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Familial hypercholesterolemia in Belgium
Genetic, clinical and epidemiologic aspects.

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Thesis for the obtention of the PhD degree in Public Health
Orientation: genetic epidemiology.
June 2007

To my wife, Caroline,

To my children, Laurence and Benoit

To my parents, Monique and Freddy

To my brothers, Stéphane and Franck.

Contents

ANNEX THESIS. BEYOND FH, INTROSPECTING NEW ASPECTS IN LIPIDOLOGY

Abbreviations

AA: aminoacid
ABCA1: ATP-binding cassette transporter A1
ACE: angiotensin-converting enzyme
APOA1: gene of apolipoprotein A-I protein (idem for ApoA2, ApoA5)
ApoA-I: apolipoprotein A-I protein (idem for ApoA-II, ApoA-V)
APOB: gene of apolipoprotein B-100
ApoB: protein of apolipoprotein B-100
APOC3: gene of apolipoprotein C-III protein
ApoC-III: apolipoprotein C-III protein
APOE: gene of the apolipoprotein E protein
ApoE: apolipoprotein E protein
ARII : angiotensin type II receptor
ASO: allele specific oligonucleotide
ATT: Achilles tendon thickness
ATX: Achille tendon
ATX: Achille tendon xanthomas
BIRNH: Belgian Interuniversity Research on Nutrition and Health
CABG: Coronary Artery Bypass Graft
CAC: coronary artery calcification
CAD: CHD when it has been angiographically assessed
CATA: “category A” of reimbursement in the Belgian system of national health insurance
CATB: “category B” of reimbursement in the Belgian system of national health insurance
CETP: cholesteryl ester transfer Protein
CHD: coronary heart disease
CR: chylomicrons
CV: cardiovascular
CVD: cardiovascular disease
CVA: cerebral vascular disease
DDD: defined daily dosage
DGGE: Denaturing Gradient Gel Electrophoresis
DLCN: Dutch Lipid Clinic Network
EBM: evidence-based medicine
ER: endothelial reticulum
FH: familial hypercholesterolemia
FDB: familial defective apolipoprotein B
FPH: familial combined hyperlipidemia
FABP: fatty acid binding protein
GP: general practitioner(s)
HC: hypercholesterolemic or hypercholesterolemia
HDL-C: serum (or plasma) concentration of the cholesterol of the high density lipoproteins
heFH: heterozygous familial hypercholesterolemia

HL (or HTGL): Hepatic lipase
HLP: hyperlipoproteinemia
hoFH: homozygous familial hypercholesterolemia
HMG-CoA reductase: 3-hydroxy-3-methylglutaryl coenzyme A
HSPG: Heparan sulfate proteoglycans
HTG: hypertriglyceridemia
HTGL (or HL): hepatic triglyceride lipase
I.N.A.M.I. / R.I.Z.I.V.: Belgian system of national health insurance: "National Institute for Sickness and Disability Insurance" (in French : Institut national d'assurance maladie invalidité; in Dutch : Rijksinstituut voor ziekte- en invaliditeitsverzekering)
IUGR: intrauterine growth restriction
LCAT: lecithin:cholesterol acyltransferase
LDL: particle(s) of low density lipoproteins (sometimes its concentration)
LDL-C: serum (or plasma) concentration of the cholesterol of the low density lipoproteins
LDLR: gene of the LDL receptor
LDLr: protein of the LDL receptor
LPL: lipoprotein lipase
LRP: LDL receptor-related protein
M: men
MEDPED FH: Make Early Diagnosis to Prevent Early Deaths in FH patients
MI: myocardial infarction
MIM: Mendelian Inheritance in Man
MLPA: multiplex ligation-dependent probe amplification
MTHFR: methylenetetrahydrofolate reductase
MTP: microsomal triglyceride transfer protein
NFH: no FH (patients)
OR: odds ratio
P90: the 90th percentile of the general population (idem P95 …)
PAD: peripheral arterial disease
PCR: polymerase chain reaction
PCSK9: proprotein convertase subtilisin/kexin type 9.
PTCA: Angioplasty, Percutaneous Transluminal Coronary
RFLP: restriction fragment length polymorphism
SBRG: Simon Broome Register Group
SNP: single nucleotide polymorphism
SR-BI: scavenger receptor class B type I
SSCP: single-strand conformation polymorphism
SMR: standardized mortality ratio
TC: serum (or plasma) concentration of total cholesterol
TG: Triglycerides or serum (or plasma) concentration of triglycerides
TRL: triglyceride rich lipoproteins
TX: tendon xanthomas
VLDL: particle of very low density lipoproteins (sometimes its concentration)
W: women
yrs: years

Preface

"What is important is not that there are uncontrollable events in our lives, but how we respond to them".

Viktor Frankl

This work summarizes my original contributions to the genetic aspects of lipidology, and more specifically, to familial hypercholesterolemia. My interest in the field of lipidology started in 1987 (20 years ago!) during my second year on internal medicine training, when I met Professor Francis Heller who invited me to collaborate to a research study on serum lipids in patients with Crohn's Disease (Descamps 1989, Heller 1993). I was so excited by the potential that opened this field to develop the skills that attracted my young curiosity (biochemistry, cell biology, statistics, mathematics, genetics) that I decided to spent three years in research on "cholesterol" at the University of Texas Southwestern Medical Centre at Dallas in the department of Molecular Genetics headed by professors Michael Brown and Joseph Goldstein (Brown & Goldstein 1986)[1]. In Dallas, with the constructive help of Professors David Bilheimer, Dennis McGarry, Helen Hobbs and Joachim Hers[2], I was introduced to the science of LDL receptor and other aspects of lipoprotein metabolism pathways and I became involved in most advanced projects studying the role of a newly discovered lipoprotein receptor, the "LRP"[3] (Descamps 1993).

At this stage, I had the naïve feeling that most was known and done about LDL-receptor and that I should focuse my efforts exploring the mysterious horizons opened by the discovery of the new members of this whole family of lipoprotein receptor, called "LDL receptor family". But, when I came back in Belgium in 1992, the exposure to the real problem of FH came to me as "clap of thunder from a clear sky" with one of my first patients at the hospital of Jolimont: a 39 year-old man who was just discovered to have very elevated cholesterol level at his admission for myocardial infarction in our hospital (Descamps 1994 & 2000a). Curiously, the patient did not express previous knowledge of this problem, but, when I asked him about his familial history, he told me that his present story resembles that of his father died suddenly (most likely from myocardial infarction) at age 43. I was shocked to hear that, at the moment of this previous drama (he was then 23 years old), nobody had taken the time to assess his cholesterol level. His present misfortune could have been prevented by this simple adequate gesture! This was unfortunately the "real life" experience of familial hypercholesterolemia (FH): a genetic disease so well understood at the molecular and biochemical level, treatable by safe and effective drugs, but greatly ignored by most medical practitioners in our

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[1] Awarded in 1985 with the Nobel Price in Medicine for their discovery of the LDL receptor.

[2] David directed all the *in vivo* studies of lipoprotein cinetics and Joachim all the *in vitro* study on cell lipoprotein metabolism. Denis Mac Garry supervised the adipocyte metabolism study.

[3] LRP or Low density lipoprotein (LDL) receptor-Related Protein

country. I soon discovered indeed that, for many doctors, FH was either completely unknown, or too rare to be bothered in routine practice or falsely considered as a "traditional bad culinary habit-induced hypercholesterolemia" culturally inherited in families and for which the only treatment was "to repeat again and again dietary advices" and "to operate a close watch on what enters the kitchen". These reactions raise in my mind a feeling of injustice with regard to these patients who are not responsible of their hypercholesterolemia, in contrast to most other cases of hypercholesterolemia induced by bad lifestyle choice. Coming from the laboratory of Michael Brown and Joseph Goldstein where was discovered the cause of FH, I realized that I could be helpful to my fellow citizens by dedicating my efforts to develop a better knowledge and awareness of this disease in my country. Taking care of FH thus appeared at that time as a real ***"rescue mission"*** in the field of preventive medicine. As a matter of fact, FH represents the perfect model of cardiovascular disease (CVD) prediction and prevention. It illustrates several important principles including the value of recognition of a strong familial tendency, the interest of an early diagnosis of atherosclerosis risk, the understanding of dietary and drug manipulation (on the LDL-receptor pathway) and the recognition of multiplicative risk in conjunction with other factors such as smoking. Even if FH account for only a small fraction of MI in the population at large, its consequences are catastrophic and deserve to be prevented more carefully.

Therefore, my first task was to develop in Belgium a program similar to the MEDPED FH program. This MEDPED FH ("Make Early Diagnosis to Prevent Early Deaths in FH patients") project was created in 1991 by Roger Williams in order to promote the identification and adequate treatment of all individuals carrying FH in the world (Williams 1993)[4]. It was launched and supported financially in many countries but, unfortunately not in Belgium. Besides this first difficulty, the challenge of identifying FH appeared very soon complicated in Belgium by some specific aspects including the rules of reimbursement of statins just promulgated two years before by our national health insurance (INAMI / RIZIV). At the start, these rules had the praiseworthy aim to facilitate the treatment of those carrying the unwilling and diet resistant hypercholesterolemic conditions of FH conditions. As a matter of fact, it appeared very soon that rather than promoting the recognition and the treatment of these patients, they paradoxically confused doctors about the disease definition. Progressively, through consecutive projects and publications, we attempted to clarify the evidences and to answer to the following questions regarding the recognition and the diagnosis of FH in Belgium:

- ***What "is" (causes and clinical presentation) "FH" in Belgium?***
  (Descamps 1997a, 1997b, 2000, 2003a, 2006b)
- ***How frequent is FH in Belgium?***
  (Descamps 1997b, 2000)
- ***Why is it important to diagnose FH?***
  (Descamps 2001a & 2003b)
- ***How to diagnose FH in Belgium?***
  (Descamps 2001b, 2006a)

[4] Dr. Roger Williams, unfortunately died in the airplane crash of Swissair Flight 111 on September 2, 1998, on his way to Geneva to chair the International MEDPED meeting.

- ***What is the best rule for statin reimbursement in FH?***
(Descamps 2002 & 2006c)

One of my biggest problems I had by writing this thesis was to communicate concepts from many different area in medicine to clinicians or researchers with no particular background in these area and to limit the number of pages to a reasonable amount despite the long-standing duration of my work in this field. Thus the idea evolved that footnotes or complementary "boxes" would be helpful to many readers not familiar with more specialized concepts and that presentations of summary of some papers and works in the present book, complemented with their more comprehensive description as full papers in an appendix would be appropriate to ease the reading while preserving the scientific approaches of these works.

I hope that the reader will find as much as sense, purpose and values as I gained by performing these works and writing this thesis.

Olivier S Descamps , 22 June 2007.

Acknowledgements

« L'empreinte d'un homme sur un autre est éternelle. Aucun destin n'a traversé le nôtre impunément ». Francois Mauriac

Life is the product of many interactions. We are the products of thousands. Many persons helped me with this thesis, wittingly or unwittingly. So many, that I cannot acknowledge individually all those who crossed my life one day, even for the 5 minutes that had the considerable influence to stimulate my thought.

If there is one person I would single out in acknowledgments, it is my wife, ***Caroline*** Daumerie. She offered me a great understanding, constant encouragement and solid emotional and wholeharted support during the 20-year period of these investigations and, specially, the last 3 years of preparation of this thesis book. My daughter ***Laurence*** and my son ***Benoit*** who, through their talents, kept me grounded in real life and reminded me ever that life is a strange combination of mathematics and arts. Without knowing it, they were a great source of inspiration for my work. I would like to thank my parents ***Monique and Freddy,*** my brothers ***Franck*** and ***Stéphane*** for the warm and kind encouragements they provided me all along my career and for their constant blessings for my success.

The patient mentoring of all the people mentioned in the preface, Professors ***Francis Heller, René Tonglet, David Bilheimer, Joachim Hers, Dennis McGarry, Helen Hobbs, Michael Brown and Joseph Goldstein***, provided me with the academic climate to stimulate my imagination, to pursuit my education in molecular biology, statistics and clinical lipidology and to start research works in the fields of "molecular lipidology" and cardiovascular epidemiology.

I am particularly grateful to my promotor ***Professor Francis Heller,*** scientific advisor to the general management of our hospital of Jolimont and past Head of the department of internal medicine, for his valuable guidance, his constant support and his huge amount of patience when he worked with me. He has accompanied me all along my career of researcher and physician and has created with the support of ***Mr Christian Faucon***, general administrator of our hospital, my original and special position (half time internist and half time researcher) which gives me the possibility and the autonomy to develop my own visions of research.

