portugueses referenciados ao Grupo de Investigação Cardiovascular do Instituto Nacional de Saúde Doutor Ricardo Jorge com um diagnóstico clínico de uma de três primordiais dislipidemias (hipercolesterolemia, hipocolesterolemia e hipertrigliceridemia).

De forma a melhor caracterizar estes indivíduos com suspeita clínica de dislipidemia familiar, o seu perfil lipídico foi determinado. A análise molecular desta amostra populacional foi feita com recurso à tecnologia de Sequenciação de Nova Geração (NGS). Para um diagnóstico e uma investigação focada apenas em dislipidemias familiares, foi utilizado um painel personalizado e composto por 57 genes envolvidos no metabolismo lipídico. Esta metodologia permitiu a análise simultânea de 57 genes, por indivíduo. Após a sequenciação massiva com recurso à plataforma NextSeq da Illumina, os resultados gerados foram analisados com recurso aos programas SureCall e wANNOVAR que permitiram identificar as diversas variantes encontradas em cada amostra. Para além destas ferramentas bioinformáticas, o programa DECoN foi utilizado para análise de variações estruturais tais como duplicações e deleções (CNVs, *copy number variation*). Cada caso index foi analisado individualmente e a relação genótipo-fenótipo foi estudada.

Neste projeto, considerando a vastidão de informação e dados gerados pela técnica de NGS, a análise genética dos casos index teve por base (como primeira abordagem) somente 18 genes previamente associados a distintas dislipidemias primárias. Os 18 genes foram subdivididos em paneis com um número de genes mais reduzido de acordo com o fenótipo clinico com o qual estão relacionados: painel FH e fenocópias (*LDLR*, *APOB*, *PCSK9*, *LDLRAP1*, *ABCG5*, *ABCG8*, *APOE*, *LIPA*), painel de hipocolesterolemia (APOB, *PCSK9*, *MTTP*, *SAR1B*, *ANGPTL3*), e painel de patologias associadas aos triglicéridos (*LPL*, *APOC2*, *APOA5*, *GPIHBP1*, *LMF*, *GPD1* , *APOE*, *LIPA*, *APOC3*). Todas as variantes raras detetadas (com uma frequência alélica menor que 1% em caso de doença autossómica dominante, ou menor que 5% para doenças autossómicas recessivas) foram classificadas de acordo com

recomendações segundo ACMG (*American College of Medical Genetics and Genomics*). Desta forma, foi possível garantir a uniformização da análise e da classificação. Todas as variantes classificadas como patogénicas ou provavelmente patogénicas foram confirmadas por reação da polimerase em cadeia (PCR) e sequenciação de Sanger. Familiares de casos index, nos quais a causa monogénica de dislipidemia foi estabelecida, foram recrutados para este projeto e estudados através de PCR e sequenciação de Sanger. Assim, para além dos 96 casos index, 33 familiares foram também estudados.

No presente estudo, foram detetadas 61 variantes raras em 96 indivíduos. Para 35 casos index, a causa monogénica de dislipidemia foi estabelecida. Uma vez que estes casos apresentavam variantes classificadas como patogénicas (ou provavelmente patogénicas) em concordância com o fenótipo clínico apresentado, foi possível conferir diagnóstico definitivo de: hipercolesterolemia familiar (22 casos), hipobetalipoproteinemia familial (3 casos), síndrome quilomicronemia familiar (2 casos), quilomicronemia multifactorial (7 casos), e hipertrigliceridemia autossómica recessiva (1 caso). O estudo dos 33 familiares incluídos neste projeto culminou no diagnóstico genético de 18 familiares.

Apesar de em aproximadamente 63% dos casos não ter sido possível estabelecer uma causa de dislipidemia familiar, um total de 31 variantes raras com significado clinico incerto (VUS) foram detetadas por NGS em 24 casos index. Este conjunto de VUS foi detetado em genes associados a FH (*APOB*, *LDLR* e *PCSK9*), sitosterolemia (*ABCG5/8*), síndrome de quilomicronemia familiar (*APOA5*) e deficiência em lipase ácida (*LIPA*). É fundamental realçar que embora não tenha sido atribuído um diagnóstico molecular com bases nestas descobertas, estas variantes podem constituir, em alguns casos, a causa de dislipidemia. As VUS são principalmente caracterizadas por grandes lacunas de informação a nível populacional e clínico. De forma a investigar o significado clínico das variantes raras detetadas (cujo significado clínico é incerto), estudos funcionais serão essenciais para testar *in vitro* o potencial impacto das mesmas.

Os 36 casos index nos quais não foram detetadas variantes raras patogénicas ou de significado clínico incerto serão analisados novamente (expandido o número de genes analisados, e/ou resequenciados) num futuro próximo, em ambiente de investigação.

No decorrer deste projeto, oito variantes raras foram detetadas pela primeira vez Portugal. Quatro estão relacionadas com o perfil clínico de hipercolesterolemia familiar: duas localizadas no gene *LDLR*, e uma em cada um dos genes *APOB* e *PCSK9*. As quatro restantes alterações foram detetadas no gene *ABCG8* (sitosterolemia) e *APOB* (associadas a hipocolesterolemia). Todas estas variantes não se encontram descritas ou reportadas em bases de dados públicas.

Estudos funcionais utilizando RNA foram iniciados, e ainda estão em curso, de forma a estudar uma variante possivelmente causadora de *splicing* (c.621G>A/p.(Gly207Gly)) no exão 4 do gene *LDLR*. Esta variante foi detetada num caso index adulto com diagnóstico clínico de FH. Apesar de se tratar de uma variante sinónima, é fundamental investigar o possível impacto da mesma, uma vez que uma alteração no mesmo nucleótido (c.621G>C/p.(Gly207Gly)) se encontra caraterizada na literatura como afetando o splicing e co segrega com o fenótipo de FH. Também para uma variante no gene *GPIHBP1* (uma grande deleção de dois exões, em homozigotia), o estudo ainda está a decorrer de forma a identificar exatamente a posição dos nucleótidos deletados e a confirmar a heterozigotia (obrigatória) dos pais deste caso index (uma criança com hipertrigliceridemia severa).

O estudo molecular de dislipidemias familiares por NGS contribuiu para uma abordagem personalizada de diagnóstico de individuos portugueses com suspeita clínica destas patologias genéticas. O estabelecimento do diagnóstico molecular de dislipidemia familiar em 35 casos foi importante para estudar e/ou identificar (através de *cascade screening*) os familiares destes index, os quais não tinham

conhecimento da sua condição até serem incluídos neste projeto. Assim, a confirmação molecular providencia a identificação precoce dos doentes, permitindo a melhor gestão do risco de desenvolvimento de complicações associadas a estas doenças genéticas (tais como doença cardiovascular, problemas neurológicos e do desenvolvimento, e doença hepática e/ou pancreática). Em suma, a abordagem aplicada neste projeto constitui uma oportunidade para direcionar a abordagem terapêutica dos doentes tendo em conta o seu genótipo, e não só o seu fenótipo, permitindo um melhor acompanhamento dos mesmos, e contribuindo para uma melhoria da qualidade de vida de doentes portuguese com dislipidemias familiares.

Palavras-chave: dislipidemia familiar, Sequenciação de Nova Geração (NGS), diagnóstico molecular, variantes.

Abbreviations

aa – Amino acids
ABC – ATP-Binding Cassette
ABL – Abetalipoproteinemia
ACMG – American College of Medical Genetics and Genomics
AP-2 – Adaptor Protein complex 2
Apo – Apolipoprotein
ARHTG – Autosomal Recessive Hypertriglyceridemia
ASCVD – Atherosclerotic Cardiovascular Disease
ATP – Adenosine triphosphate
bp – Base Pair
CAD – Coronary Artery Disease
cDNA – Complementary DNA
CE – Cholesteryl Esters
CETP – Cholesteryl Ester Transfer Protein
CHD – Coronary Heart Disease
CNV – Copy Number Variation
CPT – Cell Preparation Tube (CPT™)
CVD – Cardiovascular Disease
dL – Deciliter
DNA – Deoxyribonucleic acid
dNTP – Deoxynucleotide triphosphates
EDTA – Ethylenediamine tetraacetic acid
EGF – Epidermal Growth Factor
ER – Endoplasmatic Reticulum
FCS – Familial Chylomicronemia Syndrome
FDB – Familial Defective Apolipoprotein B
FH – autosomal codominant Familial Hypercholesterolemia
FHBL – Familial Hypobetalipoproteinemia
g – gram
GIC – Grupo de Investigação Cardiovascular
GPD1 – Glycerol-3-Phosphate Dehydrogenase
GRS – Genetic Risk Score
HDL – High-Density Lipoprotein
HeFH – Heterozygous FH
HeFHBL – Heterozygous FHBL
HGP – Human Genome Project
HMG-CoA – 3-hidroxi-3-metilglutaril coenzima A
HoFH – Homozygous FH
HTG – Hypertriglyceridemia

IC – Index case
IDL – Intermediate-Density Lipoprotein
INSA – Instituto Nacional de Saúde Doutor Ricardo Jorge
kb – Kilobases
L – Liter
LALD – Lysosomal Acid Lipase Deficiency
LCAT – Lecithin-Cholesterol Acyltransferase
LDL – Low-Density Lipoprotein
LDL-c – LDL cholesterol
LDLR – LDL receptor
LDLRAP1 – LDL Receptor Adaptor Protein 1
LOF – Loss-of-function
Lp(a) – Lipoprotein (a)
LPL – Lipoprotein Lipase
LRP – LDL Receptor-related Protein
M – Molar Concentration
MAF – Minor Allele Frequency
MCM – Multifactorial Chylomicronemia Syndrome
mg – Miligram
ml – Mililiter
mmol – Milimolar
mRNA – Messenger Ribonucleic acid
MTP – Microsomal Triglyceride transfer Protein
NGS – Next Generation Sequencing
nM – Nanomolar
ºC – Celsius degree
PCR – Polymerase Chain Reaction
PCSK9 – Proprotein Convertase Subtilisin/Kexin type 9
RNA – Ribonucleic acid
rpm – Revolutions per Minute
SNP – Single Nucleotide Polymorphism
TG – Triglycerides
V – Volt
VLDL – Very Low-Density Lipoprotein
VUS – Variant of Unknown Significance
µl – Microliter
ρmol – Picomol

Contents

Acknowledgements .... **Erro! Marcador não definido.**

Abstract iii

Resumo iv

Abbreviations vii

List of tables / List of figures xi

CHAPTER 1. INTRODUCTION

1.1. LIPID METABOLISM 1
1.1.1. Lipids, Cholesterol and Triglycerides 1
1.1.2. Lipoproteins and Apolipoproteins 1
1.1.3. Metabolic Pathways 3
1.2. DYSLIPIDEMIAS 5
1.3. HYPERCHOLESTEROLEMIA 5
1.3.1. Autosomal codominant familial hypercholesterolemia 6
1.3.2. Autosomal recessive hypercholesterolemia 7
1.3.3. Sitosterolemia/Phytosterolemia 7
1.3.4. Polygenic Hypercholesterolemia 8
1.3.5. Management and treatment of hypercholesterolemia 8
1.4. FAMILIAL HYPOCHOLESTEROLEMIA 9
1.4.1. Familial Hypobetalipoproteinemia 9
1.4.2. PCSK9 gene alterations causing hypocholesterolemia 9
1.4.3. Abetalipoproteinemia 9
1.4.4. Anderson's Disease (Chylomicron retention disease) 10
1.4.5. Management and treatment of hypocholesterolemia 10
1.5. FAMILIAL HYPERTRIGLYCERIDEMIA 10
1.5.1. Familial Chylomicronemia Syndrome 10
1.5.2. Multifactorial Chylomicronemia 11
1.5.3. Recessive Hypertriglyceridemia 11
1.5.4. Lysosomal Acid Lipase Deficiency 12
1.5.5. Dysbetalipoproteinemia 12
1.5.6. Treatment and management of hypertriglyceridemia 12
1.6. STUDY OF GENETIC DISORDERS 13
1.7. OBJECTIVES 14

CHAPTER 2. MATERIALS AND METHODS

2.1. PATIENTS RECRUITMENT 15
2.1.1. Blood sample collection 16
2.1.2. Biochemical characterization 16
2.2. MOLECULAR BIOLOGY TECHNIQUES FOR DIAGNOSTIC 16
2.2.1. Genomic dna extraction 16
2.2.2. Next generation sequencing (NGS) 17
2.2.3. Dna amplification by polymerase chain reaction (PCR) 22
2.2.4. Sanger sequencing (automated sequencing) 22
2.3. VARIANTS CLASSIFICATION 23

2.4. Co-segregation studies 23
2.5. Functional studies using rna 23
2.5.1. Isolation of peripheral blood mononuclear cells (PBMC) 23
2.5.2. RNA extraction 24
2.5.3. Reverse transcriptase reaction and cdna analysis 24
2.5.4. cDNA amplification by polymerase chain reaction (PCR) 25

CHAPTER 3. RESULTS

3.1. Clinical characterization 27
3.1.1. Hypercholesterolemia 27
3.1.2. Hypocholesterolemia 27
3.1.3. Hypertriglyceridemia 27
3.2. Molecular study 31
3.2.1. FH and phenocopies panel 31
3.2.2. Hypocholesterolemia panel 31
3.2.3. Hypertriglyceridemia panel 31
3.3. Genetic diagnostic 36
3.3.1. FH and phenocopies panel 36
3.3.2. Hypocholesterolemia panel 37
3.3.3. Hypertriglyceridemia panel 38

CHAPTER 4. DISCUSSION

4.1. NGS as a diagnosis and research tool 41
4.2. Hypercholesterolemia 41
4.2.1. Familial Hypercholesterolemia 41
4.3. Hypocholesterolemia 44
4.3.1. Hypobetalipoproteinemia 44
4.4. Hypertriglyceridemia 45
4.4.1. Familial chylomicronemia syndrome 45
4.4.2. Multifactorial chylomicronemia 46
4.4.3. Autosomal recessive hypertriglyceridemia 46
4.4.4. Lysosomal acid lipase deficiency 47
4.4.5. HTG panel negatives 47
4.5. Functional studies 48
4.5.1. *LDLR* gene 48
4.5.2. *GPIHBP1* gene 48

CHAPTER 5. CONCLUSIONS AND PERSPECTIVES 51

CHAPTER 6. REFERENCES 513

Appendix I – Patients' recruitment 59

Appendix IA. Clinical questionnaire (familial hypercholesterolemia) 59

Appendix IB. Clinical questionnaire (hypocholesterolemia and hypertriglyceridemia) 59

Appendix II - Molecular biology techniques 79

Appendix III – Variant classification 81

List of Tables

Table 1.1. Characterization of human lipoproteins present in plasma 2

Table 2.1. Simon Broome Criteria for clinical diagnosis of Familial Hypercholesterolemia 15-16

Table 3.1. Clinical and biochemical characterization of 57 index cases with clinical hypercholesterolemia 28

Table 3.2. Clinical and biochemical characterization of 5 index cases with clinical hypocholesterolemia 30

Table 3.3. Clinical and biochemical characterization of 27 index cases with clinical hypertriglyceridemia 30

Table 3.4. Summary of variants detected by NGS in FH genes, analysed within the FH and phenocopies panel 32

Table 3.5. Summary of variants detected by NGS in genes associated with sitosterolemia (ABCG5 and ABCG8), and analysed within the FH and phenocopies panel 34

Table 3.6. Summary of variants detected by NGS associated with low LDL-c levels, analysed with the hypocholesterolemia panel. 34

Table 3.7. Summary of variants detected by NGS associated with high triglyceride levels, analysed using the hypertriglyceridemia panel 35

Table 3.8. Summary of the molecular diagnosis of FH for 22 ICs 36

Table 3.9. Summary of the molecular diagnosis of FHBL for 3 ICs 38

Table 3.10. Summary of the molecular diagnosis of FCS, MCM and autosomic recessive hypertriglyceridemia for 10 IC 39

Table A. II.1. Proportion of reagents per mL of blood for genomic DNA extraction 79

Table A. II.2. SureSelectQXT P7 Indexes 1 to 12, and P5 Indexes 13 to 20, with correspondent sequence 79

Table A. II.3. List of genes associated with familial dyslipidemias analysed during this project 79

Table A. II.4. PCR and Sanger Sequencing for all genes analysed by these techniques, primers and respective annealing temperatures 80

Table A. II.5. PCR and Sanger Sequencing for studies using cDNA 81

List of Figures

Figure 1.1. Illustrative overview of the lipid metabolism 4

Figure 1.2. Illustrative representation of the LDL receptor mediated pathway 5

Figure 2.1. Gene panels for molecular diagnosis of familial dyslipidemias 21

Figure 2.2. Overview of the workflow for the project "Molecular diagnosis of familial dyslipidemias by Next Generation Sequencing" 25

Figure 3.1. Cascade screening in IC58 family 37

Figure 3.2. Cascade screening in IC67 family 38

Figure 3.3. Summary of the molecular study of familial dyslipidemias by Next Generation Sequencing 40

Figure A.III.1. Schematic representation of evidence framework necessary to classify genetic variants 81

Figure A.III.2. Combination of criteria to attribute a clinical significance to sequence variants 82

Chapter 1. Introduction

1.1. Lipid Metabolism

1.1.1. Lipids, Cholesterol and Triglycerides

Lipids are a diverse group of organic compounds enriched in carbon and hydrogen, and main characterized for being insoluble in water but soluble in nonpolar solvents [1]. These molecules can be found in all tissues and cells of the body, and generally act as hormones or hormone precursors, assist in digestion, and play a major role in metabolism function and energy storage. Furthermore, lipids serve as functional and structural components of biomembranes [2]. Cholesterol is a molecule of extreme relevance in biology and studied over more than 3 decades, being essential for steroid hormone synthesis, bile acid metabolism, and as a building block for cellular platforms such as lipid rafts. Embedded in the cell membrane, it provides rigidity to the cell thus being an integral component of cell membranes due to its insolubility [3]. However, despite cholesterol function as a barrier to protect the cell by regulating its interaction with the other components, when it accumulates within the wall of an artery, it cannot be easily mobilized. Therefore, presence of this molecule can eventually lead to atherosclerosis, as it rapidly accumulates in the body when an excess from the diet or a genetic anomaly occurs [3,4]. The cholesterol present in plasma primarily results from two pathways: the endogenous, by hepatic cholesterol synthesis; and, the exogenous in which intestinal absorption of dietary cholesterol occurs, being subsequently transported to the liver where it is processed and distributed to the tissues for metabolic use [1]. Triglycerides constitute a key energy source in the human system, being formed by free fatty acids (carbon and hydrogen-enriched molecules) hence sharing structural features with phospholipids[5]. There are two distinct pathways for the synthesis of triglycerides according to their origin. When prevenient from the diet, triglycerides are hydrolysed in the intestine (exogenous pathway) to generate fatty acids and monoglycerides [6]. On the other hand, in the endogenous pathway, the major sites of endogenous triglyceride synthesis are the liver and adipose tissue. This way, triglycerides are formed and further processed in the liver [5,6].

1.1.2. Lipoproteins and Apolipoproteins

Since lipids have relative aqueous insolubility, lipid transport throughout the bloodstream is accomplished by specialized particles known as lipoproteins. These particles are complex transport vehicles, presenting a spherical structure. Lipoproteins carry non-polar lipids (such as triglycerides and cholesterol) in their core, and are constituted by polar lipids (phospholipids and free cholesterol) oriented on the surface as a single monolayer, and one or more specific proteins termed as *apolipoproteins* [1]. Lipoproteins have different physical and chemical properties due to different containing proportions of lipids and apolipoproteins (Table 1.1). Based on lipid and protein content, size, and densities (determined by ultracentrifugation), lipoproteins are commonly categorized as chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and lipoprotein(a) [Lp(a)]. Lp(a) is a distinct lipoprotein that is structurally related to LDL, as both contain a singular apolipoprotein (apo) B-100 molecule *per* particle and a similar lipid composition [1,7]. Protein molecules known as apolipoproteins are essential components of lipoproteins and, in general, control lipoprotein metabolism. Each class of lipoproteins contains a variety of apolipoproteins in different proportions. LDL particle is an exception as it is the only lipoprotein that exclusively contains apo B-100 [1]. Collectively, apolipoproteins have three main physiologic functions: ensure the structural integrity of lipoprotein particles, activate enzymes across lipoprotein metabolism pathways, and facilitate the uptake of lipoproteins into cells through recognition by specific cell surface receptors [7].

Table 1.1 - Characterization of human lipoproteins present in plasma

Lipoprotein	Density (g/mL)	Diameter (nanometre)	Lipid – Lipoprotein ration	Major lipid content	Apolipoproteins
Chylomicron	<0.95	>70	99:1	Exogenous triglycerides	A-I, B-48, C-I, C-II, C-III
VLDL	0.95-1.006	27-70	90:10	Endogenous triglycerides	B-100, C-I, C-II, C-III, E
IDL	1.006-1.019	22-24	85:15	Endogenous triglycerides, cholesteryl esters	B-100, E
LDL	1.019-1.063	19-23	80:20	Cholesteryl esters	B-100
HDL	1.063-1.210	4-10	50:50	Phospholipids	A-I, A-II
Lp(a)	1.040-1.130	27-30	75:25-64-36	Cholesteryl esters, phospholipids	(a), B-100

VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Lp(a), lipoprotein (a). Adapted from [(1)].

Apolipoprotein AI and AII are the major structural proteins of HDL particle. Due to the relative weakness associated to the binding of their amphipathic helices to lipoproteins, these apolipoproteins can exchange linkage between different lipoproteins during metabolism. Apo A-I is synthesized in the liver and intestine, and serves as cofactor for lecithin-cholesterol acyltransferase (LCAT), an enzyme that converts free cholesterol into cholesteryl ester [(1)]. Apo A-II is also synthesised in the liver but the specific role of this apolipoprotein in metabolism remains unknown.

Apolipoprotein B is found in the body in two major forms, apo B-48 and apo B-100. Apo B-100 is produced in the liver and secreted into the bloodstream as a component of VLDL, IDL, or LDL. Apo B-100 is the major apolipoprotein of LDL, and its measurement serves as an indicator for LDL particle concentration [(1,6)]. The intestine alone produces apo B-48, which results from post transcriptional modification of internal apo B-100 mRNA, being identical to the first 48% of apo B-100 and the major apolipoprotein found in chylomicrons [(1)].

Apolipoprotein C is found in chylomicrons and VLDL, where it specifically modulates the metabolism of these triglyceride-rich lipoproteins by its effect on LPL (lipoprotein lipase) activity. Apolipoprotein C family proteins are mostly produced by the liver and found in three major forms: apo C-I, C-II, and C-III. Functionally, apo C-I inhibits VLDL uptake via hepatic receptors; apo C-II activates LPL and is required for the efficient processing of chylomicrons and VLDL in the circulation; and, Apo C-III inhibits lipolysis by interfering with the interaction of these lipoproteins with the LPL complex [(1)].

Apolipoprotein E is a lipid transport protein, primarily synthesized by liver hepatocytes, that acts as a major ligand for LDL receptors (LDLR). Furthermore, is the primary apolipoprotein of chylomicrons and VLDL, mediating lipid catabolism [(8)]. This apolipoprotein has three common alleles encoded by the *APOE* gene, which are denoted as ε2, ε3, and ε4 [(9)]. Isoform ε2 presents poor affinity for the LDL receptor resulting in the accumulation of apo E-containing lipoproteins in the circulation, thus being associated with lower concentrations of LDL-cholesterol (LDL-c) [(8,10)]. Contrarily, Apo E4 is characterized by an efficient biding of particles to the LDLR, and for promoting a preferential linkage to triglyceride-rich and very-low-density lipoproteins, which leads to LDLR downregulation. Therefore, LDL particles accumulate in plasma consequentially increasing the atherogenic risk which leads to cardiovascular disease (CVD) [(1,9)].

Lipoprotein(a) is a macromolecular complex in plasma composed by the assembly of one molecule of LDL-particle (containing apolipoprotein B-100) with a molecule of apolipoprotein (a), the second being a large and highly polymorphic glycoprotein [(11)]. Apolipoprotein (a) is synthesized in the liver, and its physiologic function remains unknown. Lp(a) levels is barely influenced by external factors as dietary or physical activity, being regulated independently from LDL. There is considerable clinical and epidemiological evidence linking high Lp(a) levels with an increased risk of atherosclerosis [(1,7)].

1.1.3. Metabolic Pathways

1.1.3.1. Exogenous Pathway

The primary function of the intestinal or exogenous pathway is the absorption of dietary lipids and their delivery to peripheral tissues and the liver. In the exogenous pathway (Figure 1.1A), ingested lipids (mostly triglycerides[5]) are synthesised by enterocytes (intestinal cells) and joint with apo B-48, thus being incorporated in chylomicrons to circulate in the lymphatic system. After secretion, apo C and apo E are incorporated into the lipoprotein within the circulation as a result of transfer from HDL, while apo A, despite playing a minor role in the structure and metabolism of chylomicrons, is included in these lipoproteins when released from the intestine [12]. LPL, which is attached to the luminal surface of capillary endothelial cells, uses apo C-II as a cofactor. LPL hydrolyses triglyceride fatty acids, allowing them to be taken up by peripheral tissues (muscle or adipose cells) or bound and transported within circulation by albumin to other tissues, including the liver. "Chylomicron remnants" are partially lipolyzed chylomicrons that contain cholesterol, phospholipids, apolipoproteins, and very little triglyceride. The majority of apo A and a portion of apo C are transferred to HDL, with the remainder available for catabolism by the liver. Because remnants contain apo B-48 and apo E, they are recognized and internalized by an LDL receptor or an LDL receptor-related protein (LRP), both of which endocyte the remnants into lysosomes, where the remaining lipoprotein components are hydrolysed. Cholesterol entering hepatocytes can be used to synthesize bile acids, incorporated into newly synthesized lipoprotein, effluxes to apo A-I particles, secreted directly into bile, or stored as cholesteryl ester. Cholesterol derived from chylomicron remnants inhibits HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [1,12].

1.1.3.2. Endogenous pathway

The endogenous pathway (Figure 1.1B), also known as hepatic, involves the delivery of hepatic synthesised lipids to peripheral cells, being very similar to the exogenous pathway [13]. However, in this pathway, chylomicrons deliver endogenously produced cholesterol and triglycerides to peripheral cells as well as the liver. This transport is accomplished by the release of VLDL particles containing apo B-100 as well as varying amounts of apo C and E apolipoproteins [1,13]. VLDL triglyceride is significantly lower than chylomicron triglyceride since VLDL particles are smaller and have fewer interactions with lipoprotein lipase. Nonetheless, when these particles interact with LPL in capillaries, the VLDL triacylglycerol core is hydrolysed (as observed in the exogenous pathway). Furthermore, apo C is transferred back to HDL during triglyceride hydrolysis of VLDL in this pathway, and the VLDL particles are converted into remnant VLDL. Some of these proteins are removed by the liver, while others continue down the lipolytic cascade, becoming IDLs (smaller, denser proteins) that contain multiple apo E proteins, and bind to the remaining hepatocyte receptors before being removed from circulation. The unprocessed IDL particles are hydrolysed several times, and the remaining triglycerides and apolipoproteins are transferred to LDL [1,12]. Apo B, the only apolipoprotein in the LDL particle, is recognized by the LDL receptor on the liver and other tissues, which internalizes the lipoprotein, releasing cholesterol for cell membrane structure and steroid hormone synthesis [12].

1.1.3.3. Reverse cholesterol transport

HDL is the primary lipoprotein responsible for reverse cholesterol transport (Figure 1.1C). Cellular cholesterol is transported to the liver to be used or excreted via this pathway. The release of apo A-I from the liver and intestines is required for HDL formation [1]. As a result, HDL particles are secreted as nascent particles by the liver and intestines, consisting primarily of phospholipids and apo A-I; and, in the extracellular environment, phospholipids, cholesterol, and some apolipoproteins are added to their surface [14]. The free cholesterol found in extrahepatic cells is transferred to the nascent HDL particles

and stratified further by the enzyme LCAT in the presence of its cofactor apo A-I. The amount of cholesterol esters accumulated and the activity of the LCAT enzyme determine the size of HDL. Circulating HDLs that are cholesterol-rich, phospholipid-rich, or triglyceride-poor have several options for dispensing their lipid cargo [14]. Because CETP (cholesteryl ester transfer protein) facilitates the transfer of CE (cholesteryl esters) to VLDL and LDL, HDL particles can exchange CE for triglycerides. As a result, after receiving CE from HDL, apo B-100 particles can travel to the liver via specific receptors for these lipoproteins. Furthermore, as HDLs acquire triglycerides via CETP, they undergo increased lipolysis, resulting in the conversion of HDL particles into smaller subspecies [1,12]. This method removes cholesteryl esters from HDL, allowing it to re-enter the lipidation cycle and be reused to perform transport functions [12].

1.1.3.4. LDL receptor mediated transport

A receptor-based mechanism can remove cholesterol from LDL particles in the blood (Figure 1.2). Specific receptors (LDLR) are found on plasma membranes, primarily in the liver, and recognize and bind to apo B-100 on the surface of LDL particles. Endocytosis then internalizes the resulting receptor–ligand complex, forming a clathrin-coated endocytic vesicle [3]. LDL Receptor Adaptor Protein 1 (LDLRAP1) aids in the internalization of the LDLR. The LDLR separates from the LDL and is recycled back to the cell surface as a result of the acid pH created by ATP-driven proton pumps, whereas the LDL particle is degraded in the lysosomal compartment, it gives rise to small peptides and amino acids[15]. When the cholesterol esters in LDL particles are hydrolysed, the cholesterol is released and can be caused for a variety of cellular functions. LDLR also binds to the hepatocyte-secreted proprotein convertase subtilisin/kexin type 9 (PCSK9). When present in an endocytic vesicle, it binds the LDLR–LDL complex extracellularly and prevents it from dissociating (Figure 1.2), thereby targeting the entire complex for degradation in the lysosomal compartment and acting as a modulator of the LDLR pathway[16].

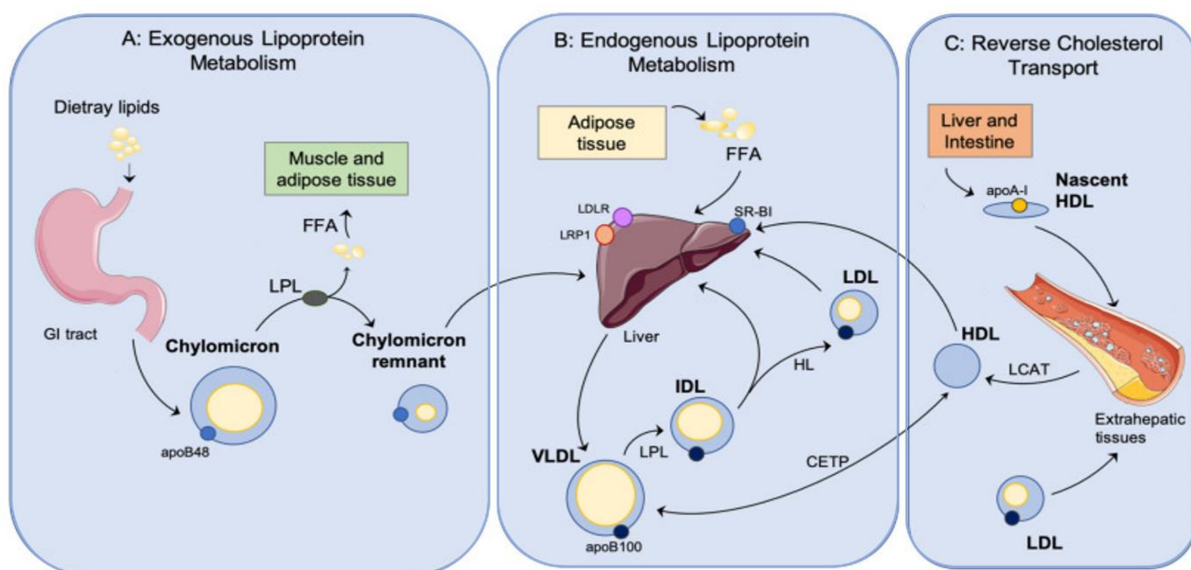

Figure 1.1 – Illustrative overview of the lipid metabolism. **(A)** Exogenous pathway. **(B)** Endogenous pathway. **(C)** Reverse cholesterol transport. GI, gastrointestinal; apoB48, apolipoprotein B-48; LPL, lipoprotein lipase; FFA, free fatty acids; VLDL, very low density lipoprotein; apoB100, apolipoprotein B-100; LRP1, LDL receptor-related protein 1; LDLR, low density lipoprotein receptor; IDL, intermediate-density lipoprotein; HL, hepatic lipase; LDL, low density lipoprotein; SR-B1, scavenger receptor B1; HDL, high density lipoprotein; CEPT, cholesteryl ester transfer protein; LCAT, cholesterol acyltransferase; apoA-I, apolipoprotein A-I. Adapted from [17].