The first draft for my thesis proposal was strongly supported and encouraged by ***Professor René Tonglet***, director of the Epidemiologic Unit at the School of Public Health of the UCL (Université Catholique de Louvain). I am grateful to him for his profound trust, wisdom and expertise in epidemiology. I learned his illness in 2004 with great sorrow and his death a few months later was a big loss for his family and friends but also for many people in the scientific world and humanitarian organisation. He is still missed but remains a significant contributor to this thesis.

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The genesis of a research facility in our hospital of Jolimont, the Centre of Medical Research, in 1992, was the main key that opened the possibility to develop original and elaborated research projects. I wish to thank all of those who had the willingness and the visionary spirit in daring and supporting such enterprise: ***Mr Christian Faucon***, ***Professor Francis Heller, Professor Marc Beauduin***, medical director of our hospital, ***Mr Alex Parfonry, Révérende Sœurs Marie-Dominique and Henri-Marie*** as well as the recently nominated members of the administrative committee, ***Mr Pascal Graux***, general manager of our hospital and ***Dr Laurent Boon-Falleur***.

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[5] Descamps OS. Ni dépendants, ni indépendants mais interdépendants. Louvain Médical 2004. 123:179-94.

Many others have also contributed to settle the basis and tools of my work. I particularly acknowledge all of those who introduced me to the field of lipidology, cell biology and molecular biology when I was a postdoctoral fellow at the Southwestern Medical Centre at Dallas, specially those who provided me with hands-on training on laboratory procedures (Scott Clark, Murphy Daniel, Weng-Ling Niu, Debra Noble, Richard Gibson). They have been wonderful persons to work with, both in the fields of science and outside it. I am also grateful to Joep Defesche and John Kastelein of the Academic Medical Centre at the University of Amsterdam and Petra Van Acker at the University of Gent for their help in setting up DGGE in 1995 for the screening of point mutations as well as for the valuable discussions on the technical and clinical aspects of genetics analysis.

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When I finished writing this thesis, my first thought went to my mother Monique and my brother Stéphane. Stéphane died in May 1997 at the age 33 in a car accident. After his death, I did not imagine that I would lose my mother too, a few months later in November 1997. Stéphane, my youngest brother and I still had a lot of things to accomplish together and therefore, I decided that, from now onwards, I would sign all my papers with his name added to my first name. The souvenir of my mother cannot be separated from that of "Love", a sweet, tender and delicious love. She surely would have been proud to assist to the finalisation of my thesis. Her love, her strong spirit, her dignity and her courage facing the difficulties assisted me all along my present work and will remain present until the end of my life.

[6] Descamps OS, Heller FR et Derue G. Programme de recherche pour les assistants de médecine interne à l'hôpital de Jolimont. Louvain Médical 2006. 125 (4); 145-148.

Familial hypercholesterolemia

LDL-receptor gene (LDLR)

1 mutant + 1 normal allele allele (>900 mut) = Heterozygote 50 % abnormal LDLr

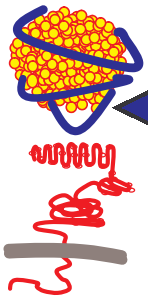
>70% HeFH

Apolipoprotein B Gene (APOB)

1 normal + 1 mutant allele allele (R3500Q) = Heterozygote 50 % abnormal apoB

~5% HeFH

Also called "familial defective apoB"

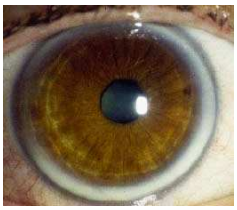


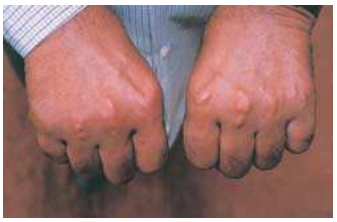
↘50% Cellular binding and uptake of LDL particles

↘50% Clearance of LDL particles

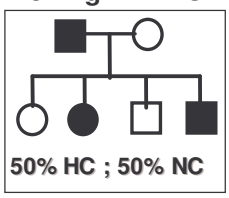
↗ LDL-cholesterol

Tendon xanthoma
Corneal arcus

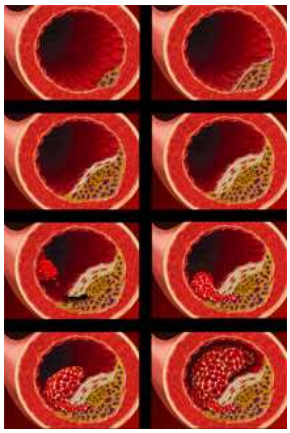




With dominant pattern of inheritance of high LDL-C



Early atherosclerosis



Chapter 1. Background on Familial hypercholesterolemia

MIM-143890

Since the first description of FH by Müller in 1930 and the discovery of the LDLR gene by Brown and Goldstein in 1979, the spectrum of clinical presentations and genetic aetiologies has extended so widely and has gained so much in nuance that it becomes necessary recently to find a new name to this entity: "autosomal dominant hypercholesterolemia" (ADH). Let us accept in the present thesis that, "ADH" and "FH" are just synonyms and refer to the same clinical syndrome without reference to the primary cause. In brief (Figure 1), FH is an autosomal dominant disorder phenotypically characterized by increased plasma levels of low-density lipoprotein cholesterol (LDL-C), the development of tendon xanthomas (TX), and the premature occurrence of cardiovascular disease (CVD), especially, coronary heart disease (CHD). It results from genetic defects in the pathway of the hepatic clearance of LDL particles. Currently, four genes (LDLR, APOB, PCSK9, ARH) encoding different actors of this pathway have clearly been incriminated. We will mainly focuse on the heterozygous form of the disease (refer as "heFH" of simply "FH") that is frequently encountered in the population. The homozygous form of FH ("hoFH") which is very rare will be presented at the end of this chapter.

Figure 1. A brief look at familial hypercholesterolemia.

In its current form, the familial hypercholesterolemia (currently abbreviated HeFH or simply FH) is due to the presence at the heterozygous state of a mutant allele of the LDL receptor gene (LDLR). The patient with heterozygous familial hypercholesterolemia (HeFH) carries an abnormal gene for the LDL-R inherited from one of his parents and one normal gene inherited from the other parent. Therefore, only half of the "LDL receptor" proteins (LDLr) located at the surface membrane of all cells function normally in this individual. The LDL particles are removed from the blood by LDLr in the liver at about half the normal rate, leading to an approximate doubling of LDL-cholesterol (LDL-C) level in the bloodstream. This elevation of blood cholesterol starts at birth and results to the accumulation of cholesterol in tendon causing tendon xanthomas (chapter 11), in the corneal stroma and anterior sclera causing an arc-shaped whitish deposit in the cornea (corneal arcus ressembling arcus senilis but occurring earlier) and in the intima-media of arteries causing early atherosclerosis and its complication such as coronary heart disease which occurs at an early age (typically 35-55 years among men and 55-75 years among women, although heart disease can occur as early as the mid-twenties). In the 80's, it was found that some families displayed a clinical pattern of FH but did not have defect of the LDLr (Soria 1989). Instead of the receptor being abnormal, the molecule apolipoprotein B (apoB) that is crucial for the binding of the LDL particles to the receptor is abnormal. These patients have a typical mutation in the gene that codes for apolipoprotein B (APOB): glutamine (abbreviated Q) is substituted for arginine (abbreviated R) at residue 3500 of the protein ("ApoB R3500Q"). This clinical cousin of FH is sometimes called "familial defective apolipoprotein B" (FDB). FDB explained 5% of FH syndrome with great variability from country to country. This form is less severe in term of LDL-C elevation and cardiovascular complications. More recently, other rarer causes of FH have been also discovered involving the gene PCSK9 orARH (see text).

Table 1-1. Summary of genetic causes of FH

Genes	LDLR	APOB	PCSK9
Denomination of the disease	Original "familial hypercholesterolemia" (FH)	"Familial ligand-defective apoB". (FDB)	No particular name
Reference MIM	FH or HCHOLAD1 (MIM 143890)	HCHOLAD2 (MIM 144010)	HCHOLAD3 (MIM 603776)
Gene product	LDL-receptor (LDLr)	Apolipoprotein B (ApoB)	NARC-1, a proprotein convertase
Reference MIM	MIM 606945	MIM 107730	MIM 607786
chromosome	19p13.1-p13.3	2p23-24	1p34.1-p32
Function	Surface receptor for LDL particles	the ligand for the LDL receptor	Control LDLr expression
Functional mutations causing ADH	> 900 (most likely any number)	1 or 2	>2 (Gain-of-function mutations)
Contribution to ADH	Most	5-10%	(approximately 2.3%)*
Other	Polymorphisms associated with LDL-C variance :	Many neutral mutants; Hypobetalipoproteinemia due to truncated APOB	Loss-of-function mutations ⇒ low cholesterol
Mutation frequency	1/500	0.08%.	?

(* in France, Allard 2005)

1. Molecular definition and pathophysiology

Mutations causing FH are mainly located in the LDL receptor gene (LDLR) (Goldstein 1974, Brown 1974), and, at a lesser extent, in the gene of apolipoprotein B-100 (APOB) (Innerarity 1987, Soria 1989). The analysis of the LDLR and APOB genes fails to detect the underlying mutation in 15-40 % FH phenotype (Austin 2004), indicating possible additional genetic cause of FH phenotypes.

1.1. Mutations of the LDLR

More than 900 different mutations (most likely, any number of mutations can occur) can produce FH phenotype. These mutations (as well as the common LDLR polymorphisms) are compiled online at two websites (Heath 2001, Villeger 2002)[7].

1.1.1. Types and classifications of mutations

Most mutations are single-nucleotide mutations, small insertions or deletions and are found in all protein domains and in the promoter region. About 5-10% mutations are large multiexon deletions. The mutations may be classified according to different criteria. 1) Based on the analysis of the LDLr activity in cultured cells[8] (mainly fibroblasts) of homozygous subjects (>5% for "receptor-defective", <5% for "receptor-negative") or heterozygous subjects (>55% for "receptor-defective", <55% for "receptor-negative") carrying the same mutation (Goldstein 2001, Hobbs 1992, Bertolini 1999). 2) Based on the biological step of the LDLr pathways (biosynthesis, maturation, trafficking, binding property, stability and lysosomal

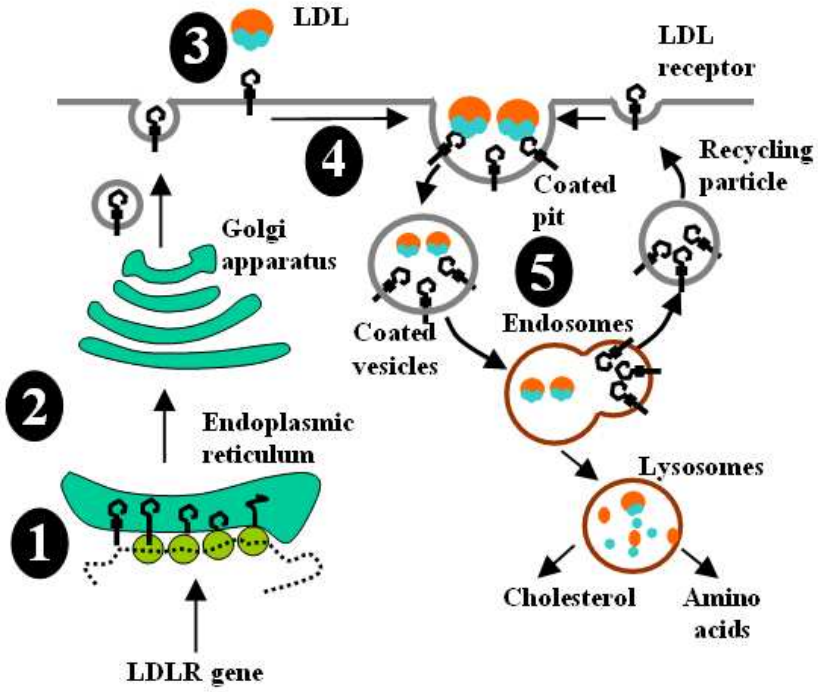
[7] http://www.ucl.ac.uk/fh/ and http://www.umd.necker.fr/LDLR/research.html.

[8] Quantitative measurement of LDL receptor activity is performed in culture of cells easily sampled from the patients like fibroblast (from cutaneous biopsy) or lymphocytes (from blood sampling). LDL-R function is assessed by measuring the stimulation of cellular acyl-CoA cholesterol acyltransferase (ACAT) activity which followed exposure to LDL in fibroblast culture (Goldstein 1974) or by measuring the binding, internalization, and degradation of 125I-labeled LDL in lymphocytes (Cuthbert 1986).

digestion of LDLr protein) that they affect preferentially (studied in fibroblast culture) (Goldstein 2001, Fig. 1-2). 3) (Most used) Based on the nature of mutations (frameshift/nonsense/rearrangement versus missense/splice defect) (Varret 1997).

Figure 1-2. Classification of mutations into 5 classes (adapted from Goldstein 2003)

As the exon organization corresponds to the LDLr domain structure and that these domains participate specifically to the different steps of receptor-mediated endocytosis and receptor cycling, there are some concordances between the location of the mutations and their cellular consequences (mutation classes). Steps of receptor cycling. "Transport" of LDLr from endoplasmic reticulum to Golgi; "binding" of apolipoprotein ligands (apoE or apoB); "clustering" of LDLr in clathrin-coated pits on the cell surface; "recycling" of the LDLr after the release of LDL particles in the endosome,



Mutation Class	Synthesis	Transport	Binding	Clustering	Recycling
1	X				
2	→	X			
3	→	→	X		
4	→	→	→	X	
5	→	→	→	→	X

Classes of mutations. Class 1: mutation located in the promoter sequence or in any other locations if the mutation causes nonsense, a frameshift or (in some cases) a splice defect. Class 2 and 3: missense in the ligand binding domain or in the epidermal growth factor precursor (EGFP) homology domain. Class 4: missense in the cytoplasmic or the membrane spanning domain (here, the defect results to the non internalization of LDL particles). Class 5: missense in the epidermal growth factor precursor (EGFP) homology domain (here the defect results to the destruction of LDLr in the lysosomes).

1.1.2. Geographical diversity

The numerous geographically based surveys of FH subjects usually reported a large spectrum of distinct LDLR mutations, with a few mutations found in a slightly

greater proportion. In certain populations, however, a small number of mutations predominate due to founder effects[9]. Such founder effects are thought to influence the spectrum of FH mutations in French Canadians (Moorjani 1989); South African Afrikaners (Seftel 1980), Christian Lebanese (Khachadurian 1973); Icelanders (Gudnason 1997); and Finns (Vuorio 2001), Tunisians (Slimane 1993); Jews (Seftel 1989), and Indians (Rubinsztein 1994). For example, three mutations cause 95% of FH in South African Afrikaners.

1.1.3. Pathophysiology

Loss of LDLr function leads logically to decreased LDL catabolism and elevated LDL levels (Brown 1986). FH patients also overproduce LDL (Soutar 1977) and VLDL particles (James 1989) because LDLr modulates the hepatic secretion of apoB-100 by promoting pre- and post secretory degradation of nascent apoB-100-containing lipoproteins (Tremblay 2004, Millar 2005). Finally, loss of LDLr function also impairs clearance of VLDL remnants which can lead also to LDL overproduction (Bilheimer 1982). LDL overproduction may explain why LDL-C concentration could be greater than twice the population average.

1.1.4. Polymorphisms of LDLR (see also box 1-1)

Some polymorphisms in LDLR have been associated with interindividual variations of cholesterol levels in normal populations (Schuster 1990, Ahn 1994, Humphries 1991, Pedersen 1989). For example, the P- variant of the Pvu II polymorphism in intron 15 is associated with a 10 % increased LDL-C compared with the P+ variant. The A370T substitution, although an amino acid substitution in the EGFP domain, does not affect the uptake and degradation of LDL and is not causing FH (Kotze 1989). However, it has a small impact as the T carriage is significantly associated with slightly higher LDLC in male Icelanders (Gudnasson 1995) and curiously, with a 3.6 fold increase (95% CI, 1.5–8.8) of stroke in the Copenhagen City Heart Study (no CHD or lipid association here) (Frikke-Schmidt 2004).

1.2. Mutations in the apolipoprotein B-100 gene (APOB)

ApoB-100 is synthesized by the liver, is carried by the LDL, VLDL and IDL, and contains the binding site essential for LDL uptake by LDLr. The first mutation of apoB reported to cause reduced binding affinity for the LDLr was the substitution of arginine by glutamine at position 3500 (Arg3500Gln or R3500Q) (Innerarity 1987, Soria 1989)[10]. One haplotype seems to predominate in several populations in Europe and North America (Ludwig 1990, Rauh 1991), though other rarer haplotypes in Germany and China (Rauh 1993, Bersot 1993) have been also reported. This has suggested that the mutation has emerged in the Mesolithic period during the Celtic migration in Europe (Miserez 2000 & 2001). The phenotype caused by this mutation

[9] A founder effect occurs when a subpopulation is formed through the immigration of a small number of "founder" subjects, followed by population expansion. If some founders had FH, then genetic drift leads to a high proportion of affected subjects who share the specific mutations introduced by the founders.

[10] Analysis in the sequence surrounding the putative receptor-binding domain of apoB has revealed several other variations. However, only R3531C and R3500W cause reductions in binding affinity for LDLr (Ludwig 1997) and a moderate hypercholesterolemic effect (30% for R3531C) in some studies but not all (Rabes 2000, Tybjarg-Hansen 1998) and less clear effects on CHD risk.

(also called “Familial ligand-defective apolipoprotein B or FDB”) is almost indistinguishable from FH caused by LDLR mutations except it is less severe (Myant 1993, Hansen 1997, Miserez 1995, Descamps 2001a & 2003b). The reasons are that mutant apoB still binds LDLr with ~ 10% normal affinity and that IDL and LDL particles carrying the mutant apoB can be cleared via the binding of LDLr to apoE that covers these particles (Innerarity 1990).

Box 1-1.Distinction between mutation and polymorphism

The distinction between “mutation” versus “polymorphism” is not as simple than it seems. Originally, a “mutation” is considered as a rare DNA change, which occurs by “accident” (an error of DNA replication caused by toxics, viruses or radiations) in the genome of a germinal cell before the fecundation and which alters strongly the function of the protein encoded by the gene. A “polymorphism” (which means "many forms", and thus the state of multiple forms), is a common (“common” defined arbitrarily as a frequency of 1% of higher in the population) DNA variation, which probably runs in the human population since its origin and affects only slightly the function of the protein encoded by the gene.
If we think about it, we might discuss very much these definitions. For example, a mutation (whether it is a point mutation, deletion, insertion, inversion, duplication, whatever), is an event that happens once in the germinal cell of an individual and changes the genome of his descent. Usually it disappears if that individual does not breed. Sometimes it causes a disease. Very rarely it might give a selective advantage, alter evolution and can increase in frequency in a population through selection. It can also increase in frequency by simple neutral genetic drift. If it increases, it can be considered as “a polymorphism”. Therefore, we may say that the mutation is the event and the polymorphism is the result of this event. But we should keep in mind that mutation is only one source of polymorphism, as recombinations can also generate new alleles of a gene and therefore new polymorphisms of that gene. Recently, because some polymorphisms have been found to alter phenotypes, because some neutral genetic variations have frequency below 1%, because some newly occurred mutations may be completely neutral and because the term “mutation” has developed a negative connotation, it has been suggested to discard these terms for more neutral terms like “sequence variant”, “alteration” or “allelic variant”. In the present work, however, we will keep the terminology of “mutation” and “polymorphism” to designate the DNA change which corresponds strictly to the definition we give above, otherwise we will use the more neutral terms.

1.3. Proprotein convertase subtilisin/kexin type 9 (PCSK9)

Recently the molecular defect responsible for an additional form of autosomal dominant hypercholesterolemic has been identified in a gene called “PCSK9” (Haddad 1999, Varret 1999). This gene encodes a protease that degrades the LDLr somewhere between its residence in the Golgi apparatus and its internalization from the plasma membrane, by either a direct mechanism or an indirect mechanism (Abifadel 2003, Seidah 2003, Maxwell 2005). This action is probably a counterregulatory mechanism that prevents excessive uptake of cholesterol into cells. Indeed, when the cholesterol pool is depleted (low intake of dietary cholesterol and of saturated fat or statins), SREBPs, which are key transcription factors, are

activated, leading to increased synthesis of the LDLr and of PCSK9. As PCSK9 may act also by decrease apoB100 lipoprotein maturation and release, its effect on plasma cholesterol homeostasis may thus be also related to an overproduction of apoB100 (Ouguerram 2004, Seidah 2003). Patients with PCSK9 missense mutations (S127R, D374Y, R218S, R357H, R469W, A443T) are clinically indistinguishable from patients with heFH and heFDB except that they have normal LDLr activity as measured in skin fibroblasts (Allard 2005). These PCSK9 mutations causing hypercholesterolemia are probably "gain-of-function" mutations, as overexpression of PCSK9 in the liver of mice produces hypercholesterolemia by reducing LDLr number. Other mutations, like the nonsense mutations Y142X and C679X (in 2.6% African Americans) or like missense R46L (in 3.2%% European Americans) are "loss-of-function" mutations that have the opposite effect with a reduction in LDL-C (28% for Y142X/C679X and 15% for R46L) and a reduction in the CHD risk (88% for Y142X/C679X and 47% for R46L) (Cohen 2006, Descamps 2006).

Table 1-2. Other very rare forms of genetic hypercholesterolemia.

	Autosomal recessive forms of HC (ARH or HCHOLARl *.	**Sitosterolemia ****	**Cholesterol 7-hydroxylase deficiency. *****
Gene and product	novel LDLr adaptor protein	ABCG5 or ABCG8 $^{+++}$	cholesterol 7-hydroxylase (CYP7A1
Function	Bind the FDNPXY of the LDLr required for internalization $^{+}$.	Control the absorption/elimination of dietary sterols (in plants and shellfish).	1° enzyme of bile acid biosynthesis.
Biology	Very low LDL clearance Normal LDLr function in fibroblasts.	↑ intestinal absorption and ↓ biliary secretion of sterols. Plasma sitosterol: 50xN.	↓ 94% bile acid content in stool. HC due to reduced hepatic LDLr activity.
Clinic	Intermediate between heFH and hoFH	LDL-C levels, xanthomas $^{++}$, atheroma^{++} , like hoFH $^{++++}$	hypertriglyceridemia or hypercholesterolemia.
Prevalence	Families in Liban and Sardinian	Rare	2 brothers and 1 sister
Treatment	Statins reduced LDL-C more strongly than in hoFH, but most required LDL apheresis	Very responsive to cholesterol restriction, bile acid resin and ezetimibe. (poorly to statin)	

* Arca 2002, He 2002; ** Berge, 2000, Lee 2001; *** Pullinger, 2002, Bhattacharyya 1974. $^{+}$ but not in fibroblasts (that is why the LDLr function was preserved in ARH fibroblasts). $^{+}$ $^{+}$ they are composed mainly of cholesterol ; $^{+++}$ two mutant alleles of ABCG5 or 2 mutant alleles of ABCG8 alleles but never one mutation in ABCG5 and one in ABCG8. $^{++++}$ Rarely, normal LDL-C planar xanthomas, aortic stenosis, hemolysis (sterols in red blood cells).

1.4. Other Mendelian forms of hypercholesterolemia

A rare autosomal recessive FH subtype results from mutations in ARH (MIM 605747), encoding a putative adaptor for the LDLr. The clinical phenotype of ARH is milder than that of receptor-negative hoFH and resembles that observed in receptor-defective hoFH: LDL-C in ARH (553 ± 89 mg/dL), in receptor-negative hoFH (830 ± 138 mg/dL) and in receptor-defective hoFH (602 ± 93 mg/dL). The

risk of coronary artery disease was 9-fold lower in ARH patients (No CHD before 20 years in ARH versus 43% in hoFH). Heterozygous ARH carriers showed higher level of LDL-C (+17%) than non-carrier family members (Pisciotta 2006).

Other very rare forms of recessive hypercholesterolemia not related to LDLr pathway resemble (phenocopy) to heterozygous or homozygous FH (Table 1-2.).

2. FH frequency

The frequency of heterozygous FH in Caucasian populations is reported as 1/500 (0,2 percent). This makes of FH the most common potentially lethal genetic disorder. This frequency of 1/500, usually reported in all textbooks and research papers, is based on the prevalence of the homozygous form of FH (hoFH), which, in principle, is more easily recognizable in childhood by paediatricians due to the presence of cutaneous xanthomas (Slack 1979). Knowing the observed frequency of hoFH in a country ($q^2 \sim 1/1.000.000$) and from the Hardy-Weinberg equation [$p^2 + 2pq + q^2 = 1$ with p and q, the prevalence of the normal allele and of the FH-causing allele, respectively], the heterozygous FH frequency (= 2pq = 2q by considering p close to one) is calculated to about "1/500". This estimate remains inaccurate, because the observed hoFH frequency is quite small and is probably underestimated as hoFH patients may not be recognized before their very early death.