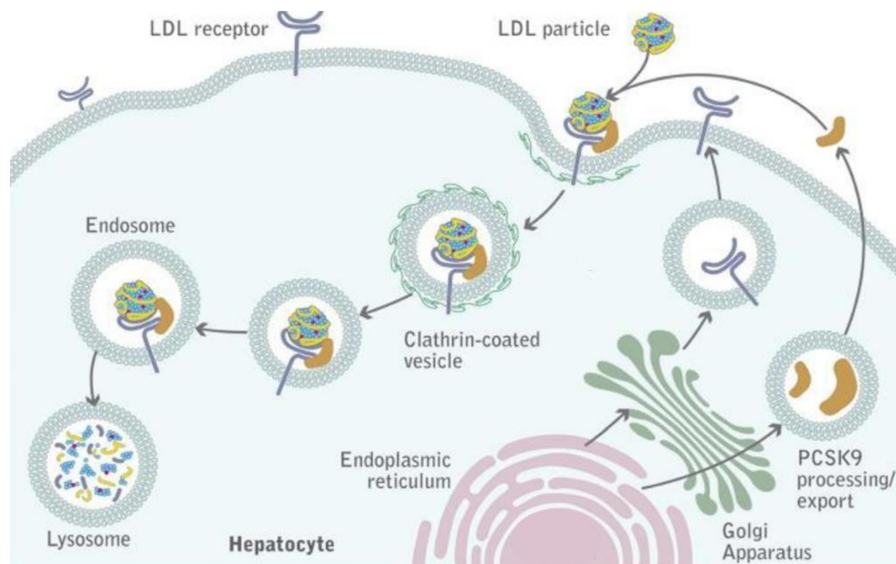

Figure 2.2 – Illustrative representation of the LDL receptor mediated pathway. LDL, low density lipoprotein; PCSK9, proprotein convertase subtilisin/kexin type 9. Adapted from [18].

1.2. Dyslipidemias

Dyslipidemia is a commonly encountered clinical condition defined as an abnormal concentration of lipids in the blood and ranges from raised to low plasma concentrations of total cholesterol, LDL cholesterol, HDL cholesterol or, triglycerides [19,20]. Dyslipidemias can be classified as hyperlipidemias, hypolipidemias or hypertriglyceridemias depending on whether there is an increase or decrease in plasma lipid levels, and thus depending on the metabolic pathway affected. Disorders in lipid metabolism are not exclusively related to lifestyle and can have a genetic etiology [19]. Therefore, dyslipidemias can be: genetically determined (known as primary or familial dyslipidemias) with or without physical manifestations; or secondary, being related with other conditions (such as diabetes mellitus, obesity, hypothyroidism, pregnancy, and others), the latter being more common. Familial dyslipidemias typically follow codominant, autosomal dominant, or autosomal recessive inheritance patterns [20]. Several rare variants have been discovered through population studies, thus being pointed as the cause of the disease after correlation between genotype and phenotype [21,22]. According to the origin of the lipid disturb, disease complications are different and vary among the population [20]. Hyperlipidemias are particularly important in the cardiovascular medicine perspective, as an excess of plasma lipids (specifically LDL and total cholesterol levels) promotes the development of atherosclerosis, one of the major cardiovascular risk factors. Neurological manifestations and poor weight progression related to hypocholesterolemia (low LDL cholesterol levels) are usually observed, and an increased risk of pancreatitis is often verified in cases of high triglyceride concentration in plasma[23,24]. Therefore, it is particularly important to investigate the molecular base of primary dyslipidemias since the etiology determines management and treatment for each subject affected [23].

1.3. Hypercholesterolemia

Hypercholesterolemia is characterized by abnormally high serum levels of LDL-c or apolipoprotein B-100 from birth (untreated LDL-c levels upper than 190 mg/dL) [23]. Physical manifestations include of xanthomas (lipid deposits), xanthelasmas (lipid deposits beneath the skin on or around the eyelids), or *arcus cornealis* (a white or grey opaque ring in the corneal margin) [23,25]. Patients with hypercholesterolemia, regardless of the underlying genetic defect, have abnormal lipid levels and a family history of elevated LDL-c levels and/or CVD. This subset of dyslipidemias is strongly linked to early atherosclerotic and coronary artery disease (CAD) [26].

1.3.1. Autosomal codominant familial hypercholesterolemia

Autosomal codominant familial hypercholesterolemia, usually referred as FH, is the most common genetic disorder associated with lipid metabolism, affecting individuals of all ethnic backgrounds (20,27). Nevertheless, this disorder is severely underdiagnosed and undertreated despite its high prevalence of 1/300 individuals (27). The majority of those with genetically confirmed heterozygous FH or homozygous FH have one or two altered alleles of the LDL receptor gene (*LDLR*), respectively, resulting in either defective or null LDL receptor functionality. Variants in other genes such as *APOB* and *PCSK9* account for less than 10% of cases of heterozygous FH. In fact, heterozygous FH is more common than homozygous FH, the last being estimated to affect one in every 160 000-300 000 subjects, globally (28). When untreated, this genetic disorder of cholesterol metabolism can lead to premature atherosclerosis, CVD, and often death in the first decade of life. Diagnosis of FH primarily depends on clinical assessment according to specific criteria concerning LDL and total cholesterol levels, physical manifestations, and family history of CVD (Simon Broome Criteria, *section 2.1*) (29). Therefore, genetic assessment is fundamental for a correct diagnosis of the disease since clinical severity depends on the nature of the gene that harbours the causative variant among other intraindividual aspects (30).

Elevated LDL-c levels were showed to be a hallmark and a cause of disease for FH in early 70's by Goldstein and Brown, who reported scientific evidence of alterations in *LDLR* gene as the underlying defect in FH (31,32). The LDL receptor is expressed preferentially on hepatocytes and binds to apo B-100 (present on LDL) and apo E (found on VLDL, IDL, HDL, and chylomicrons). *LDLR* gene is located on the distal short arm of chromosome 19 (p13.1-p13.3), span 45 kilobases (kb), and is comprised of 17 introns and 18 exons, coding a protein of 860 amino acids (aa). The mature form is formed by 830 aa and presents with five distinct domains (33). Exon 1 of the gene encodes the signal peptide (21 aa), which function is to direct the receptors synthesised in the ribosomes to the membrane of the endoplasmic reticulum, where the protein becomes mature. Exons 2-6 code the ligand-binding domain that ensure apo B-100 and apo E binding stability. Exons 7 to 14 code for the second domain (EGF precursor homolog) required for receptor-bound lipoprotein dissociation in the endosome during recycling (in acidic medium) (15,33). It also allows the APOB receptor-binding domain to be placed on the cell surface. Exon 15 codes a third domain that functions as an attachment site for various oxygen-linked carbohydrate chains. The fourth domain is a transmembrane domain, which anchor the protein to the cell membrane (exon 16 and the 5'-end of exon 17). Finally, the cytoplasmic domain is coded by the final of exon 17 and the 5'-end of exon 18, and contains the signal for receptor clustering in clathrin-coated pits, allowing it to be internalized via receptor-mediated endocytosis (15,33).

In some cases, the hypercholesterolemic phenotype presented by individuals is explained by an inherited defect in the ability of the patient's LDL to bind to the LDL receptor (34). Therefore, since apoB-100 is a key component for LDL-LDLR biding, the concept of familial defective apolipoprotein B (FDB) arose, pointing that defects in the *APOB* gene could represent the cause of this disease. FDB is mainly defined by elevated LDL-c serum concentration due to decreased clearance of apolipoprotein B-containing particles. *APOB* gene is located on the short arm of chromosome 2 (2p23-2p24), codes both apo B isoforms (48 and 100), spans 43 kilobases (kb), and is comprised of 29 exons coding 4563 amino acids (1,34). Genetic alterations in *APOB* causing hypercholesterolemia are essentially variants leading to amino acid substitutions (35). Of the *APOB* well-known variants, p.(Arg3527Gln), is the most frequently observed, and disrupts the interaction of apolipoprotein B with the LDL receptor (36). About 50 additional likely pathogenic *APOB* variants are associated with hyperlipidemia, many involving arginine residues within the receptor-binding domain that are encoded mainly by exon 26 (37). Nonetheless, several variants outside the LDL-binding region were additionally found to cause familial hypercholesterolemia (35). The phenotype of patients with FDB is usually more moderate when

compared with patients suffering from heterozygous FH due to a pathogenic variant in the *LDLR* gene. However, FDB has also been associated with adverse clinical effects, such as increased rates of both mild and significant coronary artery calcification and myocardial infarction [38,39].

PCSK9 gene was first shown to be related to hypercholesterolemia in 2003, through linkage studies in French families, as genetic alterations co-segregated with the phenotype of severe hypercholesterolemia [40]. This gene codes proprotein convertase subtilisin/kexin type 9 (PCSK9), a protease able to destroys LDL receptors in the liver, thereby regulating plasmatic LDL-c levels [41]. The *PCSK9* gene is found on the short arm of chromosome 1 (1p34-2p32) and is formed by 12 exons that code for 692 aa [42]. *PCSK9* alterations can result in hypercholesterolemia by promoting the degradation of LDLRs in hepatocytes (gain-of-function), resulting in LDL particle accumulation in the plasma [43]. Although the fact that the underlying mechanism of the gain-of-function mutations is unknown, *PCSK9* is a sterol-regulated gene, indicating involvement in cholesterol metabolism [41]. More than 30 gain-of-function variants in *PCSK9* have been reported in patients with FH in several countries, including Portugal. In Portugal, the missense variant p.(Asp374His) was reported in patients who presented a severe phenotype, with extremely high levels of total cholesterol [44]. However, together, variants in *PCSK9* account for less than 1% of all cases of autosomal dominant familial hypercholesterolemia, representing an extremely rare cause of hypercholesterolemia [23].

1.3.2. Autosomal recessive hypercholesterolemia

Autosomal recessive hypercholesterolemia is, nowadays, known as a lipid metabolism genetic disorder caused by disruptive null variants in both alleles (homozygosity) of the low-density lipoprotein receptor adaptor protein-1 (*LDLRAP1*) gene. The worldwide prevalence of this disorder is estimated to be less than 1/5 000 000, hence being considered a rare metabolism disorder. The *LDLRAP1* gene codes a cytosolic protein which is required for LDLR-mediated internalization of LDL in hepatocytes [45]. This protein binds the LDLR, clathrin, and the clathrin-associated adaptor protein complex 2 (AP-2), and is mainly required for LDLR endocytosis into hepatocytes. The *LDLRAP1* gene is located on the short arm of chromosome 1 (1p35), is formed by 9 exons that code for 308 amino acids, and spans 25kb. LDLRAP1 is composed by three main domains necessary for efficient ARH-dependent LDLR endocytosis: a phosphotyrosine binding domain required for binding to the cytoplasmic tail of the LDL receptor as well as the membrane; a clathrin box required for the recruitment or retention of LDL receptors to clathrin-coated pits (LDLLE domain); and, AP-2 adaptor complex that contributes to the localization of ARH protein to clathrin-coated structures at the cell surface [46]. Due to the similarity between phenotypes presented, patients with *LDLRAP1* alterations are frequently clinically diagnosed with homozygous familial hypercholesterolemia [45]. Nonetheless, the phenotype can be milder and less severe in some cases, even within affected individuals from the same family. The development of premature coronary heart disease (CHD) is frequently delayed, and has not been documented in patients younger than 20 years old, in contrast to FH homozygotes [47].

1.3.3. Sitosterolemia

Other rare dyslipidemias can have a clinical presentation similar to FH, such as sitosterolemia, an autosomal recessive disorder due to alterations in *ABCG5* and *ABCG8* genes [48]. This disease was identified in patients with severe hypercholesterolemia, showing a recessive inheritance pattern of phenotype [49,50]. *ABCG5* and *ABCG8* genes encode the ATP-binding cassette (ABC) sub-family G members 5 and 8. The *ABCG5/8* genes are located in chromosome 2, both formed by 13 exons that code for 651 and 673 amino acids, respectively. ABC transporters constitute a ubiquitous protein superfamily that requires energy derived from ATP hydrolysis to translocate substrates across membranes [51].

Dietary cholesterol is released by lysosomes in enterocytes and selectively esterified by acyl-CoA cholesteryl acyltransferase 2 before incorporation into chylomicrons. Excess free cholesterol in enterocytes, as well as absorbed plant sterols, are excreted again into the intestinal lumen [(6)]. ABCG5 and ABCG8 hemi-transporters of the ATP binding cassette transporter family are responsible for the apical excretion of cholesterol and other sterols back into the gut lumen. These are also in charge of hepatocytes excreting cholesterol and other sterols into the bile. Cholesterol feeding stimulates the expression of ABCG5 and ABCG8 in hepatocytes and enterocytes [(6)]. Pathogenic variants in genes coding ABCG5/8 result in an extremely rare condition in which plant sterols accumulate in the blood and tissues [(48)]. In the absence of a functional ABCG5/8 heterodimer, subjects over absorb cholesterol and non-cholesterol sterols from enterocytes [(49)]. Consequently, plant sterols in addition to cholesterol accumulate across body tissues causing tendon xanthomas, premature atherosclerosis, and CVD [(6).]

1.3.4. Polygenic Hypercholesterolemia

The understanding of some monogenic dyslipidemias is limited due to the rarity of causative variants. However, common genetic variants with high population frequency have been already identified as having phenotypical contribution. These variants are single nucleotide polymorphisms (SNPs) that have a minor impact on lipid traits; however, together, SNPs comprise a “polygenic trait”[(52,53)]. Consequently, this concept arose the possibility of a “polygenic (risk) score” for hypercholesterolemia which aims to quantify the burden of common SNP alleles (in an individual's genome) associated with a phenotype, being designed to associate unfavourable lipid phenotypes that can lead to dyslipidemia. A higher risk score indicates a greater burden or accumulation of risk-associated SNP alleles [(53)]. The first SNP-score was developed to study hypercholesterolemic patients who did not have a causative monogenic variants in either *LDLR*, *APOB*, *PCSK9* genes, showing that a significant 20–30% of them had a high polygenic risk score for LDL-c [(54)]. Individuals can inherit multiple LDL-c-raising alleles, which can raise LDL-c to levels observed in patients with heterozygous FH [(25)]. As such, patients who do not have a monogenic cause for their lipid phenotype may benefit from the estimation of their CHD risk, using risk algorithms to improve their management in general practice[(55)].

1.3.5. Management and treatment of hypercholesterolemia

Patients suffering from homozygous FH (HoFH) have a well-established treatment approach, including lipoprotein apheresis, dietary counselling, and combined pharmacotherapy with statins, ezetimibe, and/or PCSK9 inhibitors besides the lifestyle adaptations [(24)]. Statins are an HMG-CoA reduction agent, and the most frequent therapy used in hypercholesterolemia, acting by blocking HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase activity thus decreasing intrahepatic cholesterol, and increasing hepatic clearance of LDL particles [(56)]. Ezetimibe is a cholesterol absorption inhibitor that blocks Niemann-Pick C1-like protein 1 which mediates the uptake of cholesterol by enterocytes. Combined pharmacotherapy is also frequently required in HeFH (heterozygous FH) patients since plasma LDL cholesterol targets are rarely achieved through diet and lifestyle changes [(25,56)]. Homozygous FDB have CVD risk profiles similar to HeFH. Therefore, treatments used in HeFH are usually effective in FDB as well. Additionally, individuals with *PCSK9* gain-on-function alterations can generally be managed in the same manner as HeFH. Treatment for patients suffering from autosomal recessive hypercholesterolemia differs significantly from HoFH since for these patients bile acid sequestrants are usually indicated, without the need for plasmapheresis or LDL-apheresis [(24)]. In cases of sitosterolemia, patients do not require apheresis and their hyperlipidemia often responds well to dietary sterol intake reduction, and to treatment with ezetimibe or bile acid sequestrants [(23)].

1.4. Familial Hypocholesterolemia

Primary hypobetalipoproteinemia is an inherited trait of dyslipidemia characterized by extremely low or absent plasma LDL-c and ApoB concentrations. This group of diseases include familial hypobetalipoproteinemia (FHBL), *PCSK9* derived hypocholesterolemia, abetalipoproteinemia (ABL), or Anderson disease [(24)]. General clinical manifestations include acanthocytosis, anaemia from birth, fat malabsorption, steatorrhea, diarrhea and growth failure in early childhood. Later onset of features of fat-soluble vitamin deficiency include atypical retinitis pigmentosa, and peripheral neuropathy [(57,58)].

1.4.1. Familial Hypobetalipoproteinemia

Hypobetalipoproteinaemia (FHBL) is a rare autosomal codominant disorder involving the *APOB* gene and is characterized by low concentrations of apo B (lower than 38mg/dL) [(59)]. Variants in *APOB* gene causing FHBL compromise the integrity of the lipoprotein particle due to structural protein defects originated by protein truncation. As a result, secretion of truncated forms of apolipoprotein B of various sizes affect the apo B ability to form plasma lipoproteins (in the liver and intestine) and to export lipids. Consequently, decreased VLDL secretion and increased catabolism of VLDL and LDL, results in lower circulating cholesterol concentrations [(60)]. Patients with heterozygous FHBL have increased risk of hepatic steatosis, but concurrently reduced risk of ASCDV [(58)]. Hepatic consequences in these individuals might be explained since decreased production of LDL apo B-100, increased catabolism of VLDL, and extremely low secretion of the truncated apo-B might result in decreased triglyceride export from the liver, thus developing fatty liver [(60)]. Frequently, homozygous FHBL individuals have clinical manifestation such as acanthocytosis, deficiency of fat-soluble vitamins and malabsorption, atypical retina pigmentosa and neuromuscular abnormalities. Although HeFHBL also exhibit these manifestations, these individuals can present a less severe phenotype [(61)].

1.4.2. *PCSK9* gene alterations causing hypocholesterolemia

Despite the association of PCK9 variants to autosomal dominant FH, variants in this gene can also contribute to the opposite phenotype [(62)]. Several loss-of-function (LOF) variants have been described and associated with hypocholesterolemia, the large majority being missense and nonsense alterations that prevent PCSK9-mediated LDLR degradation, and thus increase LDL uptake by the liver, resulting in a 40% reduction in plasma LDL-c levels [(63)]. LOF PCSK9 variants are not associated, at moment, with specific clinical symptoms, despite possibly being linked to a lower risk of CVD [(64)].

1.4.3. Abetalipoproteinemia

Abetalipoproteinemia (ABL) is a rare autosomal recessive disease caused by LOF alterations in the *MTT* gene that result in truncated forms of the coded protein [(65,66)]. This condition is generally characterized by the absence of VLDL and chylomicron production thus conferring undetectable concentration of LDL cholesterol in plasma, low total cholesterol concentration (<30 mg/mL) [(23)]. ABL patients demonstrate a phenotype similar to FHBL patients. However, in ABL, heterozygous subjects frequently have normal lipid profiles, consistent with autosomal recessive inheritance pattern [(58)]. *MTTP* gene is located on chromosome 4, is composed of 18 exons, and codes an 894 aa protein known as microsomal triglyceride transfer protein (MTP) [(57)]. MTP is a transfer protein required in the liver and intestine for the assembly and secretion of apo B-containing lipoproteins. In the assembly of VLDL triglyceride in the rough endoplasmic reticulum, this protein catalyses the transfer of triglyceride to the nascent apo B particles while apo B is co-translationally translocated across the endoplasmic reticulum membrane [(57,66)].

1.4.4. Anderson's Disease (Chylomicron retention disease)

Anderson's disease, also nominated as chylomicron retention disease, is a rare autosomal recessive disease caused by biallelic loss-of-function alterations in the *SAR1B* gene that lead to failure of chylomicron secretion from enterocytes (66,67). Often, failure to thrive is observed in childhood, along with severe malabsorption, steatorrhea, and fat-soluble vitamin deficiency (67). Homozygous patients present absence of apo B-48 and chylomicrons, as heterozygous subjects have normal lipid profiles (23). *SAR1B* gene is located on chromosome 5, contains 7 exons, and results in a protein with 178 aa long. This gene codes SAR1B, a small GTPase that regulates the formation and assembly of ER-derived COPII vesicles during protein export from the endoplasmatic reticulum to the Golgi. SAR1B, as the GDP-to-GTP exchanger, is a critical element in the final step of assembling this vesicular transport complex. Consequently, alterations in this gene affect pre-chylomicron trafficking from the ER to the Golgi apparatus, leading to the absence of chylomicrons and a marked accumulation of lipids in enterocytes (67,68).

1.4.5. Management and treatment of hypocholesterolemia

The overall management principles for FHBL, ABL and Anderson's disease include a fat-restricted diet, essential fatty acid supplementation, and high oral doses of vitamins A, D, E, and K. Carriers of biallelic loss-of-function variants in *PCSK9*, in contrast, do not require any specific treatment. It is critical to identify and treat symptomatic primary hypocholesterolemia as soon as possible (23,59). Patients with FHBL and ABL should be advised to rule out other potential risk factors (alcohol consumption, obesity, metabolic syndrome, diabetes mellitus, hypertriglyceridemia) (23).

1.5. Familial Hypertriglyceridemia

Hypertriglyceridemia (HTG) is a clinical condition observed in individuals with elevated triglyceride concentrations in plasma. Fasting triglyceride plasmatic concentrations between 175-885 mg/dL are usually considered as "mild to moderate" hypertriglyceridemia, and when above 885 mg/dL is nominated as "severe hypertriglyceridemia" (69). Familial hypertriglyceridemia typically presents recessive inheritance, often being the result of homozygosity or compound heterozygosity for large effect variants (70,71). HTG clinical features include xanthomas, abdominal pain, hepatomegaly, hepatosplenomegaly, *lipemia retinalis*, and pancreatitis. In general, patients suffering from HTG (particularly in the severest form) have an increased risk of developing atherosclerotic complications and stroke, and even an increased risk of mortality by ASCVD (72).

1.5.1. Familial Chylomicronemia Syndrome

Familial chylomicronemia syndrome (FCS) is a classical monogenic disorder that follows autosomal recessive inheritance and results from rare biallelic variants in one of five canonical genes (*LPL*, *APOC2*, *GPIHBP1*, *APOA5*, and *LMF1*), causing impaired hydrolysis of triglyceride-rich lipoproteins (71). FCS has an estimated prevalence of 1 in 100 000 – 1 000 000 thus being considered an extremely rare form of familial dyslipidemia. It is estimated that only 1-2% of adults with hypertriglyceridemia are affected due to a monogenic cause of disease (23). *LPL* gene alterations are the most common molecular base of disease, representing over 80% of the cases (73). This gene codes lipoprotein lipase, is located on chromosome 8, and is composed of 10 exons coding a total of 475 aa, spanning 30 kb (74). LPL catalyses the hydrolysis of triglycerides in chylomicrons and VLDL particles, thus having an important role in receptor mediated uptake of chylomicron remnants and lipoproteins (75). LOF alterations in these gene reduce or eliminate LPL activity in the homozygous state, preventing hydrolysis and resulting in accumulation of TG-rich lipoproteins (74,75).

Biallelic *GPIHBP1* alterations, including large-scale deletions, are the second most common cause of FCS, being detected in 5-10% of the cases [(23)]. This gene codes glycosylphosphatidylinositol-anchored HDL binding protein 1, which translocates newly secreted LPL across capillary endothelium, and stabilises the enzyme on the endothelial surface, where it interacts with chylomicrons and VLDL. GPIHBP1 is a short protein, with only 184 aa in length, coded by 4 exons [(76,77)]. Consequently, *GPIHBP1* LOF variants impaired the lipid metabolism efficiency.

APOC2 and APOA5 LOF alterations cause about 5-10% of FCS cases [(23)]. *APOC2* gene codes apolipoprotein C-II, a required co-activator of LPL. This gene is located on chromosome 19 and comprehends 4 exons [(78)]. Deficiency of apo C-II due to biallelic LOF variants results in the malfunction of the LPL-apoC-II system, leading to accumulation of chylomicrons in the plasma. Homozygous patients for these alterations can present a similar phenotype of homozygous LPL deficiency [(75,78)]. *APOA5* gene is located on the long arm of chromosome 11, and contains four exons that code for a 366 amino acid protein called apolipoprotein A-V [(79)]. Apo A-V modulate triglyceride concentrations by trafficking lipid particles and binding to GPIHBP1, which ensures interaction with LPL to hydrolyse triglycerides and chylomicrons, and promotes interaction at the capillary endothelium's surface [(80)]. Nonetheless, patients with biallelic LOF alteration in *APOA5* show less severe clinical and biochemical phenotype [(81)].

At last, *LMF1* gene alterations constitute the least frequent cause of FCS, representing only 1-2% of the cases [(23)]. The *LMF1* gene is found on chromosome 16 and codes for a 567 aa protein (lipase maturation factor 1) required for proper LPL folding and intracellular trafficking [(82)]. Despite being a transmembrane protein, the mechanism underlying LMF1's lipase chaperone function remains unknown. It has been suggested that LMF1 possibly modulates lipase assembly, thereby controlling lipase expression [(82)]. LMF1 deficiency leads to markedly reduced LPL secretion, causing a severe hypertriglyceridaemia phenotype, similarly to LPL deficiency [(75)].

1.5.2. Multifactorial Chylomicronemia Syndrome

Clinical features in multifactorial chylomicronemia syndrome (MCM) include *lipemia retinalis*, hepatosplenomegaly, eruptive xanthomas, and abdominal pain. However, MCM is a much more common condition, compared to FCS, with an estimated frequency of 1 in 600-1000 worldwide [(71,83)]. Contrarily to FCS, the genetic basis of MCM is not defined by biallelic alterations in the five canonical genes [(71)]. Instead, genetic factors are not deterministic, the presence of pathogenic variants (mainly in heterozygosity, in FCS related genes) does not ensure the phenotypical expression, but their presence might increase the susceptibility for severe hypertriglyceridemia [(81)]. Furthermore, as a multifactorial disorder, a high burden of common small-effect SNP alleles can create a polygenic trait of hypertriglyceridemia [(70)]. Despite the hardship of determinate functional mechanisms underlying lipid disorders, several SNPs associated with triglyceride high levels have been identified giving rise, recently, to polygenic risk scores for hypertriglyceridemia [(84)].

1.5.3. Autosomic Recessive Hypertriglyceridemia

Transient Infantile Hypertriglyceridemia is a particularly rare dyslipidemia (with autosomal recessive inheritance) caused by pathogenic compound heterozygous or homozygous alterations in *GPD1* gene, leading to GPD1 LOF. This disorder is characterized by early onset hepatomegaly, hypertriglyceridemia, moderately elevated transaminases, hepatic steatosis, and hepatic fibrosis. To date, few cases of this disease are known, and the correlation between a phenotype and a homozygous LOF variant in *GPD1* gene was observed in 2012 for the first time [(85)]. The identified cause of disease

was a splice aberrant alteration in *GPD1* resulting in complete loss of activity of glycerol-3-phosphate dehydrogenase (GPD1) probably leading to increased hepatic secretion of VLDL triglycerides. The *GPD1* gene is mapped on chromosome 12 (12q12-q13) [86]. Functionally, together with mitochondrial GPD2, GPD1 form G3P shuttle, which transfers reducing equivalents resulting from redox between dihydroxy-acetone phosphate and reduced nicotine adenine dinucleotide (NADH), from the cytosol to the mitochondria thus being relevant in lipid metabolism. However, despite genotype/phenotype correlation, the exact mechanism of hypertriglyceridemia in GPD1 deficiency remains unclear and should be further investigated [87].

1.5.4. Lysosomal Acid Lipase Deficiency

Lysosomal Acid Lipase Deficiency (LAL-D) is a rare autosomic recessive disorder characterized by mild cholesterol and triglycerides levels increase together with clinical manifestations of hepatosplenomegaly, failure to thrive, steatorrhea, and anaemia [23]. LAL-D is caused by alteration in *LIPA* gene (located on chromosome 10, and constituted by10 exons) which codes LAL, an enzyme that functions as a responsible for intracellular hydrolysis of TG and cholesteryl esters. Variants causing LIPA LOF, when in homozygous or compound heterozygous, lead to this lipid storage disease characterized by intracellular accumulation of unhydrolyzed lipids [88]. Cholesterol and triglyceride levels are not particularly elevated in this disease, nevertheless due to poor hydrolysis ability, TG and cholesterol accumulation is observed in liver, spleen aid intestinal lining [23,88].

1.5.5. Dysbetalipoproteinemia

Dysbetalipoproteinemia is a rare inherited dyslipidemia affecting 1 to 2 individuals *per* 20 000. Clinically, both triglycerides and cholesterol are increased, and physical manifestations include palmar and tuberoeruptive xanthomas on the knees and elbows [23]. Some of these patients have an increased risk of develop premature ASCVD [24]. *APOE* gene (located on chromosome 19, 3 exons) codes apo E, which is found in chylomicrons, VLDL, IDL, and HDL. This apolipoprotein is crucial in lipid metabolism as it enables the biding of these lipoproteins to LDLR in hepatic and extrahepatic tissues[89]. Pathogenic variants in *APOE* gene are responsible for the cause of disease which presents a complex inheritance pattern. Some rare *APOE* variants have been identified and implicated in a dominant mode of inheritance since heterozygous subjects for those alterations displayed the phenotype. In these cases, *APOE* modifications were mainly amino acid substitution in the LDLR binding region [89]. Nonetheless, large-effect dominant rare missense variants in APOE affects only about 10% of patients suffering from dysbetalipoproteinemia [23]. In addition, other rare *APOE* variants were observed to cause this phenotype, despite displaying an autosomic recessive inheritance pattern and variable clinical severity [89]. The majority off affected individuals are homozygous for the ε2 isoform, which lacks an effective binding to the LDL receptor, resulting in an accumulation of apo B-48 chylomicron remnants in the blood [24]. Contrarily, heterozygosity patients for apo E deficient alleles are usually clinically silent since the clinical manifestation also depends on the other allele [19,89].

1.5.6. Treatment and management of hypertriglyceridemia

Patients suffering from hypertriglyceridemia, particularly in the severest form are recommended to have an extremely controlled and restricted diet. In cases of FCS, patients' treatment is seriously limited [56]. This group of patients has extremely elevated triglyceride concentrations even under dietary restrictions and pharmacological treatment. The risk of developing pancreatitis is always present, and during acute episodes patients are limited to complete fasting during the first days of treatment, which has demonstrated to be very effective [56,90]. Usually, fibrates (which increase LPL activity) are an

ineffective therapy in patients with FCS. Fibrates and omega-3 fatty acids (which decrease VLDL levels and possibly chylomicron secretion) are recommended and beneficial to individuals with possible polygenic hypertriglyceridemia [91]. To date, there is no specific treatment for LAL-D. Frequently these patients have a pharmacotherapeutic treatment with statins, ezetimibe, and enzyme replacement by infusion of sebelipase alfa. Dysbetalipoproteinemia can be managed with a calory-restricted diet, and either a statin or a fibrate to correct the predominant abnormality lipoprotein concentrations [23].

All patients with rare or common familial dyslipidemias should be followed-up by clinicians and specialists in genetic counselling in order to avoid disease complications [24]. Management of familial dyslipidemias also includes cascade screening in families with individuals who present phenotype of any type of familial dyslipidemia, especially at an early age, since family history dyslipidemia suggests a genetic basis of the clinical manifestation [23,83].

1.6. Study of Genetic Disorders

In the last 40 years, the evolution of techniques and technologies in the genomic field allowed a greater understatement of the human genome. Such advances greatly improved the knowledge of the molecular mechanisms underlying monogenic and polygenic diseases [92]. The human genome project (HGP) was the turning point for the insight into genetics and for the improvement of molecular biology techniques. HGP aimed to accurately sequence the entire human genome, identify all genes present and, ultimately accomplish a genetic and physical mapping of the genome, proving that whole genome sequencing could be achieved [93].

Genome sequencing constituted an advance in the diagnoses of rare lipoprotein disorders and associated dyslipidaemias [23]. Due to interindividual variability in the genetic causes and the phenotypical differences among populations, genomic analysis plays a crucial role in the diagnosis and management of these conditions. In Portugal, the interest and the awareness of rare metabolism disorders started with the implementation of the "Portuguese Familial Hypercholesterolemia Study" in 1999 at National Health Institute Doutor Ricardo Jorge (Lisbon) [94]. The principal aim was to characterize patients suffering from the condition. The characterization of FH patients and other patients who inherited any genetic dyslipidemia contributed to correct identification of the genetic cause of disease, and enabled a personalized treatment based on the genotype and not only on the phenotype. Initially, patients were molecularly studied according to the clinical phenotype presented. The core methodologies applied in the initial study (1999) were: amplification of *LDLR* (18 exons, total) and *APOB* (exons 26 and 29) genes by polymerase chain reaction; highly sensitive denaturing high-pressure liquid chromatography (DHPLC); and, multiplex ligation-dependent probe amplification (MLPA) for assessment of large rearrangements in the *LDLR* gene. Since 2005, the analysis of all 12 exons of the *PCSK9* gene was also included for FH molecular diagnosis. Exome sequencing by Next Generation Sequencing (using pyrosequencing) for *LDLR*, *APOB* and *PCSK9* was introduced for FH, in 2014 [95]. Additionally, functional assays to study variants affecting splicing, mainly in *APOB* and *LDRL* genes have been developed in Portugal, since 2008 to date [35]. From 2017 to nowadays, Next Generation Sequencing with target gene panels has been improved and optimized. The implementation started with gene panels containing 24, 48, and currently 57 genes related to different metabolic pathways in lipid metabolism. Until now, more than 1000 families have been characterized in Portugal, suffering from all different rare lipid disorders, and the number of patients recruited and studied is rising. A better characterization of Portuguese population is being accomplished, not only of FH patients but also of patients with other rare familial dyslipidemias.

1.7. Objectives

The presented project was integrated within the Portuguese Familial Hypercholesterolemia Study (EPHF) and the "Study of Monogenic and Polygenic rare causes of Familial Dyslipidemia", currently developed by the Cardiovascular Research Group (GIC) at National Health Institute Doutor Ricardo Jorge (INSA). Both projects aim to characterize, clinically and molecularly, Portuguese individuals with one type of genetic dyslipidemia. The main goals of the presented research project were:

a) Biochemically characterize the phenotype of 96 portuguese patients with a clinical diagnosis of at least one type of genetic dyslipidemia referred to the GIC-INSA (evaluation of individuals based on the phenotype presented);
b) Identify the genetic cause of dyslipidemia in 96 Index Cases;
c) Perform cascade-screening by genetic testing relatives of confirmed affected Index Cases;
d) Investigate variants found in this project, in order to access their pathogenicity and possible clinical significance.

Chapter 2. Materials and Methods

This project was conducted during 12 months at the Cardiovascular Research Group (GIC), National Health Institute of Portugal, Lisbon (INSA). The project was integrated within the Portuguese Familial Hypercholesterolemia Study (EPHF) which was approved by INSA Ethics Committee [94] (workflow overview in Figure 2.2, at the end of chapter 2, page 25).