More direct estimate of heterozygous FH frequency have been attempted by approaches using more or less stringent clinical criteria or molecular definition to identify FH individuals. The problems are that the clinical criteria are unspecific and that the genetic tests are too expensive to screen the whole mutation panel in population based surveys. Furthermore, prevalence varies with age. In principle, birth prevalence must be higher than prevalence at old age (a significant loss of FH carriers occurs after 50 years) but, practically the prevalence studied in physicians' records increases with age (FH people are diagnosed after 50 years) (Neil 2000).

Estimates based on clinical criteria. Amongst the survivors of myocardial infarction, the prevalence was estimated to about 10% in US (Goldstein 1973) and in UK (Patterson 1972). Using patients' records in lipid clinics or counties, the frequency was estimated to 0.22% (134 cases) in the Ostford County (Norway; Heiberg 1976), 0.11% (=3 cases) amongst the 2,700 patients of lipid clinics (Japan; Mabuchi 1977), 0.19 % (39 cases) amongst 21,000 patients in Hungary (Kalina 2001) or 0.53% in the exhaustive searching for cases in Oxfordshire, UK (involving doctors, lipid clinic and hospital records) (Neil 2000) and 1/232 in 8800 adults of 4 general practices in Hoofddorp (NL, Lansberg 2000). In a study of consecutive Danish newborns, very elevated cholesterol was found in 11 infants out of 10440 (birth prevalence = 0.11%) (Andersen 1979).

Estimates based on molecular genetics. Population-based screening of one or more specific mutations using direct tests at a reasonable cost have been performed for the "FDB" mutation (R3500Q) in California and Denmark or for the most frequent LDLR mutations in populations with founder gene effect. In 5160 bank employees in California (Bersot 1993), 9255 participants in the Copenhagen City Heart Survey (Tybjarg-Hansen 1998) and 5,000 newborns from the Denmark (Hansen 1994), the frequency of the R3500Q APOB variants has been estimated to about 0.08%. This

frequency may vary geographically as, for example, R3500Q was shown to contribute to a greater proportion of FH suspected patients in Switzerland (Miserez 2000). In populations with a founder gene effect, the frequency of the "common" LDLR mutations was estimated to 1/411 (0.24%) for North Karelians of Finland (Vuorio 2001), 1/67 for Ashkenazi Jews in South Africa (Meiner 1991), 1/70 for Afrikaners in South Africa (Kotze 1991) and 1/200 for French Canadians (Simard 1994). These frequencies appear higher than the usual "1/500".

3. Clinical manifestations of FH

As the LDLR mutations are the major causes of heFH (the heterozygous state of FH), we focuse first on the clinical description of "LDLR related FH". The other forms of FH (FDB and due to PSCK9 mutations) and the homozygous form (hoFH) of FH will be presented later.

3.1. Lipid parameters

In adults, the mean values of total cholesterol (TC) and LDL-C differ strongly between studies and these differences reflect selection bias or environmental and genetic differences between different countries (Table 1-3). Unfortunately, an ideal cohort for examining the clinical characteristics which would consist of individuals collected by population based screening of FH mutations, does not exist.

Table 1-3. Lipid values in FH men and FH women in several studies.

	Hill 1991	**SBRG 1999**	**Umans-Eckenhausen 2002**	**de Sauvage 2003**	**Bertolini 2004**
Country	Canada	UK	Netherlands	Netherlands	Italy
Criteria	LDL-C > P95th and TX	Clinical definite FH (SBRG)	Genetic test	Genetic test or >5 pts in DLCN algorithm	Italian criteria
Population index patients or relatives	364 patients from 283 families	Index patients	2nd, 3rd, 4th d° relatives No index	Index patients in lipids clinics	Index patients in lipids clinics
Women N	208	580	204	229	119
Age	45 (17)	43.9	35 (1.6)	48 (13)	50 (13)
TC	350 (66)	348 (93)	294 (65)	419 (91)	394 (76)
LDL-C	270 (62)	271 (93)	222 (60)	334 (89)	316 (76)
HDL-C	49 (12)	50 (12)	44 (14)	52 (14)	55 (13)
TG	131 (71)	106	129 (79)	150	127 (58)
% CVD				35%	24%
Men N	156	605	195	287	102
Age	40 (17)	40.3	35 (1.6)	48 (13)	49 (13)
TC	328 (58)	329 (81)	279 (52)	396 (77)	371 (62)
LDL-C	256 (58)	255 (81)	213 (48)	315 (76)	299 (62)
HDL-C	42 (8)	43 (12)	37 (12)	43 (11)	44 (12)
TG	136 (80)	123	134 (84)	159	143 (63)
% CVD				39%	42%
TX				44%	

Values are given as mean (standard deviation).

Close to this ideal, are the data of Umans-Eckenhausen et al. (2002) who collected 1695 "free-living" FH relatives through cascade screening from 66 index patients

refereed to lipid clinics and genetically diagnosed for FH. In this cohort, the average values were quite lower, due to the better representativeness of FH patients but perhaps also because the individuals on lipid-lowering medication (with a high proportion of most severe cases) were excluded from the average calculation.

The elevation of LDL-C is already evident at birth (Table 1-4.) in cord blood (Kwiterovich 1973, Glueck 1971, Tsang 1974, Darmady 1972, Boulton 1979, Goldstein 1974, Andersen 1979, Blades 1988, Vuorio 1997). Thereafter, the cholesterol levels increase rapidly during the first week, more slowly during the ensuing month, and not any more from 6 months to 2 years (Van Biervliet 1980 & 1981, Srinivisan 1982, Lane 1986). The increment of serum cholesterol takes place as quickly but with much greater amplitude compared to non-FH children (Vuorio 1997, Darmady 1972, Boulton 1979, Kwiterovich 1973 & 1993).

HDL-C and apoA1 levels are usually slightly lower in FH adults (Hill 1991, Miltiadous 2002, Umans-Eckenhausen 2002, Streja 1978) as well as in FH children (Kwiterovich 1974, Torres 1996, Wiegman 2003) and in cord blood (Kwiterovich 1973). The reason(s) for low HDL-C in FH have not been fully elucidated but may be due to the greater transfer of cholesterol from HDL to the greater pool of VLDL/IDL via cholesteryl ester transfer protein (CETP) (Imazu 1992, Gillian-Daniel 2002) or to the increased pool size of apoE (less efficiently cleared by the defective LDLr) (Schaefer 1992), leading to apoE-enriched HDL particles which are more rapidly cleared. Rare FH patients present severely elevated triglycerides, with a "type V hyperlipidemia" lipid profile, often due to an additional factors such as heavy alcohol consumption, endocrinological disorder like diabetes mellitus or genetic factors like E2/E2 genotype or APOE mutants (Kobayashi 2005).

Table 1-4. Lipid values in FH and non FH children

References	**Kwiterovich 1973&1993**		**Vuorio 1997**		**Wiegman 2003**
Criteria	Clinical FH diagnosis in parents		DNA test [1]		DNA or clinical heFH parents [2]
Age	Cord blood	1-19 years	Cord blood	1 years	2-18 years
FH infants	N=16	N=105	N=11	N=8	N=742
TC	100 (15)	299 (63)	101 (27)	324 (46)	281 (63)
LDL-C	62 (16)	242 (60)	68 (22)	271 (41)	217 (63)
% > cut-off	80% TC>92	92% LDL>164	NA	NA	96% LDL>135
NFH infants	N=13	N=131	N=11	N=9	N=189
TC	73 (13)	176 (28)	60 (9)	170 (26)	166 (27)
LDL-C	33 (5)	112 (25)	30 (6)	112 (26)	99 (27)
% > cut-off	0% TC>92	7% LDL>164	NA	NA	4%[3] LDL>135

Values are given as mean (standard deviation); [1] only 2 mutations : North Karelia and FH-Helsinki ; [2] LDL-C > 95th in families with early CVD in conjunction with TX; [3] estimated according to the figure 1 of the paper but not given in the text ; "by ROC": best cut off established by ROC curve; P95 : the 95th percentiles; NA : not available

3.2. Cardiovascular diseases (CVD)

FH is associated with premature CVD, especially CHD and with decreased life expectancy, most likely because of the high LDL-C levels from birth onwards but perhaps also because of the induction of other risk factors such as low HDL-C, high lp(a) and higher susceptibility of LDL to oxidation.

3.2.1. Coronary heart disease (CHD)

Several studies (Mabuchi 1978, Gagné 1979, Hill 1991) have examined the CHD prevalence in various age categories but subsequent studies (Jensen 1967, Slack 1969, Stone 1974 , Jansen 2004) have attempted to careful establish the life curve of coronary event or death. In Table 1-5, I display the data of some of the most interesting studies regarding the cumulative risk of CHD associated with FH. Despite their difference (selection criteria, availability of statins, clinical endpoints, proportion of relatives ad index), most yield similar life tables of CHD events, with the exception of the earliest studies which selected the most severe forms of FH by using criteria such as tendon xanthomas. For instance, the two largest studies (Stone 1974 and Jansen 2004) were quite consistent in term of cumulative CHD or CVD. Only the data of extreme ages in men seem to diverge slightly: Stone et al. found a greater CHD incidence at younger age (< 30 years) and a lower CHD incidence at older age (60 years) compared to Jansen et al. Although this difference was not commented by the authors, this could partly be caused by the recent statin prescriptions in young FH patients that delay the occurrence of CVD to older age, by the differences of endpoints examined in the two studies (cerebro-vascular diseases included in Jansen et al. but not in Stones et al., occurred later in life than coronary diseases) or by the differences in times (secular trend of progression of CVD, Sijbrands 2001, Williams 1986, Descamps 1997) or in geographical area (with different environmental or genetic factors; smoking prevalence was 45% in Stone's study and 70% in Jansen's study). In 1995, using data from 5 different studies (>1000 FH individuals), Brown and Goldstein (Goldstein 2001) have estimated that FH men have a 20% chance of developing CHD by age 40, a 45% chance by age 50, and a 75% chance by age 60. Chances of FH men dying from CHD are 25% by age 50, 50% by age 60, and 80% by age 70. Risks are lower for FH women, but still far higher than risk among the general population.

Here are some of the statements and references most currently reported in papers about FH :

1. At least 50% of FH men and about 30% of FH women develop CHD by the age of 60 (Slack 1969).
2. 16% of FH men have developed CHD by the age of 40 years. 52% of FH men and 33% of FH women have developed CHD by the age of 60 years with a marked increase occurring post-menopausally (Stone 1974). Only 20% of FH-men and 70% of FH-women , reach the age of 70 years.
3. The mean age of onset of CVD is between 40 and 45 years in male FH patients, and 10 years later in female FH patients (SBRG 1991).
4. In 20-39 year-old subjects, the relative risk of fatal coronary event was increased 125-fold (95% CI: 15-451) in women and 48-fold (95%: 17-105) in men compared to the general population and the annual coronary mortality was 0.17% in women and 0.46% in men. The relative risk decreased with age but the absolute risk increased (SBRG 1999).
5. The overall SMR of FH is 1.34 (95% CI : 1.16-1.55) compared to the general population. High excess mortality occurred in males between age 40 and 54 (SMR 2.34, 95% CI 1.60-3.31). (Sijbrands 2000)

Table 1-5 Various studies examining the cumulative incidence of CVD in FH patients

	Number of patients (index and relatives)	criteria of FH in index patients criteria for inclusion or exclusion of relatives	endpoint	Follow-up	N CHD	Cumulative incidence of CHD in Men	Cumulative incidence of CHD in Women
Jensen 1967	181 index patients and relatives from 11 families	TC > 350 mg/dL for age above 15 yeras	"CHD" according to clinical history and diagnsis by the authors	up to 20 years		<40 yrs : 19,3% <50 yrs : 45,1%	<40 yrs : 25% <50 yrs : 75%
Hill 1991	364 patients from 283 families Age 0-79 years, 156 men and 208 women	LDL-C > P95 for age/sex AND TX in the patients or a FDR.	"CAD" = AP, MI, angiographically proven disease or history of bypass.	no	73 CAD (47 M; 26 W)	<25 yrs : 3% <35 yrs : 9% <45 yrs : 26% <55 yrs : 30%	<25 yrs : 0% <35 yrs : 5% <45 yrs : 8% <55 yrs : 11%
Gagné 1979	558 patients (209 families), 50% index, 50% relatives Age > 20 years (mean 30+/-19 years). 264 men and 311 women.	see criteria Lipid research clinic of the Laval University Hospital Center in table 2		no	124 (72 M; 52 W)		<25 yrs : 0% <35 yrs : 5% <45 yrs : 8% <55 yrs : 11%
Slack 1969	44 index patients + 60 affected relatives	HC > 325 mg/dl AND TX Relatives included if HC (> 2 SD) excluded if triglycerides>200 mg/dl	Ischemic heart disease (typical symptoms and ECG)	up to 10-years		<30 yrs : 5,4% <40 yrs : 23,7% <50 yrs : 51,4% <60 yrs : 85,4% <50 yrs : 100%	<30 yrs : 0% <40 yrs : 0% <50 yrs : 12,2% <60 yrs : 57,5% <50 yrs : 74,4%
Stone **1974**	116 index patients and 1023 relatives diagnosed during the period 1964-1970 at the National Institutes of Health Clinical Center Age 0 to 59 years	LDL-C > P95 for age PLUS either LDL-C>P95 in a FDR or TX Relatives included if HL type II, excluded if HL type IV	Fatal and non fatal CAD events : AP, MI, abnormal Q wave, narrowing of major coronary vessel (>75%), coronary death.	up to 9 years (1964-1973)	30 % (23 % AP, 10% CAD death or MI, 7% abnormal Q wave)	<20 yrs : 2% <30 yrs : 7% <40 yrs : 16% <50 yrs : 34% <60 yrs : 52%	<20 yrs : 1% <30 yrs : 2% <40 yrs : 8% <50 yrs : 19% <60 yrs : 32%
Jansen 2004	2400 patients from 27 lipid clinics (26% with CVD and 8% with signs possibly related to CVD).	I. LDL receptor mutation or II LDL-C > P95 for age/sex PLUS TX in the patient or a FDR; LDL-C > P95 in a FDR; CAD < 60years in patient or a FDR; Exclusion of secondary HC or other genetic causes (FDB)	Fatal and non fatal CHD (AP, MI, coronary death) PLUS Cerebrobascular and peripheral arterial diseases *	Followed since 1969 (90% after 1988)	782 35 died	<20 yrs : 0% <30 yrs : 2% <40 yrs : 14% <50 yrs : 41% <60 yrs : 61% <70 yrs : 86%	<20 yrs : 0% <30 yrs : 1% <40 yrs : 4% <50 yrs : 14% <60 yrs : 31% <70 yrs : 52%

P95 age/sex: 95th percentile corrected for sex and age ; FDR: First degree relative; TX: tendnous xanthomas; FDB: familial detective apolipoprotein B; MI: myocardial infarction, AP: angor pectoris; HL: hyperlipoproteinemia; * documented ischaemic stroke or transient attack or peripheral arterial diseases documented by a surgical or percutaneous invasive intervention or by clinically defined intermittent claudication confirmed by a usual diagnosis procedure.

3.2.2. Cerebro-vascular diseases

The association of stroke with FH is unclear (Hutter 2004). Two studies conducted in the 1980s indicated an increased risk of stroke in FH subjects (Bansal 1986, Goldstein 1973), whereas two others found no higher risk (Mabuchi 1986, Frikke-Schmidt 1999). All these studies had methodological limitations due to small sample sizes, differences in FH definition, possible inclusion of hemorrhagic strokes and lack of control groups. A recent large prospective study of FH by the UK-based Simon Broome Register Group (1405 men and 1466 women, 22992 person-years) did not find an excess risk of stroke mortality for subjects with clinical FH (Huxley 2003), but, once again the statistical power was low due to the small number of events observed (9 deaths from stroke), the use of stroke mortality rather than ischemic stroke incidence as endpoint and the widespread use of statins in the population. The difficulty to associate stroke and FH may be related to the controversial association between elevated cholesterol levels and stroke also seen in the general population (Truelsen 2003): associations between cholesterol levels and stroke were found in some observational studies (Neaton 1993, Hachinski 1996) but not in more recent large-scale cohort studies (Shahar 2003, PSC 1995); the decrease in cholesterol levels is accompanied by a decrease in the risk of stroke events in some but not all clinical trials of statins (Vaughan 2003).

3.2.3. Peripheral arterial diseases (PAD)

The prevalence of PAD is increased from 5- to 10-fold in FH subjects compared with NFH controls. Symptomatic PAD such as intermittent claudication is present in 8% to 16% in FH patients (Slack 1969, Stone 1974, Beaumont 1976). Presymptomatic PAD is demonstrated in 30% FH patients (with the severity increasing with age) by reduced blood flow measured by echo-Doppler methods (Postiglione 1985, Kuo 1987) and in 65% FH patients (versus 7% in controls) by abnormal ankle/arm blood pressure ratio (Rubba 1988, Perhoniemi 1989, Kroon 1995). Abnormality of ankle/arm blood pressure ratio is apparent in FH patients as young as 30 years, and in contrast to CHD, the age of PAD onset is similar for males and females (Kroon 1995). In addition, the intima-media thickness of the carotid and/or femoral artery is increased in FH subjects (Hutter 2004, Chapter 8).

3.3. Tendinous xanthomas

3.3.1. Description

Tendinous xanthomas (TX) are accumulations of free cholesterol and foam cells (macrophages containing cholesterol esters) (Mabuchi 1977). They are usually located in the Achilles tendon (AT), in the tendons of the hand (extensor tendons) and of the elbow. They may cause achillodynia and rarely spontaneous tendon rupture (Haacke 1979). In FH, they develop by the age of 20 years (Myant 1973) with a proportion that increases with age. Other xanthomatous lesions such as tuberous, eruptive and planar xanthomas and palpebral xanthelasma, all characterized by cholesterol ester accumulation in dermal foam cells (Vermeer 1982) are usually not found in heFH although flat xanthomas in the interdigital webs of the hands may be found in some patients with FH with other genetics disorders (Feussner 1996).

3.3.2. Mechanism of formation

The increase in tendon size is caused not only by the intratendinous lipids, but also by oedema and inflammation (Vermeer 1982). Its mechanism of formation is probably identical than atherosclerosis: LDL derived from plasma is trapped in collagen and glycosaminoglycans of the tendon matrix and oxidized by macrophages or other cells of the tendon. These OxLDL are taken up mainly by macrophages, promoting the formation of foam cells (Rapp 1983, Mori 2001) and promoting, by chemotactism, the accumulation of lymphocytes and neutrophil polymorphs. To limit the intracellular cholesterol accumulation in macrophages (which is toxic), a macrophage enzyme, the sterol 27-hydroxylase or CYP27A1 oxidizes cholesterol into more polar metabolites (27-hydroxycholesterol and cholestenoic acid), which can flux out from the cells to the circulation (Lund 1996). Another protective mechanism is operated by apoE that facilitates the efflux of cholesterol from macrophages and prevents them from transforming into foam cells.

3.3.3. Risk factors of XT

Achilles tendon thickness (ATT) is positively correlated with cholesterol, age, cholesterol year score, (specially studied in homozygous FH), BMI and physical activity and negatively correlated with HDL-C and apoA1 levels (Murano 1993, Schmidt 1996, Lehtonen 1981, Civeira 1998). TX is also correlated positively with the titre of auto-antibodies against oxLDL (Okada 2000) and negatively with HDL_3–C and CETP (Inazu 1999, Mack 1996). In the most recent study (Civeira 2005), TC and LDL-C were higher in TX+ than in TX- subjects (439 and 363 mg/dL versus 400 and 323 mg/dL) whereas HDL-C was lower in TX+ than in TX- subjects (50 versus 53 mg/dL). Lp(a) and APOE genotype showed no difference between groups (Civeira 2005). ATT is usually more frequent in men than women (Murano 1993, Civeira 1998) in receptor-negative subjects than receptor-defective subjects (2-fold greater in Bertolini 2000). Possibly also specific susceptibility genes for TX may exist: amongst 6 hoFH individuals of a consanguineous Syrian kindred, half had giant xanthomas, and half did not, even though their LDL-C were similar degrees (> 540 mg/dL), suggesting an autosomal mode of TX inheritance (Vergopoulos 1997).

3.4. Clinical (phenotypic) variability and risk factors in FH

The biochemical phenotype "elevated LDL-C" is present in almost all FH-individuals since birth, but LDL-C varies, even among patients who carry the same LDLR mutation and cardiovascular consequence of LDL-C accumulation are even more variable, in part due to several risk factors that we mentioned here.

3.4.1. Lipid levels

Despite the fact that LDL-C is the most pivotal determinant of CVD in FH, cholesterol or LDL-C does not emerge as risk factors in several studies (Hill 1991, Hopkins 2001, de Sauvage 2003, Jansen 2004, Neil 2004, Holmes 2005). Reasons may be that, above a certain threshold level, any increase in LDL-C (about 300 mg/dL LDL-C according to Bertolini 2000) do not worsen any more the development of atherosclerosis or simply be the too low statistical power to observe an effect from LDL-C difference due to the narrow range of LDL-C in this population (Jansen 2004) or the unavailability of numerous pre-treatment values

(Humphries 2007, personal comment). In contrast, low HDL-C (<35 mg/dl in men and < 40 in women) (Jansen 2004, Neil 2004, Real 2001, Hirobe 1982, Slack 1969), high ratio TC/HDL-C or LDL-C/HDL-C (Real 2001, Panagiotakos 2003) and high triglycerides (Gaudet 1998, Vuorio 1997) are important risk factor in FH.

Table 1-6. Odd ratio or relative risk of risk factors in multivariate analysis

	Jansen 2004	**de Sauvage 2003**	**Panagiotakos 2003 ***	**Neil 2004**
Recruitment	Relatives of index patients	Index patients of Lipid Clinics	Index patients of Lipid Clinics *	FH patients treated with statins
N	2400	526	639	410
Sex (M vs W)	2.82	1.70	3.84	2.5
Age, years		1.10	NA	-
Smoking	1.67	NS	2.45	2.5
Hypertension	1.36	1.82	3.02	NS
Diabetes	2.19	NS (u)	NS	NS (glycemia)
BMI, kg/m²	NS (u)	1.07	NS	NA
TC	NS	NS	NS	NS
LDL-C	NS	NS	NS	NS
HDL-C mg/dl	NA	0.39	?	0.94 (adj/sex&age)
HDL-C risk ***	1.37			
TG	NS (u)	NS	NS	NS
LDL-C/HDL-C		NA	1.17	
Fibrinogen	MS	NA	1.04	NS
Lp(a) >30mg/dl	1.50	NS	NS	NS
Homocystein	NS (u)	NS (u)		NS

NS: not significant; NA: not available; (u) significant in univariate analysis only; HDL-C risk *** = HDL-C < 35 mg/dL in men and < 40 mg/dL in women.

3.4.2. Classical risk factors

Except cholesterol, several studies in many countries demonstrated that CVD in FH dependent upon the same risk factors than in general populations: male gender, smoking, hypertension, diabetes mellitus (Hill 1991, Carmena 1996, Panagiotakos 2003, Jansen 2004, Neil 2004, Holmes 2005). In the retrospective, multicentre study in Netherlands (Jansen 2004, 782 patients with CVD and 1618 without, included in 1969-2002, 112.943 person years,), these risk factors were associated in multivariate analysis with relative risk respectively of 3.0, 1.7, 1.4 and 2.2 (Table 1-6).

3.4.3. Tendon xanthomas

In FH-patients, the prevalence of CVD or atherosclerosis is strongly associated to the presence of Achilles tendon xanthomas (ATX) or thickening (ATT) (Murano 1993, Yamamoto 1986, Hata 1981, Beaumont 1976, Hirobe 1982, Ferrieres 1995, Mabuchi 1978, Civeira 2005, Table 1-7). However in a study on the mortality in a large FH cohort (1569 with TX+ and 1302 with TX-; follow-up>10.000 person-years), the CHD mortality risk is similar whether or not patients had TX (Neil 2003).

As a matter of fact, TX and atherosclerosis share the same mechanisms of lipid accumulation, similar histological features and some similar risk factors. In both lesions, calcification can occur (Kruth 1985) and histochemical analysis shows the accumulation of a high amount of free cholesterol (>50%; predominantly extracellularly), of cholesterol esters (extra- and intracellurarly in macrophages), of

OxLDL and of Apo(a) (von Bahr 2002, Sugiyama 1992). Cholesterol derives from the circulation as demonstrated by noninvasive imaging experiments with 99mTechnetium-labeled LDL (Elmaleh 1998), but appears more rapid exchangeable in TX (Bhattacharyya 1976) than in atheroma. Both lesions regress under treatment by statins and fibrates, whereas probucol which does not affect atherosclerosis produced a marked regression of xanthomas (Regnstrom 1996).

Table 1-7. Tendon xanthomas (XT) or Achilles tendon thickness (ATT) as CVD risk factor.