2.1. Patients Recruitment

A total of 96 Portuguese index cases (IC, probands with the severest phenotype) were included in the presented research study. Individuals were recruited by several clinicians (cardiologists, paediatricians, geneticists, endocrinologists) all over the country (including Madeira and Açores), and referred to the Cardiovascular Research Group (National Health Institute of Portugal, Lisbon). All index cases fulfilled clinical diagnostic criteria for three different types of genetic dyslipidemias: very high LDL cholesterol levels (including FH and related conditions), very low LDL cholesterol levels (including hypobetalipoproteinemia and abetalipoproteinemia) and very high levels of triglycerides (including familial chylomicronemia syndrome and autosomal recessive hypertrygliceridemia) . For FH, since diagnostic criteria is well established, index cases fulfilled Simon Broome criteria [29]. These criteria categorises index cases as having "definite FH" or "possible FH" according to their cholesterol levels and presence of CVD and a family history of hypercholesterolemia or CVD (Table 2.1). Regarding hypocholesterolemia, index cases with LDL cholesterol levels below P10th (considering sex and age) were included in this study. Individuals presenting a clinical scenario of triglyceride levels above 200 mg/dL, normal cholesterol levels, and a personal and/or familial history of hypertriglyceridemia complications (particularly cardiovascular events and pancreatitis) were also referred to this study.

Participants completed a clinical questionnaire (addressing lipid lowering therapeutic, cardiovascular events and family history of CVD) (Appendix IA and IB), and were informed about study timeline, research process and molecular result communication. Participants signed a written informed consent declaration before the inclusion in the study. All information concerning the individuals was registered in a confidential database in which a confidential number was assigned for each sample, according to legal requirements. In cases where a pathogenic or likely pathogenic variant was identification, the results were reported to the clinician responsible for patient referral. Frequently, clinicians cooperate with the Cardiovascular Research Group in order to identify relatives for cascade screening and co-segregation studies. On behalf of this research project, and the Portuguese Familial Hypercholesterolemia Study (EPHF), 33 index relatives were included as participants. Therefore, an overall total of 129 Portuguese individuals participated in the presented research study.

Table 2.1 – Simon Broome Criteria for clinical diagnosis of Familial Hypercholesterolemia

Confirmed familial hypercholesterolemia is defined as: Child under 16 with total cholesterol over 260 mg/dL (6.7 mmol/L) or LDL cholesterol over 155 mg/dL (4 mmol/L); Adult with total cholesterol over 290 mg/dL (7.5 mmol/L) or LDL cholesterol over 190 mg/dL (4.9 mmol/L), **and**: - Tendon xanthoma in the index case or relative (parents, siblings, grandparents, children, aunts/uncles); **or** - Genetic evidence of a variant in *LDLR*, *APOB* or *PCSK9* genes.

Possible familial hypercholesterolemia is defined as:
Child under 16 with total cholesterol over 260 mg/dL (6.7 mmol/L) or LDL cholesterol over 155 mg/dL (4 mmol/L); Adult with total cholesterol over 290 mg/dL (7.5 mmol/L) or LDL cholesterol over 190 mg/dL (4.9 mmol/L), **and**:
- Familial history of myocardial infarction before the age of 50 in grandparents and/or aunts/uncles; or, before the age of 60 in parents, siblings and children.; **or**
- Familial history of elevated cholesterol levels (>290 mg/dL) in parents, siblings and/or children; or, elevated cholesterol levels over 290 mg/dL in grandparents and/or aunts/uncles.

2.1.1. Blood sample collection

For each index case and respective relatives, fasting blood samples were collected in order to perform DNA extraction (3 x 2.7 mL in EDTA tubes for adults; 2 x 2.7 mL in EDTA tubes for children participants) and biochemical determination (7.5 mL in serum tubes for adults; 5 mL for children). In order to perform RNA extraction from peripheral blood mononuclear cells, a CPT was used to collect approximately 7 mL of fresh blood (of Index Case Patients).

2.1.2. Biochemical characterization

Biochemical parameters such as total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), triglycerides (TG), apolipoprotein AI (ApoAI), apolipoprotein B (ApoB) and lipoprotein (a) (Lp(a)), were determined by enzymatic and colorimetric methods for all participants (after blood collection to a serum tube), at INSA (UDR, Diagnostic and Reference laboratory Unit) using the COBAS Integra device.

2.2. Molecular Biology Techniques for Diagnostic

2.2.1. Genomic DNA extraction

Genomic DNA was extracted from leucocytes in peripheral blood samples collected in EDTA tubes (~3 mL). DNA extraction was performed for 2 collected tubes, in independent days, providing 2 different DNA samples for diagnosis confirmation and investigation purpose. This technique was executed as an adaptation of the protocol described in [96], and the proportion of each reagent to mL of blood is presented in Appendix II, Table A I.1. The blood was well homogenised and transferred to a 15 mL falcon tube. For leucocyte cell membrane lysis, an equal volume of TKM X-100 solution (containing 10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM MgCl2, 2 mM EDTA and 25 mL Triton-X 100/L) was added and mixed several times by inversion. In addition, IGEPAL was added and mixed until total solubilization. The tubes were centrifuged at room temperature for 10 min at 2200 rpm (5810 R *Eppendorf* centrifuge) and the supernatant was disposed by decantation. The resulting pellet (in each falcon tube) was washed in an equal volume of TKM1 solution (TKM-X100 without the Triton-X 100) and centrifuged at room temperature for 10 mins at 1600 rpm. After repeating this wash, the supernatant was discarded and the pellet was resuspended with TKM2 solution (containing 10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 0.4 M NaCl and 2 mM EDTA) and 10% of SDS. The suspension was mixed and followed by a 10 mins incubation at 55ºC, for protein denaturation. Subsequently, the falcon tube content was transferred to a 2 mL Eppendorf tube containing NaCl 5M, for protein precipitation. The tubes were centrifuged (in 5415D *Eppendorf* microcentrifuge) at room temperature for 20 mins at 13200 rpm, forming a reddish pellet. Each supernatant was transferred to a clean falcon tube containing absolute ethanol and was gently mixed by inversion in order to precipitate DNA. The DNA fibrils were removed with a loop and washed in a 70% ethanol solution. The DNA was then resuspended in 150 µL of TE (10 mM Tris-HCL, 1 mM EDTA pH 8.0). DNA content was quantified using a spectrophotometer

(NanoDrop 1000, Thermo Scientific). The quality of DNA was assessed by agarose gel electrophoresis using 1% agarose gel, prepared with 100 mL of TBE buffer 1x (Invitrogen), 1.0 g of NuSieve™ 3:1 Agarose (Lonza) and with 0.01% of SYBR Safe DNA gel stain (Invitrogen). The samples loaded into 1% agarose gel were prepared by dilute 1 µL of genomic DNA in 6 µL of bidistilled water and 3 µL of gel loading dye (bromophenol blue). The electrophoresis was performed in a Bio-Rad Power Pac 3000 equipment, at 90V for 40 mins, and the gel was visualized in a Safe Imager™ transilluminator (Invitrogen).

2.2.2. Next Generation Sequencing (NGS)

Next Generation Sequencing was used for massive parallel sequencing of 96 samples. Gene panels were customised for monogenic disorders of lipid metabolism and included a total of 57 genes related with metabolic pathways: 4 small panels of 24 genes strongly associated with at least one type of dyslipidemia, and a larger panel including 33 candidate genes. Sample libraries were prepared using the Sureselect^{QXT} Target Enrichment for Illumina Multiplex Sequencing (Version F0, Agilent Technologies) and enriched samples were sequenced on a NextSeq platform (Illumina). The protocol was performed simultaneously for 48 samples and executed two times in the course of this project. The experimental protocol for next generation sequencing is divided into 5 main steps: genomic DNA samples preparation; genomic DNA libraries preparation; libraries hybridization and capture; indexing, and sample processing for multiplex sequencing; massive parallel sequencing.

2.2.2.1. Genomic DNA preparation

Preparation of the samples included the dilution of all 96 samples in order to obtain a final concentration of 25 ng/µL. This is considered as a critical step of the protocol, as it is extremely important to obtain a precise sample concentration, which will allow a perfect fragmentation of the DNA. The present step of the protocol has a duration of approximately 3 days. Quantification and dilution of the samples was accomplished using the Qubit dsDNA BR Assay kit in a Qubit fluorometer (Invitrogen). The initial concentration of the samples was determined, following the manufacturer instruction for fluorometric quantification. The DNA samples were diluted by adding nuclease-free water to a final concentration of 100 ng/µL, in a final volume of 25 ul. After an overnight homogenization, diluted samples (100 ng/µL) were quantified using the same invitrogen kit. After this step, each sample was diluted again with nuclease-free water to a final concentration of 25 ng/µL, in a final volume of 25 ul. Subsequently, diluted DNA was quantified with Qubit dsDNA BR reagent kit and the Qubit fluorometer, after an overnight homogenization. This last quantification had a maximum error of 2 ng/µL (samples had an overall concentration of 23-27 ng/µL). Finally, diluted gDNA samples at 25 ng/µL were stored at -20ºC.

a) Fragmentation and adaptor-tagging the genomic DNA samples

The gDNA is enzymatically fragmented in this step, and adaptors are added to the ends of the fragments in a single reaction. A library was prepared for each DNA sample. Fragmentation reaction were set on ice, using PCR strips. To each sample well, 17 µL of SureSelect QXT Buffer were added, followed by 2 µL each DNA at 25 ng/ µL and 2 µL of SureSelect QXT Enzyme Mix ILM. Strips were then placed in the Applied biosystems thermal cycler for an incubation step during 10 mins at 45ºC, and 1 min at 4ºC. After the incubation, to each fragmentation reaction, 32 µL of SureSelect QXT Stop Solution (containing 25% ethanol) were added. Samples were incubated at room temperature for 1 minute.

b) Purification of the adaptor-tagged library (using AMPure XP beads)

After 1 min incubation at room temperature, 52 µl of the homogeneous AMPure XP bead suspension were added to each well containing the DNA samples. Thereafter, samples were incubated

for 5 mins at room temperature. Strips were placed on the magnetic stand at room temperature until the solution was cleaned (approximately 3 to 5 minutes). Next, while in the magnetic stand, the clear solution was carefully removed and discarded (without disturbing the beads) and washed with 200 µL of fresh 70% ethanol. After one minute, the ethanol was removed. After two washes, 11 µL of nuclease-free water were added. Upon 2 min incubation at room temperature, tubes were placed in the magnetic stand, each cleared supernatant was removed (10 µL approximately) to a fresh strip tube.

c) Amplification of the adaptor-tagged DNA library

In this step, the adaptor-tagged gDNA library is repaired and PCR-amplified. To each strip tube, containing 10 µL of purified DNA library sample, 40 µL of pre-capture PCR reaction mix were added. PCR mix contained (for each sample): 25 µl of nuclease-free water, 10 µl of Herculase II 5× Reaction Buffer, 0.5 µl of 100 mM dNTP Mix (25 mM each dNTP), 2.5 µl of DMSO, 1 µl of SureSelect QXT Primer Mix, and 1 µl of Herculase II Fusion DNA Polymerase. Pre-capture PCR reaction was held in the thermal cycler over 37 mins, as follow: one min at 68 ºC; one min at 98 ºC; 8 cycles of three steps: denaturation for 30 sec at 95 ºC; annealing for 30 sec at 57 ºC; and elongation for 1 min at 72 ºC; and final extension for 5 min at 72 ºC.

d) Purification of the amplified library (using AMPure XP beads)

After removing from the thermal cycler, samples were transferred to room temperature and 50 µl of the homogeneous AMPure XP bead suspension were added to each amplified DNA sample. Amplified libraries were purified as described above (step b). Nuclease-free water was added (13 µL to each sample) To the samples and incubated 2 mins at room temperature. Until being placed again in the magnetic stand in which each supernatant was removed (approximately 13 µL) to a fresh strip tube For each sample (1 µL) was separated to a fresh strip to further quantity and quality assessment. Samples were stored at 4 ºC until the following day.

e) DNA library quantification and quality assessment

In this stage, the D1000 ScreenTape sample plate, and associated reagents, was used to analyse the amplified libraries, in the Tapestation Automated Electrophoresis System (Agilent). The assay for DNA quantification and quality assessment was performed according to the Agilent D1000 Assay Quick Guide. Each amplified DNA library (1 µl) was diluted in 3 µL of D1000 buffer. Strips containing the mixture (sample and buffer) were agitated during 1 minute in IKA MS3 *vortex* (Agilent) and spined. Sample strips were loaded into the Tapestation as instructed in the reagent kit guide. This *run* as a duration of approximately 1 minute *per* sample. Once the *run* in the Tapestation was over, electropherograms were generated for each sample. All the 96 electropherograms showed nearly the same profile: the peak of DNA fragment size positioned between 245 to 325 bp (as desired, demonstrating a good quality of the fragments. After this step, samples were stored at 4ºC (overnight) in order to execute the following steps of the protocol on the following day.

2.2.2.2. Libraries hybridization and capture

At this point of the protocol, each prepared genomic DNA library was hybridized with a set of probes (capture library) specific for the target genes (customised panel). The target molecules were captured on streptavidin-coated beads, after hybridization.

a) Hybridization of DNA samples to the capture library

To each sample (comprising 12 µL of adaptor-tagged DNA), 5 µL of SureSelect QXT Fast Blocker Mix were added and then samples were placed in the thermal cycler (Applied biosystems). Samples were incubated according to the hybridization programme, starting with 5 min at 95 ºC and 10 mins for 65 ºC. Then, without removing the strip tubes from the thermal cycler, 13 µl of probe hybridization mix were added to each sample well. This reacting mix was prepared using: 2 µl of 25% RNase Block

solution, 6 µl of SureSelect Fast Hybridization Buffer, 2 µl of probe and 3 µl of nuclease-free water. I thermal cycler programme then continues with 60 cycles of: 1 min at 65 ºC followed by 3 sec at 37 ºC.

b) <u>Preparation of streptavidin-coated magnetic beads (for DNA hybrid capture)</u>

For each hybridization sample, 50 µl of the Dynabeads MyOne Streptavidin T1 magnetic beads were washed with 200 µl of SureSelect Binding Buffer and repeated two more times. The beads were then resuspended in 200 µl of SureSelect Binding Buffer.

c) <u>Capture the hybridized DNA using streptavidin-coated beads</u>

After the conclusion of the hybridization step, samples were transferred to room temperature and the entire volume of each hybridization mixture was transferred to the wells containing 200 µl of washed streptavidin beads. Tubes were incubated at room temperature on a plate mixer, at 1400 rpm, for 30 mins. Subsequently, samples were disposed into the magnetic separator and the supernatant was discarded. Beads were washed with 200 µl of Wash Buffer 1 . Samples were disposed into the magnetic separator and the supernatant was removed. To each sample was added 200 µL of Wash Buffer 2 (pre-warmed at 65 ºC), and incubated for 10 mins at 65 ºC (Applied biosystems thermal cycler). Samples were placed in the magnetic separator and the supernatant was discarded. This step was repeated in a total of 3 washes. Thereafter, 23 µL of nuclease-free water were added to each sample well. In this phase, the supernatant is not separated from the beads, since during the post-capture amplification step, the captured DNA will be retained on the streptavidin beads.

2.2.2.3. Indexing and sample processing for Multiplex Sequencing

a) <u>Captured libraries amplification (and addition of index tags)</u>

In this step, DNA libraries enriched with the target genes were amplified throughout a PCR reaction using the appropriate pair of double-indexing primers. For each DNA library, an index amplification reaction was prepared. Different combinations of indexing primers were used for samples sequenced simultaneously. Indexing primers sequences are presented in Appendix Table A.II.2. A post-capture PCR mix was prepared with 10 µl of Herculase II 5× Reaction Buffer, 0.5 µl of 100 mM dNTP Mix (25 mM each dNTP), 1 µl of Herculase II Fusion DNA Polymerase and nuclease-free water was added to make a final volume of 25 µl. Therefore, the PCR mix was added to each microtube containing 23 µl of captured DNA in bead suspension. Next, 1 µl of the appropriate P7 dual indexing primer (P7 i1 to P7 i12) and also 1 µl of the appropriate P5 dual indexing primer (P5 i13 to P5 i20) were added to each PCR reaction mixture well. Post-capture PCR reaction was held in the thermal cycler over 45 mins as follow: 2 mins at 98 ºC; 12 cycles of: 30 secs at 98 ºC, 30 secs at 58 ºC and 1 min at 72 ºC; and finally, 5 mins at 72 ºC and 1 min at 4 ºC. When the PCR amplification programme was completed, the streptavidin-coated beads were discarded, after placing the strip on the magnetic stand (at room temperature) and remove the 50 µl of supernatant to a fresh tube strip.

b) <u>Purification of amplified captured libraries (using AMPure XP beads)</u>

To each 50 µl of amplified DNA samples, 60 µl of the homogeneous AMPure XP bead suspension were added and amplified libraries were purified as described before (*section 2.2.2.2.b*). Samples were incubated at room temperature with 25 µl of nuclease-free water for 2 mins and placed in the magnetic stand for 2 mins. The clean supernatant (nearly 25 µl) was removed to a new fresh strip, and beads were discarded. For each sample 2 µL was separated to a fresh strip to further quantity and quality assessment. Samples were stored at 4 ºC until the *pooling* step.

c) <u>Indexed library DNA quantification and quality assessment</u>

In this stage, the Tapestation Automated Electrophoresis System was used to analyse the amplified

libraries using the High Sensitivity D1000 ScreenTape sample plate and associated reagents (Agilent). The assay for DNA quantification and quality assessment was carried out following the manufacturer's instructions. Each amplified DNA library (2 µL) was diluted in 2 µL of High Sensitivity D1000 buffer. Strips containing the mixture (sample and buffer) were spined after 1 minute of agitation in an IKA MS3 vortex (Agilent). Sample strips were loaded into the Tapestation. This run lasts approximately 1 minute per sample. Electropherograms were generated for each sample after the run in the Tapestation was completed. The same profile was visible in all 96 electropherograms, the peak of DNA fragment size was located between 325 and 450 bp (indicating a successful procedure).

2.2.2.4. Library processing for Multiplex Sequencing

a) Indexed libraries quantitative assessment

Indexed DNA libraries were quantified using the Qubit dsDNA HS (High Sensitivity) Assay kit (Invitrogen), following the manufacturer instruction.

b) Libraries *pooling*

This step of the protocol aimed to perform the junction (*pooling*) of all the previously prepared indexed DNA libraries in equimolar amounts to a final concentration of 4 nM. Molar concentration of each sample was calculated using the Equation 2.1.

Equation 2.1:

$$\text{Molar concentration (nM)} = \frac{\text{Qubit concentration (ng/µl) x 1 000 000}}{[\text{Peak size (pb) x 660}] + 157.9}$$

The volume of each indexed library was determined using the Equation 2.2.

Equation 2.2:

$$\text{Indexed library volume (µl)} = \frac{\text{Pool's final volume (µl) x Pool's final concentration (nM)}}{\text{number of libraries x Indexed library concentration (nM)}}$$

Indexed library pool was prepared by adding each calculated volume of indexed library, and Low TE Buffer 1X (0.1 mM EDTA) to obtain a final concentration of 4 nM. The final concentration of the pool was confirmed using the Qubit dsDNA HS Assay kit (invitrogen), following the manufacturer instruction. The molar concentration of the *pool* was validated by applying the formula presented in Equation 2.3. Pool was stored at 4 ºC until the performance of Multiplex Sequencing in a NextSeq Illumina Platform at the Technology and Innovation Unit of the Human Genetic Department at INSA-Lisbon (UTI-DGH, INSA).

Equation 2.3:

$$\text{Molar concentration (nM)} = \frac{\text{Qubit concentration (ng/µl) x 1 000 000}}{[\text{Mean peak size (pb) x 660}] + 157.9}$$

2.2.2.5. NGS analysis

NGS generated files were analysed individually to each Index Case, ensuring a focused research on the possible cause(s) of their dyslipidemia. The molecular research was focused on 18 genes divided into three small panels (figure 2.1) (Appendix II, Table A.II.3).

Familial Dyslipidemias			
Phenotype	High LDL cholesterol levels	Low LDL cholesterol levels	High triglyceride levels
Genes	*LDLR* *APOB* *PCSK9* *LDLRAP1* *APOE* *ABCG5* *ABCG8* *LIPA*	*ANGPTL3* *MTTP* *SAR1B* *APOB* *PCSK9*	*LPL* *APOC2* *APOA5* *APOC3* *GPIHBP1* *LMF1* *GPD1* *APOE* *LIPA*

Figure 2.1 – Gene panels for molecular diagnosis of familial dyslipidemias. Three panels with a total of 18 genes, according to clinical phenotype (hypercholesterolemia, hypocholesterolemia, and hypertriglyceridemia).

a) Bioinformatic analysis for single-nucleotide variants

The resulting sequencing files are generated in FASTQ format for each sample. For bioinformatic processing, SureCall Software Application from Agilent Technologies was utilized as the first approach. Sequencing reads were aligned to the human reference genome (according to Genome Reference Consortium Human Build 37, GRCh37)). VCF (variant calling) and BAM files (target region coverage statistic) were generated for each sample, containing information on the genomic position and zygosity of identified variants, as well as the depth of coverage for each sequencing read. wANNOVAR was used as a tool for the annotation and analysis of variants, as it is a reliable open-sourced tool [97,98]. Files resulting from wANNOVAR analysis were in CSV format, the analysis focused on rare variants with potential for protein-altering effects, such as missense, nonsense, insertion, deletions, splice-donor, and splice-acceptor variants. Files contain approximately 200 to 700 variants *per* sample, which were filtered by minor allele frequency (MAF), reducing the number of variants to 50-200 per sample. Rare variants (MAF <1% in case of dominant or codominant disorders, and MAF between 1-5% for recessive disorders) or variants absent from publicly available databases were selected as primary variants of interest.

b) Copy number variants (CNVs) analysis

CNVs were detected using the DECoN 1.0.2 Software [99]. This software required the BAM files previously originated (by SureCall software), a BED file (created by SureDesign Illumina only containing the panel genes and the correspondent reference sequence), and a FASTA file (assembled in SureCall) for the analysis. DECoN resulting files were generated in txt format containing all detected CNVs (a single txt file with all CNVs for all studied samples), and a single PDF file for each CNV detected.

c) Polygenic Risk Score

The LDL-c GRS (genetic risk score), for each individual, was calculated using the six SNPs previously reported in the characterization of polygenic hypercholesterolemia [100]: rs629301 (*CELSR2*), rs1367117 (*APOB*), rs4299376 (*ABCG5/8*), rs6511720 (*LDLR*), rs429358 and rs7412 (*APOE*). The SNPs were genotyped using the NGS methodology and the GRS was calculated by summing the effect sizes (weight) of the raising alleles of the respective SNPs. For interpretation of the GRS, scores were divided into quarters. Individuals above P75th were considered to have a high polygenic risk score, between P25th and P75th an intermediate polygenic score, and below P25th a low polygenic score.

d) Lp(a) Score

The Lp(a) GRS was calculated for each index case by genotyping *LPA* gene, using the NGS methodology. Two SNPs (rs3798220 and rs10455872) are the molecular basis of the score since these risk alleles contribute to high Lp(a) levels, and thus increasing the risk of development of CVD [101]. The score was calculated according to the existence of the number of risk alleles for each of the two SNPs implicated in increased Lp(a) concentrations. Score ranges between 0 and 4 that correspond to the sum of variant alleles (C for rs3798220, and G for rs10455872) in either of the two SNPs, therefore individuals with a Lp(a) score above 1 were considered to have a high *LPA* GRS.

2.2.3. DNA Amplification by Polymerase Chain Reaction (PCR)

Rare variants detected by Next Generation Sequencing were confirmed through Polymerase Chain Reaction (PCR). Fragments containing parts of *LDLR*, *APOB*, *PCSK9*, *APOA5*, *LPL*, *GPDI*, and *GPIHBP1* exons were amplified by this methodology. All primers used, as well as respective annealing temperatures and fragment length are presented in Appendix II, Table A.II.4. For fragment amplification of genomic DNA, PCR was performed with BIOTAQ™ DNA Polymerase kit (Bioline). In each reaction tube (0.2 µl) was added, according to Bioline kit established protocol: 4 µl of dNTPs (100 nM dNTP mix containing 100 nM of dATP, dCTP, dGTP and dTTP), 2.5 µl NH_4 buffer (10x, Bioline), 0.75 µl of Mg^{2+} (50 mM, Bioline), 1 µl of each primer required (forward and reverse) (10 ρmol/µl , Invitrogen), 1.25U of BioTaq polymerase (Bioline) and bidistilled water up to a final volume of 24 µl. At last, 1 µl of genomic DNA (100-300 ng) was added to the previously prepared PCR mix. As control, a replicate without DNA was used for each exon amplification reaction. PCR reaction was held in a thermal cycler (Applied biosystems 2720) follow: initial denaturation for 3 min at 95 ºC; 35 cycles of three steps: denaturation for 45 sec at 94 ºC; annealing for 30 sec at an ideal estimated temperature depending on the primers used (Appendix II, Table A.II.4); and elongation for 1 min at 72 ºC; and final extension for 30 min at 72 ºC. All PCR products were assessed by an agarose gel electrophoresis using a 1.5% agarose gel, prepared with 100 mL of TBE buffer 1x (Invitrogen), 1.5 g of SeaKem™ 3:1 Agarose (Lonza) and with 0.02% of SYBR Safe DNA gel stain (Invitrogen). A gene ruler marker (1 µL Thermofisher GeneRuler - 0.5 µg/µL - diluted in 5 µl of water and 4 µl of blue dye) was used for approximate estimation of fragment length. Samples loaded into 1.5% agarose gel were prepared by adding 5 µL of PCR product to 3 µL of gel loading dye (bromophenol blue). The electrophoresis was performed in a Bio-Rad Power Pac 3000 equipment, at 90V for 40 mins, and the gel was visualized in a Safe Imager™ transilluminator (Invitrogen).

2.2.4. Sanger Sequencing (Automated Sequencing)

Before Sanger Sequencing, PCR products were purified in order to remove the excess of primers and dNTPs originated by the amplification reaction. The purification was realized using ExoStar (Illustra™ ExoStar™), which allowed an enzymatic digestion. For purification, 1 µL of ExoStar was added in each reaction tube containing 2.5 µL of PCR product. Tubes were placed in a thermal cycler (Applied Biosystems 2720) for an incubation at 37ºC for 15 mins, followed by an enzyme activation at 80ºC for 15 mins. The reaction mix for Sanger sequencing was prepared as follow: 2 ρmol of primer (forward or reverse) (Invitrogen), 1 µL of BigDye™ Terminator v3.1 Cycle Sequencing Kit (Themo Fisher) and bidistilled water was added up to a final volume of 9 µL. At last, 1 µL of the purified product was added. The sequencing reaction was also held in the Applied Biosystems thermal cycler: initial denaturation for 30 sec at 96 ºC; 25 cycles of three steps: denaturation for 10 sec at 96 ºC, annealing for 5 sec at 50 ºC and elongation for 4 min at 60ºC. Resulting products were sequenced in a 3500 Genetic Analyser (Applied Biosystems) at the at the Technology and Innovation Unit of the Human Genetic Department at INSA-Lisbon (UTI-DGH, INSA). Automated sequencing .AB files were analysed using Staden Package software (Gap5 Alignment program). Sequences were read compared with a “control” without variant and following the reference sequence of transcripts for each gene (Ref Seq number, Appendix II, Table A.II.3). Variants were numbered according to Human Genetic Variation Society (HGVS) guidelines. The coding nomenclature was described using the “A” of the ATG translation initiation codon as position number 1.

2.3. Variants Classification

Variants were classified according to the guidelines and recommendations of the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology [102]. Particularly for variants in the LDLR gene, variants were curated according to the Clinical Genome Resource (ClinGen) Familial Hypercholesterolemia Variant Curation Expert Panel consensus guidelines specifically designed for *LDLR* classification [103], since the AMCG guidelines are generalizable for Mendelian disorders.

Overall, both guidelines were elaborated to promote the use of standard terminology for classifying sequence variants using available evidence weighted according to a system developed through expert opinion, workgroup consensus, and community input. Each rare variant was individually classified using a scoring algorithm (Appendix III, Figure A.III.1 and A.III.2), in which points are attributed, given in consideration the amount of information available in public and private databases regarding several aspects. Briefly, the main information accessed to classify variants included population data; computational and predictive *in silico* tools; functional data; segregation and *de novo* information; allelic data; and other additional information. Data was obtained after bioinformatic research using GnomAD (allele frequency and populational information), Ensembl (for transcript confirmation), Mutalyzer (nomenclature confirmation), REVEL (*in silico* tool), ClinVar and PubMed (for populational and clinical information). Data regarding the same variant was gathered, and critically analysed to attribute "strong" or "supporting" points (in case of benign contribution), and "supporting", "moderate", "strong" or "very strong" points (in case of pathogenic contribution) as presented in Appendix III, Figure A.III.1. The combination of these different points allowed variant classification according to the recommendations that promoted the use of the following terminology: "pathogenic", "likely pathogenic", "variant with uncertain significance", "likely benign", and "benign" (Appendix III, Figure A.III.2) for clinical significance.

2.4. Co-segregation Studies

To perform cascade screening, co-segregation studies were carried in relatives of index cases with a putative variant causing genetic dyslipidemia. After the confirmation of the causative variant in the index (by NGS followed by PCR and Sanger Sequencing), 33 relatives were firstly enrolled this research project for sample collection. Index relatives' samples were collected for lipid profiling and biochemical characterization (7.5 mL in serum tubes for adults; 5 mL for children), and DNA extraction (3 x 2.7 mL in EDTA tubes for adults; 2 x 2.7 mL in EDTA tubes for children participants). Subsequently, after DNA extraction, quantification and quality assessment, we exclusively searched for the specific variant discovered in the respective family index case. The exonic or intronic location of variants (in relatives) was accessed by PCR and Sanger Sequencing. All these laboratory procedures were conducted in the same condition as described in previous sections: *2.2.1. "Genomic DNA Extraction", 2.2.3. "Polymerase Chain Reaction",* and *2.2.4. "Sanger Sequencing".* Families' pedigree was design using Cyrillic Software.

2.5. Functional Studies using RNA

2.5.1. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral Blood Mononuclear Cells (PBMC) were isolated from CPT tubes (BD Vacutainer® CPT™, Cell Preparation Tube containing Sodium Citrate anticoagulant) containing fresh blood. After blood collection, tubes were well homogenised by slow inversion and centrifuged at room temperature for 30 minutes at 2800 rpm, acceleration 9 and break 3 (5810 R *Eppendorf* centrifuge). This procedure

must be performed during the first 2 hours after blood collection. PBMC were resuspended though inversion of CPT tubes. Each supernatant (upper layer, containing plasma) was transferred to a 15 mL falcon tube and PBMC were obtained by centrifugation at 4ºC for 10 minutes at 1600 rpm, acceleration 9 and break 7. The mononuclear cells were resuspended in sterile 1 mL PBS 1X (Dulbecco's phosphate buffered saline – 0.0095M (PO4) without Ca and Mg) stored at 4ºC. After separating 1 μl of cellular suspension and adding 9 μl of Trypan Blue (Trypan blue stain 0.4%, GIBCO), cells were counted by applying the 10 μl in an Neubauer counting chamber. The remaining cell resuspension was transferred to a tube and centrifuged at 4ºC for 10 minutes at 3000 rpm (microcentrifuge). The supernatant was disposed, and the pellet was resuspended in 350 μl ($<5x10^6$ cells) or in 600 μl ($\geq 5x10^6$ to $1x10^7$ cells) of RTL Buffer (Quiagen) containing 1% Mercaptoethanol (Merk). Resuspension was subsequently homogenised with a syringe (20G, 0,6 mm, 1mL) in order to enable cellular lysis, and the lysate was centrifuged using QIAShredder mini column at 4ºC for 2 minutes at 13200 rpm. Tubes containing PBMC were stored at -80ºC for posterior RNA extraction or for immediate RNA extraction.

2.5.2. RNA extraction

Thawed samples were used to extract total RNA (with RNeasy® Mini Kit, Quiagen). After adding an equal volume of ethanol 70% (-20ºC) to the lysate, samples were homogenised and transferred to a RNeasy column for a 15 secs centrifugation at 1100 rpm (at room temperature). The eluted was discarded and 350 μl - 500 μl of ethanol 70% (-20ºC) were added to the columns, which were centrifuged at room temperature, for 15 secs at 1100 rpm (as earlier). The columns were transferred to new collector tubes and 350 μl of RW1 buffer solution (Kit RNeasy) were added. Tubes were centrifuged at room temperature for 15 secs at 1100 rpm, after gently mixing of samples by inversion, and the eluted was disposed. Mix DNase I (containing 70 μl of RDD Buffer plus 10 μl of DNase I) was mixed and applied in the centre of the membrane, for each column. Samples incubated at room temperature (18ºC) for 15 mins. DNase I was removed by adding 350 μl of RW1 buffer solution and by centrifugation of the tubes, at the same conditions as previously. Eluted volume was poured off, 500 μl of RPE buffer solution were added and mixed, and a centrifugation was performed at 18ºC, 1100 rpm for 15 secs. After repeating this step of the protocol once, tubes were centrifuged (with the same conditions) in order to remove all RPE buffer. The column was transferred to a 1.5 mL Eppendorf and 50 μl of RNase free water were applied in the centre of each membrane. Eppendorf tubes containing the columns were centrifuged at 1300 rpm for 1 min at 4ºC for DNA elution. Columns were discarded and 6 μl - 10 μl of the samples were transferred to a tube for quantitative and qualitative analysis of RNA. The remaining volume of RNA was stored at -80ºC. RNA content was quantified using a spectrophotometer (NanoDrop 1000, Thermo Scientific). The quality of RNA was assessed by agarose gel electrophoresis using 1% agarose gel, prepared with 100 mL of TBE buffer 1x (Invitrogen), 1.0 g of Nusivier ® agarose and with 0.01% of SYBR Safe DNA gel stain (Invitrogen). The samples loaded into 1% agarose gel were prepared by mixing 5 µL of RNA with 5 µL of gel loading dye (bromophenol blue). The electrophoresis was performed in a Bio-Rad Power Pac 3000 equipment at 90V for 40 mins, and the gel was visualized in a Safe Imager™ transilluminator (Invitrogen).