1, CHD – XT				
Hopkins 2001	N=262 (30-88 y)	All	OR=1,98 (p=0,02)	(Univariate)
	(clinical criteria)	Early CHD	OR=3,17 (p=0,02)	(Multivariate)
Ferrieres 1995	N=263 (41±12 y.)	In women	OR=4,14 (p<0,01)	(Univariate)
	(all 10 kb del)	In Men	OR=2,01 (p=0,14)	(Univariate)
Civeira 2005	N=952 (DNA)	In women	OR=4,49 (p<0,01)	(Univariate)
	20-30 (38%)	In Men	OR=2,26 (p<0,01)	(Univariate)
	51-60 (	In young women	OR=17.1 (p<0,01)	
		In young men	OR=3.60 (p<0,01)	
2, CHD – ATT (by RX)				
Hirobe 1982	N=52 (45±4 y.)	<13 mm	OR=1,00	(Univariate)
	(clinical criteria)	13-21 mm	OR=4,32 (p=0,03)	
		>21mm	OR=24,0 (p<0,01)	
3, Carotid IMT – XT				
Tonstad 1998	N=79 (26-46 y.)	Correlation	R = 0,29 (p<0,05)	(Multivariate)
	(genetic test)	with IMT		

3.4.4. Emerging risk factors

Contradictory results were found with homocystein (Neil 2004, Hopkins 2001, Sauvage 2003, Jansen 2004), fibrinogen (Neil 2004, Panagiotakos 2003), oxidation parameters or circulating antibodies to oxidized LDL (van Tits 2004, Paiker 2000), smaller LDL (Hopkins 2001), white blood cell count (Neil 2004, Hopkins 2001), C-reactive protein (Mohrschladt 2000, Hopkins 2001), abdominal obesity (waist circumference > 95 cm) and hyperinsulinemia (Gaudet 1998), exercise tolerance test indices (exercise capacity, heart rate recovery at 1 min, and peak pulse pressure levels) (Pitsavos 2004) or Lp(a) (Jansen 2004, Holmes 2005, Hopkins 2001, Seed 1990, Carmena 1996). It is possible that some of these factors are generated by the status of FH disease itself or are marker of early atherosclerosis (CRP, fibrinogen, anti-oxLDL, WBC) or marker of altered lipid metabolism (Lp(a)) rather than real causal factors. For example, FH patients have higher fibrinogen (Otto 2001) and higher lipoprotein(a) (Lingenhel 1998, Kraft 2000, Bowden 1994, Utermann 1989).

3.4.5. Type of FH causing mutations

Most authors (Yamamoto 1989, Gudnason 1994, Sijbrands 1998, Graham 1999, Gaudet 1999, Vohl 1997, Umans-Eckenhausen 2002, Brorholt-Petersen 2002, Descamps 2001a, Torres 1996, Heath 1999, Gaudet 1998, Real 2003, Bertolini 2000) agree that phenotypic comparison of severe versus less severe mutations demonstrates a difference of LDL-C but this difference is quite small compared to the large variability of LDL-C levels observed in each cluster. Regarding the risk of CVD, although some found an increased CHD risk in patients with receptor-

defective mutations compared with receptor-negative mutations (Gaudet 1999, Vohl 1997, Bertolini 2000, Umans-Eckenhausen 2002), others did not (Sijbrands 2000).

3.4.6. Modifyer genes

Modifier genes may influence either the lipid metabolism or the CVD risk.

3.4.6.1. Genes involved in lipid metabolism

APOE. Curiously, apoE4 allele which is generally associated with CVD in the genral population was not associated with CVD in most studies in FH (Mozas 2003, De Knijff 1990, Kitahara 1990, Hill 1991, Gylling 1991, Kotze 1993, Ferrieres 1994, Lindahl 1994, Tonstad 1995, Vuorio 1997, Carmena-Ramon 2000). Only a few studies found an association of apoE4 with slightly higher LDL-C (Eto 1988, Friedlander 1996, Bertolini 2000). But, even in these positive studies, apoE4 accounted for only 4% variation of LDL-C (Bertolini 2000) versus 8-11% in the general population (Frikke-Schmidt 2000), reflecting a very weak association. It is likely that, FH in which the LDLR locus is responsible for most of the LDL-C elevation and in which the normal LDL-R allele is highly up-regulated, the LDL-C rising effect of apoE4 due to the down-regulation of the LDL-R activity is completely masked (Chapter 17). The E4 allele has been associated with lower HDL-C in adults (Miltiadous 2002) as well as children where apoE4 account of 74 % of HDL-C variance (Wiegman 2003). The apoE2 allele has also been associated with lower LDL-C in some studies, especially FH women. FH patients with E2E2 (occurrence of this coexistence = 1/500 x 1/100 = 2/100.000) have higher VLDL-C and triglycerides, especially in men and may develop dysbetalipoproteinaemia with very elevated TC, TG and CVD risk (Carmena-Ramon 2000).

OTHERS. In FH, the influence of **lipoprotein lipase (LPL)** activity may be complicated: theoretically, an increase activity may accelerate the catabolism of large TG-rich VLDL and subsequently improves hyperlipidemia but it may also enhance the generation and accumulation of small dense LDLs, which are more atherogenic. In animal models (Watanabe heritable hyperlipidemic (WHHL) rabbits), transgenic overexpression of human LPL ameliorated the hypertriglyceridemia and hypercholesterolemia, but increased small dense LDL and atherosclerosis (Koike 2005). In humans, low postheparin LPL levels (Dugi 1997) or LPL defective polymorphisms (D9N, N291S) were associated with reduced HDL-C, elevated TG and higher CVD risk (OR: 2.8 for D9N and 3.9 for N291S) in FH patients to the same extent than in non-FH patients (Wittekoek 1999 & 1998). In HeFH subjects, **cholesterol ester transfer protein (CETP)** TaqlB variant is associated with higher HDL-C levels, lower LDL-C, lower CVD risk but also lower appearance of arcus cornealis and xanthomata (Mohrschladt 2005, Carmena-Ramon 2001). **The microsomal triglyceride transfer protein (MTP)** gene promoter variant (MTP-493T) which is associated with lower LDL-C in non FH-subjects, is associated with lower triglycerides in FH (Bjorn-Lundahl 2000). This is probably explained by an effect on VLDL synthesis as the MTP plays a role in intracellular apoB lipidation.

MULTIPLE GENE ANALYSIS. A large panel of common polymorphisms have been examined in a recent study (Bertolini 2004) : APOE (2, 3, 4), MTP (-93G/T), APOB (-516C/T), APOA-V (-1131T/C), HL (-514C/T, -250G/A), FABP-2 (A54T), LPL (D9N, N291S, S447X) and ABCA1 (R219K). The following significant and

independent association were found: (a) plasma LDL-C with APOE, MTP and APOB; (b) plasma HDL-C with HL, FABP-2 and LPL S447X; (c) plasma triglycerides with APOE and APOA-V) and (d) CAD positively with FABP-2*54TT and negatively with ABCA1*19RK+KK. In multivariate analysis, independent predictors of high CHD risk were male sex, age, arterial hypertension, LDL-C and FABP-2 54TT genotype, and absence of the 219RK and KK genotypes of ABCA1.

Another recent study (van Aalst-Cohen 2005) tried to determine the extent to which common genetic variants (ABCA1, APOA-IV, APOC3, APOE, CETP, HL, LPL, and two paraoxonases) can explain the variation of HDL-in heFH patients : multiple linear regression showed that, together, these polymorphisms explain only 3.9% of the variation of HDL-C plasma levels, 12.5% when significant two-way interactions between polymorphisms were taken into account, and to 32.5% when sex, smoking, alcohol use, BMI, and concomitant beta-blocker were incorporated in the models.

3.4.6.2.Genes not related to lipid metabolism.

One example is Beta-thalassemia (Calandra 2004, Deiana 2000). Carriers of this disease have lower TC and LDL-C than noncarriers, and, this LDL-lowering effect was also present in FH-subjects where a reduction of 30% of LDL-C is observed. The LDL-lowering effect may be related to (a) the erythroid hyperplasia, which increases the LDL removal by the bone marrow, and (b) the chronic activation of the monocyte-macrophage system, causing an increased secretion of cytokines (interleukin-1 and -6, and TNF□) known to affect the production and clearance of LDL. It is not known yet whether this LDL-lowering effect slows the development of CVD in FH.

Other genes variant such as Growth hormone receptor variant (L526I) has been shown to modify HDL-C (Leu/Leu : 37 +/- 2 ; Leu/Ile : 41 +/- 2 and Ile/Ile : 50 +/- 4 mg/dl) in FH (Takada 2003). Growth hormone plays a role in the regulation of lipoprotein metabolism and is an independent determinant of TC and triglycerides. In defect of growth hormone receptor (Laron syndrome), short stature, specific facial appearance, elevated serum growth hormone levels, and decreased insulin-like growth factor I levels is associated with increased CVD risk due to elevated LDL-C.

3.4.6.3.Gene involved in atherosclerosis without lipid effect

Allelic variations in the genes for ACE (O'Malley 1996), for ARII (Wierzbicki 2000) and for MTHFR (Kawashiri 2000) increase predisposition to early CAD in FH-subjects, but these effects are relatively small and not always confirmed (Jansen 2005). The ACE was also associated with lipoprotein changes in FH-patient (O'Malley 1996). A multigenetic study examining the genetic determinant of CVD in a large cohort of FH found that the A allele of G20210A polymorphism in the prothrombin gene (Jansen 2005) was strongly associated with CVD risk.

3.3.6.4. Lipid lowering genes that neutralise FH ?

There are several descriptions of FH-families where carriers of the LDL-R mutation had normal cholesterol (Nora 1985, Hobbs 1989, Sass 1995, Knoblauch 2000). These observations suggest the existence of strong “lipid-lowering” gene(s). We also identified a similar family with FH whose one FH-affected member had normal LDL-C and does not manifest atherosclerosis. Recently, mutation in the PCSK9 gene has been incriminated to explained low levels of LDL-C in the population

(Cohen 2006). One of this mutation explained a case of normal cholesterol in at least one of the FH family previously described by Helen Hobbs in 1989 (Helen Hobbs , personal communication).

3.4.7. Interaction with risk factors?

There is no evidence of interaction between risk factors and FH. What is observed on lipid parameters indicates that the loss of half of LDL receptor modulates the magnitude of LDL-C variation but does not change the pattern of variation (simple additive effects) of lipid profile (LDL-C, HDL-C and TG) associated with other factors such age or gender. The lack of evidence of interaction may of course result from the lack of statistical power due to the difficulty to collect large sample sizes and to reduce genetic variability at the LDLR locus by including only patients with the same mutation. In fact, the only known example of interaction occurring in presence of LDLR mutations is the one with APOE2 homozygosity which produces the very rare phenotype of dysbetalipoproteinemia type III.

3.4.8. Consequence of FH variability

Due to this phenotypical variability, it is difficult to set clear criteria for suspecting of FH, to establish a clear prognosis only based on a mutation and to recognise a clear dominant pattern of inheritance in some familes. In reality, FH resembles often to a more complex pattern with an important genetic influence and with a more modest but yet significant environmental influence. This is the case for the phenotype “high LDL-C” which differs in individuals with the same mutation, even in the same family. This is still more pronounced for CVD which does not necessarily segregate with the mutation (or even with HC) as it exists also commonly in the general population. On the other hand, this great phenotypic variability is a proof of the highly modifiable character of the prognosis of FH. Although there is no clinical endpoint study in FH, there is indirect evidence that treating FH by statins can help (SBRG 1999). Even environmental factors like diet are able to mitigate its severity[11]. The finding that classical risk factors still play a role in FH patients, even in those who are on lipid lowering medication (Neil 2004), underscores the importance of vigorous correction of these factors in these patients.

3.5. Other clinical findings

Attempts to link FH with Alzheimer's disease were unsuccessful (Bertram 2007). Untreated and simvastatin-treated FH patients have greater increase of CK levels after exercises, compared to normocholesterolemic controls (Smit 1995).

3.6. Familial defective apolipoprotein B (FDB)

FDB is considered as less severe than LDLR-related FH (Myant 1993, Brugger 1996, Real 2003, Hansen 1997 & 1998, Miserez 1995). Compared to controls, TC is rised by 35-45% in FDB patients and by more than 70% in LDLR-FH patients (Miserez 1995, Ludwig 1990, Descamps 2001a & 2003b) whereas CVD risk is increased by 2.7-fold in FDB patients and by 8.5-fold in LDLR-FH patients. In the

[11] Chinese heFH patients who were living in Canada had higher LDL-C (289±50 mg/dL versus 169±42 mg/dL), greater prevalence of TX and of early CHD than those with similar mutations living in Canton, China (Pimstone 1998). These differences could be ascribed to differences in dietary fat consumption (calories consumed as fat was 34% in Canadian and < 20% in the poor rural areas around Nanjing).

large Danish population study where R3500Q, R3531C and R3500W were screened, only the R3500Q was associated with severe HC and increased risk of CHD (OR=7.0; 95%CI: 2.2 to 22; P=0.003) (Tybjarg-Hansen 1998). FDB homozygotes have LDL-C comparable to those in LDLR-FH heterozygotes and lives longer (most are between 40 and 60 years of age) than LDLR-FH homozygotes (Myant 1993).