2.5.3. Reverse transcriptase reaction and cDNA analysis

In order to prepare cDNA for reverse transcriptase (RT) reaction, RNA samples (stored at -80ºC) were thawed, as all reagents from the kit (Roche, reverse transcription kit) needed to prepare the RT Mix. Aliquots containing 1µg of RNA were prepared by adding RNA and RNase free water, to a total volume of 19.25 μl *per* sample. To each sample, 1.25 μl of Oligo d(T) were added, and an incubation step of 5 mins at 65ºC followed by 2 mins at 4ºC was performed at Applied Biosystems 2720 thermal cycler. During the incubation of samples, RT Mix was prepared by mixing firstly non-enzymatic

reagents (5 µl of 10X TaqMan RT buffer, 11 µl of MgCl2 (25mM), 10 µl dNTP Mix and 1.25 µl Random hexamers primer) and thereafter the enzymatic components of the mix were added (1 µl of RNase inhibitor (20U/µl) and 1.25 µl of Reverse Transcriptase (50U/µl)). RT Mix was gently homogenised, and then 29.5 µl of mix were distributed to each pre-incubated sample containing RNA plus Oligo d(T). Tubes with a total volume of 50 µl were incubated (10 minutes at 25ºC), followed by RT synthesis phase (30 mins at 48ºC) and lastly a synthesis inactivation phase (5 mins at 95ºC). After this process, RNA samples were stored at -80ºC once again, and cDNA was stored at -20ºC.

2.5.4. cDNA amplification by Polymerase Chain Reaction (PCR)

For one specific case in this project, PCR was performed using synthetised cDNA for an Index case with a variant in *LDLR* gene, exon 4. The polymerase chain reaction followed the same protocol (amount of reagents and thermal cycler program) as described in section 2.2.3. "*Polymerase Chain Reaction*". Primers and annealing temperatures are detailed in (Appendix II, Table A.II.5). PCR products were assessed by an agarose gel electrophoresis using a 2.5% agarose gel, prepared with 100 mL of TBE buffer 1x (Invitrogen), 2.5 g agarose mixture (2g Nusivier ®+ 0.5g of MetaPhor ®) and with 0.02% of SYBR Safe DNA gel stain (Invitrogen). A stained gene ruler marker (1 µL Thermofisher GeneRuler - 0.5 µg/µL - diluted in 5 µl of water and 4 µl of blue dye) was used for approximate estimation of fragment length. Samples loaded into 2.5% agarose gel were prepared by adding 5 µL of PCR product to 3 µL of gel loading dye (bromophenol blue). The electrophoresis was performed in a Bio-Rad Power Pac 3000 equipment, at 50V for 2 hours, and the gel was visualized in a Safe Imager™ transilluminator (Invitrogen).

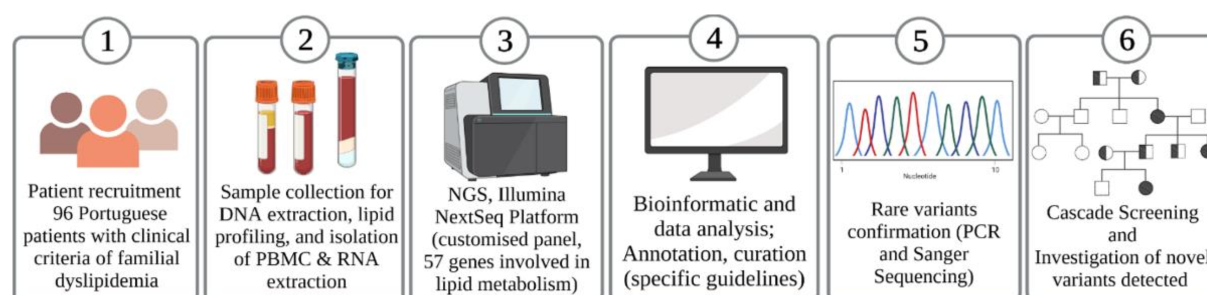

Figure 2.2 – Overview of the workflow for the project "Molecular diagnosis of familial dyslipidemias by Next Generation Sequencing". **1)** 96 Portuguese patients, referred to GIC-INSA, were clinically diagnosed with at least one type of familial dyslipidemia; **2)** Blood collection was performed to: access lipid profile (for biochemical characterization) using serum (first tube), DNA extraction using EDTA tubes (second tube), and isolation of PBMC using a CPT tube (third tube) (to perform RNA extraction if necessary in case of functional studies); **3)** Molecular analysis was performed using NGS technique with a customised panel of 57 genes related to lipid metabolism, the samples were run in an Illumina NextSeq Platform. **4)** Generated data was analysed using bioinformatic tools, such as clinical and population databases, to investigate the pathogenicity or possible previous report of rare variants identified (MAF 1-5%). Additionally, variants were classified according to the ACMG Guidelines and specific adaptations if existent; **5)** Rare variants detected were confirmed using PCR and Sanger Sequencing; **6)** After study the index patient, in case of detection of a pathogenic or likely pathogenic variant or VUS, cascade screening was performed to identify affected relatives. In this project, some variants (detected for the first time in Portugal) were investigated in a deeper level (studies of mRNA splicing). EDTA, Ethylenediamine tetraacetic acid; PBMC, peripheral blood mononuclear cells; CPT, Cell Preparation Tube (BD Vacutainer® CPT™) with Sodium Citrate; NGS, next generation sequencing; MAF, minor allele frequency; ACMG, *American College of Medical Genetics*; PCR, polymerase chain reaction; VUS, variant of uncertain significance. Workflow designed with BioRender.com

Chapter 3. Results

3.1. Clinical Characterization

A total of 96 unrelated Portuguese individuals were analysed using NGS in order to find a monogenic cause of their dyslipidemia. All the subjects admitted to this project fulfilled clinical criteria for a familial dyslipidemia associated to the three traits under study (hypercholesterolemia, hypocholesterolemia, and hypertriglyceridemia). According to their lipid profile (high cholesterol, low cholesterol, and high triglycerides), subject were divided into three main groups.

In addition, a total of 33 relatives, affected and unaffected, were also recruited.

3.1.1. Hypercholesterolemia

The group of individuals presenting elevated cholesterol levels comprised 64 unrelated individuals, 44 adults (with ages comprehended between 18 and 73 years old) and 20 children (with ages comprehended between 5 and 17 years old). Clinical and biochemical characterization is summarized in Table 3.1.

3.1.2. Hypocholesterolemia

The group of individuals presenting low cholesterol levels was constituted by 5 unrelated individuals, 4 adults (with ages comprehended between 28 and 53 years old) and 1 child (16 years old). Clinical and biochemical characterization is summarized in Table 3.2.

3.1.3. Hypertriglyceridemia

The group of individuals presenting elevated triglyceride levels comprised 27 unrelated individuals, 18 adults (with ages comprehended between 21 and 66 years old) and 9 children (with ages comprehended between 2 months and 17 years old). Clinical and biochemical characterization is summarized in Table 3.3.

Table 3.1 - Clinical and biochemical characterization of 64 index cases with clinical hypercholesterolemia.

Patient	Sex	Age (years)	Biochemical characterization at referral							Medication	Physical manifestations and/or CV events
			TC (mg/dL)	LDL-c (mg/dL)	HDL-c (mg/dL)	TG (mg/dL)	Lp(a) (mg/dL)	ApoA1 (mg/dL)	ApoB (mg/dL)		
IC 1	F	65	323	255	45	256	64	154	196	No	No
IC 2	M	12	268	190	73	62	10.8	184	129.2	No	No
IC 3	F	48	277	196	60	88	2.6	153	131.1	Atorvastatin + Ezetimibe	Xanthelasma
IC 4	F	5	226	166	53	52	15.7	125.9	112.8	Stanol + Phytosterol	No
IC 5	F	46	328	227	62	252	23.9	202.1	168.4	No	No
IC 6	F	18	379	302	37	158	23.4	118.9	228.2	No	No
IC 7	M	5	270	205	39	77	8.3	110	146	Stanol + Phytosterol	No
IC 8	F	23	183	124	58	52	16.2	137	96.6	Atorvastatin + Ezetimibe	No
IC 9	F	25	272	190	68	123	-	195	147	No	No
IC 10	M	23	209	166	39	55	10.9	103.5	138.2	Rosuvastatin	No
IC 11	M	11	207	153	29	135	8.3	92	141	Stanol + Phytosterol	No
IC 12	F	14	162	104	42	75	8.3	129	95	Rosuvastatin	No
IC 13	F	15	199	118	56	118	15	153	104	Pravastatin	Angina, 15y
IC 14	M	14	237	172	38	87	8.3	115	138	No	No
IC 15	F	19	311	201	45	247	27	166	204	No	No
IC 16	F	9	252	168	70	54	8.3	168	116	No	No
IC 17	F	61	184	101	60	110	173	180	87	Rosuvastatin + Ezetimibe	MI 44y, PTCA (y NK)
IC 18	F	30	360	179	23	276	8.3	78	184	No	No
IC 19	F	20	172	95	56	141	59.6	101.7	62.7	Rosuvastatin	No
IC 20	F	28	280◆	209◆	51◆	98◆	-	-	-	Atorvastatin + Ezetimibe	No
IC 21	F	62	245	162	44	198	139	146	147	Rosuvastatin	No
IC 22	F	50	300◆	228◆	55◆	87◆	-	-	-	Atorvastatin + Ezetimibe	MI 42y, PTCA 42y
IC 23	M	9	176	102	56	67	8.3	159	77	Pravastatin	No
IC 24	F	61	305	200	53	195	101	159	165	Rosuvastatin + PCSK9i	No
IC 25	F	52	228	133	66	89	69	191	110	Rosuvastatin + Ezetimibe	No
IC 26	F	43	393	323	47	83	66	139	237	No	No
IC 27	F	61	159	93	35	159	33	125	105	Rosuvastatin + Ezetimibe	Angina, MI 54y, PTCA (y NK), CABG 56y
IC 28	F	18	237	168	60	70	-	-	-	No	No
IC 29	F	5	231	150	48	149	103	150	136	No	No
IC 30	F	16	224	114	94	46	70	203	94	Simvastatin	No
IC 31	F	18	246	158	61	61	8.3	154	115	No	No
IC 32	M	73	211	130	54	101	8.3	160	120	Rosuvastatin + Ezetimibe	No
IC 33	F	37	281	195	73	54	70	162	141	No	No
IC 34	M	30	255	198	38	97	14	119	156	No	No

Table 3.1 - Clinical and biochemical characterization of 64 index cases with clinical hypercholesterolemia (continuation).

IC 35	F	57	403◆	274◆	63◆	332◆	-	-	-	Rosuvastatin + Ezetimibe	No
IC 36	F	20	268◆	184◆	40◆	221◆	-	-	-	No	No
IC 37	M	57	282◆	219◆	35◆	142◆	-	-	-	Rosuvastatin + Ezetimibe	Angina 37y
IC 38	F	39	381	295	54	158	82	134	113	Statin (NK)	No
IC 39	F	11	239◆	168◆	51◆	98◆	-	-	-	No	No
IC 40	F	>18	196	91	84	60	8.3	232	88	Atorvastatin	No
IC 41	F	44	346	270	39	121	8.3	120	216	Statin (NK)	No
IC 42	M	8	244	187	44	47	8.3	125	143	Stanol + Phytosterol	No
IC 43	F	19	274	164	55	356	10	175	133	No	No
IC 44	F	59	143	61	56	98	16	165	75	Rosuvastatin + Ezetimibe	No
IC 45	F	72	151	79	52	111	-	-	-	NK	No
IC 46	M	16	221	152	50	60	8.3	131	113	Simvastatin	No
IC 47	F	11	339	265	50	126	104	128	135	NK	No
IC 48	M	56	278	198	46	126	11	158	158	Pravastatin	No
IC 49	M	35	263	192	46	139	70	145	156	Atorvastatin + Ezetimibe	No
IC 50	M	49	128	70	29	171	8.3	119	78	Atorvastatin + Ezetimibe	MI 49y, PTCA 49y
IC 51	F	62	185	100	51	134	17	156	93	Atorvastatin + Ezetimibe	No
IC 52	M	53	160	90	29	152	114	99	104	Rosuvastatin + Ezetimibe	No
IC 53	F	50	220	156	47	115	132	140	115	Lovastatin + Ezetimibe + Fibrate	No
IC 54	M	47	204	145	36	97	13	110	122	Statin (NK) + Ezetimibe	No
IC 55	M	29	297	218	64	61	48	148	129	Atorvastatin + Ezetimibe	No
IC 56	M	33	161	93	47	100	181	133	81	Rosuvastatin	No
IC 57	F	49	249	137	63	192	12	219	125	Rosuvastatin + Ezetimibe + Fenofibrate	No
IC 58	F	67	209	128	63	144	-	-	-	Atorvastatin + Ezetimibe	No
IC 59	F	13	213	156	47	59	-	-	-	Simvastatin	No
IC 60	M	12	270	185	71	61	10,5	184	129.2	No	No
IC 61	F	7	246	188	39	87	41.5	115.7	139.5	Phytosterol	No
IC 62	F	14	306	248	51	150	-	145	162	Simvastatin	No
IC 63	F	13	205	134	56	82	-	158	99	Atorvastatin	No
IC 64	F	53	224	150	65	84	-	-	-	Atorvastatin + Ezetimibe	MI 28y

IC, index case; F, female; M, male; y, years; CV, cardiovascular disease; TC, total cholesterol; LDL-c, cholesterol LDL; HDL-c, cholesterol HDL; TG, triglycerides; Lp(a), lipoprotein a; ApoA1, apolipoprotein A-I; ApoB, apolipoprotein B; NK, not known; PCSK9i, PCSK9 inhibitor; MI, myocardial infarction; PTCA, percutaneous transluminal coronary angioplasty; CABG, coronary artery bypass graft. ◆values provided through the clinical questionnaire.

Table 3.2 - Clinical and biochemical characterization of 5 index cases with clinical hypocholesterolemia.

Patient	Sex	Age (years)	Biochemical characterization at referral							Medication	Physical/clinical features
			TC (mg/dL)	LDL-c (mg/dL)	HDL-c (mg/dL)	TG (mg/dL)	Lp(a) (mg/dL)	ApoA1 (mg/dL)	ApoB (mg/dL)		
IC 65	F	28	69	18	52	13	-	115	15	No	No
IC 66	F	32	< P5th ◆	< P5th ◆	-	-	-	-	-	-	No
IC 67	F	16	30	<4	28	9	<8.3	82.5	<20	No	No
IC 68	F	38	132	78	40	37	19	115	69	No	Hepatic Steatosis and Hypothyroidism
IC 69	M	53	62	11	48	18	38	110	<20	No	Hepatomegaly and Hepatic Steatosis

IC, index case; F, female; M, male; TC, total cholesterol; LDL-c, cholesterol LDL; HDL-c, cholesterol HDL; TG, triglycerides; Lp(a), lipoprotein a; ApoA1, apolipoprotein A-I; ApoB, apolipoprotein B. ◆values provided through the clinical questionnaire.

Table 3.3 - Clinical and biochemical characterization of 27 index cases with clinical hypertriglyceridemia.

Patient	Sex	Age (years)	Biochemical characterization at referral							Medication	CVD	Hepatic complications
			TC (mg/dL)	LDL-c (mg/dL)	HDL-c (mg/dL)	TG (mg/dL)	Lp(a) (mg/dL)	ApoA1 (mg/dL)	ApoB (mg/dL)			
IC 70	F	2 m	116	23	21	536	8	139	45	No	No	Hepatomegaly
IC 71	M	3 m	310	-	5	1102	<8	18	240	No	No	Hepatomegaly
IC 72	F	53	280	182	24	378	<8.3	118	186	No	MI 49y	No
IC 73 ■	M	58	156	73	57	176	60.4	169	89	Statin + PCSK9i	MI 46y, PTCA	No
IC 74 ■	F	32	318	121	30	851	9.9	108	180	Statin + Fibrates	No	Pancreatitis
IC 75	M	43	>300◆	-	-	1110◆	-	-	-	NK	No	No
IC 76	M	40	127	7	6	1824	-	-	-	NK	NK	Pancreatitis
IC 77	M	44	263	90	30	494	-	-	-	Statin + Fibrates	MI 40y	No
IC 78	M	71	242	66	20	1469	-	-	-	Statin + Fibrates	No	No
IC 79	F	1	192◆	-	19◆	1158◆	-	-	-	No	No	No
IC 80	F	17	78	45	11	542	-	-	-	Statin + Fibrates + PCSK9i + DHA	No	Pancreatitis
IC 81	M	47	258	169	28	349	-	-	-	Atorvastatin + Ezetimibe	MI 45y, CABG	No
IC 82	M	44	207	101	42	354	-	-	-	Fibrates + Ezetimibe	No	Hepatic Steatosis
IC 83	F	54	297	15	9	2445	-	-	-	Rosuvastatin + Fibrates	No	No
IC 84 ■	M	40	122	27	23	584	<8.3	122	56	Rosuvastatin + Fibrates + Omega 3	No	Yes (NK)
IC 85	F	21	111	56	42	99	100.5	128.2	62.7	Rosuvastatin	No	No
IC 86	F	17	268	148	47	446	52.4	199.5	144.7	No	No	No
IC 87	M	66	198	121	36	262	-	142	122	Atorvastatin + Ezetimibe + Fibrates	No	Hepatomegaly and Hepatic Steatosis (63y)
IC 88	F	13	165	82	44	187	156	137	83	Statin	No	No
IC 89	F	9	221	137	39	258	<8.3	130	133	Stanols + Phytosterols	No	No
IC 90	F	47	190	6	9	2259	26	91	67	Rosuvastatin + Fibrates	No	Pancreatitis (34y)
IC 91	M	57	377	281	32	264	61	139	236	Rosuvastatin + Ezetimibe	No	No
IC 92	F	6 m	669◆	181◆	6*	6744◆	-	-	-	No	No	Hepatomegaly (3 m)
IC 93	F	32	162	46	21	622	<8.3	117	103	Atorvastatin + Fibrates	No	Pancreatitis (25y), Hepatis steatosis (30y)
IC 94	F	6	287	200	25	250	26	89	164	No	No	No
IC 95	M	62	124	43	18	433	<8.3	71	86	NK	NK	NK
IC 96	M	46	153	88	33	137	<8.3	109	94	Statin + Fibrates	NK	NK

IC, index case; F, female; M, male; m, months; y, years; CV, cardiovascular disease TC, total cholesterol; LDL-c, cholesterol LDL; HDL-c, cholesterol HDL; TG, triglycerides; Lp(a), lipoprotein a; ApoA1, apolipoprotein A-I; ApoB, apolipoprotein B; NK, not known; PCSK9i, PCSK9 inhibitor; DHA, docosahexaenoic acid; MI, myocardial infarction; PTCA, percutaneous transluminal coronary angioplasty; CABG, coronary artery bypass graft. ■, present xanthelasma; ◆values provided through the clinical questionnaire.

3.2. Molecular Study

In this project, 96 IC were studied using NGS panels according to three main dyslipidemic traits (18 genes in total). After bioinformatic analysis of NGS-generated data, rare variants with a minor allele frequency below 1% (for autosomic codominant disorders) and below 5% (for autosomic recessive disorders) were classified according to ACMG general criteria or specific recommendations, if existent, to establish patient's cause of disease [102–104]. A total of 61 rare variants were detected in these ICs.

3.2.1. FH and phenocopies panel

FH and phenocopies panel covered a total of 8 genes (*LDLR*, *APOB*, *PCSK9*, *ABCG5/8*, *LDLRAP1*, *LIPA*, and *APOE*). Regarding 64 index cases, 48 different rare variants were found in five different genes (*LDLR*, *APOB*, *PCSK9*, *ABCG5/8*). Variants detected were located in genes associated with two different genetic disorders: FH (Table 3.4), and sitosterolemia (Table 3.5).

After applying ACMG criteria, 15 variants were classified as pathogenic, 4 as likely pathogenic, and the majority (29) as variants of unknown significance (VUS). It is worth highlighting that among 29 VUS, five missense variants (two in *ABCG8;* one in *PCSK9, APOB* and *LDLR*) were found for the first time within the Portuguese population, and are not reported to date in publicly available databases (in grey, Table 3.4). Regarding the findings in the FH panel, for two alterations (in blue, Table 3.4), functional studies were designed and initiated during this project.

3.2.2. Hypocholesterolemia panel

The hypocholesterolemia panel covered a total of 5 genes (*APOB*, *PCSK9*, *MTTP, SAR1B,* and *ANGPTL3*). Rare variants were only detected in the *APOB* gene in five ICs (Table 3.6). Three pathogenic variants included: one frameshift alteration c.3367del/p.(Val1123Leufs*62), one splicing alteration c.12087+1G>A/p.(?), and one nonsense variant c.4651C>T/p.(Gln1551*). In addition, two variants were classified as likely pathogenic: a large deletion of two *APOB* exons, and one splicing alteration c.2604+1G>A/p.(?). Two variants ((c.3367del/p.(Val1123Leufs*62), and (c.12087+1G>A/p.(?)) were found for the first time within the Portuguese population, and are not reported to date in publicly available databases (in grey, Table 3.6).

3.2.3. Hypertriglyceridemia panel

The panel used for analysis of rare disorders associated with triglyceride levels included 9 genes. A total of 27 different cases were studied, and 7 different variants were found in 4 genes (*APOA5*, *LPL*, *GPD1* and *GPIHBP1*). Variants detected were located in genes associated with FCS, MCM, and autosomal recessive hypertriglyceridemia (Table 3.7). After applying ACMG criteria, four variants (in 3 genes) were classified as pathogenic: c.289C>T/p.(Gln97*) and c.990_993del/p.(Asp332Valfs*4) both in the *APOA5*, c.701C>T/p.(Pro234Leu) in the *LPL*, and a deletion of 2 exons in the *GPIHBP1*. Two variants were classified as likely pathogenic: c.590G>T/p.(Arg197Leu) in the *LPL* gene, and c.895G>A/p.(Gly299Arg) in the *GPD1* gene. One alteration, c.644C>T/p.(Pro215Leu) in *APOA5* gene, was classified as VUS.

The variant in the *GPIHBP1* was found for the first time within the Portuguese population. For this variant functional studies were designed and initiated during this project (in blue, Table 3.7). In addition, in IC87, clinically characterized with hypertriglyceridemia, was found a variant c.449A>G/p.(Tyr150Cys), located in exon 5 of the *LIPA* gene in heterozygosity (VUS).

Table 3.4 – Summary of variants detected by NGS in FH genes, analysed within the FH and phenocopies panel.

Gene	Alteration			MAF (%)	First described	Functional Studies	Revel	Co segregation	ACMG		Patients
	Loc	cDNA	Protein						Criteria	Classification	
LDLR	**ex 1 - int 1**	**c.-57_67+56 del**	**p.(?)**	**-**	**Not described**	**No**	**NA**	**NA**	**PVS1, PM2, PP4**	**Pathogenic**	IC 20
	prom	c.-135 C>G	p.(?)	-	(33)	(33)	NA	1/1; 0/0	PS4, PP1_Strong, PM2, PS3_Moderate, PP4	**Pathogenic**	IC 62
	ex 2	**c.170 A>G**	**p.(Asp57Gly)**	**-**	**Not described**	**No**	**0.954**	**NA**	**PM2, PP3, PP4**	**VUS**	IC 52
	ex 4	c.326 G>T	p.(Cys109Phe)	-	(44)	(GIC, unpublished data)	0.951	2/2; 0/0	PP1_moderate, PM1, PM2, PP3, PP4	**Likely Pathogenic**	IC 10
	ex 4	c.369_393del	p.(Arg124Glyfs*74)	-	(44)	No	NA	2/2; 0/2	PVS1, PM2, PP1, PP4, PS4_Supporting	**Pathogenic**	IC 26
	ex 4	c.551 G>A	p.(Cys184Tyr)	0.009	(105)	(106)	0.854	NA	PS3, PP1_Strong, PM2, PM1, PS4_moderate, PP3, PP4	**Pathogenic**	IC 24, 64
	ex 4	**c.621 C>A**	**p.(Gly207Gly)**	**-**	**Not described**	**(GIC, ONGOING)**	**NA**	**NA**	**PM2, PP4, BP4, BP7**	**VUS**	IC 37
	ex 4	c.666 C>G	p.(Cys222Trp)	-	(95)	(GIC, unpublished data)	0.918	1/1; 0/0	PM1, PM2, PP3, PP4, PS3_Supporting	**Likely Pathogenic**	IC 32
	ex 4	c.670 G>A	p.(Asp224Asn)	-	(33)	(33)	0.833	3/3; 0/1	PS4, PP1_Strong, PM2, PM1, PM3, PS3_Moderate, PP3, PP4	**Pathogenic**	IC 3, 58
	ex 4	c.693 C>G	p.(Cys231Trp)	-	(107)	No	0.708	NA	PM2, PM1, PP3, PP4, PS4_Supporting	**Likely Pathogenic**	IC 6
	int 5	c.818-2 A>G	p.Val273Glyfs*31	-	(44)	(108)	NA	1/1; 0/0	PVS1, PM2, PP1_Moderate, PS3_Supporting, PS4_Supporting, PP4	**Pathogenic**	IC 7
	ex 6	c.862 G>A	p.(Glu288Lys)	0.004	(109)	(106,110)	0.85	NA	PP1_Strong, PM2, PS4_Moderate, PS3_Supporting, PP3, PP4	**Pathogenic**	IC 53
	ex 6	c.939 C>A	p.(Cys313*)	-	(33)	(33)	NA	NA	PVS1, PS3_Moderate, PM2, PM1, PP1	**Pathogenic**	IC 57
	ex 7	c.1027 G>A	p.(Gly343Ser)	0.003	(33)	(33)	0.929	1/1; 0/0	PP1_Strong, PM2, PS4_Moderate, PP3, PP4, PS3_Supporting	**Pathogenic**	IC 31
	ex 9	c.1291 G>A	p.(Ala431Thr)	0.0004	(111)	(111,112)	0.914	1/1; 0/0	PS3, PS4, PP1_Strong, PM2, PM3, PP3, PP4	**Pathogenic**	IC 4
	ex 9	c.1322 T>C	p.(Ile441Thr)	-	(113)	(114)	0.907	1/1; 0/0	PS3, PP1_Strong, PM2, PS4_Moderate, PP3, PP4	**Pathogenic**	IC 59
	ex 11	c.1618_1620del	p.(Ala540del)	-	(95)	No	NA	1/1; 0/0	PM2, PM4, PP4, BP4	VUS	IC 47
	ex 11	c.1646 G>A	p.(Gly549Asp)	0.002	(111)	(33,115,116)	0.902	NA	PS4, PM2, PS3_Moderate, PP3, PP4	**Pathogenic**	IC 27
	ex 12	c.1775 G>A	p.(Gly592Glu)	0.006	(33)	(106,115,116)	0.938	1/2; 0/0 1/1; 0/0	PS4, PP1_Strong, PM2, PS3_Moderate, PP3, PP4	**Pathogenic**	IC 33, 41, 61

Table 3.4 - Summary of variants detected by NGS in FH genes, analysed within the FH and phenocopies panel (continuation).

PCSK9	ex 1	c.136 C>T	p.(Arg46Cys)	0.002	Not described	No	-	NA	PP4	VUS	IC 49
	ex 1	c.185 C>A	p.(Ala62Asp)	-	(117)	(117)	-	2/2; 0/1	PS3, PM2, PP4	**Likely Pathogenic**	IC 16
	ex 3	**c.416 A>G**	**p.(His139Arg)**	**-**	**Not described**	No	**-**	**NA**	PM2, PP4	**VUS**	IC 27
	ex 5	c.709 C>T	p.(Arg237Trp)	0.07	(43)	(118)	-	1/2; 0/0	PM8, PP4, BS3	VUS	IC 38
	ex 9	**exon 9 deletion**	**p.(?)**	**-**	**-**	**(GIC, ONGOING)•**	**NA**	**NA**	**PM2, PP4**	**VUS**	IC 1
APOB	ex 10	c.1213 C>A	p.(Pro405Thr)	0.0008	Not described	No	-	NA	PP4	VUS	IC 26
	ex 19	c.2938 G>A	p.(Ala980Thr)	0.006	(119)	No	-	NA	PP4	VUS	IC 50, 35
	ex 19	c.2950 G>A	p.(Ala984Thr)	0.001	Not described	No	-	NA	PP4	VUS	IC 9
	ex 22	c.3427 C>T	p.(Pro1143Ser)	0.2	(120)	No	-	NA	PP4, PM8	VUS	IC 44
	ex 26	c.4696 T>C	p.(Tyr1566His)	0.002	Not described	No	-	NA	PP4	VUS	IC 16
	ex 26	c.5599 C>T	p.(Arg1867Trp)	0.02	(35)	No	-	2/2; 0/0	PM8, PP4	VUS	IC36
	ex 26	c.6639_6641del	p.(Asp2213del)✦	0.5	(35)	No	NA	1/1; 0/0	PM4, PP4, BS2	VUS	IC51
	ex 26	c.6895 G>C	p.Asp2299His	0.2	Not described	No	-	NA	PP4	VUS	IC40
	ex 26	c.7853 T>C	p.(Ile2618Thr)	0.003	(35)	No	-	1/1; 0/0	PP4	VUS	IC 57
	ex 26	c.8045 G>T	p.(Ser2682Ile)	-	Not described	No	-	NA	PM2, PP4	VUS	IC 47
	ex 26	c.9835 A>G	p.(Ser3279Gly)✦	0.5	(121)	No	-	NA	PM1, PP4, BS2	VUS	IC 18, 34, 50
	ex 26	c.10580 G>A	p.(Arg3527Gln)	0.04	(36)	(122,123)	-	1/1; 0/0	PS3, PM5, PM8, PM9, PP4	**Pathogenic**	IC 42
	ex 29	c.12382 G>A	p.(Val4128Met)✦	0.6	(124)	No	-	NA	PP4, BS2	VUS	IC 17
	ex 29	c.12794 T>C	p.(Val4265Ala)	0.5	(125)	No	-	NA	PM1, PM8, PP4	VUS	IC 45
	ex 29	c.12940 A>G	p.(Ile4314Val)	0.5	(125)	No	-	NA	PM1, PP4	VUS	IC 53, 56
	ex 29	c.13177 A>G	p.(Ile4393Val)	0.004	Not described	No	-	NA	PM1, PP4	VUS	IC 33
	ex 29	c.13395 G>C	p.(Lys4465Asn)	0.0004	Not described	No	-	NA	PM1, PP4	VUS	IC 17
	ex 29	**c.13556 G>C**	**p.(Arg4519Thr)**	**-**	**Not described**	No	-	NA	PM1, PM2, PP4	**VUS**	IC 18

Each column presents information concerning variants detected by NGS in genes associated with FH. Information is presented by gene, alteration localization, nomenclature, proteins effect, frequency (percentage) first report (reference), functional information, *in silico* prediction using REVEL score (variant score >0.75 = probable do have a pathogenic effect), co-segregation (with alteration/with phenotype; with alteration/without phenotype), ACMG classification, and IC. Loc, localization (ex, exon; int; intron; prom, promotor); NA, not applicable; IC, index cases. Variants in *LDLR* were classified according to specific guidelines for this gene [103], and in *APOB* and *PCSK9* using general recommendations [104] (*in silico* predictors are not recommended for both genes). ✦ indicate variants detected in normolipidemic cohorts. Blue highlight, functional studies were initiated during this project (• Currently, functional studies are being designed and optimized). Grey highlight, novel variants, not reported in publicly available databases, and found for the first time in Portugal.

Table 3.5 – Summary of variants detected by NGS in genes associated with sitosterolemia (*ABCG5* and *ABCG8*), analysed within the FH and phenocopies panel.

Alteration				MAF (%)	First described	Functional Studies	Revel	Co segregation	Cis/trans	ACMG		Patients
Gene	**Loc**	**cDNA**	**Protein**							Criteria	Classification	
ABCG5	ex 5	c.593 G>A	p.(Arg198Gln)	0.1	(126)	No	0.776	NA	NA	PP3, PP4	VUS	IC 15
ABCG8	ex 4	c.434 G>C	p.(Arg145Thr)	0.0008	Not described	No	0.150	NA	NA	BP4, PM2, PP4	VUS	IC 21
	ex 7	c.1106 A>T	p.(Asp369Val)	0.003	Not described	No	0.160	NA	NA	BP4, PM2, PP4	VUS	IC 11
	ex 8	**c.1177 C>T**	**p.(Pro393Ser)**	**-**	**Not described**	**No**	**0.347**	**NA**	**?**	**BP4, PM2, PP4**	**VUS**	IC14
	ex 11	c.1608G>A	p.(Trp536*)	0.002	Not described	No	NA	NA	?	PVS1, PM2, PM4, PP4	**Pathogenic**	
	ex 11	**c.1688 G>A**	**p.(Ser563Asn)**	**-**	**Not described**	**No**	**0.274**	**NA**	**NA**	**BP4, PM2, PP4**	**VUS**	IC 34

Each column presents information concerning variants detected by NGS in genes associated with sitosterolemia. Information is presented by gene, alteration, localization, nomenclature, protein effect, frequency (percentage), frequency (percentage), first report (reference), functional information, *in silico* prediction using REVEL score (variant score >0.75 = probable do have a pathogenic effect), cis/trans expression, ACMG classification, and IC. Loc, localization (ex, exon); MAF, minor allele frequency; NA, not applicable; IC, index cases. Variants in were classified according to the ACMG guidelines [102]. Grey highlight, novel variants, not reported in publicly available databases, and found for the first time in Portugal.

Table 3.6 – Summary of variants detected by NGS associated with low LDL-c levels, analysed with the hypocholesterolemia panel.

Alteration				Zygosity	MAF (%)	First described	Functional Studies	Revel	Co segregation	ACMG		Patients
Gene	**Loc**	**cDNA**	**Protein**							Criteria	Classification	
APOB	int 17	c.2604+1 G>A	p.(?)	CpHtz	0.0003	Not described	No	-	NA	PSV1, PP4	**Likely Pathogenic**	IC 69
	ex 22	**c.3367del**	**p.(Val1123Leufs*62)**	**Htz**	**-**	**Not described**	**No**	**-**	**NA**	**PSV1, PM2, ,PP4**	**Pathogenic**	IC 65
	int 27	**c.12087+1 G>A**	**p.(?)**	**Hmz**	**-**	**Not described**	**No**	**-**	**3/3; 0/0**	**PSV1, PM2, PP4**	**Pathogenic**	IC 67
	ex 26	c.4651 C>T	p.(Gln1551*)	CpHtz	-	Not described	No	-	NA	PSV1, PM2, PP4	**Pathogenic**	IC 69
	Deletion of exons 27 to 29		p.(?)	Htz	-	-	-	-	**NA**	PSV1, PP4	**Likely Pathogenic**	IC 68

Each column presents information concerning variants detected by NGS in genes associated with familial hypobetalipoproteinemia. Information is presented by gene, alteration, localization, nomenclature, protein effect, zygosity, frequency (percentage), first report (reference), functional information, co-segregation (with alteration/with phenotype; with alteration/without phenotype), ACMG classification, and IC. Loc, localization (ex, exon; int; intron); MAF, minor allele frequency; Hmz, homozygous; Htz, heterozygous, CpHtz, compound heterozygous; NA, not applicable. IC, index case; Variants were classified according to the ACMG guidelines [102]. Grey highlight, novel variants, not reported in publicly available databases, and found for the first time in Portugal.

Table 3.7 – Summary of variants detected by NGS associated with high triglyceride levels, analysed using the hypertriglyceridemia panel.

Alteration				Zygosity	MAF (%)	First described	Functional Studies	Revel	Co segregation	Cis/trans	ACMG		Patients
Gene	**Loc**	**cDNA**	**Protein**								**Criteria**	**Classification**	
APOA5	ex3	c.289 C>T	p.(Gln97*)	Htz	0.008	(127)	(128)	NA	NA	NA	PSV1, PS3, PM2, PM4, PP4	**Pathogenic**	IC 89
		c.644 C>T	p.(Pro215Leu)	Htz	0.02	Not described	No	0.415	NA	NA	BP4, PP4	VUS	IC 93
		c.990_993del	p.(Asp332Valfs*4)	Htz	0.0008	Not described	No	NA	NA	NA	PSV1, PM2, PM4, PP4	**Pathogenic**	IC 79, 84, 88, 94
LPL	ex 5	c.590 G>T	p.(Arg197Leu)	Htz	0.0004	(129)	No	0.671	NA	NA	PS4_supportive, PM1, PM2, PP4	**Likely Pathogenic**	IC 78
		c.701 C>T	p.(Pro234Leu)	Htz	0.004	(130)	(130)	0.818	NA	NA	PS4_supportive, PS3, PM2, PM3⋆, PP3, PP4	**Pathogenic**	IC 96
		c.701 C>T	p.(Pro234Leu)	Hmz						?			IC 76
LIPA	ex 5	c.449A>G	p.(Tyr150Cys)	Htz	0.0008	Not described	No	0.370	NA	NA	BP4, PM2, PP4	VUS	IC 87
GPD1	ex 7	c.895 G>A	p.(Gly299Arg)	Hmz	-	(131)	No	0.907	NA	?	PS4_suportive, PM2, PM3⋆, PP3, PP4	**Likely Pathogenic**	IC 70
GPIHBP1	Deletion of exon 3 and 4		p.(?)	Hmz	-	(132)	(132)	NA	NA	?	PSV1, PS3, PM2, PM3⋆, PM4, PP4	**Pathogenic**	IC 92

Each column presents information concerning variants detected by NGS in genes associated with hypertriglyceridemia. Information is presented by gene, alteration, localization, nomenclature, protein effect, zygosity, frequency (percentage), first report (reference), functional information, *in silico* prediction using REVEL score (variant score >0.75 = probable do have a pathogenic effect), cis/trans expression, ACMG classification, and IC. Loc, localization (ex, exon); MAF, minor allele frequency; Hmz, homozygous; Htz, heterozygous; NA, not applicable; IC, index case. Variants in were classified according to the ACMG guidelines [102]. ⋆ attributed to variants detected in homozygosity. Blue highlight, functional studies were initiated during this project.

3.3. Genetic Diagnostic

From the totality of Portuguese individuals included in this project, a definite diagnosis of monogenic familial dyslipidemia was successful for 35/96 cases. The overall results of these ICs and additional findings among the totality of participants is summarized in Figure 3.3 (at the end of Chapter 3, page 39).

3.3.1. FH and phenocopies panel

In 22 ICs of the 64 individuals with clinical suspicion of genetically determined hypercholesterolemia, a monogenic cause of disease was identified (Table 3.8). Approximately 90% of the rare pathogenic variants proving a genetic diagnosis of FH were detected in *LDLR* gene (16 variants) whereas only 10 % were identified in other genes associated, such as *APOB* and *PCSK9* (1 variant in each gene). All patients have heterozygous FH. Despite the majority of genetic alterations comprise exonic missense alterations in the *LDLR* gene, NGS also identified one point substitution in the promotor region, two frameshift deletions, and a deletion of the first exon of the *LDRL* gene which constituted the identification of a novel variant causing of FH.

Table 3.8 – Summary of the molecular diagnosis of FH for 22 ICs.

Suspicion	Diagnosis	IC	Gene	Variant	Protein
FH	FH	IC 3, 58	*LDLR*	c.670 G>A	p.(Asp224Asn)
		IC 4	*LDLR*	c.1291 G>A	p.(Ala431Thr)
		IC 6	*LDLR*	c.693 C>G	p.(Cys231Trp)
		IC 7	*LDLR*	c.818-2 A>G	p.Val273Glyfs*31
		IC 10	*LDLR*	c.326 G>T	p.(Cys109Phe)
		IC 16	*PCSK9*	c.185 C>A	p.(Ala62Asp)
		IC 20	*LDLR*	c.-57_67+56 del	p.(?)
		IC 24, 64	*LDLR*	c.551 G>A	p.(Cys184Tyr)
		IC 26	*LDLR*	c.369_393del	p.(Arg124Glyfs*74)
		IC 27	*LDLR*	c.1646 G>A	p.(Gly549Asp)
		IC 31	*LDLR*	c.1027 G>A	p.(Gly343Ser)
		IC 32	*LDLR*	c.666 C>G	p.(Cys222Trp)
		IC 33, 41, 61	*LDLR*	c.1775 G>A	p.(Gly592Glu)
		IC 42	*APOB*	c.10580 G>A	p.(Arg3527Gln)
		IC 53	*LDLR*	c.862 G>A	p.(Glu288Lys)
		IC 57	*LDLR*	c.939 C>A	p.(Cys313*)
		IC 59	*LDLR*	c.1322 T>C	p.(Ile441Thr)
		IC 62	*LDLR*	c.-135 C>G	p.(?)

However, in 42/64 Portuguese ICs with clinically diagnosed hypercholesterolemia, a definite diagnosis was not reached (Figure 3.3). Rare pathogenic or likely pathogenic alterations, in homozygosity and/or heterozygosity, in genes closely related to autosomic recessive and/or dominant disorders causing hypercholesterolemia were not found. Nonetheless, we were able to find several rare variants in genes related to sitosterolemia (*ABCG5/8*), in heterozygosity (Table 3.5).

A total of 29 variants were detected in *LDLR*, *APOB*, *PCSK9*, *ABCG5* and A*BCG8* genes and were classified as VUS according to ACMG criteria. Among 22 individuals without a definite diagnose of

monogenic dyslipidaemia, 24 variants were found: 17 ICs presented variants in *LDLR*, *APOB*, and *PCSK9* genes; and, 5 IC in *ABCG5* and *ABCG8* genes. Considering the ACMG criteria applied for classification of this wide range of variants, a definite molecular diagnosis was not reached for these subjects due to the uncertainty of variants clinical significance (Figure 3.3). Additionally, 5 VUS (four in *APOB*, and one in *PCSK9* genes) were identified in patients with an LDLR or PCSK9 pathogenic or likely pathogenic variant.

In addition, segregation was accessed for 12 families in which the IC was found to have a monogenic cause of FH. Therefore, cascade screening was possible to perform in a total of 27 relatives. Co segregation studies (exemplified in Figure 3.1) showed that 14 of the 27 recruited relatives also present the same variant identified in the index case.

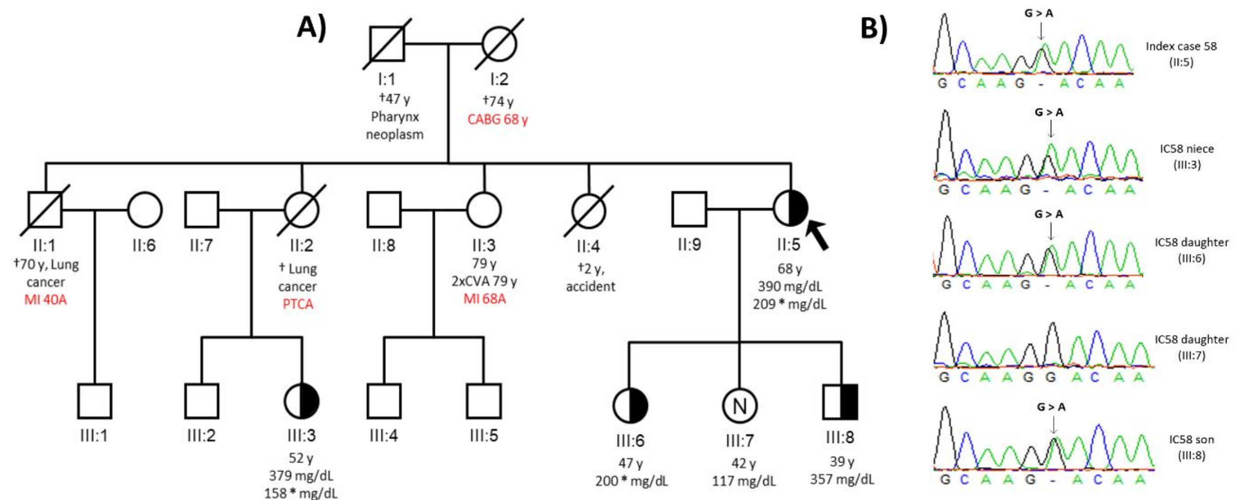

Figure 3.1 – Cascade screening in IC58 family. (**A**) Family pedigree. Arrow indicates the IC58 (II:5). Age (in years) and total cholesterol levels (mg/dL) are under each symbol (*corresponds to levels under pharmacotherapeutic treatment). Half black symbols represent heterozygous individuals for a variant in *LDLR* gene, c.670 G>A/p.(Asp224Asn). "N" represents a normolipidemic individual who did not inherit the variant in study. Deceased relatives are represented, † represents individuals cause of death, and age. Cardiovascular complications are highlighted (red) and respective age of CVD detection (CABG, coronary artery bypass graft; MI, acute myocardial infarction; PTCA, percutaneous transluminal coronary angioplasty; AVC, cerebrovascular accident). (**B**) Partial sequence of exon 4 of *LDLR* gene of IC58 and respective relatives, where the alteration G→A is located in position c.670, resulting in the amino acid change from an Aspartic acid to an Asparagine at protein position 224.

In 20 index cases, no pathogenic, likely pathogenic, or VUS were found. Thereby, in these specific cases, their genetic risk score and Lp(a) score was evaluated independently. Using the LDL-c GRS (genetic risk score), calculated using six SNPs [100], it was found that 13 out of 20 ICs had a GRS above P75th, and 7 ICs presented a moderated GRS, between P25th and P75th. Regarding the Lp(a) score, the analysis was performed by genotyping the *LPA* gene, and score values ranged between 0 and 4. Considering this specific group of "FH negative" patients, only 5 out of 20 ICs presented an elevated LPA score.

3.3.2. Hypocholesterolemia panel

We reached a monogenic cause of FHBL (familial hypobetalipoproteinemia) for 3 ICs considering the 5 individuals with clinical suspicion of FHBL or ABL (Table 3.9). NGS detected: one variant in homozygosity, one in heterozygosity, and two in compound heterozygosity, all *APOB* rare variants. No rare variants were discovered regarding *PCSK9*, *MTTP*, *SAR1B*, or *ANGPTL3*.

Additionally, one CNV in *APOB* gene (deletion of exon 27 to 29) was detected in one case, and its confirmation (through alternative sequencing techniques) is ongoing. For the 5th case, no pathogenic, likely pathogenic or VUS were detected in the five analysed genes (*APOB*, *PCSK9*, *MTTP*, *SAR1B*, and *ANGPTL3*). For both individuals a definite diagnose was not possible at the time.

Table 3.9 – Summary of the molecular diagnosis of FHBL for 3 ICs.

Suspicion	Diagnosis	IC	Gene	Variant	Protein
ABL or FHBL	**FHBL**	IC 65	*APOB*	c.3367del	p.(Val1123Leufs*62)
		IC 67		(c.12087+1 G>A; c.12087+1G>A)	p.(?)
		IC 69		c.2604+1 G>A	p.(?)
				c.4651 C>T	p.(Gln1551*)

In addition to the molecular study of index cases clinically diagnosed with hypocholesterolemia, cascade screening was possible only for one family. The co-segregation study performed enabled to confirm the IC67 as a true homozygous for the c.[(12087+1 G>A); (12087+1G>A)] variant, and respective parents as obligated heterozygotes (Figure 3.2). Efforts were made in order to recruit more relatives, mainly pertaining to IC65 and IC69 families.

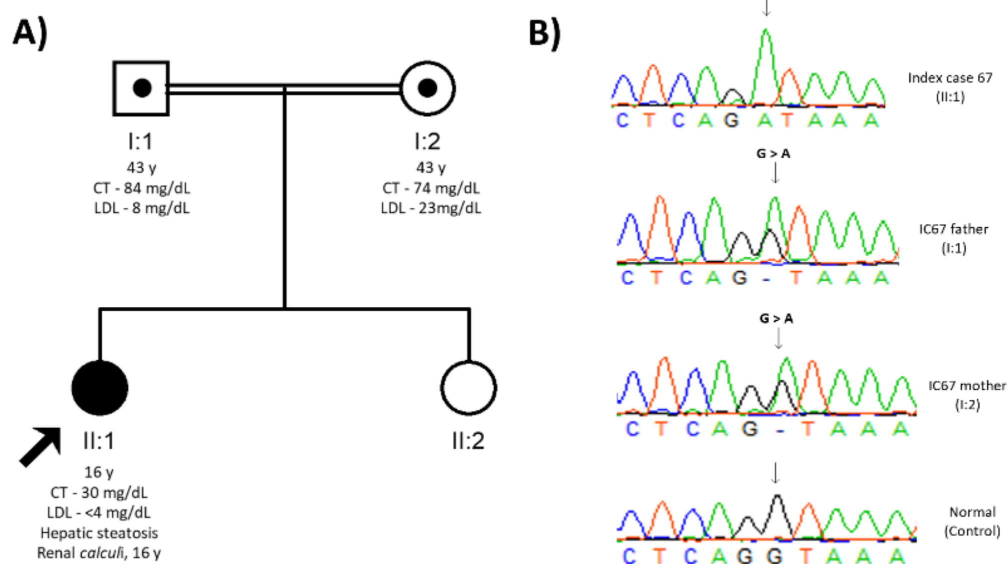

Figure 3.2 – Cascade screening in IC67 family. **(A)** Family pedigree. Arrow indicates the IC67 (II:1). Age (in years) and total cholesterol levels and LDL-c levels (mg/dL) are under each symbol. IC67 hepatic complications are reported under lipid profile. Consanguineous relation between I:1 and I:2 (parents of IC67). Point symbols represent heterozygous individuals for a pathogenic alteration in *APOAB* gene, c.12087+1G>A. Black filled symbol represents homozygosity for this variant [(c.12087+1 G>A; c.12087+1G>A)]. **(B)** Partial sequence of intron 27 of *APOB* gene of IC58 and respective relatives, and a normal control (without alteration) where the alteration G→A is located in position c.12087+1 (intron 17).

3.3.3. Hypertriglyceridemia panel

Regarding the 27 Portuguese individuals clinically diagnosed with hypertriglyceridemia, a definite monogenic cause of disease was possible to achieve in 10 cases.-The majority of variants were located in the five canonical genes associated to FCS (*APOA5, LPL, LMF1, APOC2* and *GPIHBP1*).

Rare pathogenic variants only provide a definite diagnosis of FCS when found in homozygosity. Therefore, only two index cases were diagnosed with this disorder due to a pathogenic missense variant

in the *LPL* gene (IC76), and a large pathogenic deletion alteration in the *GPIHBP1* gene (IC92) (Table 3.10). Family co-segregation studies for IC76 were not possible since relatives samples were not available. However, IC92 relatives, and the index herself, were further studied during the course of this project (section 4.5). This *GPIHBP1* deletion was detected in Portugal for the first time.

A total of 7 ICs were diagnosed with MCM. Four different variants were detected by NGS among these ICs: two missense alterations in *LPL*, one frameshift deletion, and one nonsense variant both in *APOA5*. Curiously, the majority of patients diagnosed with MCM presented the same frameshift variant (in heterozygosity) in *APOA5* gene, despite being unrelated. In addition, one subject, presented a nonsense variant in *APOA5* resulting in the generation of a premature stop codon (Table 3.10). For these IC, cascade screening was not performed since no relatives were recruited to date.

In one IC, NGS analysis revealed a rare likely pathogenic variant in the *GPD1* gene, in homozygosity (Table 3.10). Therefore, a definite molecular diagnosis of recessive hypertriglyceridemia was provided to this proband. Co segregation studies were not performed yet; however, IC70 parents will be recruited for cascade screening.

Table 3.10 – Summary of the molecular diagnosis of FCS, MCM and autosomic recessive hypertriglyceridemia for 10 ICs.

Suspicion	Diagnosis	IC	Gene	Variant	Protein
FCS	**FCS**	IC 76	*LPL*	(c.701 C>T; c.701 C>T)	p.[(Pro234Leu); (Pro234Leu)]
		IC 92	*GPIHBP1*	Deletion of exon 3 and 4	p.(?)
	MCM	IC 89	*APOA5*	c.289 C>T	p.(Gln97*)
		IC 79		c.990_993del	p.(Asp332Valfs*4)
		IC 84		c.990_993del	p.(Asp332Valfs*4)
		IC 88		c.990_993del	p.(Asp332Valfs*4)
		IC 94		c.990_993del	p.(Asp332Valfs*4)
		IC 78	*LPL*	c.590 G>T	p.(Arg197Leu)
		IC 96		c.701 C>T	p.(Pro234Leu)
	Autosomic Recessive Hypertriglyceridemia	IC 70	*GPD1*	(c.895 G>A; c.895 G>A)	p.[(Gly299Arg); (Gly299Arg)]

Clinical Characterization
Molecular Study
Genetic Diagnosis
Future Investigation

96 ICs

Hypercholesterolemia
64 ICs
Detection of pathogenic variants
22 ICs
Monogenic Familial Hypercholesterolemia (FH)
22 ICS
No pathogenic variants
42 ICs
FH negatives
42 ICS
VUS
22 ICs
FH associated genes (*LDLR*, *APOB*, *PCSK9*)
17 ICs
Sitosterolemia associated genes (*ABCG5*, *ABCG8*)
5 ICs
Without VUS ✦
20 ICs
Elevated Lp(a) Score *
5 ICs
GRS *
[P25th – P75tth] - 7 ICs
> P75th - 13 ICs

Hypocholesterolemia
5 ICs
Detection of pathogenic variants
4 ICs
Familial Hypobetalipoproteinemia (FHBL)
3 ICs
Large deletion in the *APOB* gene (Likely Pathogenic)
1 IC
Variant confirmation
If confirmed: Definite diagnose of FHBL in 4/5 ICs
No pathogenic variants
1 IC
Negative genetic test
1 IC
Without VUS ✦
1 IC

Hypertriglyceridemia
27 ICs
Detection of pathogenic variants
10 ICs
Familial Chylomicronemia Syndrome - 2 ICs
Multifactorial Chylomicronemia - 7 ICs
Autosomal Recessive Hypertriglyceridemia - 1 IC
No pathogenic variants
17 ICs
Negative genetic test
17 ICs
VUS
2 ICs
FCS associated gene (*APOA5*)
1 IC
LAL-D associated gene (*LIPA*)
1 IC
Without VUS ✦
15 ICs
Polygenic cause of dyslipidemia? New causes of hypertriglyceridemia, or other genes associated?

Figure 3.3 – Summary of the molecular study of familial dyslipidemias by Next Generation Sequencing, through distinct project stages: clinical characterization of 96 ICS, molecular study using small panels according to clinical characterization; genetic assessment and diagnosis of ICs; and additional findings and observations to further investigate. IC, index case; VUS, variant with uncertain significance; FCS, familial Chylomicronemia Syndrome; LAL-D, Lysosomal acid lipase deficiency; GRS, LDL-c genetic risk score. ✦Without VUS/Negative genetic test after analysis of an exclusive number of genes for each phenotype; * GRS and Lp(a) Score were analysed separately for those 20 ICs (without VUS in analysed genes related to hypercholesterolemia).

Chapter 4. Discussion

4.1. NGS as a diagnosis and research tool

The field of lipid metabolism and cardiovascular genetics is growing worldwide. Since the recognition of dyslipidemias as a possible genetic condition and not only as a consequence of lifestyle options or other secondary to other pathologies, the classification of primary dyslipidemias in monogenic and polygenic, became essential to further understand this group of diseases [(23)].

Serious clinical complications resulting from genetic dyslipidemias awakened the scientific community for the need to investigate these disorders. NGS technology emerged allowing massive parallel DNA sequencing, enabling the rapid and cost-effective sequencing of the entire genome hence altering the traditional laboratory approach to genetic testing and research (Sanger Sequencing) [(92)]. As a research tool, NGS customized panels allow a focused investigation of rare familial dyslipidemias by combining genes associated with familial dyslipidemias and genes that are candidates for an important role in lipid metabolism. This technology is extremely useful in studies (with large cohorts) for SNPs association to a specific lipid trait, such as high levels of LDL-c or triglycerides [(133)].

In this project, genetic testing through NGS showed to be advantageous in the genetic diagnosis of these conditions since it was able to identify several disease-causative variants at once and in a short period of time. NGS allowed to achieve the correct diagnosis which is relevant information for therapeutic management of patients suffering from common or rare genetic dyslipidemias. Nevertheless, the large amount of NGS-generated data constitutes an obstacle to reach a clear diagnosis. Given that, we decided, as a first approach, to focus our research exclusively on 18 genes previously associated with three main dyslipidemic traits, and thus organized into 3 panels: familial hypercholesterolemia and phenocopies panel, hypocholesterolemia panel, and hypertriglyceridemia panel. Overall, this approach enabled a successful diagnose of 35 out of 96 Portuguese patients with clinical diagnosis of at least one familial dyslipidemia: 22 patients with FH, 3 with FHBL, 2 with FCS, 7 with MCM, and 1 with autosomic recessive hypertriglyceridemia.

4.2. Hypercholesterolemia

4.2.1. Familial Hypercholesterolemia

4.2.1.1. FH Positives

A definite diagnosis of FH was possible to achieve in 34% of the cases referred to GIC-INSA with a clinical diagnosis of familial hypercholesterolemia. It is worth mentioning that every FH patient included into this molecular study is included in the Portuguese Familial Hypercholesterolemia Study, in which the positive diagnose rate observed has been reported to be between 30-50% depending on clinical criteria stringency [(95,108)].

Using an NGS analysis panel with 8 genes for FH and phenocopies, a total of 18 rare pathogenic or likely pathogenic variants were found in 22 unrelated index patients (out of 64). In these 22 ICs the molecular findings were consistent with their clinical diagnosis [(29)]. The majority of pathogenic variants were detected across several exons in the *LDLR* gene, as expected, since *LDLR* rare variants correspond to the most common monogenic cause of FH, particularly in heterozygosity, being identified about 90% of FH cases [(23)]. A large percentage of these variants have already been described and many shown to affect LDLR function thus conferring their pathogenic clinical implication. One pathogenic variant, a partial deletion of the *LDLR* gene (exon 1) was detected and identified as a novel cause of FH, detected

for the first time in Portugal. Clinical and molecularly diagnosed FH patients mandatorily need lifestyle adaptations and adequate pharmacotherapeutic to manage their increased risk of premature CVD since cardiovascular events are often observed in patients with FH and among their families [23]. Cascade screening is fundamental to identify IC relatives who might benefit from early prevention of serious complications associated with FH [134]. FH individuals are generally asymptomatic until performance of biochemical screening or development of cardiac disorders so early identification is crucial [25]. We recruited several relatives of ICs, resulting in the confirmed molecular diagnosis of FH in 14 individuals, who were not aware of their condition until their recruitment. Therefore, we provided an early identification of undiagnosed FH patients who can now be managed and advised, thus improving patient's prognosis by preventing the development of cardiovascular diseases [134].

4.2.1.2. FH with VUS

In this research study, a monogenic cause of dyslipidemia was not identified in 42 unrelated individuals presenting clinical criteria of probable FH. However, several VUS were identified in FH and sitosterolemia associated genes (*LDLR*, *APOB*, *PCSK9*, *ABCG5* and *ABCG8*), within 22 of 42 cases.

Concerning variants detected in FH-associated genes, a large percentage of VUS were found in the *APOB* gene, specifically in exons 26 and 29, which reenforces the concept of *APOB* as a very polymorphic gene but also one has to be aware that APOB can harbour other unknown pathogenic variants [35]. The great majority of detected VUS lack functional data, and available information mostly concerns variant frequency among different populations (found through publicly available databases, such as GnomAD), thus conferring the rare character of these alterations. The exact influence of VUS on metabolism remains unknown and their functional impact, in the majority of the cases, has to be evaluated. This is especially true for APOB variants. If a VUS, is found in exclusivity in an index case, this could represent a monogenic cause of FH but pathogenicity must be proved. In fact, functional data could be determinant in order to improve variants classification from VUS to likely pathogenic or pathogenic as shown before [103]. As such, as an example of the fundamental role of functional assessment, in this research project, the performance of functional assays could possibly improve the diagnosis rate, from 34% to at least 50%, if half of detected VUS were proven to affect LDLR pathway. Co segregation information could also provide more evidence for the pathogenicity assessment and characterization of these variants together with functional data.

Regarding autosomal recessive disorders associated with hypercholesterolemia only variants associated to sitosterolemia were found. In *ABCG5/ABCG8* genes, six rare variants were detected, one in *ABCG5* gene and five in *ABCG8*. Poor clinical and populational information was found after bioinformatics analysis, which was expected due to the rarity of sitosterolemia. Four missense variants in *ABCG5* and *ABCG8* were found in heterozygosity. The missense variant detected in *ABCG5* gene was reported to be associated to higher levels of cholesterol and higher risk of CAD in a large cohort including subjects from the UK, Denmark and Iceland [135,136]. Similarly to IC15, the alteration was reported in a hypercholesterolemic carrier, who was FH-negative (absence of a pathogenic variants in *LDLR*, *APOB*, and *PCSK9* genes), thus suggesting a possible involvement of ABCG5 variants in patients' hypercholesterolemia [135]. Nonetheless, the functional impact of this variant on metabolism is yet unknown. From the three missense variants detected in *ABCG8* gene, two were present in publicly available databases (despite the lack of functional data) and one is novel, hence being found for the first time within the portuguese population.

In one index case (IC14), we found other two distinct variants in *ABCG8* gene: one pathogenic nonsense alteration, and one missense alteration (VUS) detected for the first time within the Portuguese population. It is plausible to question if this patient (who presents clinical expression of a heterozygous

FH) could represent a case of compound heterozygosity resulting in sitosterolemia, in case of confirmed pathogenicity of the detected VUS. Serum of the patient was sent to be analysed by sterol chromatography to confirm or exclude sitosterolemia. Sitosterolemia, as FH, is mainly characterized by elevated LDL-c levels, presence of xanthomas, and history of CHD [50,137]. However, contrarily to homozygous FH, patients with sitosterolemia do not require apheresis, and their hyperlipidemia often responds well to dietary sterol reduction, pharmacotherapeutic treatment [23].

Therefore, NGS can be a fundamental tool in precision medicine since the enlightenment regarding the possible molecular cause of hypercholesterolemia in these patients is essential considering that a diagnosis of sitosterolemia (instead of clinical diagnosed FH) dramatically changes the therapeutic approach selected for patients affected [23]. Cascade screening was not possible to performed for this family with possible sitosterolemia after genetic testing. However, co segregation studies will be fundamental to investigate the existence of any case of variant homozygosity (considering the pathogenic variant detected), and to enable the confirmation if both detected variants are located in different or in same alleles (cis or trans). The finding of VUS variants in *ABCG5/8* genes in patients with a clinical diagnosis of FH could be relevant if these variants are proven to affect cholesterol metabolism. It has been described that ABCG5/8 pathogenic variants in heterozygosity can contribute to increase cholesterol levels leading to misdiagnosis of FH [135,138]. Other FH cohorts have described the same findings in a small percentage of their clinical FH patients with no pathogenic variants in *LDLR*, *APOB* and *PCSK9* genes [139].

4.2.1.3. FH Negatives

For 20 index cases, rare variants classified as pathogenic, likely pathogenic or VUS were not found. Even though a large number of variants in FH-associated genes have been described, several studies demonstrated that approximately 50% of cases with a clinical diagnosis of FH do not have a pathogenic or likely pathogenic alteration in one of the eight genes analysed, implying that there might be other undescribed genes or mechanisms underlying FH phenotype [95,140]. Considering the scenario of a hypothetically undetected or novel monogenic cause of FH in these cases, they will be re-evaluated in an investigation environment. This re-evaluation may benefit from the analysis of the remaining 39 candidate genes of the 57 comprehended in the NGS panel.

In these 20 index cases, we evaluated patient's genetic risk score and their Lp(a) score to analyse if these could be the cause of the phenotype. Thirteen ICs presented a high polygenic SNP score (above P75th), indicating an accumulated burden of trait-raising SNPs for elevated LDL-c. Patients with high GRS frequently present increased cholesterol levels and a family history of CVD, possibly resulting from their high genetic burden and life-long exposure to elevated LDL-c [25,134]. Therefore, it is probable that these particular group of 13 ICs present clinical manifestations of heterozygous FH as a consequence of a polygenic dyslipidemia instead a monogenic cause of FH, which explains their inclusion upon clinical FH suspicion [55]. In addition, 7/20 ICs presented a moderate genetic risk score, between P25th and P75th. Despite a probable polygenic contribution to their clinical phenotype, these are more in the grey zone. Pharmacotherapeutic treatment in patients with polygenic dyslipidemia is frequently adjusted to each patient, as they respond well to life-style changes and may be able to be managed in general practice clinics. Thereby, the polygenic risk score calculation using NGS technology is a potential tool for refinement diagnosis and possible prediction of CV risk in clinically diagnosed FH patients, leading to a personalized therapeutic approach [55,134].

High lp(a) levels have been described as an FH phenocopy [39]. A lp(a) score has been described and associated with lp(a) levels [101]. For this reason, the evaluation of the Lp(a) score within this group of 20 individuals was performed and showed that 5 ICs presented an elevated score [141]. Lp(a)

cholesterol is more atherogenic than LDL cholesterol, contributing to atherosclerosis and, in the long term, leading to CV events. In particular, unlike LDL cholesterol, it is unknown whether genetic factors related to lipoprotein(a) may provide information about lifetime exposure relevant to ASCVD risk prediction [141]. Additionally, external factors such as diet or physical activity have a poor influence on Lp(a) levels, which are predominantly inherited (75-90 percent) and have been associated to single-nucleotide variants in the *LPA* gene [141,142]. The applicability of LPA genetic testing is not completely established and needs better assessment but could be of diagnostic value. In case of the individuals found with a high LPA score, instead of a monogenic cause of disease, their Lp(a) might acted as confounding factor, meaning that patient's cholesterol levels and family history of CVD could be related with Lp(a) and not with rare FH-causative variants [25,141].

4.3. Hypocholesterolemia

4.3.1. Hypobetalipoproteinemia

In this research project, 5 out of 96 ICs were clinically diagnosed with a hypocholesterolemia. Despite several syndromes could underlie their cause of disease, ABL and FHBL were the main clinical suspicion as these disorders are the most frequent cause of hypocholesterolemia, although both being rare lipid disorders. ABL and FHBL are characterized by LDL-c above the $P5^{th}$ and low total cholesterol levels, and are clinically indistinguishable [23].

A definite diagnosis of FHBL was possible to achieve in 3 out of 5 cases (5/96 cases). FHBL is a co dominant disorder, so it has a heterozygous and a homozygous form. All the detected variants were identified in the *APOB* gene resulting in either splicing errors or premature stop codons leading to protein truncations, thus impairing APOB function [58]. None of the detected variants are reported or described in the literature. The variants found prevent the complete translation of apoB mRNA, resulting in the production of truncated dysfunctional apoB proteins that are most probably degraded by the cell. Without apoB, new lipoproteins are not formed in the liver and/or intestine and exported, resulting in low or absent LDL levels in plasma [143,144].