3.7. Homozygous FH (hoFH)

The homozygous FH patients have the devastating phenotype of full-blown FH with extreme characteristics (called « malignant atherogenesis » Hoeg 1994). In general, LDL-C levels (>600 mg/dl) are inversely related to the residual LDLr activity. They develop cutaneous (planar) xanthomas in the first years of life on the knees, elbows and between the webs of the fingers, whereas TX and CHD develops later in childhood (Goldstein 2001). Some rare patients present also giant ectopic cholesterol xanthomas in the brain, in the mediastinum and in the muscles of the buttock (Yamada 1989, Francis 2005, Bhattacharyya 1980). These arise silently or may clinically simulate neoplasic tumours. Atherosclerosis develops initially in the aortic root, causing supravalvular aortic stenosis (Rallidis 1998) and extends into the coronary ostia (Srimannarayana 2004). The severity of atherosclerosis is proportional to the extent and duration of elevated LDL-C levels (calculated as the cholesterol-year score) (Hoeg 1994). Based on the LDLr activity measured in skin fibroblasts, receptor-defective hoFH patients (2–25% normal LDLr activity) who usually develop clinically significant CVD by age 30, have better prognosis than receptor-negative hoFH patients (<2% normal LDLr activity) who rarely survive beyond the second decade (Goldstein 2001). Extremely severe HC (above 1000 mg/dl) can lead also to pseudohyponatremia, retinal cholesterol thromboembolism and cholesteroloma of the lung[12].

[12] Hyperviscosity has been described in primary biliary cirrhosis with extreme HC (Rosenson 1990).

Chapter 2. Historical context of FH in Belgium.

In the early's 1990's, our interest in the field was stimulated by the fact that despite the impressive progress in the understanding of the pathophysiology and treatment of FH and a preferential regimen of reimbursement for statins in Belgium, many affected individuals were not diagnosed and adequately treated.

1. Only few Belgian FH patients were identified in the early 90's

Given the special rule of statin reimbursement for Belgian FH patients (Box 2-1) and the expected frequency of FH (we supposed 1/500 like in most countries), we could assume in the early 90's that most FH patients would have been already identified by their doctors and that, thus, most general practitioners knew at least one or two FH patients[13]. As a matter of fact, we could only strongly suspect FH in a dozen of patients in our lipid clinics and in a first survey in 1994 (by phone and mail) amongst more than 50 general practitioners and cardiologists (data not published), we found that none of their patients benefited of such reimbursement. More surprisingly, only very few doctors appeared to really suspect the existence of such disease. Amongst those who knew the disease, most claimed their difficulty to identify such patients on the basis of the criteria (specially the "tendon xanthoma criterion") established for the statin reimbursement. A second survey in 1995 near some regional "Mutualité" confirmed the fact that less than 25 patients in our region of 250000 inhabitants had the CATA reimbursement.

2. FH patients were undertreated

Most patients in whom we confirmed the diagnosis of FH along our works had been followed for many years by their doctors before visiting us. During these years, these patients were regularly tested for blood cholesterol and the blood test repetitively confirmed their high cholesterol levels. However, most had never been treated or were treated with inadequate drugs (fibrates) or insufficient dosage of statins (20 mg simvastatin) (see data in Chapter 6). The usual claimed arguments for not treating them properly were that they were too young, that hypolipemic treatments were too expensive and that it was not clear whether these treatments did really any good. In these early 1990's, various studies indeed raised the problem that cholesterol level below 160 mg/dL was associated with a significantly increased risk of death from cancer, hepatic cirrhosis, suicide, respiratory disease, trauma and alcohol dependence syndrome (Neaton 1992, Jacobs 1992). In these years, the only available statin was simvastatin (Zocor®) marketed at 10 and 20 mg tablet in package of 28 tablets. To reduce enough the very high cholesterol ("enough" at this time was to obtain cholesterol level below 250 mg/dl), required usually to prescribe

[13] 9000 out of the 17947 doctors registered as GP are considered to be active, that means, that they have more than 500 patient contacts per year (the remaining are retired, involved in administrative work or advisor doctors for private or national health insurance organisations, for workplaces or schools). Theoretically, there are 20.000 FH patients. Therefore, there may be on average 2 FH patients per GP.

80 mg of simvastatin (4 tablets a day and thus four 28-tablet packages a month). Such treatment was difficult to comply for individuals who were totally asymptomatic and was expensive (about 60 euros a month), especially in families where several young FH adults were financially dependent of their parents,

3. Family screening was rarely performed

We met also numerous cases of patients just recently discovered by chance for hypercholesterolemia although their affected father or mother died of early CVD several years prior to their visits. It was surprising that, at the time of the drama, no physician had taken the time to assess risk factors in the rest of the family. The reason for such omission was probably the ignorance of FH but, perhaps also the fact that, in the early 90's, CVD prevention only emerged in clinical practice and that thus, the idea to search for CVD risk factors in the young offsprings of parents suffering from CVD was not obvious for most doctors. Finally, economic factors were probably another reason: to screen and treat multiple family relatives of an affected index "in one visit" is a time consuming job, not reimbursed by medical financing systems.

4. Rules for statin reimbursement were not appropriate

In our clinical practices, we discovered many FH patients who did not meet the "Belgian criteria of statin reimbursement for FH" (Box 2-1). Very often, the criteria "tendon xanthomas" or "family history of (very) early CVD" were absent or only present in one relative. There was also the problem of small family size, of parents dead earlier from other causes, and for some rare cases, the problem linked to adoption or doubt about paternity. We were especially worried to observe that, amongst doctors, the criteria designed by our national health insurance for statin reimbursement in FH became the gold standard diagnostic test to identify or exclude FH, perversely contributing to diffuse a wrong idea about the disease.

Therefore … Nine years since Michael Brown and Joseph Goldstein received the Nobel Prize of Physiology and Medicine (1985) for discovering the defective genetic mechanism causing FH, six years since the "miracle" drugs ("statins") were made available for routine clinical use in Belgium (1988) and four years since the establishment of the preferential regimens of reimbursement for FH in Belgium (1990), our impression was that, in Belgium, very few persons with FH were clearly diagnosed and furthermore even fewer were taking any cholesterol lowering medication. Two years after my coming back from the Brown's and Goldstein's laboratory (1992), I decided thus to undergo this work on familial hypercholesterolemia in my own country.

Box 2-1. Payment of hypolipemic drugs in the Belgian health care system

Some of the principles of our health insurance system of Belgium and of the reimbursement rules for statins are reminded here. In Belgium, the National Health Insurance (in French, "I.N.A.M.I." or "Institut National d'assurance des Maladies et Invalidités" and in Dutch, "R.I.Z.I.V." or "Rijksinstituut voor Ziekteen Invaliditeitsverzekering", in English, "National Institute for Sickness and Disability Insurance") established the rules for drug and medical care reimbursement.

1. The structure of the Belgian health care system. Belgium has a system of compulsory health insurance. It is organized through non-profit sickness funds (called "Mutualités"). The sickness funds developed historically along political and religious lines. Membership of a sickness fund is compulsory, but the choice of sickness fund and care providers (GP, specialists, pharmacists, hospitals…) is free. Payment arrangement is different for service (visit to the doctor) for which patients usually pay the complete fee to the providers and are reimbursed partially by their sickness fund on submission of the bill, and for hospital care or pharmaceuticals where financing occurs mainly through "third-payer arrangements", in which the sickness funds pay the providers and the patients are only responsible for the co-payment (called "ticket modérateur", which is a percentage of the bill but with a maximal amount, that vary with time and with the type of care service).

2. The payment of lipid lowering drugs in Belgium. Lipid-lowering drugs are reimbursed by the National Health Insurance under certain conditions, first established in 1990 and changed successively in December 2003 and in June 2006[14]. Before 2003, patients who benefited of reimbursement felt into 2 possible categories. In **"category B" (CATBR)** which included most patients treated with statins[15] or others[16], the patient received the drug with a maximal expense of 10 to 16 euros ("ticket modérateur") by package of the drug. In **"category A" (CATAR)**, which is designated to profit FH patients, the patients received the drug at no expense at all (complete reimbursement by the national health insurance). First criteria for CATAR and CATBR in 1991 were the following.

- CATAR (only statins) required three simultaneous criteria: **1/** TC above 300 mg/dL, **2/** palpable tendon xanthomas in the patient and/or two closed parents (father, mother, brother, sister, grand-parents, uncles and aunts), and **3/** early coronary disease before 50 years (for men or women) in the patient and/or two closed parents (idem TX). In principle, the doctors had the obligation to provide (if requested by the medical adviser of the "mutualité") documents supporting the diagnosis and the age of occurrence of the CVD events in the relatives.
- CATBR (statins and others) required simply that, after a non-specified diet of at least three months, fasting TC remained higher than 250 mg/dL (for statins or fibrates) or TG remains higher than 200 mg/dL (for fibrates only).

[14] Another change occurred in June 1 (concerned only CATB reimbursement).

[15] Statins: in 1992, simvastatine (ZOCOR®), pravastatine (PRAVASINE®); in 1997, fluvastatine (LESCOL®) and atorvastatine (LIPITOR®); in 2003, rosuvastatine (CRESTOR®).

[16] Fibrates: fenofibrate (LIPANTHYL®), ciprofibrate (HYPERLIPEN®), bezafibrate (CEDUR®, EULITOP®), Acipimox (OLBETAM®), Bile acid sequestrants: cholestyramine (QUESTRAN®), colestipol (COLESTID®).

In both categories, the practical procedures were as follows:

- The physician had to fill a special form mentioning the particular condition of the patient and to send it to the local sickness fund of the patients ("Bureau local de Mutualité").
- All requests were centralised in the regional offices of the "mutualités" where medical advisers decided to approve or not the reimbursement. They sent the authorisation for CATA or CATB reimbursement to the patients.
- The patient brought the authorisation to the pharmacy along with the prescription of his doctor, each time he needed a new package of drug.
- Authorisation needed to be renewed at 12-monthly intervals by filling a new prolongation form mentioning the previous category and the current biological data under treatment. Whether or not authorisation was renewed however depended on the demonstration of a reduction of lipoprotein levels during treatment[17].

3. Where did these rules come from? No one could tell me (possibly the main actors are retired or deceased) on which basis the CATAR criteria were established in 1991. It is likely that the criteria were based on the early definition of FH used in papers published before 1990, like those of Gagne et al in Canada (1979), where FH was defined by the following criteria: (a) TC > the upper normal limits of TC in the population; (b) detection of HC or TX in at least two first-degree relatives; (c) exclusion of FCHL by the presence of type IIa lipoprotein pattern or TX in the relatives; (d) exclusion of secondary HC by the presence of TX or the persistence of HC despite the correction of the secondary cause. **In the Simon Broome register published in 1991** (SBRG 1991, but probably presented in congress before 1991), the "definite FH" was defined as: (a) TC > 290 mg/dl in adults or > 259 mg/dl in children or LDL-C > 190 mg/dl in adults or > 155 mg/dl in children; (b) PLUS TX in the patient or a first-degree relative. The "probable FH" was defined as : (a) the same lipid criterion; (b) PLUS family history of MI before age 50 years in a second-degree relative or before age 60 years in a first-degree relative OR family history of TC above 290 mg/dl a first- or second-degree relative.

Although this is not the subject of this thesis, it is worth to mention that the rule of CAT.B was as much surprising. From the start, this rule was viewed by most Belgian experts as obsolete. Indeed, in 1990, several guidelines (Consensus 1985, EAS 1987, EAS 1988, NCEP 1988) already stated that decisions concerning management of elevated lipid levels should be influenced by the overall cardiovascular risk (based on gender, smoking, blood pressure, diabetes, family history of CHD and age) and not by their levels of lipids alone. In these guidelines, the level of 250 mg/dl was the limit above which drug therapy should be indicated. This level was chosen because most European countries have a mean population cholesterol level approaching 230 mg/dl and because TC of 270 mg/dl was the inclusion criterion of most large drug trials of this time.

[17] We heard from some patients that their doctors believed firmly that this prolongation of reimbursement was also dependent on the condition that the cholesterol level remained above 250 mg/dL even under treatment. We heard about curious stories: some doctors used to stop the medication before measuring cholesterol for the renewal of the reimbursement; other simply claimed to the patient that he could not have any more reimbursement because he had cholesterol too low under the level of 250 mg/dL.