Rare variants of potential interest were not found in IC66, thus in this project it was not possible to find the cause of this patient's hypocholesterolemia. All the genes known to be associated with hypocholesterolemia were analyzed in this project since all were covered using the customized NGS target panel for familial dyslipidemias. This case is a representation of the hardship of diagnosing some disorders, such as FHBL and other rare inherited dyslipidemias since the genetic bases for most of them remain largely unknown. FHBL is nowadays known and understood as a disorder characterized by a major heterogeneity of causative variants and possible genetic bases of disease [23,59].

After CNV analysis for these five cases, in IC68, a copy number variation was discovered in heterozygosity. The detected alteration in this subject may result in a large exonic deletion in the *APOB* gene, spanning exon 28 to exon 29, a crucial region for APOB structure and function [1]. After applying ACMG criteria, this variant was classified as likely pathogenic. Nonetheless, the alteration is being further confirmed and investigated. Despite the current lack of a definitive diagnosis for IC68, the detected CNV (if confirmed) will be considered as the cause of this IC hypocholesterolaemia.

Functional assessment of NGS detected variants, in these cases, is relevant to understand the effect of these specific truncations since the length of the truncated protein may influence clinical significance [102]. In addition, in order to access variant pathogenicity, cascade screening is essential to study inherited disorders. Particularly in FHBL, due to its co-dominant inheritance pattern, heterozygous relatives of ICs molecularly studied will also be affected with FHBL. Therefore, relatives also benefit from genetic

assessment, and counselling [23]. APOB truncating variants can lead to liver steatosis, so carriers of these variants need to be evaluated for this condition.

Studies focused on FHBL subjects demonstrated by correlation that this group of patients is relatively protected by the (life-long) reduced levels of exposure to apo B-containing lipoproteins, suggesting that apo B-containing particles may constitute a central factor in atherogenesis [143]. Given that, it is plausible to consider that FHBL patients constitute a unique cohort to evaluate the impact of life-long exposure to unusually low levels of apoB-containing atherogenic lipoproteins. Nonetheless, despite low cardiovascular risk of FHBL patients, other serious conditions are associated with this disorder, such as neurological complications and hepatic steatosis [58]. Early identification of patients and early treatment of symptomatic primary hypocholesterolemia is crucial.

4.4. Hypertriglyceridemia

4.4.1. Familial Chylomicronemia Syndrome

In this research project, despite the suspicion of FCS as the genetic condition underlying the clinical diagnosis of hypertriglyceridemia in 27 patients, only 2 were diagnosed with FCS.

Pathogenic rare variants providing this molecular diagnose were located in the *LPL* and *GPIHBP1* genes, both in homozygosity. In patient IC76, who presented pancreatitis, a pathogenic variant in *LPL* was detected. Lipoprotein lipase is essential for the hydrolysis of triglycerides in plasma lipoproteins [74,75]. Therefore, an impairment of the activity of this enzyme as a result of an exonic sequence alteration clearly explains the clinical phenotype of hypertriglyceridemia exhibit by FCS patients, particularly IC76 [71]. We found a CNV in IC92, a large deletion in homozygosity spanning from exon 3 and exon 4 of the *GPIHBP1* gene. This deletion was detected for the first time in Portugal during this project in a child presenting hepatomegaly. A similar deletion was detected and characterized in 2014 in patients from Pakistan as the case of our IC [132]. Due to GPIHBP1 fundamental role in the translocation of LPL across capillary endothelium [76,77], a shortening of this protein (caused by the deletion of two exons) impairs its metabolic efficiency, thus causing severe hypertriglyceridemia.

It is worthy of mention that, due to the variable penetrance of variants causing FCS, heterozygotes for these variants also might display a variable expression of hypertriglyceridemia, in these cases are described as having multifactorial chylomicronemia (MCM) [71,81]. Given that, parents of these index cases are obligated heterozygous patients (both have MCM). Therefore, cascade screening will be performed in a near future, particularly for relatives of the index case presenting the genetic LPL alteration.

Clinical manifestations of FCS, despite abnormally high levels of triglycerides in plasma, include early symptoms such as nausea, vomiting, failure to thrive along with frequent abdominal pain [71]. Patients suffering from FCS should be carefully managed with long-term diet and pharmacological treatment since the main complication associated with this disorder include severe hypertriglyceridemia, failure to thrive, and gastrointestinal complications such as abdominal pain. Often, recurrent episodes of pancreatitis are observed in patients with FCS [23,91].

Therapeutic management of these individuals is extremely relevant, but it is limited [23,91]. Often, approaches focus on dietary restrictions, fasting during acute pancreatitis episodes, and in some cases fibrates besides the observed inefficiency of this intervention [23,56]. A new treatment exists and has been approved by the European Medicine Agency. Carriers of heterozygous variants have biochemical phenotypes ranging from completely normal to severe hypertriglyceridemia within the same family [71].

Therefore, due to the lipid profile heterogeneity of clinical FCS patients and their relatives, cascade screening is crucial to an early identification of this condition, improving their prognosis by the implementation of counselling and/or adequate therapeutic approaches [(23)].

4.4.2. Multifactorial Chylomicronemia

Contrarily to FCS, multifactorial chylomicronemia is a much more common and it is a complex disorder [(71)]. MCM encompasses a much broader population with similar markedly increased TG concentrations and physical features as FCS. Nonetheless, pancreatitis episodes occur less frequently in MCM [(83)]. In addition, in adults who primarily express MCM, the pediatric features as failure to thrive were not typically observed. MCM is strongly believed to have a genetic basis, but unlike FCS, where recessive or biallelic variants in the five canonical genes are disease-causative, the genetic factors in MCM are not deterministic [(69,71)]. Nevertheless, we have observed that the vast majority of severe hypertriglyceridemia cases that are not diagnosed with FCS present variants in heterozygosity in the canonical genes related with this condition, thus having MCM [(71)]. It is worth mentioning that a diagnose of MCM do not fully explain patients' conditions, but provides information regarding possible genetic contribution for their clinical manifestations, thus showing a possible codominant inheritance pattern.

Therefore, in the course of this research project, we were able to provide a diagnose of MCM to 7 out of 27 patients. Within the 7 ICs, two missense alterations in *LPL*, and two variants (missense and frameshift) in *APOA5* genes were detected. These findings, *a priori*, demonstrate that rare heterozygous variants in FCS canonical genes, in patients with clinical hypertriglyceridemia, can have variable penetrance [(81)]. Overall, the fact that these seven heterozygous ICs express clinical hypertriglyceridemia together with hepatic complications, such as pancreatitis and hepatic steatosis in a low percentage of these cases, is congruent with the definition of MCM [(81,83)].

In addition to these patients, in IC93 (who also presented clinical hypertriglyceridemia and hepatic complications) a missense variant classified as VUS was detected in the *APOA5* gene. Given the lack of data regarding this variant, a diagnose of MCM was not possible for IC93. Nonetheless, in case of proven pathogenicity of this variant, the diagnose rate of MCM would improve, thus suggesting (as discussed before) a contribution of heterozygous variants in FCS associated genes to the manifestation of hypertriglyceridemia [(71,81)].

The majority of detected variants were not functionally accessed to date, which main constitute a step forward in FCS/MCM investigation. Patients with suspected FCS, who are found to have MCM should be followed-up to avoid disease complications, as well as all patients with rare familial dyslipidemias (such as FCS). Specific therapeutic approaches for MCM are not defined to date. Nonetheless, patients are usually managed and treated as FCS patients [(23)].

4.4.3. Autosomal Recessive Hypertriglyceridemia

In IC70, a 2 month old female, we found a homozygous variant in the *GPD1* gene (c.895G>A)/p.(Gly299Arg). This gene is associated with Transient infantile hypertriglyceridemia (or autosomal recessive hypertriglyceridemia, ARHTG), a condition characterized by severe hypertriglyceridemia and hepatic complications [(23)], which correlates to the clinical phenotype observed in IC70. The variant detected by NGS in this case was classified as likely pathogenic, thus being consistent with a diagnose of ARHTG. This variant was reported before in a child (16 years old) with persistent severe hypertriglyceridemia [(131)]. In this case, the use of fibrates as therapeutic approach showed to be efficient for the reduction of triglyceride levels after only 1 year of follow-up. Despite the genotype/phenotype link, few cases of this disease are known to date. Moreover, the exact mechanism

of hypertriglyceridemia in GPD1 deficiency remains unknown, and more research is needed [87]. Index parents were not recruited to date, but efforts are being made to include these relatives in further diagnostic and research studies to confirm their heterozygosity. Cascade screening might be crucial in this family since autosomal recessive hypertriglyceridemia is mainly characterized by serious hepatic complications such as early-onset hepatomegaly (which is observed in IC70), hypertriglyceridemia, hepatic steatosis, and hepatic fibrosis [23]. An early diagnosis in cases possibly affected by such rare disorders allows and early counselling and therapeutic intervention, improving patient's prognosis. It is worth mentioning that, if this variant is pathogenic, this will be the first Portuguese patient to be found diagnosed with this rare disorder.

4.4.4. Lysosomal acid lipase Deficiency

Upon NGS analysis, using a wide gene panel for familial dyslipidemias, a particularly interesting case is worth highlighting. In IC87, the variant c.449A>G/p.(Tyr150Cys) was detected in the *LIPA* gene in heterozygosity. Poor information regarding this variant was found in publicly available databases (such as GnomAD and ClinVar) leading to a VUS classification. Despite LALD being categorised as an autosomal recessive dyslipidemia, this heterozygous variant in the *LIPA* gene if functional assessed and proven to be pathogenic, might suggest an involvement in the observed LALD phenotype. Nonetheless, the role of heterozygosity LIPA variants as cause of LALD phenotype is yet to be further studied, and the effect of this specific variant is yet to be assessed [145]. LALD is a condition in which individuals affected with pathogenic variants, when in homozygosity, usually present alterated triglyceride levels, together with high liver enzymes and hepatic steatosis [88,145].

Regarding the phenotype, IC87 presents clinical manifestations of LALD, such as hyperlipidemia along with hepatic steatosis and hepatomegaly [145]. Since the patient presents some of the clinical characteristics of LALD, the determination of LAL has been requested to confirm or exclude the LALD diagnosis. Considering that only a variant in heterozygosity was found, if the enzymatic test comes positive, other variants in *LIPA* gene can have been missed or other variant in undescribed genes are the cause of the phenotype. Heterozygous carriers of LIPA variants have not been described to present such a severe phenotype. Overall, so far, taking into consideration the molecular findings and the resulting effects of the pharmacological management of IC87, it is possible that abnormal triglyceride levels arose as a consequence of IC dietary lifestyle.

4.4.5. HTG panel negatives

Overall, concerning the group of 27 individuals with clinical hypertriglyceridemia, approximately 55% of patients do not have a definite diagnosis for their dyslipidemia (15 ICs). Non-mendelian genetic factors, such as epigenetics or mitochondrial genetics, or the possible existence of genes with unreported associations to triglyceride levels, can perhaps underly determinants in the remaining undefined patients with clinical phenotype [23]. In order to investigate the possible molecular cause of dyslipidemia in these patients, additional non-canonical genes associated with TG should be analyzed besides the genes contemplated in this project. Moreover, other genes that underly other medical conditions, such as diabetes, in which elevated TG levels are found secondarily, could contribute to the proportion of genetically undefined patients [71].

Such as observed in familial hypercholesterolemia (a common dyslipidemia), rare disorders of triglycerides metabolism might have a polygenic base. Nowadays, stemming from applied genetic risk scores for FH, it is consensual in the scientific community that the quantification of heterozygous rare variants effect and excess of SNPs accumulation is crucial, and that additional studies are needed [71].

Polygenic risk scores for hypertriglyceridemia are being already used, despite their recent development [70]. Studies showed that approximately 32% of patients with highly severe hypertriglyceridemia presented a high polygenic score [70]. A polygenic score for hypertriglyceridemia will be introduced in the future in the NGS panel in order to improve the patient diagnosis.

Nonetheless, scores must be carefully used and developed since they do not represent a certain or absolute cause of dyslipidemia, it possibly indicates a genetic contribution to the phenotypical traits or a predisposition [23,84]. A high polygenic score cannot fully explain patient's trait, but can highlight a genetic contribution. Given that, in a large percentage of cases, we were unsuccessful to find a clear genetic cause of dyslipidemia in patients with a clinical diagnosis of hypertriglyceridemia. As such, the development and implementation of a genetic risk score for hypertriglyceridemia (using SNPs related with TG-raising alleles) would probably largely contribute to the increase of justified phenotypes in Portugal.

4.5. Functional Studies

4.5.1. *LDLR* gene

In index case 37, a heterozygous synonymous variant in the *LDLR* gene was detected by NGS: c.621C>A/p.(Gly207Gly). This IC, a 57 year old male suffering from angina during the last 20 years, presented a lipid profile mainly characterized by elevated total cholesterol and LDL cholesterol despite being managed using combined pharmacotherapeutic (Rosuvastatin and Ezetimibe). Hence, IC37 was clinically diagnosed with FH.

After NGS performance and data analysis of IC37, this rare synonymous variant was the only one detected in genes within the FH and phenocopies panel. Frequently, synonymous variants are known to have a silent effect in protein products, thus not being relevant for disease [102,103]. Nonetheless, synonymous variants in the *LDLR* gene have been discovered to have influence in splicing events and a pathogenic significance. Including a pathogenic synonymous variant in the same cDNA position codon as the one identified in this project, described by Defesche and colleagues: c.621C>T/p.(Gly207Gly) [146]. Therefore, the variant detected in this project should be properly taken into consideration and studied.

As a consequence of this described alteration, aberrant splicing occurs originating partially deleted transcripts. Considering the effect of the variant already characterized, the investigation of the synonymous variant detected in IC37 is extremely relevant. Therefore, the investigation of the possible occurrence of a splicing event originated by c.621C>A alteration is ongoing, using IC37 RNA; however, currently, we do not have concrete data to formulate a hypothesis concerning this subject. Since co segregation data would be extremely relevant in this study, IC37 son was already recruited for genetic analysis and it was possible to confirm that he has inherited the same variant. Nonetheless, IC37 son RNA sample is not available to date, which would be important to complement the RNA studies initiated with the father's sample. Therefore, efforts are being made for a novel sample collection of IC37 son in order to obtain his RNA, and to better characterize this variant.

4.5.2. *GPIHBP1* gene

During this project, we found a CNV (through NGS) located in the *GPIHBP1* gene, in IC92. The detected variant consisted in a large deletion, in homozygosity, spanning from exon 3 and exon 4 of the *GPIHBP1* [132]. Index case 92 was a 6 month old child clinically diagnosed with severe hypertriglyceridemia, presenting hepatomegaly at a such young age.

This variant was classified as pathogenic, thus conferring a molecular diagnosis of FCS to IC92. Therefore, since *GPIHBP1* is one canonical gene causing FCS, parents of this homozygote patient are obligate heterozygous, being molecularly diagnosed with MCM [(71)]. To approach this scenario, and as an effort to confirm if this deletion corresponds to the already identified Pakistanease variant, PCR protocols are being optimized to provide a clear visualization of the cut points of the alteration found within this family. IC92 parents were also recruited for this study.

Chapter 5. Conclusions and Perspectives

The presented project allowed the application of NGS technology which enabled a personalised investigation of familial dyslipidaemias. This methodology allowed the identification of genetic alterations that affect a relevant pathway and cause a genetic disorder in 35 of 96 individuals clinically diagnosed with hypercholesterolaemia, hypocholesterolaemia and/or hypertriglyceridaemia. However, one of the main observations during this research study was that there is lack of knowledge regarding functional and structural impact of several alterations. A third of the patients present with VUS. Several variants have insufficient scientific evidence to be considered as causes of disease, resulting in the negative or inconclusive diagnosis of patients in whom the monogenic cause of their dyslipidaemia could have been discovered if more that would be available.

This work will be continued in the future in a research environment in order to obtain results regarding the functional studies started during this project, namely regarding the synonymous alteration in the *LDLR* gene and the deletion detected in the *GPIHBP1* gene. In addition, future work will focus mainly on the functional study of VUS found in the Portuguese population to broaden the information regarding the spectrum of alterations associated to several diseases such as FH, FHBL and FCS/MCM. The performance of functional assays as an integrated part of molecular diagnosis should be implemented for all cohorts, as it will contribute for the elucidation of the molecular basis of genetic dyslipidemias.

The molecular study of individuals with clinical suspicion of familial dyslipidaemia contributed to an early identification of Portuguese patients, as a personalized approach, and in order to provide guidance for an adequate therapy, adjusted to patients' genotype. Furthermore, this work contributed to identify family members (that may be affected by the familial dyslipidemias despite being asymptomatic) through cascade screening, in which the co-segregation of the phenotype/genotype in the family is evaluated by genetic testing in several generations.

Collaboration between the clinical and research/diagnosis environment is essential for better management of Portuguese patients since a molecular diagnosis can change patients' treatment and improve risk monitoring (associated to disease-complications). Extended research efforts in Portuguese patients allows a better characterization of the population and should be pursued. Considering the fact that rare disorders addressed in this project are underestimated within the Portuguese population, it is essential to increase the awareness of this group of inherited diseases in order to contribute to a better quality of life of the Portuguese patients with these disorders.

Chapter 6. References

1. Remaley AT, Dayspring TD, Russel Warnick G. Lipids, lipoprotein and apolipoprotein. In: Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. 6th ed. 2017. p. 539–603.
2. Fahy E, Cotter D, Sud M, Subramaniam S. Lipid classification, structures and tools. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. 2011;1811(11):637–47. DOI: 10.1016/j.bbalip.2011.06.009.
3. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science. 1986;232(4746):34–47. DOI: 10.1126/science.3513311
4. Schade DS, Shey L, Eaton RP. Cholesterol review: A metabolically important molecule. Endocrine Practice. 2020;26(12):1514–23. DOI: 10.4158/EP-2020-0347.
5. Burdge GC, Calder PC. Introduction to fatty acids and lipids. World Review of Nutrition and Dietetics. 2015;112:1–16. DOI: 10.1159/000365423.
6. Bayly GR. Lipids and disorders of lipoprotein metabolism [Internet]. Third Edit. Clinical Biochemistry: Metabolic and Clinical Aspects: Third Edition. Elsevier Ltd.; 2014. 702–736 p. Available from: http://dx.doi.org/10.1016/B978-0-7020-5140-1.00037-7.
7. Dominiczak MH. Apolipoproteins and lipoproteins in human plasma. Rifai N, Warnick RG, Dominiczak MH, editors. Vol. 125, Handbook of Lipoprotein Testing. American Association for Clinical Chemistry, Inc.; 2001. 1–29 p. DOI: 10.5858/2001-125-1130a-holt.
8. Mahley RW. Apolipoprotein E : Cholesterol Transport. Science. 1988;240:622–30.
9. Zannis VI, Breslow JL, Utermann G, Mahley RW, Weisgraber KH, Havel RJ, et al. Proposed nomenclature of apoE isoproteins, apoE genotypes, and phenotypes. Journal of Lipid Research [Internet]. 1982;23(6):911–4. Available from: http://dx.doi.org/10.1016/S0022-2275(20)38094-9.
10. Weisgraber KH. Apolipoprotein E: Structure-function relationships. Advances in Protein Chemistry. 1994;45:249–302. DOI: 10.1016/s0065-3233(08)60642-7
11. Ehnholm C, Garo H, Renkonen O, Simons K. Protein and Carbohydrate Composition of Lp (a) Lipoprotein from Human Plasma. Biochemistry. 1972;11(17):3229–32. DOI: 10.1021/bi00767a015.
12. Davis PG, Wagganer JD. Lipid and lipoprotein metabolism. In: Lipid metabolism and health. 2006. p. 47–60. DOI: 10.1021/bi00767a015.
13. Gurr MI, Harwood JL, Frayn KN. Lipid biochemistry, an introduction. Food Biochemistry and Food Processing. 2007. 319 p. DOI: 10.1021/bi00767a015.
14. Barter PJ, Kastelein JJP. Targeting cholesteryl ester transfer protein for the prevention and management of cardiovascular disease. Journal of the American College of Cardiology. 2006;47(3):492–9. DOI: 10.1021/bi00767a015.
15. Goldstein JL, Brown MS, Anderson RGW, Russell DW, Schneider WJ. ENDOCYTOSIS : Concepts Receptor System. Receptor. 1985;1–39.
16. Zhang DW, Lagace TA, Garuti R, Zhao Z, McDonald M, Horton JD, et al. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. Journal of Biological Chemistry. 2007;282(25):18602–12. DOI: 10.1074/jbc.M702027200.
17. Stemmer K, Finan B, DiMarchi RD, Tschöp MH, Müller TD. Insights into incretin-based therapies for treatment of diabetic dyslipidemia. Advanced Drug Delivery Reviews [Internet]. 2020;159:34–53. Available from: https://doi.org/10.1016/j.addr.2020.05.008.
18. Stoekenbroek RM, Kastelein JJP, Huijgen R. PCSK9 inhibition: The way forward in the treatment of dyslipidemia. BMC Medicine [Internet]. 2015;13(1):1–6. Available from: http://dx.doi.org/10.1186/s12916-015-0503-4.
19. Ramasamy I. Update on the molecular biology of dyslipidemias. Clinica Chimica Acta [Internet]. 2016;454:143–85. Available from: http://dx.doi.org/10.1016/j.cca.2015.10.033.
20. Pirillo A, Casula M, Olmastroni E, Norata GD, Catapano AL. Global epidemiology of dyslipidaemias. Nature Reviews Cardiology [Internet]. 2021;18(10):689–700. Available from: http://dx.doi.org/10.1038/s41569-021-00541-4.
21. Kathiresan S, Willer CJ, Peloso GM, Demissie S, Musunuru K, Schadt EE, et al. Common variants at 30 loci contribute to polygenic dyslipidemia. Nature Genetics. 2009;41(1):56–65. DOI: 10.1038/ng.291.
22. Teslovich TM, Musunuru K, Smith A v., Edmondson AC, Stylianou IM, Koseki M, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature [Internet]. 2010;466(7307):707–13. Available from: http://dx.doi.org/10.1038/nature09270.
23. Hegele RA, Borén J, Ginsberg HN, Arca M, Averna M, Binder CJ, et al. Rare dyslipidaemias, from phenotype to genotype to management: a European Atherosclerosis Society task force consensus statement. The Lancet Diabetes and Endocrinology [Internet]. 2020;8(1):50–67. Available from: http://dx.doi.org/10.1016/S2213-8587(19)30264-5.
24. Hegele RA. Lipoprotein and lipid metabolism. Emery and Rimoin's Principles and Practice of Medical Genetics. 2013;1–33. DOI: 10.1016/B978-0-12-383834-6.00100-2.
25. Defesche JC, Gidding SS, Harada-Shiba M, Hegele RA, Santos RD, Wierzbicki AS. Familial hypercholesterolaemia. Nature reviews Disease primers [Internet]. 2017;3:17093. Available from: http://dx.doi.org/10.1038/nrdp.2017.93.
26. Nelson RH. Hyperlipidemia as a Risk Factor for Cardiovascular Disease. Primary Care - Clinics in Office Practice [Internet]. 2013;40(1):195–211. Available from: http://dx.doi.org/10.1016/j.pop.2012.11.003

27. Hu P, Dharmayat KI, Stevens CAT, Sharabiani MTA, Jones RS, Watts GF, et al. Prevalence of Familial Hypercholesterolemia among the General Population and Patients with Atherosclerotic Cardiovascular Disease: A Systematic Review and Meta-Analysis. Circulation. 2020;141(22):1742–59. DOI: 10.1161/CIRCULATIONAHA.119.044795.
28. Cuchel M, Bruckert E, Ginsberg HN, Raal FJ, Santos RD, Hegele RA, et al. Homozygous familial hypercholesterolaemia: New insights and guidance for clinicians to improve detection and clinical management. A position paper fromthe Consensus Panel on Familial Hypercholesterolaemia of the European Atherosclerosis Society. European Heart Journal. 2014;35(32):2146–57. DOI: 10.1093/eurheartj/ehu274.
29. Betteridge DJ, Broome K, Durrington PN, Mann JI, Miller JP, Neil HAW, et al. Risk of fatal coronary heart disease in familial hypercholesterolaemia. British Medical Journal. 1991;303(6807):893–6. DOI: 10.1093/eurheartj/ehu274.
30. Alves AC, Medeiros AM, Francisco V, Rato Q, Bourbon M. Molecular diagnosis of familial hypercholesterolemia: an important tool for cardiovascular risk stratification. Revista Portuguesa de Cardiologia. 2010;29.
31. Goldstein JL, Brown MS. Familial hypercholesterolemia: identification of a defect in the regulation of 3 hydroxy 3 methylglutaryl coenzyme A reductase activity associated with overproduction of cholesterol. Proceedings of the National Academy of Sciences of the United States of America. 1973;70(10):2804–8. DOI: 10.1073/pnas.70.10.2804.
32. Goldstein JL, Brown MS. Expression of the familial hypercholesterolemia gene in heterozygotes: model for a dominant disorder in man. Transactions of the Association of American Physicians. 1974;87:120–31.
33. Hobbs HH, Brown MS, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Human Mutation. 1992;1(6):445–66. DOI: 10.1002/humu.1380010602.
34. Innerarity TL, Weisgraber KH, Arnold KS, Mahley RW, Krauss RM, Vega GL, et al. Familial defective apolipoprotein B-100: Low density lipoproteins with abnormal receptor binding. Proceedings of the National Academy of Sciences of the United States of America. 1987;84(19):6919–23. DOI: 10.1073/pnas.84.19.6919.
35. Alves AC, Etxebarria A, Soutar AK, Martin C, Bourbon M. Novel functional APOB mutations outside LDL-binding region causing familial hypercholesterolaemia. Human Molecular Genetics. 2014;23(7):1–12. DOI: 10.1093/hmg/ddt573.
36. Soria LF, Ludwig EH, Clarke HRG, Vega GL, Grundy SM, McCarthy BJ. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(2):587–91. DOI: 10.1073/pnas.86.2.587.
37. Iacocca MA, Chora JR, Carrié A, Freiberger T, Leigh SE, Defesche JC, et al. ClinVar database of global familial hypercholesterolemia-associated DNA variants. Human Mutation. 2018;39(11):1631–40. DOI: 10.1002/humu.23634.
38. Andersen LH, Miserez AR, Ahmad Z, Andersen RL. Familial defective apolipoprotein B-100: A review. Journal of Clinical Lipidology [Internet]. 2016;10(6):1297–302. Available from: http://dx.doi.org/10.1016/j.jacl.2016.09.009.
39. di Taranto MD, Giacobbe C, Fortunato G. Familial hypercholesterolemia: A complex genetic disease with variable phenotypes. European Journal of Medical Genetics [Internet]. 2020;63(4):103831. Available from: https://doi.org/10.1016/j.ejmg.2019.103831.
40. Abifadel M, Varret M, Rabès JP, Allard D, Ouguerram K, Devillers M, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nature Genetics. 2003;34(2):154–6. DOI: 10.1038/ng1161.
41. Horton JD, Cohen JC, Hobbs HH. Molecular biology of PCSK9: its role in LDL metabolism. Trends in Biochemical Sciences. 2007;32(2):71–7. DOI: 10.1016/j.tibs.2006.12.008.
42. Kotowski IK, Pertsemlidis A, Luke A, Cooper RS, Vega GL, Cohen JC, et al. A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol. American Journal of Human Genetics. 2006;78(3):410–22. DOI: 10.1086/500615.
43. Benjannet S, Rhainds D, Essalmani R, Mayne J, Wickham L, Jin W, et al. NARC-1/PCSK9 and its natural mutants: Zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. Journal of Biological Chemistry. 2004;279(47):48865–75. DOI: 10.1074/jbc.M409699200.
44. Bourbon M, Alves AC, Medeiros AM, Silva S, Soutar AK. Familial hypercholesterolaemia in Portugal. Atherosclerosis. 2008;196(2):633–42. DOI: 10.1016/j.atherosclerosis.2007.07.019.
45. Fellin R, Arca M, Zuliani G, Calandra S, Bertolini S. The history of autosomal recessive hypercholesterolemia (ARH): From clinical observations to gene identification. Gene [Internet]. 2015;555(1):23–32. Available from: http://dx.doi.org/10.1016/j.gene.2014.09.020.
46. D'Erasmo L, di Costanzo A, Arca M. Autosomal recessive hypercholesterolemia: update for 2020. Current opinion in lipidology. 2020;31(2):56–61. DOI: 0.1097/MOL.0000000000000664.
47. Pisciotta L, Oliva CP, Pes GM, di Scala L, Bellocchio A, Fresa R, et al. Autosomal recessive hypercholesterolemia (ARH) and homozygous familial hypercholesterolemia (FH): A phenotypic comparison. Atherosclerosis. 2006;188(2):398–405. DOI: 0.1016/j.atherosclerosis.2005.11.016.
48. Patel SB. Recent advances in understanding the STSL locus and ABCG5/ABCG8 biology. Current Opinion in Lipidology. 2014;25(3):169–75. DOI: 0.1097/MOL.0000000000000071.
49. Rios J, Stein E, Shendure J, Hobbs HH, Cohen JC. Identification by whole-genome resequencing of gene defect responsible for severe hypercholesterolemia. Human Molecular Genetics. 2010;19(22):4313–8. DOI: 10.1093/hmg/ddq352.
50. Wang J, Joy T, Mymin D, Frohlich J, Hegele RA. Phenotypic heterogeneity of sitosterolemia. Journal of Lipid Research. 2004;45(12):2361–7. DOI: 10.1194/jlr.M400310-JLR200.
51. Lee JY, Kinch LN, Borek DM, Wang J, Wang J, Urbatsch IL, et al. Crystal structure of the human sterol transporter ABCG5/ABCG8. Vol. 533, Nature. 2016. p. 561–4. DOI: 10.1038/nature17666.
52. Dron JS, Hegele RA. Polygenic influences on dyslipidemias. Current Opinion in Lipidology. 2018;29(2):133–43. DOI: 10.1097/MOL.0000000000000482.

53. Willer CJ, Schmidt EM, Sengupta S, Peloso GM, Gustafsson S, Kanoni S, et al. Discovery and refinement of loci associated with lipid levels. Nature Genetics. 2013;45(11):1274–85. DOI: 10.1038/ng.2797.
54. Talmud PJ, Shah S, Whittall R, Futema M, Howard P, Cooper JA, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: A case-control study. The Lancet [Internet]. 2013;381(9874):1293–301. Available from: http://dx.doi.org/10.1016/S0140-6736(12)62127-8.
55. Futema M, Bourbon M, Williams M, Humphries SE. Clinical utility of the polygenic LDL-C SNP score in familial hypercholesterolemia. Atherosclerosis [Internet]. 2018;277:457–63. Available from: https://doi.org/10.1016/j.atherosclerosis.2018.06.006.
56. Hegele RA, Tsimikas S. Lipid-lowering agents targets beyond PCSK9. Circulation Research. 2019;124(3):386–404. DOI: 10.1161/CIRCRESAHA.118.313171.
57. Welty FK. Hypobetalipoproteinemia and abetalipoproteinemia. Current Opinion in Lipidology. 2014;25(3):161–8. DOI: 10.1097/MOL.0000000000000072.
58. Lee J, Hegele RA. Abetalipoproteinemia and homozygous hypobetalipoproteinemia: A framework for diagnosis and management. Journal of Inherited Metabolic Disease. 2014;37(3):333–9. DOI: 10.1007/s10545-013-9665-4.
59. Moutzouri E, Elisaf M, Liberopoulos EN. Hypocholesterolemia. Current Vascular Pharmacology. 2011;9:200–12. DOI: 10.2174/157016111794519354.
60. Parhofer KG, Barrett PHR, Bier DM, Schonfeld G. Lipoproteins containing the truncated apolipoprotein, apo B-89, are cleared from human plasma more rapidly than apo B-100-containing lipoproteins in vivo. Journal of Clinical Investigation. 1992;89(6):1931–7. DOI: 10.1172/JCI115799.
61. Huang LS, Kayden H, Sokol RJ, Breslow JL. ApoB gene nonsense and splicing mutations in a compound heterozygote for familial hypobetalipoproteinemia. Journal of Lipid Research [Internet]. 1991;32(8):1341–8. Available from: http://dx.doi.org/10.1016/S0022-2275(20)41964-9.
62. Seidah NG. Proprotein Convertase Subtilisin Kexin 9 (PCSK9) Inhibitors in the Treatment of Hypercholesterolemia and other Pathologies. Current Pharmaceutical Design. 2013;19(17):3161–72. DOI: 10.2174/13816128113199990313.
63. Lopez D. PCSK9: An enigmatic protease. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. 2008;1781(4):184–91. DOI: 10.1016/j.bbalip.2008.01.003.
64. Cohen JC, Boerwincle E, Mosley TH, Hobbs HH. Sequence Variations in PCSK9, Low LDL, and Protection against Coronary Heart Disease. Heart Disease. 2006;1264–72.
65. Wetterau JR, Aggerbeck LP, Bouma ME, Eisenberg C, Munck A, Hermier M, et al. Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science. 1992;258(5084):999–1001. DOI: 10.1126/science.1439810.
66. Hooper AJ, van Bockxmeer FM, Burnett JR. Monogenic hypocholesterolaemic lipid disorders and apolipoprotein B metabolism. Critical Reviews in Clinical Laboratory Sciences. 2005;42(5–6):515–45. DOI: 10.1080/10408360500295113.
67. Levy E, Poinsot P, Spahis S. Chylomicron retention disease: Genetics, biochemistry, and clinical spectrum. Current Opinion in Lipidology. 2019;30(2):134–9. DOI: 10.1097/MOL.0000000000000578.
68. Georges A, Bonneau J, Bonnefont-Rousselot D, Champigneulle J, Rabès JP, Abifadel M, et al. Molecular analysis and intestinal expression of SAR1 genes and proteins in Anderson's disease (Chylomicron retention disease). Orphanet Journal of Rare Diseases. 2011;6(1):1–16. DOI: 10.1186/1750-1172-6-1.
69. Hegele RA, Ginsberg HN, Chapman MJ, Nordestgaard BG, Kuivenhoven JA, Averna M, et al. The polygenic nature of hypertriglyceridaemia: Implications for definition, diagnosis, and management. The Lancet Diabetes and Endocrinology [Internet]. 2014;2(8):655–66. Available from: http://dx.doi.org/10.1016/S2213-8587(13)70191-8.
70. Dron JS, Wang J, Cao H, McIntyre AD, Iacocca MA, Menard JR, et al. Severe hypertriglyceridemia is primarily polygenic. Journal of Clinical Lipidology [Internet]. 2019;13(1):80–8. Available from: https://doi.org/10.1016/j.jacl.2018.10.006.
71. Dron JS, Hegele RA. Genetics of Hypertriglyceridemia. Frontiers in Endocrinology. 2020;11(July). DOI: 10.3389/fendo.2020.00455.
72. Neil HAW, Cooper J, Betteridge DJ, Capps N, McDowell IFW, Durrington PN, et al. All-cause and cardiovascular mortality in treated patients with severe hypertriglyceridaemia: A long-term prospective registry study. Atherosclerosis [Internet]. 2010;211(2):618–23. Available from: http://dx.doi.org/10.1016/j.atherosclerosis.2010.03.006.
73. Baass A, Paquette M, Bernard S, Hegele RA. Familial chylomicronemia syndrome: an under-recognized cause of severe hypertriglyceridaemia. Journal of Internal Medicine. 2020;287(4):340–8. DOI: 10.1111/joim.13016.
74. Enerbäck S, Gimble JM. Lipoprotein lipase gene expression: Physiological regulators at the transcriptional and post-transcriptional level. Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism. 1993;1169(2):107–25. DOI: 10.1016/0005-2760(93)90196-G.
75. Hegele RA. Multidimensional regulation of lipoprotein lipase: Impact on biochemical and cardiovascular phenotypes1. Journal of Lipid Research. 2016;57(9):1601–7. DOI: 10.1194/jlr.C070946.
76. Beigneux AR, Weinstein MM, Davies BSJ, Gin P, Bensadoun A, Fong LG, et al. GPIHBP1 and lipolysis: An update. Current Opinion in Lipidology. 2009;20(3):211–6. DOI: 10.1097/MOL.0b013e32832ac026.
77. Gin P, Yin L, Davies BSJ, Weinstein MM, Ryan RO, Bensadoun A, et al. The acidic domain of GPIHBP1 is important for the binding of lipoprotein lipase and chylomicrons. Journal of Biological Chemistry. 2008;283(43):29554–62. DOI: 10.1074/jbc.M802579200.
78. Wolska A, Dunbar RL, Freeman LA, Ueda M, Amar MJ, Sviridov DO, et al. Apolipoprotein C-II: New findings related to genetics, biochemistry, and role in triglyceride metabolism. Atherosclerosis [Internet]. 2017;267:49–60. Available from: https://doi.org/10.1016/j.atherosclerosis.2017.10.025.

79. Kluger M, Heeren J, Merkel M. Apoprotein A-V: An important regulator of triglyceride metabolism. Journal of Inherited Metabolic Disease. 2008;31(2):281–8. DOI: 10.1007/s10545-008-0863-4.

80. Forte TM, Sharma V, Ryan RO, Kluger M, Heeren J, Merkel M. Apolipoprotein A-V gene therapy for disease prevention / treatment: A critical analysis. Journal of Biomedical Research. 2008;30(2):88–93. DOI: 10.7555/JBR.30.20150059.

81. Johansen CT, Hegele RA. Genetic bases of hypertriglyceridemic phenotypes. Current Opinion in Lipidology. 2011;22(4):247–53. DOI: 10.1097/MOL.0b013e3283471972.

82. Péterfy M. Lipase maturation factor 1: A lipase chaperone involved in lipid metabolism. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids [Internet]. 2012;1821(5):790–4. Available from: http://dx.doi.org/10.1016/j.bbalip.2011.10.006.

83. Paquette M, Bernard S, Hegele RA, Baass A. Chylomicronemia: Differences between familial chylomicronemia syndrome and multifactorial chylomicronemia. Atherosclerosis. 2019;283(November):137–42. DOI: 10.1016/j.atherosclerosis.2018.12.019.

84. Dron JS, Hegele RA. The evolution of genetic-based risk scores for lipids and cardiovascular disease. Current Opinion in Lipidology. 2019;30(2):71–81. DOI: 10.1097/MOL.0000000000000576.

85. Basel-Vanagaite L, Zevit N, Zahav AH, Guo L, Parathath S, Pasmanik-Chor M, et al. Transient infantile hypertriglyceridemia, fatty liver, and hepatic fibrosis caused by mutated GPD1, encoding glycerol-3-phosphate dehydrogenase 1. American Journal of Human Genetics [Internet]. 2012;90(1):49–60. Available from: http://dx.doi.org/10.1016/j.ajhg.2011.11.028.

86. Ou X, Ji C, Han X, Zhao X, Li X, Mao Y, et al. Crystal structures of human glycerol 3-phosphate dehydrogenase 1 (GPD1). Journal of Molecular Biology. 2006;357(3):858–69. DOI: 10.1016/j.jmb.2005.12.074.

87. Li JQ, Xie XB, Feng JY, Chen L, Abuduxikuer K, Lu Y, et al. A novel homozygous mutation in the glycerol-3-phosphate dehydrogenase 1 gene in a Chinese patient with transient infantile hypertriglyceridemia: A case report. BMC Gastroenterology. 2018;18(1). DOI: 10.1186/s12876-018-0827-6.

88. Grabowski, Gregory A, Valayannopoulos, Vassili ; Goodman, Zachary D. ; Balwani M. Lysosomal Acid Lipase Deficiency: The Continuous Spectra of Disease Variants. In: he Online Metabolic and Molecular Bases of Inherited Disease. McGraw Hill; 2019.

89. Walden CC, Hegele RA. Apolipoprotein E in hyperlipidemia. Annals of Internal Medicine. 1994;120(12):1026–36.

90. Berberich AJ, Ziada A, Zou GY, Hegele RA. Conservative management in hypertriglyceridemia-associated pancreatitis. Journal of Internal Medicine. 2019;286(6):644–50. DOI: 10.1111/joim.12925.

91. Brahm AJ, Hegele RA. Chylomicronaemia-current diagnosis and future therapies. Nature Reviews Endocrinology. 2015;11(6):352–62. DOI: 10.1038/nrendo.2015.26.

92. Shendure J, Balasubramanian S, Church GM, Gilbert W, Rogers J, Schloss JA, et al. DNA sequencing at 40: Past, present and future. Nature [Internet]. 2017;550(7676). Available from: http://dx.doi.org/10.1038/nature24286.

93. Collins FS, Morgan M, Patrinos A. The Human Genome Project: Lessons from large-scale biology. Science. 2003;300(5617):286–90. DOI: 10.1126/science.1084564.

94. Bourbon M, Rato Q, Investigadores do Estudo Português de Hipercolesterolemia Familiar. Portuguese Familial Hypercholesterolemia Study: presentation of the study and preliminary results. Revista Portuguesa de Cardiologia. 2006;25(11):999–1013.

95. Medeiros AM, Alves AC, Bourbon M. Mutational analysis of a cohort with clinical diagnosis of familial hypercholesterolemia: Considerations for genetic diagnosis improvement. Genetics in Medicine. 2016;18(4):316–24. DOI: 10.1038/gim.2015.71.

96. Lahiri DK, Numberger JI. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Research. 1991;19(19):5444. DOI: 10.1093/nar/19.19.5444.

97. wANNOVAR [Internet]. Available from: https://wannovar.wglab.org/

98. Chang X, Wang K. Wannovar: Annotating genetic variants for personal genomes via the web. Journal of Medical Genetics. 2012;49(7):433–6. DOI: 10.1136/jmedgenet-2012-100918.

99. Fowler A, Mahamdallie S, Ruark E, Seal S, Ramsay E, Clarke M, et al. Accurate clinical detection of exon copy number variants in a targeted NGS panel using DECoN. Wellcome Open Research. 2016;1(May):1–12. DOI: 10.12688/wellcomeopenres.10069.1.

100. Futema M, Shah S, Cooper JA, Li K, Whittall RA, Sharifi M, et al. Refinement of variant selection for the LDL cholesterol genetic risk score in the diagnosis of the polygenic form of clinical familial hypercholesterolemia and replication in samples from 6 countries. Clinical Chemistry. 2015;61(1):231–8. DOI: 10.1373/clinchem.2014.231365.

101. Clarke R, Peden JF, Hopewell JC, Kyriakou T, Goel A, Heath SC, et al. Genetic Variants Associated with Lp(a) Lipoprotein Level and Coronary Disease. New England Journal of Medicine. 2009;361(26):2518–28. DOI: 10.1056/nejmoa0902604.

102. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in Medicine. 2015;17(5):405–24. DOI: 10.1038/gim.2015.30.

103. Chora JR, Iacocca MA, Tichý L, Wand H, Kurtz CL, Zimmermann H, et al. The Clinical Genome Resource (ClinGen) Familial Hypercholesterolemia Variant Curation Expert Panel consensus guidelines for LDLR variant classification. Genetics in Medicine. 2022;24(2):293–306. DOI: 10.1016/j.gim.2021.09.012.

104. Chora JR, Medeiros AM, Alves AC, Bourbon M. Analysis of publicly available LDLR, APOB, and PCSK9 variants associated with familial hypercholesterolemia: Application of ACMG guidelines and implications for familial hypercholesterolemia diagnosis. Genetics in Medicine [Internet]. 2018;20(6):591–8. Available from: http://dx.doi.org/10.1038/gim.2017.151.

105. Lee WK, Haddad L, Macleod MJ, Dorrance AM, Wilson DJ, Gaffney D, et al. Identification of a common low density lipoprotein receptor mutation (C163Y) in the west of Scotland. Journal of Medical Genetics. 1998;35(7):573–8. DOI: 10.1136/jmg.35.7.573.

106. Alves AC, Azevedo S, Benito-Vicente A, Graça R, Galicia-Garcia U, Barros P, et al. LDLR variants functional characterization: Contribution to variant classification. Atherosclerosis [Internet]. 2021;329(February):14–21. Available from: https://doi.org/10.1016/j.atherosclerosis.2021.06.001.

107. Marduel M, Carrié A, Sassolas A, Devillers M, Carreau V, di Filippo M, et al. Molecular spectrum of autosomal dominant hypercholesterolemia in France. Human Mutation. 2010;31(11):1811–24. DOI: 10.1002/humu.21348.

108. Medeiros A, Alves AC, Francisco V, Bourbon M, Investigators of FH study. Update of the Portuguese Familial Hypercholesterolaemia Study. Atherosclerosis. 2010;212(2):553–8. DOI: 10.1016/j.atherosclerosis.2010.07.012.

109. Ebhardt M, Schmidt H, Doerk T, Tietge U, Haas R, Manns MP, et al. Mutational analysis in 46 German families with familial hypercholesterolemia: identification of 8 new mutations. Human Mutation. 1999;13(3):257.

110. Etxebarria A, Palacios L, Stef M, Tejedor D, Uribe KB, Oleaga A, et al. Functional characterization of splicing and ligand-binding domain variants in the LDL receptor. Human Mutation. 2012;33(1):232–43. DOI: 10.1002/humu.21630.

111. Hobbs HH, Russell DW, Brown MS, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: Mutational analysis of a membrane protein. Annual Review of Genetics. 1990;24:133–70. DOI: 10.1146/annurev.ge.24.120190.001025.

112. Chang JH, Pan JP, Tai DY, Huang AC, Li PH, Ho HL, et al. Identification and characterization of LDL receptor gene mutations in hyperlipidemic Chinese. Journal of Lipid Research. 2003;44(10):1850–8. DOI: 10.1194/jlr.M200470-JLR200.

113. Fouchier SW, Defesche JC, Umans-Eckenhausen MA, Kastelein JJ. The molecular basis of familial hypercholesterolemia in the Netherlands. Human Genetics. 2001;109(6):602–15. DOI: 10.1007/s00439-001-0628-8.

114. Benito-Vicente A, Alves AC, Etxebarria A, Medeiros AM, Martin C, Bourbon M. The importance of an integrated analysis of clinical, molecular, and functional data for the genetic diagnosis of familial hypercholesterolemia. Genetics in Medicine. 2015;17(12):980–8. DOI: 10.1038/gim.2015.14.

115. Bertolini S, Cassanelli S, Garuti R, Ghisellini M, Simone ML, Rolleri M, et al. Analysis of LDL receptor gene mutations in Italian patients with homozygous familial hypercholesterolemia. Arteriosclerosis, Thrombosis, and Vascular Biology. 1999;19(2):408–18. DOI: 10.1161/01.ATV.19.2.408.

116. Romano M, di Taranto MD, Mirabelli P, D'Agostino MN, Iannuzzi A, Marotta G, et al. An improved method on stimulated T-lymphocytes to functionally characterize novel and known LDLR mutations. Journal of Lipid Research. 2011;52(11):2095–100. DOI: 10.1194/jlr.D017772.

117. Alves AC, Etxebarria A, Medeiros AM, Benito-Vicente A, Thedrez A, Passard M, et al. Characterization of the First PCSK9 Gain of Function Homozygote. Journal of the American College of Cardiology [Internet]. 2015;66(19):2152–4. Available from: http://dx.doi.org/10.1016/j.jacc.2015.08.871.

118. Cameron J, Holla ØL, Ranheim T, Kulseth MA, Berge KE, Leren TP. Effect of mutations in the PCSK9 gene on the cell surface LDL receptors. Human Molecular Genetics. 2006;15(9):1551–8. DOI: 10.1093/hmg/ddl077.

119. Futema M, Plagnol V, Whittall RA, Neil HAW, Humphries SE. Use of targeted exome sequencing as a diagnostic tool for Familial Hypercholesterolaemia. Journal of Medical Genetics. 2012;49(10):644–9. DOI: 10.1136/jmedgenet-2012-101189.

120. Leren TP, Berge KE. Identification of mutations in the apolipoprotein B-100 gene and in the PCSK9 gene as the cause of hypocholesterolemia. Clinica Chimica Acta. 2008;397(1–2):92–5. DOI: 10.1016/j.cca.2008.07.025.

121. Johansen CT, Wang J, Lanktree MB, Cao H, McIntyre AD, Ban MR, et al. Excess of rare variants in genes identified by genome-wide association study of hypertriglyceridemia. Nature Genetics [Internet]. 2010;42(8):684–7. Available from: http://dx.doi.org/10.1038/ng.628.

122. Innerarity TL, Mahley RW, Weisgraber KH, Bersot TP, Krauss RM, Vega GL, et al. Familial defective apolipoprotein B-100: A mutation of apolipoprotein B that causes hypercholesterolemia. Journal of Lipid Research [Internet]. 1990;31(8):1337–49. Available from: http://dx.doi.org/10.1016/S0022-2275(20)42605-7.

123. Fernández-Higuero JA, Etxebarria A, Benito-Vicente A, Alves AC, Arrondo JLR, Ostolaza H, et al. Structural analysis of APOB variants, p.(Arg3527Gln), p.(Arg1164Thr) and p.(Gln4494del), causing Familial Hypercholesterolaemia provides novel insights into variant pathogenicity. Scientific Reports [Internet]. 2015;5(June):1–8. Available from: http://dx.doi.org/10.1038/srep18184.

124. Bourbon M, Alves AC, Alonso R, Mata N, Aguiar P, Padró T, et al. Mutational analysis and genotype-phenotype relation in familial hypercholesterolemia: The SAFEHEART registry. Atherosclerosis [Internet]. 2017;262:8–13. Available from: http://dx.doi.org/10.1016/j.atherosclerosis.2017.04.002.

125. Radovica-Spalvina I, Latkovskis G, Silamikelis I, Fridmanis D, Elbere I, Ventins K, et al. Next-generation-sequencing-based identification of familial hypercholesterolemia-related mutations in subjects with increased LDL-C levels in a latvian population. BMC Medical Genetics [Internet]. 2015;16(1):1–9. Available from: http://dx.doi.org/10.1186/s12881-015-0230-x.

126. Lek M, Karczewski KJ, Minikel E v., Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature [Internet]. 2016;536(7616):285–91. Available from: http://dx.doi.org/10.1038/nature19057.

127. Oliva CP, Carubbi F, Schaap FG, Bertolini S, Calandra S. Hypertriglyceridaemia and low plasma HDL in a patient with apolipoprotein A-V deficiency due to a novel mutation in the APOA5 gene. Journal of Internal Medicine. 2008;263(4):450–8. DOI: 10.1111/j.1365-2796.2007.01912.x.

128. Charrière S, Cugnet C, Guitard M, Bernard S, Groisne L, Charcosset M, et al. Modulation of phenotypic expression of APOA5 Q97X and L242P mutations. Atherosclerosis. 2009;207(1):150–6. DOI: 10.1016/j.atherosclerosis.2009.04.021.

129. Rabacchi C, Pisciotta L, Cefalù AB, Noto D, Fresa R, Tarugi P, et al. Spectrum of mutations of the LPL gene identified in Italy in patients with severe hypertriglyceridemia. Atherosclerosis [Internet]. 2015;241(1):79–86. Available from: http://dx.doi.org/10.1016/j.atherosclerosis.2015.04.815.

130. Ma Y, Henderson HE, Ven Murthy MR, Roederer G, v. Monsalve M, Clarke LA, et al. A Mutation in the Human Lipoprotein Lipase Gene as the Most Common Cause of Familial Chylomicronemia in French Canadians. New England Journal of Medicine. 1991;324(25):1761–6. DOI: 0.1056/nejm199106203242502.

131. Matarazzo L, Ragnoni V, Malaventura C, Leon A, Colavito D, Vigna GB, et al. Successful fenofibrate therapy for severe and persistent hypertriglyceridemia in a boy with cirrhosis and glycerol-3-phosphate dehydrogenase 1 deficiency. JIMD Reports. 2020;54(1):25–31. DOI: 10.1002/jmd2.12125.

132. Berge KE, Retterstøl K, Romeo S, Pirazzi C, Leren TP. Type 1 hyperlipoproteinemia due to a novel deletion of exons 3 and 4 in the GPIHBP1 gene. Atherosclerosis [Internet]. 2014;234(1):30–3. Available from: http://dx.doi.org/10.1016/j.atherosclerosis.2014.02.005.

133. Johansen CT, Dubé JB, Loyzer MN, MacDonald A, Carter DE, McIntyre AD, et al. LipidSeq: A next-generation clinical resequencing panel for monogenic dyslipidemias. Journal of Lipid Research. 2014;55(4):765–72. DOI: 10.1194/jlr.D045963.

134. Futema M, Taylor-Beadling A, Williams M, Humphries SE. Genetic testing for familial hypercholesterolemia—past, present, and future. Journal of Lipid Research [Internet]. 2021;62:100139. Available from: https://doi.org/10.1016/j.jlr.2021.100139.

135. Lamiquiz-Moneo I, Baila-Rueda L, Bea AM, Mateo-Gallego R, Pérez-Calahorra S, Marco-Benedí V, et al. ABCG5/G8 gene is associated with hypercholesterolemias without mutation in candidate genes and noncholesterol sterols. Journal of Clinical Lipidology. 2017;11(6):1432-1440.e4. DOI: 10.1016/j.jacl.2017.09.005.

136. Helgadottir A, Thorleifsson G, Alexandersson KF, Tragante V, Thorsteinsdottir M, Eiriksson FF, et al. Genetic variability in the absorption of dietary sterols affects the risk of coronary artery disease. European Heart Journal. 2020;41(28):2618–28. DOI: 10.1093/eurheartj/ehaa531.

137. Williams K, Segard A, Graf GA. Sitosterolemia: Twenty years of discovery of the function of abcg5 abcg8. International Journal of Molecular Sciences. 2021;22(5):1–14. DOI: 10.3390/ijms22052641.

138. Reeskamp LF, Volta A, Zuurbier L, Defesche JC, Hovingh GK, Grefhorst A. ABCG5 and ABCG8 genetic variants in familial hypercholesterolemia. Journal of Clinical Lipidology [Internet]. 2020;14(2):207-217.e7. Available from: https://doi.org/10.1016/j.jacl.2020.01.007.

139. Corral P, Geller AS, Polisecki EY, Lopez GI, Bañares VG, Cacciagiu L, et al. Unusual genetic variants associated with hypercholesterolemia in Argentina. Atherosclerosis. 2018;277:256–61. DOI: 10.1016/j.atherosclerosis.2018.06.009.

140. Alves AC, Chora JR, Bourbon M. Genomics of familial hypercholesterolaemia. Current Opinion in Lipidology. 2019;30(2):148–50. DOI: 10.1097/MOL.0000000000000584.

141. Trinder M, Uddin MM, Finneran P, Aragam KG, Natarajan P. Clinical Utility of Lipoprotein(a) and LPA Genetic Risk Score in Risk Prediction of Incident Atherosclerotic Cardiovascular Disease. JAMA Cardiology. 2021;6(3):287–95. DOI: 10.1001/jamacardio.2020.5398.

142. Boerwinkle E, Leffert CC, Lin J, Lackner C, Chiesa G, Hobbs HH. Apolipoprotein(a) gene accounts for greater than 90% of the variation in plasma lipoprotein(a) concentrations. Journal of Clinical Investigation. 1992;90(1):52–60. DOI: 10.1172/JCI115855.

143. Sankatsing RR, Fouchier SW, de Haan S, Hutten BA, de Groot E, Kastelein JJP, et al. Hepatic and cardiovascular consequences of familial hypobetalipoproteinemia. Arteriosclerosis, Thrombosis, and Vascular Biology. 2005;25(9):1979–84. DOI: 10.1161/01.ATV.0000176191.64314.07.

144. Rabacchi C, Simone ML, Pisciotta L, di Leo E, Bocchi D, Pietrangelo A, et al. In vitro functional characterization of splicing variants of the APOB gene found in familial hypobetalipoproteinemia. Journal of Clinical Lipidology [Internet]. 2019;13(6):960–9. Available from: https://doi.org/10.1016/j.jacl.2019.09.003.

145. Reiner Ž, Guardamagna O, Nair D, Soran H, Hovingh K, Bertolini S, et al. Lysosomal acid lipase deficiency - An under-recognized cause of dyslipidaemia and liver dysfunction. Atherosclerosis. 2014;235(1):21–30. DOI: .1016/j.atherosclerosis.2014.04.003.

146. Defesche JC, Schuurman EJM, Klaaijsen LN, Khoo KL, Wiegman A, Stalenhoef AFH. Silent exonic mutations in the low-density lipoprotein receptor gene that cause familial hypercholesterolemia by affecting mRNA splicing. Clinical Genetics. 2008;73(6):573–8. DOI: 0.1111/j.1399-0004.2008.00999.x.

Appendix I – Patients Recruitment

Appendix IA - Clinical Questionnaire (Familial Hypercholesterolemia)

REPÚBLICA PORTUGUESA
SAÚDE

SNS SERVIÇO NACIONAL DE SAÚDE

Instituto_Nacional de Saúde Doutor Ricardo Jorge

Estudo Português de Hipercolesterolemia Familiar

Adaptado do "Simon Broome Heart Research Trust"

Confidencial

Número do processo: ____
Número da família: ____
(A preencher pelo INSA)

Identificação do caso-índex

Nome completo: ______________________________

Morada: ______________________________

____

Telefone: ____________ Email: ____________

Data de Nasc.: ____________

Estado Civil: ____________

Natural de: ____________ Naturalidade dos pais: Pai ____________/ Mãe ____________

Sexo: Masculino ☐ Feminino ☐

Origem étnica (assinalar a opção adequada):

☐ Caucasiano ☐ Asiático ☐ Africano ☐ Outro

Condições de colheita e envio das amostras

Devem ser feitas as seguintes colheitas em jejum:

CASO-INDEX:

Adultos:

7.5 mL de sangue em tubo de soro com gel separador - após centrifugação (3500rpm/10minutos)

~11 mL de sangue total (4x tubos de EDTA 2.7mL)

Crianças:

5 mL de sangue em tubo de soro com gel separador - após centrifugação (3500rpm/10minutos)

~11 mL de sangue total (4x tubos de EDTA 2.7mL)

FAMILIARES (adultos e crianças):

5 mL de sangue em tubo de soro com gel separador - após centrifugação (3500rpm/10minutos)

~8 mL de sangue total (3x tubos de EDTA 2.7mL)

O formulário clínico deve ser sempre acompanhado pela Declaração de Consentimento

REPÚBLICA PORTUGUESA SAÚDE

SNS SERVIÇO NACIONAL DE SAÚDE

Instituto_Nacional de Saúde Doutor Ricardo Jorge

Estudo Português de Hipercolesterolemia Familiar

Inquérito adaptado do "Simon Broome Heart Research Trust" Confidencial

Número do processo:
Número da família
(A preencher pelo INSA)

Razão primária de inclusão no estudo:
(assinalar a opção adequada)

- ☐ Parente afectado
- ☐ Parente com doença coronária crónica
- ☐ Caso-index tem doença coronária crónica
- ☐ Caso-index sofre de outras doenças vasculares
- ☐ Sinais físicos
- ☐ Screening
- ☐ Outros

Critérios para admissão no estudo (segundo os critérios abaixo mencionados):

- ☐ Hipercolesterolemia familiar confirmada
- ☐ Hipercolesterolemia familiar possível

Critérios para diagnóstico:

Hipercolesterolemia familiar confirmada é definida como:

Crianças menores de 16 anos: Colesterol total acima de 260 mg/dL (6,7 mmol/L) ou LDL colesterol acima de 155 mg/dL (4.0 mmol/L)

Adultos: Colesterol total acima de 290 mg/dL (7,5 mmol/L) ou LDL colesterol acima de 190 mg/dL (4.9 mmol/L).

e

(a) Xantomas nos tendões no caso-index ou parente (pais, filhos avós, irmãos, tios)

ou

(b) Evidência genética de mutação nos genes *LDLR*, *APOB* ou *PCSK9*.

Hipercolesterolemia familiar possível é definida como: (a)

e

(b) História familiar de enfarte do miocárdio antes dos 50 anos em avós e tios ou antes dos 60 anos nos pais, irmãos e filhos

ou

(c) História familiar de nível elevado de colesterol nos pais, irmãos ou filhos; ou colesterol total acima de 290 mg/dL (7,5 mmol/L) nos avós e/ou tios.

Identificação do Médico Assistente:

Nome: ____

Telefone: ____________________ Email: ____________________________

Hospital: ______________________________

Serviço: ______________________________

Morada: ______________________________
____
____

História médica do caso-index

Valores antes do tratamento

Data	Colesterol mg/dl	LDL. mg/dl	HDL mg/dl	TG mg/dl	ApoB mg/dl	ApoAI mg/dl	Lp(a) mg/dl

Xantomas nos tendões:

(assinale se o caso index alguma vez apresentou xantomas)

	Presente	Ausente		Presente	Ausente
Dorso das mãos	☐	☐	Pretibiais	☐	☐
Cotovelos	☐	☐	Dorso dos pés	☐	☐
			Tendão de Achilles	☐	☐

Olhos

	Presente	Ausente
Lipaemia retinalis	☐	☐
Arco corneano	☐	☐
Xantelasma	☐	☐

Assinalar se o caso-index tem ou teve algumas das seguintes situações:

	Confirmado	Possível		
Angina	☐	☐	Idade de início (anos):	_________
Enfarte do miocárdio	☐	☐	Idade do 1º enfarte:	_________
CABG	☐	☐	Se sim, quando?	_________
Angioplastia	☐	☐	Se sim, quando?	_________
Outras doenças vasculares	☐	☐	Se sim, quando?	_________
Hipertensão	☐	☐		
A.V.C.	☐	☐	Idade do 1ºA.V.C.	_________
A.I.T.	☐	☐		
Claudicação	☐	☐		
Pancreatite	☐	☐		
Doença da tiroide	☐	☐		
Doença renal	☐	☐		
Diabetes	☐	☐	Idade de início (anos):	_________

Tratamento da Diabetes:
- Insulina ☐
- Oral ☐
- Insulina e oral ☐
- Dieta ☐

Informação médica da consulta mais recente

Data: ________________________ Terapêutica: Sim ☐ Não ☐

Data	Colesterol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TG (mg/dl)	ApoB (mg/dl)	ApoAI (mg/dl)	Lp(a) (mg/dl)

Altura: ________________________(m)
Peso: ________________________(kg) IMC: _________________(kg/m^2)

Pressão arterial: Sístole___________________ Diástole ___________________

Álcool: Número de unidades por semana: ________________________________
(1 unidade = 1 cerveja ou um copo de vinho)

Fumador? Sim ☐ Quantos cigarros por dia? _________________
Não ☐ Se é um ex-fumador há quantos anos deixou de fumar?________________

Faz exercício? Sim ☐ Que tipo de exercício?________________Quantas vezes por semana? _______
Não ☐

História familiar do caso-índex (preenchimento obrigatório)

Parentesco	Colesterol elevado	Presença de xantomas	Triglicerideos elevado	Idade do 1º enfarte do miocardio	Vivo (V) Morto (M) Desc.(?)	Idade presente ou na morte	Causa de morte
Pai Nome:	mg/dl		mg/dl				
Mãe Nome:	mg/dl		mg/dl				
Irmão M/F Nome:	mg/dl		mg/dl				
Irmão M/F Nome:	mg/dl		mg/dl				
Irmão M/F Nome:	mg/dl		mg/dl				
Filho M/F Nome:	mg/dl		mg/dl				
Filho M/F Nome:	mg/dl		mg/dl				
Filho M/F Nome:	mg/dl		mg/dl				
Cônjuge Nome:	mg/dl		mg/dl				

Preencher com SIM e NÃO quando os valores não são conhecidos

NOTA: Preencher também o Anexo A caso envie amostras de familiares

Árvore genealógica

Tratamento:

Dieta ☐ Estanois e Fitoesterois ☐

LDL Aferese ☐ Medicamentos ☐

Terapia actual para baixar os lípidos

Estatinas ☐ Qual?____________________ Dosagem _______

Data do início do tratamento ____________________

Resinas ☐ Qual?____________________ Dosagem _______

Data do início do tratamento ____________________

Fibratos ☐ Qual?____________________ Dosagem _______

Data do início do tratamento ____________________

Ácido nicotínico ou derivado ☐ Qual?____________________ Dosagem _______

Data do início do tratamento Dosagem _______

____

Inibidor da absorção intestinal do colesterol ☐ Qual?____________________

Data do início do tratamento ____________________

Outras observações:

DECLARAÇÃO DE CONSENTIMENTO

Considerando a "Declaração de Helsínquia" da Associação Médica Mundial
(Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996 e Edimburgo 2000)

Estudo Português de Hipercolesterolemia Familiar

ENQUADRAMENTO DO ESTUDO

Para que se possa compreender e atuar a nível da prevenção das doenças cardiovasculares é necessário a realização de estudos de investigação nesta área. Alguns dos fatores de risco mais importantes para doenças cardiovasculares são a hipertensão arterial, a diabetes, o tabagismo e a dislipidemia. Algumas dislipidemias têm origem genética, nomeadamente a Hipercolesterolemia Familiar (FH). A identificação precoce da causa genética da hipercolesterolemia permite obter o diagnóstico correto da dislipidemia fundamentando a introdução atempada de medidas terapêuticas e aconselhamento de estilos de vida de modo a melhorar o prognóstico do doente. Doentes com Hipercolesterolemia Familiar corretamente identificados e tratados podem ver a sua esperança de vida aumentada em 20/30 anos.

O QUE É O ESTUDO PORTUGUÊS DE HIPERCOLESTEROLEMIA FAMILIAR?

O Estudo Português de Hipercolesterolemia Familiar é um estudo de investigação, cujo objetivo principal é determinar a causa genética da hipercolesterolemia em indivíduos com o diagnóstico clínico de Hipercolesterolemia Familiar, assim como aumentar o conhecimento sobre os mecanismos do desenvolvimento da doença cardiovascular nas pessoas afetadas. Este estudo está autorizado pela Comissão de Ética do Instituto Nacional de Saúde Doutor Ricardo Jorge e pela Comissão Nacional de Proteção de Dados.

O QUE TEREI DE FAZER PARA PARTICIPAR E QUE ANÁLISES IRÃO SER REALIZADAS?

No âmbito deste estudo será realizada uma colheita de sangue, cerca de 15-20 ml no total, para a determinação de parâmetros laboratoriais de relevância e extração de DNA/RNA para estudos moleculares. Será ainda preenchido um questionário onde serão pedidas informações sobre alguns dos seus dados pessoais e elementos da sua história clínica importantes para este estudo.

As análises efetuadas serão gratuitas, porém, não haverá qualquer tipo de compensação financeira pela sua participação ou deslocação.

Estes estudos são confidenciais e o anonimato dos participantes está assegurado de acordo com as normas éticas dos estudos genéticos: os dados referentes à sua identidade não constarão das bases de dados do estudo uma vez que esta informação estará codificada. Só o investigador responsável e o seu assistente é que conhecem a chave de descodificação do participante. As amostras e informações recolhidas serão utilizadas apenas para o presente trabalho de investigação cujo objetivo está descrito acima; as amostras serão guardadas pelo tempo que este estudo estiver em curso, seja para encontrar a causa da dislipidemia, ou para estudar a relação bioquímica/genética, de forma a aumentar o conhecimento dos mecanismos da doença.

IREI TER ACESSO AOS RESULTADOS DAS ANÁLISES PARA AS QUAIS TIREI SANGUE?

Como participante no estudo irá ter acesso aos seus dados clínicos e moleculares, podendo exigir ser retirado deste estudo se assim o desejar. Todos os resultados obtidos serão enviados para o seu médico assistente. Os resultados das análises bioquímicas serão enviados no prazo de 2 semanas e os resultados moleculares serão enviados no prazo máximo de 12 meses.

IMPORTÂNCIA DOS REGISTOS NACIONAIS E INTERNACIONAIS

Os registos nacionais e internacionais de doenças específicas são importantes para a observação da realidade nacional, europeia ou internacional, permitindo o aumento do conhecimento específico de determinada doença, neste caso da Hipercolesterolemia Familiar, em termos da sua prevalência, distribuição e controlo. Este conhecimento irá permitir desenhar estratégias nacionais e/ou internacionais para melhorar a qualidade e esperança de vida destes doentes nomeadamente através da publicação de orientações sobre a identificação e tratamento precoce destes doentes de forma a reduzir o seu elevado risco cardiovascular.

Os dados pessoais como nome, morada e outros contactos, não serão inseridos nestes registos, sendo por

Instituto Nacional de Saúde
Doutor Ricardo Jorge

egistos anonimizados.

DECLARAÇÃO DE CONSENTIMENTO

Considerando a "Declaração de Helsínquia" da Associação Médica Mundial
(Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996 e Edimburgo 2000)

Estudo Português de Hipercolesterolemia Familiar

Eu, abaixo-assinado, (nome completo do participante do estudo)
____,

compreendi a explicação que me foi fornecida, por escrito e verbalmente, acerca da investigação que se tenciona realizar, bem como do estudo em que irei participar. Foi-me dada oportunidade de fazer as perguntas que julguei necessárias, e para todas obtive resposta satisfatória. Tomei conhecimento de que, de acordo com as recomendações da Declaração de Helsínquia, a informação ou explicação que me foi prestada versou os objetivos, os métodos, os benefícios previstos, os riscos potenciais, o eventual desconforto e quais os resultados que me serão comunicados e de que modo. Além disso, foi-me afirmado que tenho o direito de recusar a todo o tempo a minha participação no estudo, sem que isso possa ter como efeito qualquer prejuízo na assistência que me é prestada. Foi-me dado todo o tempo de que necessitei para refletir sobre esta proposta de participação. Nestas circunstâncias:

	Sim	Não
Declaro que fui informado dos objetivos deste estudo e aceito participar nele.		
Autorizo o **tratamento anonimizado e automatizado** dos meus dados pessoais		
Autorizo a **inserção** dos meus dados **anonimizados** num **Registo Nacional de Hipercolesterolemia Familiar**		
Autorizo a **inserção** dos meus dados **anonimizados** num **Registo Internacional de Hipercolesterolemia Familiar**		
Autorizo a **publicação** dos **resultados** obtidos **anonimizados** em artigos em revistas nacionais e internacionais		
Caso não seja possível **contactar diretamente** com o meu médico, autorizo o contacto pela equipa de investigação para o seguimento deste estudo		
Compreendo que este é um **estudo de investigação** cujo tempo de resposta dependerá das condições de financiamento atuais, mas o meu médico assistente receberá um resultado no prazo máximo de 12 meses		

Localidade:____________________________Data:_______/_______/_______

Nome do Participante: ____

Assinatura:____

(Em caso do caso-índex ser menor de 18 anos, os pais devem assinar para dar o seu consentimento)

Nome do Médico Assistente ou do Investigador: ____ Assinatura: ______

Investigadora Responsável do Estudo Português de Hipercolesterolemia Familiar
Doutora Mafalda Bourbon. Tel: 217 508 130 / 217 508 126; Email: mafalda.bourbon@insa.min-saude.pt

NOTA: DEVE SER TIRADA UMA COPIA DA DECLARAÇÃO DE CONSENTIMENTO E ENTREGAR AO PARTICIPANTE OU IMPRIMIR E ASSINAR EM DUPLICADO

Appendix I – Patients Recruitment

Appendix IB - Clinical Questionnaire (Hypocholesterolemia and Hypertriglyceridemia)

REPÚBLICA PORTUGUESA
SAÚDE

SNS SERVIÇO NACIONAL DE SAÚDE

Instituto_Nacional de Saúde Doutor Ricardo Jorge

Nº. Processo INSA	Data	Nº Processo DF	Família

(Campos a preencher pelo INSA)

Identificação ☐ Caso-índex ☐ Familiar (Grau de parentesco:) ________________
Nome do caso-índex: ________________________

____

Identificação do Propositus

Nome completo: ____

Morada: ____

____

Telefone:____________________ Email: ___________________

Data de Nasc.: _______________

Estado Civil: ________________

Natural de: __________________ Naturalidade dos pais: Pai______________Mãe ______________

Sexo: Masculino ☐ Feminino ☐

Origem étnica (assinalar a opção adequada):

☐Caucasiano ☐ Asiático ☐ Africano ☐ Outro

Condições de colheita e envio das amostras

Devem ser feitas as seguintes colheitas em jejum:

CASO-INDEX:

Adultos:

7.5 mL de sangue em tubo de soro com gel separador - após centrifugação (3500rpm/10minutos)

~11 mL de sangue total (4x tubos de EDTA 2.7mL)Crianças:

5 mL de sangue em tubo de soro com gel separador - após centrifugação (3500rpm/10minutos)

~8 mL de sangue total (3x tubos de EDTA 2.7mL)

FAMILIARES (adultos e crianças):

5 mL de sangue em tubo de soro com gel separador - após centrifugação (3500rpm/10minutos)

~8 mL de sangue total (3x tubos de EDTA 2.7mL)

Estudo de dislipidemias familiares de causa monogénica rara e poligénica

Critérios de Inclusão e de Exclusão

Deficiência Familiar em LPL

A Deficiência Familiar em LPL (FLLD) é uma doença autossómica recessiva, sendo a sua prevalência 1 caso em 1 milhão e a consanguinidade comum nas famílias afetadas. Os indivíduos com esta patologia não possuem ou possuem muito pouca atividade da LPL. A dislipidemia apresenta-se geralmente na infância com episódios de dor abdominal e soro anormalmente lipémico, valores de triglicéridos elevados (>1000 mg/dL) e pancreatite **e/ou** hepatomegalia **e/ou** xantomas eruptivos **e/ou** lipemia retinal. Os genes associados à FLLD são: *LPL* e *APOC2*. **Critérios de Exclusão:** diabetes e distúrbios da Tiróide.

Dislipidemia Familiar Combinada

A dislipidemia familiar combinada (FCHL) é uma doença autossómica dominante comum, afetando 1-3% da população. É caracterizada por níveis elevados de colesterol e/ou triglicéridos (CT >250 mg/dL (6.5 mmol/L) **e/ou** TGs >200 mg/dL (2.3 mmol/L)) em pelo menos dois membros da mesma família, apresentando variedade intra-individual e inter-familiar do fenótipo lipídico, prevalência elevada de VLDL e/ou LDL principalmente relacionados com um aumento da ApoB (> 120 mg/dL) e risco aumentado para doença coronária prematura. A FCHL pode ser causada por diferentes genes, não tendo sido até ao momento identificado um gene responsável pela maioria dos casos, tratando-se desta forma de uma doença poligénica. **Critérios de Exclusão:** Presença de xantomas, diabetes, Hipercolesterolemia Familiar e distúrbios da tiróide.

Hipertrigliceridemia Familiar

A hipertrigliceridemia familiar (HTG) é uma doença autossómica dominante, caracterizada por valores de triglicéridos entre 200 mg/dL e 1000 mg/dL e colesterol total normal, apresentando um risco aumentado para doença coronária prematura. Os genes mais fortemente associados à HTG são: *APOA5*, *LMF1* e *GPIHBP1*. Critérios de Exclusão: Diabetes e Distúrbios da Tiróide.

Deficiência em lipase ácida lisossomal

Deficiência em lipase ácida lisossomal (LALD) é uma doença autossómica recessiva, caracterizada por uma deficiência na enzima lipase ácida lisossomal que origina um aumento de ésteres de colesterol e triglicéridos no lisossoma. A prevalência desta patologia é de 1/150 000-300 000 (em algumas populações é de 1/60 000). Mutações no gene *LIPA* originam LALD. **Critérios de Exclusão:** Diabetes e Distúrbios da Tiróide.

Lipodistrofia familiar tipo Dunnigan

A lipodistrofia familiar tipo Dunnigan é uma doença autossómica dominante rara. É caracterizada pelo desaparecimento progressivo de tecido adiposo subcutâneo nos membros, região do glúteo, abdómen e tronco, acompanhado pela acumulação de gordura na face, pescoço, supraclavicular fossa e região intra-abdominal, conferindo o aspeto de hipertrofia. Verificam-se também alterações do metabolismo lipídico, como hipertrigliceridemia, diabetes, esteatose hepática e hipertensão. Mutações no gene lamina A/C (*LMNA*) encontram-se associadas à lipodistrofia familiar tipo Dunnigan.

Hipocolesterolemia:

A Hipobetalipoprotenemia e Abetalipoprotenemia são doenças autossómicas recessivas, com uma prevalência de 1 em 1 milhão e caracterizam-se por valores muito baixos de colesterol total, LDL e apoB, bem como, má absorção de vitaminas solúveis em lípidos que conduzem à degeneração da retina, neuropatia, e coagulopatia. A esteatose hepática pode também ser comum. São causadas por mutações nos genes *MTP*, *APOB*, *SARB1* ou *ANGPTL3* que não permitem o correto empacotamento e secreção de apoB. Os pais dos casos-índex com abetalipoprotenemia, apresentam normalmente valores normais de c-LDL, enquanto os pais dos casos-índex com Hipobetalipoprotenemia apresentam valores reduzidos, para metade, de c-LD.

Instituto_Nacional de Saúde
Doutor Ricardo Jorge

Estudo de dislipidemias familiares de causa monogénica rara e poligénica

Questionário

Família | Nº Processo DF

(Campos a preencher pelo INSA)

Motivo de inclusão no estudo

☐ Caso-índex ☐ Familiar

☐ Deficiência Familiar em LPL
☐ Dislipidemia Familiar Combinada
☐ Hipertrigliceridemia Familiar
☐ Deficiência em Lipase Ácida Lisossomal
☐ Lipodistrofia Familiar tipo Dunnigan
☐ Hipocolesterolemia
☐ Outro – Qual?________________

Identificação do Médico Assistente

Nome do Médico Assistente: ____

____________________________________ Telefone: ____________________________

____________________________________ ____________________________

Hospital/Serviço :

História Clínica do Caso-índex/Familiar

Tensão Arterial:

Sistólica:______________
Diastólica:_____________
Data:_/_/

Índice de Massa Corporal (IMC):

Altura (m): ______________________
Peso (Kg): ______________________
IMC (Kg/m2): ____________________
Cintura (cm): ____________________
Anca (cm):______________________

Características físicas :

☐ Xantomas cutâneos eruptivos ☐ *Lipaemia retinalis*
☐ Degeneração da retina ☐ subdesenvolvimento
☐ Outros Quais? ____

Pancreatite:

Presente ☐ Ausente ☐ Idade de inicio (anos):_______________________

Hepatomegalia:

Presente ☐ Ausente ☐ Idade de inicio (anos):_______________________

Esteatose hepática:

Presente ☐ Ausente ☐ Idade de inicio (anos): ____________________

Neuropatia:

Presente ☐ Ausente ☐ Idade de inicio (anos): ____________________

História de doença cardiovascular:

☐ Angina — Idade de início (anos): ______________
☐ Enfarte do miocárdio — Idade (anos): ____________________
☐ CABG — Se sim, quando? __________________
☐ Angioplastia — Se sim, quando? __________________
☐ AVC / AIT — Idade (anos): ____________________
☐ Doença carotídea
☐ Doença arterial periférica
☐ Outras doenças vasculares — Quais? ________________________
Quando? ______________________

Diabetes:

☐ Não
☐ Sim Tipo 1 ☐ Tipo 2 ☐ Idade de início (anos): ______________

Doença Renal:

Presente ☐ Ausente ☐
Qual? ____________________
Idade de início (anos): ________

Doença da Tiroide:

Presente ☐ Ausente ☐
Qual? ____________________
Idade de início (anos): _______

Hábitos Diários do Caso-índex/Familiar

Consumo de Álcool:

Número de unidades por semana: ________
(1 unidade = 1 cerveja ou um copo de vinho)

Hábitos Tabágicos:

Fumador? Sim ☐ Quantos cigarros por dia? ____

Não ☐ Se é ex-fumador, há quanto tempo deixou de fumar? ______________________
Quantos cigarros fumava/dia? ______ Durante quanto tempo fumou? ___________

Exercício Físico:

☐ Não
☐ Sim → Que tipo de exercício? ________________________________

Quantas vezes por semana? ______________________________

Dados Bioquímicos:

	Data	CT (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)	Apo B (mg/dL)	AST (U/L)	GGT (U/L)	ALT (U/L)
Sem tratamento (análises disponíveis)									
Em tratamento (últimas análises)									

Atividade da enzima lipase ácida lisossomal:____________nmol/punch/hour

Terapêutica Actual:

☐ Sem tratamento   Dieta ☐   LDL Aferese ☐   inicio em:___/___/_____

☐ Em terapêutica. Indique qual:

Estatinas ☐ Qual?______________Dosagem ___________
Data do início do tratamento __________________

Resinas ☐ Qual?______________Dosagem ___________
Data do início do tratamento __________________

Fibratos ☐ Qual?______________Dosagem ___________
Data do início do tratamento __________________

Ácido nicotínico ou derivado ☐ Qual?______________Dosagem ___________
Data do início do tratamento __________________

Inibidor da absorção intestinal do colesterol ☐ Qual?______________Dosagem ___________
Data do início do tratamento __________________

Outro ☐ Qual?______________Dosagem ___________
Data do início do tratamento __________________

História familiar do caso-índex (preenchimento obrigatório)

Parentesco	Colesterol	Presença de xantomas	Triglicerídeos	Idade DCV (indicar qual)	Vivo (V) Morto (M) Desc.(?)	Idade presente ou na morte	Causa de morte
Pai Nome:	mg/dl		mg/dl				
Mãe Nome:	mg/dl		mg/dl				
Irmão M/F Nome:	mg/dl		mg/dl				
Irmão M/F Nome:	mg/dl		mg/dl				
Irmão M/F Nome:	mg/dl		mg/dl				
Filho M/F Nome:	mg/dl		mg/dl				
Filho M/F Nome:	mg/dl		mg/dl				
Filho M/F Nome:	mg/dl		mg/dl				
Cônjuge Nome:	mg/dl		mg/dl				

Preencher com SIM e NÃO quando os valores não são conhecidos

Árvore genealógica

Considerando a "Declaração de Helsínquia" da Associação Médica Mundial
(Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996 e Edimburgo 2000)

Estudo de dislipidemias familiares de causa monogénica rara e poligénica

ENQUADRAMENTO DO ESTUDO

Para que se possa compreender e atuar a nível da prevenção das doenças causadas por alterações no metabolismo dos lípidos, nomeadamente doenças cardiovasculares e neurológicas, é necessário a realização de estudos de investigação nesta área. Alguns dos fatores de risco mais importantes para estas doenças são a hipertensão arterial, a diabetes, o tabagismo e a dislipidemia. Algumas dislipidemias têm origem genética, nomeadamente: a hipertrigliceridemia familiar, deficiência familiar em LPL, deficiência de lipase ácida lisossomal, lipodistrofia, hipocolesterolemia (hipobetalipoprotenemia e abetalipoprotenemia), que se incluem no grupo das dislipidemias monogénicas raras, e a dislipidemia familiar combinada, que é um exemplo de uma dislipidemia poligénica. Uma dislipidemia monogénica é causada por alterações em apenas um gene e as dislipidemias poligénicas resultam do efeito cumulativo de alterações em vários genes. A identificação precoce da causa genética da dislipidemia permite obter o diagnóstico correto, fundamentando a introdução atempada de medidas terapêuticas e aconselhamento de estilos de vida de modo a melhorar o prognóstico do doente.

O QUE É O ESTUDO DE DISLIPIDEMIAS FAMILIARES?

O estudo de dislipidemias familiares é um estudo realizado em pessoas que apresentem diagnóstico clínico de dislipidemia monogénica rara ou poligénica, cujo objetivo principal é determinar a causa genética da dislipidemia, assim como aumentar o conhecimento sobre os mecanismos do desenvolvimento da doença nas pessoas afetadas.

O QUE TEREI DE FAZER PARA PARTICIPAR E QUE ANÁLISES IRÃO SER REALIZADAS?

No âmbito deste estudo será realizada uma colheita de sangue, cerca de 15-20 ml no total, para a determinação de parâmetros laboratoriais de relevância e extração de DNA/RNA para estudos moleculares.
Será ainda preenchido um questionário onde serão pedidas informações sobre alguns dos seus dados pessoais e elementos da sua história clínica importantes para este estudo.
As análises efetuadas serão gratuitas, porém, não haverá qualquer tipo de compensação financeira pela sua participação ou deslocação.
Estes estudos são confidenciais e o anonimato dos participantes está assegurado de acordo com as normas éticas dos estudos genéticos: os dados referentes à sua identidade não constarão das bases de dados do estudo, uma vez que esta informação estará codificada. Só o investigador responsável e o seu assistente é que conhecem a chave para descodificação do participante. As amostras e informações recolhidas serão utilizadas apenas para o presente trabalho de investigação cujo objetivo está descrito acima; as amostras serão guardadas pelo tempo que este estudo estiver em curso, seja para encontrar a causa da dislipidemia, ou para estudar a relação bioquímica/genética, de forma a aumentar o conhecimento dos mecanismos da doença.

IREI TER ACESSO AOS RESULTADOS DAS ANÁLISES PARA AS QUAIS TIREI SANGUE?

Como participante no estudo irá ter acesso aos seus dados clínicos e moleculares (apenas a pedido do médico), podendo exigir ser retirado deste estudo se assim o desejar. Todos os resultados obtidos serão enviados para o seu médico assistente. Os resultados das análises bioquímicas serão enviados no prazo de 2 semanas; para os resultados moleculares, não haverá um prazo para a comunicação dos resultados ao seu médico assistente, embora a pedido do médico possa ser enviada informação sobre os genes estudados.

IMPORTÂNCIA DOS REGISTOS NACIONAIS E INTERNACIONAIS

Os registos nacionais e internacionais de doenças específicas são importantes para a observação da realidade nacional, europeia ou internacional, permitindo o aumento do conhecimento científico de determinada doença, neste caso das dislipidemias monogénicas raras ou poligénicas, em termos da sua prevalência, distribuição e controlo. Este conhecimento irá permitir desenhar estratégias nacionais e/ou internacionais para melhorar a qualidade e esperança de vida destes doentes, nomeadamente através da publicação de orientações sobre a identificação e tratamento precoce destes doentes de forma a reduzir o seu elevado risco cardiovascular e neurológico. Os dados pessoais como nome, morada e outros contactos, não serão inseridos nestes registos, sendo por esta razão chamados de **registos anonimizados.**

Considerando a "Declaração de Helsínquia" da Associação Médica Mundial
(Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996 e Edimburgo 2000)

Estudo de dislipidemias familiares de causa monogénica rara e poligénica

Eu, abaixo-assinado, (nome completo do participante do estudo)

____,

compreendi a explicação que me foi fornecida, por escrito e verbalmente, acerca da investigação que se tenciona realizar, bem como do estudo em que irei participar. Foi-me dada oportunidade de fazer as perguntas que julguei necessárias, e para todas obtive resposta satisfatória. Tomei conhecimento de que, deacordo com as recomendações da Declaração de Helsínquia, a informação ou explicação que me foi prestada versou os objetivos, os métodos, os benefícios previstos, os riscos potenciais, o eventual desconforto e quaisos resultados que me serão comunicados e de que modo. Além disso, foi-me afirmado que tenho o direito de recusar a todo o tempo a minha participação no estudo, sem que isso possa ter como efeito qualquer prejuízo na assistência que me é prestada. Foi-me dado todo o tempo de que necessitei para refletir sobre esta proposta de participação. Nestas circunstâncias:

	Sim	Não
Declaro que fui informado dos objetivos deste estudo e aceito participar nele.		
Autorizo o **tratamento anonimizado e automatizado** dos meus dados pessoais		
Autorizo a **inserção** dos meus dados **anonimizados** num **Registo Nacional de Dislipidemias Familiares (monogénicas raras ou poligénicas)**		
Autorizo a **inserção** dos meus dados **anonimizados** num **Registo Internacional de Dislipidemias Familiares (monogénicas raras ou poligénicas)**		
Autorizo a **publicação** dos **resultados** obtidos **anonimizados** em artigos em revistas nacionais e internacionais		
Caso não seja possível **contactar diretamente** com o meu médico, autorizo o contacto pela equipa de investigação para o seguimento deste estudo		
Compreendo que este é um **estudo de investigação** cujo tempo de resposta dependerá das condições de financiamento atuais, sendo assim não haverá um prazo para a comunicação dos resultados ao meu médico assistente, embora a pedido do médico possa ser enviada informação sobre os genes estudados.		

Localidade:___________________________Data:_______/_______/_______

Nome do Participante: ____

Assinatura:____

(Em caso do caso-índex ser menor de 18 anos, os pais devem assinar para dar o seu consentimento)

Nome do Médico Assistente ou do Investigador: ______________________________________

Assinatura: ____

Investigadora Responsável pelo Estudo de Dislipidemias Familiares de causa monogénica rara e poligénica

Doutora Mafalda Bourbon. Tel: 217 508 130 / 217 508 126; Email: mafalda.bourbon@insa.min-saude.pt

NOTA: DEVE SER TIRADA UMA COPIA DA DECLARAÇÃO DE CONSENTIMENTO EENTREGAR AO PARTICIPANTE OU IMPRIMIR E ASSINAR EM DUPLICADO

Appendix II - Molecular Biology Techniques

Table A. II.1 – Proportion of reagents per mL of blood for genomic DNA extraction (section 2.2.1. “Genomic DNA extraction”)

Blood	TKM X100	IGEPAL	TKM1	TKM2	SDS	NaCl	EtOH
1 mL	1 mL	25 µl	1 mL	160 µl	10 µl	60 µl	460 µl

Table A. II.2 – SureSelect^{QXT} P7 Indexes 1 to 12, and P5 Indexes 13 to 20, with correspondent sequence.

Index Number	Sequence	Index Number	Sequence
P7 Index 1 (P7 i1)	TAAGGCGA	**P7 Index 1 (P7 i11)**	AAGAGGCA
P7 Index 1 (P7 i2)	CGTACTAG	**P7 Index 1 (P7 i12)**	GGACTCCT
P7 Index 1 (P7 i3)	AGGCAGAA	**P5 Index 13 (P7 i13)**	GCGATCTA
P7 Index 1 (P7 i4)	TCCTGAGC	**P5 Index 14 (P7 i14)**	ATAGAGAG
P7 Index 1 (P7 i5)	GTAGAGGA	**P5 Index 15 (P7 i15)**	AGAGGATA
P7 Index 1 (P7 i6)	TAGGCATG	**P5 Index 16 (P7 i16)**	TCTACTCT
P7 Index 1 (P7 i7)	CTCTCTAC	**P5 Index 17 (P7 i17)**	CTCCTTAC
P7 Index 1 (P7 i8)	CAGAGAGG	**P5 Index 18 (P7 i18)**	TATGCAG
P7 Index 1 (P7 i9)	GCTACGCT	**P5 Index 19 (P7 i19)**	TACTCCTT
P7 Index 1 (P7 i10)	CGAGGCTG	**P5 Index 20 (P7 i20)**	AGGCTTAG

Table A. II.3 – List of genes associated with familial dyslipidemias analysed during this project.

Gene	Chromosome	Ref Seq number	Phenotype
LDLR	19	NM_000527	FH
APOB	2	NM_000384	FH, FHBL
PCSK9	1	NM_174936	FH, low LDL
LDLRAP1	1	NM_10627	ARH
APOE	19	NM_000041	Dysbetalipoproteinemia
ABCG5	2	NM_022436	Sitosterolemia
ABCG8	2	NM_022437	Sitosterolemia
LIPA	10	NM_001127605	LAL-D
ANGPTL3	1	NM_014495	FCH
MTTP	4	NM_000253	ABL
SAR1B	5	NM_001033503	Anderson Disease
LPL	8	NM_000237	HTG
APOC2	19	NM_000483	HTG
APOA5	11	NM_052968	HTG
APOC3	11	NM_000040	HTG
GPIHBP1	8	NM_178172	HTG
LMF1	16	NM_22773	HTG
GPD1	12	NM_005276	HTG

Columns indicate (for each gene) chromosome location, Ref Seq number for variant annotation, and associated phenotype. FH, Familial hypercholesterolemia; FHBL, Familial hypobetalipoproteinemia; LDL, low-density lipoprotein; ARH, autosomic recessive hypercholesterolemia; LAL-D, lysosomal acid lipase deficiency; FCH, familial combined hyperlipidemia; ABL,

Table A. II.4 – PCR and Sanger Sequencing for all genes analysed by these techniques, primers and respective annealing temperatures.

Gene	Loc.	Primer Sequence (5'-3')	Amplified region (pb)	Annealing temperature	Sequencing primer
LDLR	Prom + Ex 1	(F) GGGTTAAAAAGCCGATGTCA	397	60 ºC	F
		(R) GGGTTAAAAAGCCGATGTCA			
	Ex 4	(F) GTACAGATGAGGAAACTGAG	677	57 ºC	F
		(R) TTGGCATGTTGTTGGAAATCC			
	Ex 6	(F) TGAATGAGTGCCAAGCAAAC	277	59 ºC	F
		(R) TTCCCAAAACCCTACAGCAC			
	Ex 7	(F) GCGAAGGGATGGGTAGGG	440	59 ºC	R
		(R) CCCAGAGGCAAGAAACTCTG			
	Ex 9 + 10	(F) GGAGGTCTTTTCCACCCTCT	880	60 ºC	F
		(R) CTTGATGATCCACCCACCTT			
	Ex11	(F) GCCACATTTGGAGTTTGGGGTTC	335	60 ºC	F
		(R) AGCAGCTTGGGCTTGTCCCAGA			
	Ex 12	(F) CTCACATGTGGTTGGAGCTG	375	60 ºC	R
		(R) CTGGGAAACGAGAGCAAAAC			
APOB	Ex 22	(F) GGTTTTGATCACCACAAATGG	658	60 ºC	F
		(R) CTTTGGAAACCTTCCTGCAC			
	Ex 26	(F) TGAGGTTCTTCAGCCTGCTT	458	59 ºC	F
		(R) GCCTGCAATGTTCAGACTGT			
	Ex 27-28	(F) AGTGTGAGCTGTACATTGCA	662	59 ºC	F
		(R) TTACCGCCTGTCTTTCACCT			
PCSK9	Ex 1	(F) CCGAAACCTGATCCTCCAG	739	60 ºC	F
		(R) GCGACCTGCACTCCACTT			
	Ex 9	(F) CACTGGCAGGAGTCCCCTGC	1827	63 ºC	F & R
		(R) GAGTATGGAACTGCAAGTCAGG			
APOA5	Ex 3	(F) GAGTTGGAGGAGGTGAAGG	845	59 ºC	F
		(R) AGACAAGGAGCTGGGAATG			
LPL	Ex 5	(F) AGTGCATTCAAATGATGAGC	433	57 ºC	F
		(R) TGGCCAAATGTGTATATGAAA			
GPD1	Ex 6+7	(F) GAGTATGGGGCAGGGCTTA	869	62 ºC	F
		(R) CCCCTTTCTGTCCACTTCAC			
GPIHBP1	Ex 1-2	(F) TGTAAAACGACGGCCAGTCATCCCACTTACCGCAGCTC	694	60 ºC	F & R
		(R) CAGGAAACAGCTATGACCCCTCGTCGTAGTCATCTGGC			
	Ex 2	(F) TGTAAAACGACGGCCAGTGACCACGGGCCAGATGACTA	609	60 ºC	F & R
		(R) CAGGAAACAGCTATGACCCTCTGCTTTTCTCGGGATGG			
	Int 2 – Ex 3	(F) TGTAAAACGACGGCCAGTGGAACCGCTTAGAGCAGAGC	755	60 ºC	F & R
		(R) CAGGAAACAGCTATGACCAATGAGGGTTGTGCAGGTCT			
	Ex 3 – Ex 4	(F) TGTAAAACGACGGCCAGTTGACGCAGAACTGCTCACAT	574	60 ºC	F & R
		(R) CAGGAAACAGCTATGACCGATCGCCCAAGACACTCCAA			
	Ex 4	(F) TGTAAAACGACGGCCAGTGCAATGGGGGCCAGGAG	717	60 ºC	F & R
		(R) CAGGAAACAGCTATGACCACTTTCCAAGCCAGGGTCTC			
	Ex 4	(F) GTAAAACGACGGCCAGTACAGGCACCAAGTATGAAGAGG	680	60 ºC	F & R
		(R) CAGGAAACAGCTATGACCTGGTGGGGATCTAGGGGTTC			

First column indicates several gene, in which rare variants were detected. Each exon and respective primer sequence for PCR is presented in the second and third columns, with the respective fragment size (bp) and annealing temperature (ºC). Loc, location; ex, exon; int, intron; bp, base pairs; F, forward; R, reverse.

Table A. II.5 – PCR and Sanger Sequencing for studies using cDNA.

Gene	Loc.	Primer Sequence (5’-3’)	Amplified region (pb)	Annealing temperature	Sequencing primer
LDLR	Ex 1-6	(F) GGCTGGAAATTGCGCTGGAC	840	62 ºC	F & R
		(R) TGACCAGTCCCGGCAGTCTCTAGC			
	Ex 2-6	(F) CAGCGCTGAGTGCCAGGATGG	690	62 ºC	F & R
		(R) TGACCAGTCCCGGCAGTCTCTAGC			

Primer combinations for study of mRNA splicing possibly caused by the variant Gly207Gly (*LDLR*, exon 4), primer sequence for PCR, with the respective fragment size (bp) and annealing temperature (ºC). Loc, location; ex, exon; int, intron; bp, base pairs; F, forward; R, reverse.

Appendix III – Variant Classification

Population data	MAF is too high for disorder BA1/BS1 **OR** observation in controls inconsistent with disease penetrance BS2			Absent in population databases PM2	Prevalence in affecteds statistically increased over controls PS4	
Computational and predictive data		Multiple lines of computational evidence suggest no impact on gene /gene product BP4 Missense in gene where only truncating cause disease BP1 Silent variant with non predicted splice impact BP7 In-frame indels in repeat w/out known function BP3	Multiple lines of computational evidence support a deleterious effect on the gene /gene product PP3	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before PM5 Protein length changing variant PM4	Same amino acid change as an established pathogenic variant PS1	Predicted null variant in a gene where LOF is a known mechanism of disease PVS1
Functional data	Well-established functional studies show no deleterious effect BS3		Missense in gene with low rate of benign missense variants and path. missenses common PP2	Mutational hot spot or well-studied functional domain without benign variation PM1	Well-established functional studies show a deleterious effect PS3	
Segregation data	Nonsegregation with disease BS4		Cosegregation with disease in multiple affected family members PP1	Increased segregation data →		
De novo data				De novo (without paternity & maternity confirmed) PM6	De novo (paternity and maternity confirmed) PS2	
Allelic data		Observed in *trans* with a dominant variant BP2 Observed in *cis* with a pathogenic variant BP2		For recessive disorders, detected in trans with a pathogenic variant PM3		
Other database		Reputable source w/out shared data = benign BP6	Reputable source = pathogenic PP5			
Other data		Found in case with an alternate cause BP5	Patient’s phenotype or FH highly specific for gene PP4			

Figure A.III.1 – Schematic representation of evidence framework necessary to classify genetic variants. Criteria is organized by the types of evidence and the strength of the criteria for benign and pathogenic significance (left and right side, respectively). BS, benign strong; BP, benign supporting; FH, family history; LOF, loss of function; MAF, minor allele frequency; path., pathogenic; PM, pathogenic moderate; PP, pathogenic supporting; PS, pathogenic strong; PVS, pathogenic very strong. Adapted from [102].

Pathogenic	(i) 1 Very strong (PVS1) *AND* (a) ≥1 Strong (PS1–PS4) *OR* (b) ≥2 Moderate (PM1–PM6) *OR* (c) 1 Moderate (PM1–PM6) and 1 supporting (PP1–PP5) *OR* (d) ≥2 Supporting (PP1–PP5) (ii) ≥2 Strong (PS1–PS4) *OR* (iii) 1 Strong (PS1–PS4) *AND* (a)≥3 Moderate (PM1–PM6) *OR* (b)2 Moderate (PM1–PM6) *AND* ≥2 Supporting (PP1–PP5) *OR* (c)1 Moderate (PM1–PM6) *AND* ≥4 supporting (PP1–PP5)
Likely pathogenic	(i) 1 Very strong (PVS1) *AND* 1 moderate (PM1–PM6) *OR* (ii) 1 Strong (PS1–PS4) *AND* 1–2 moderate (PM1–PM6) *OR* (iii) 1 Strong (PS1–PS4) *AND* ≥2 supporting (PP1–PP5) *OR* (iv) ≥3 Moderate (PM1–PM6) *OR* (v) 2 Moderate (PM1–PM6) *AND* ≥2 supporting (PP1–PP5) *OR* (vi) 1 Moderate (PM1–PM6) *AND* ≥4 supporting (PP1–PP5)
Benign	(i) 1 Stand-alone (BA1) *OR* (ii) ≥2 Strong (BS1–BS4)
Likely benign	(i) 1 Strong (BS1–BS4) and 1 supporting (BP1–BP7) *OR* (ii) ≥2 Supporting (BP1–BP7)
Uncertain significance	(i) Other criteria shown above are not met OR (ii) the criteria for benign and pathogenic are contradictory

Figure A.III.2 – Combination of criteria to attribute a clinical significance to sequence variants. Adapted from [102].