Chapter 3. Brief presentation of my personal projects

In this Belgian context, we initiated a project aimed to obtain objective information on the situation of FH subjects in Belgium and to develop better means for identifying these subjects amongst the population. The plan of our investigations has been organized in this sense of mission aimed to promote the best management of the Belgian FH citizens. Five questions have been currently approached.

1. What is FH in Belgium?

1.1. The spectrum of Belgian mutations causing FH

The spectrum of mutations causing FH was not known when we started our projects. From 1994 till now, we analysed the DNA of more than 650 patients. The tested patients come from various recruitment designs, from different parts of the country and from different ethnic groups (including not only French-speaking and Flemish-speaking Belgians but also Italians for example). In this part, I describe an updated list of the mutations causing FH in our country and comment briefly their distribution in Belgium and their particularities in comparison to other countries. The data presented here are an update of the data presented in the published review (Descamps 2000), papers (Descamps 2001a, 2001b 2003b), abstract (Descamps 1997b), papers in collaboration (Varret 1998, Van Leuven 2001) and of the data shared in the websites (www.umd.necker.fr and http://ucl.ac.uk/fh). Other issues regarding the reactions of our patients (and the social problems) associated with their genetic testing are presented in brief. This work was also a preliminary work to recruit genetically ascertained FH patients who helped us later to answer to the more epidemiologic and clinical questions.

1.2. The clinical characteristics of the FH patients of our lipid clinics

During these last 10 years, we tried to collect as much clinical characteristics as possible in the genetically ascertained FH patients recruited in our lipid clinics[18]. This gives an idea of the clinical characteristics of Belgian FH patients referred to lipid clinics and the evolution of our ability to treat these patients, including the feasibility of the application of the new FH guidelines in typical Belgian lipid clinics given the situation of FH in Belgium (Descamps 2006b). I also remind briefly the data of two previous works: the description of a group of patients with the most common mutation found so far in the French part of the country (C122X) (Descamps 1997a) and the systematic analysis of coronary status in FH patients recruited in our hospital over a 2-year period (Descamps 2003a).

[18] The lipid clinics included initially all patients refereed by their GP for suspicion of high CVD risk.

2. How frequent is FH in Belgium?

The frequency of FH subjects has never been examined in Belgium. The estimate of the frequency of FH is important to appreciate the Public Health impact as well as the extent to which diagnosis of FH is correctly performed in a country. As a matter of fact, it is often reported that FH is under-diagnosed worldwide. From the patients recruited in our lipid clinics and their relatives for whom a FH diagnosis (Descamps 2006b) was confirmed by genetic test, we tried to deduce a minimal estimate of the prevalence of FH in our region (Descamps 2000). From the result of this first survey amongst our patients, it became clear that any doctor (especially general practitioners (GP)), could easily encounter several FH cases in his routine practice. To establish this fact and diffuse this message to GP, we started in 1996 a project (Descamps 1997b) aiming to characterise and identify the families with FH in the province of Hainaut ("Genetic Hypercholesterolemia in Hainaut" or "GHHainaut" project).). As secondary purpose, this project aimed also at studying the feasibility of cascade screening using the collaboration of GP.

3. Why is it important to identify FH?

Because there were some doubts amongst many general practitioners about the necessity to identify these patients, we paid a special attention to develop arguments to convince them about the importance to diagnose FH. There are several reasons why a precise diagnosis may help to manage FH patients: clear diagnosis motivates the patients and the doctors for initiating early and intensive treatment, for screening actively other members in families (especially children) and for undergoing regular follow-up at the search of cardiovascular complications. However, another reason which was often questioned by GP and not yet explored in epidemiologic studies, concerned the difference of prognosis of FH patients compared to other patients that are clinically similar and confoundable with FH patients (but do not have a mutation). It is a fact that not all patterns of extreme hypercholesterolemia and family history of early CVD are the result of a monogenic disorder such as FH. Some may result from other causes such as the interaction of (poly)genic susceptibility and environmental conditions (excess of cholesterol consumption or diseases such as metabolic syndrome or diabetes, ...).

To answer this question, we explored the following hypothesis: amongst patients with clinical characteristics of FH (elevated total cholesterol and familial history of early cardiovascular diseases), do those who carried FH have greater atherosclerosis than those without FH? In patients of our lipid clinics, we examined their genetic status and their degree of atherosclerosis using carotid and femoral ultrasonography to measure intima-media thickness, thoracic computed tomography to search coronary calcification and stress test to demonstrate the presence of silent ischemia. (Descamps 2001a & 2003b). This is the first study that underwent a comparison of atherosclerosis between FH patients and patients who are clinically similar to them, because similar levels of cholesterol and similar family history of early CVD.

4. How to identify FH in current practice?

We developed new accessible tools and clinical criteria on which doctors, especially GP, could rely to identify FH. The main objective was to improve the criteria "tendon xanthomas (TX)" included in the current rule of statin reimbursement. Although the TX is a hallmark of FH, it is often not recognised by most doctors, because of misknowledge on how and where to search it but also because it is often not visible or palpable and it remains a subjective clinical sign. That is why we developed a test based on the measurement of thickness of Achilles tendon using ultrasonography (US) (Descamps 2001b). In order to establish the best tendon criteria for diagnosing FH, we compared the tendon data of 127 genetically ascertained FH patients with those of 160 NFH patients (presenting with various lipid profiles) and used receiver operating characteristics (ROC) curves.

It was also important to examine the diagnostic performance of other usual criteria such as cholesterol cut off and the presence of family history of early CVD. In most studies, their diagnostic performances are impossible to appreciate for the obvious reason that these criteria are used to recruit the cohort of suspected patients (selection bias). To examine more objectively the sensitivity and specificity, we used the data of the very large cohort of the Netherlands on lipid levels and on CVD morbid/mortality and developed a mathematical approach to evaluate the probabilities of finding FH associated to different levels of cholesterol (adapted in our country) or to different definitions of "family history" in variable circumstances (Descamps 2006a).

5. What would be the best rule of statin reimbursement for FH?

As the project was ongoing, the Ministry of Health organised the first "consensus meeting" on the use of lipid-lowering drugs in 2001. This was the occasion for us to pledge for changing the previous rule of reimbursement, in order to facilitate the recognition of FH by GP, to stimulate the awareness of FH patients about their special conditions and also to make justice by allowing more FH patients to take advantage of this preferential reimbursement (Descamps 2002). As I was writing this thesis, I felt important to examine the consequence of these new rules in term of efficiency of FH diagnosis and treatment with the two following questions: "1. How well do these criteria really apply to identify FH individuals today?" "2. How many individuals do benefit of CATA reimbursement today?" The first question can only be answered by probabilities based on a mathematical approach and some assumptions. In contrast, the second question was examined from the Pharmanet data from the National Insurance Health Organization, regarding the prescription of statins reimbursed for the specific indication of FH (Descamps 2006c). This allowed deducing some interesting conclusions about the evolution of the habit of prescriptions in this category. Moreover, the careful examination of Pharmanet data offered some supports to the theoretical conclusions developed in the first question.

Chapter 4. Methodology

Our plan was to select the patients suspected of FH on the basis of clinical criteria excluding tendon xanthomas and to use the tools of molecular genetic to confirm the diagnosis of FH. The project started in 1994 and is still ongoing today. During this period, there has been some minor evolutions in the criteria of selection to suspect FH amongst our patients and in the methods used to establish the DNA diagnosis of FH.

1. Subjects

Based on various sets of criteria used in the research papers of this time[19], we selected first (1994-1995) our patients on the basis of 3 criteria (Descamps 1997a).

- Cholesterol levels above the 95th percentile (equal to 300 mg/dl as we calculated from the data of the BIRNH study (Kornitzer 1989) (Table 4-1)) after standard diet and after correction of a eventual secondary cause of hyperlipidemia.
- Dominant pattern of inheritance of hypercholesterolemia.
- "TG<250 mg/dl" (to exclude familial combined hyperlipidemia)

Table 4-1. Values of total cholesterol in the Belgian population.

Age	Men					Women			
	M (SD)	Obs P90	Calc P90	Calc P95		M (SD)	Obs P90	Calc P90	Calc P95
25-34	211 (42)	268	265	280		199 (40)	243	250	265
35-44	229 (43)	285	284	300		215 (37)	264	262	276
45-54	239 (41)	296	292	306		240 (45)	298	298	314
55-64	241 (42)	291	295	310		264 (46)	323	323	340

"Obs. P90"= 90th percentiles given by BIRNH. "Calc. P90" = 90th percentiles calculated by us using the mean and standard deviation of BIRNH and assuming the normal distribution. As the observed and calculated P90 are very similar, we were confident to use the same calculation for the P95 ("Calc P95"). Notice that the concentrations observed in BIRNH were obtained without preliminary diet. However, a Belgian survey in 1999 demonstrated that the median cholesterol reduction obtained after standard hypolipemic diet advised by general practitioners was only 3.5% (Muls 1999).

Because the information to establish the "dominant pattern of inheritance of hypercholesterolemia" was difficult to obtain in a reasonable time or impossible in many families (too small or too scattered family), we modified the criteria:

- Cholesterol levels as above PLUS one of the following conditions :
- "Self reported family history of <u>any</u>[20] early cardiovascular disease" (first- and second-degree relatives; before 55 years for men and 65 years for women)
- "Self-reported family history of hypercholesterolemia" (the patient has heard that at least one of his relatives takes a lipid-lowering drug or has "very elevated" cholesterol).

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[19] When we started in 1994, most diagnostic algorithm like those of the "Simon Broome Register" in UK" (SBR 1991) included the criteria of "tendon xanthomas" or "family history of early CVD" that we did not want to include as obligatory criteria for the reasons mentioned above.

[20] NB: it was not known at this time that stroke was not clearly associated with FH

- Tendon xanthomas or thickening of Achilles tendon at US mainly for the patients of our lipid clinics (we had a hard time to have information on TX from GP).
- Hypercholesterolemia in children defined according to the American guidelines: LDL-C > 135 mg/dl (Ose 1995, Ose 2004)
- "LDL-C > 200 mg/dl" (based on the data of a cohort of 268 individuals sampled in 1996 from medical check-up in the out-patient clinics (Table 4-2).

"TG>250mg/dl" was also suppressed (because we found frequently hypertriglyceridemia (due to diabetes, metabolic syndrome or alcohol) in true FH patients.

Table 4-2. Lipids of 268 non FH-patients at the outpatient clinics of Jolimont.

	140 Women	128 Men
Age (years)	47 ± 15	47 ± 15
BMI (kg/m²)	25.6 ± 5.2	25.9 ± 4.0
TC (mg/dl)	231 ± 45	229 ± 46
HDL-C (mg/dl)	69 ± 20	54 ± 15
TG (mg/dl)	111 ± 55	146 ± 89
LDL-C (mg/dl)	140 ± 39	146 ± 42 [124]

This survey was necessary because the BIRNH survey (Kornitzer 1989) did not evaluate LDL-C. Our cohort had however the same distribution of TC, HDL-C, BMI and age than this previous study.
The 95th percentile of LDL-C was 198 mg/dl and the upper limit was 240 mg/dl.

2. Genetics tests

Here are some brief explanations of the methods that we currently use to identify LDLR and APOB mutations.

2.1. The R3500Q mutation of APOB gene

In APOB Gene, we only look for the G to A mutation at nucleotide 10,708 in exon 26 creating a substitution of glutamine for arginine in the codon for amino acid 3500 (R3500Q), as the other mutations of APOB gene have not been fully linked to severe hypercholesterolemia (Chapter 2). The R3500Q mutation of APOB gene is identified by RFLP after a mutagenic polymerase chain reaction (PCR) to introduce restriction sites at the FDB gene locus (Mamotte 1993).

2.2. Point mutations, small deletions/insertions in the LDLR gene

An exon-by-exon screening was performed using DGGE protocols according to the papers of Lombardi and Nissen (Lombardi 1995, Nissen 1995 & 1996). Each time that the electrophoretic pattern alerts that a PCR-amplified fragment of the gene was abnormal, we sequenced the corresponding exons. This was performed by radioactive cycle sequencing during the year 1995 to 2001 and by automatic sequencing afterwards. In both cases, the sequences are analysed manually. In some rare cases where the clinical diagnosis was clear but no mutation was found by DGGE we also performed the complete sequencing of all exons.

Strict measures were taken to assure that the nucleotide change was a true result by repeating the DGGE and the sequencing in a second PCR product of the DNA sample of patient (if possible from a new blood sample), or, if available, of DNA

sample from an affected relative. When possible, we also confirmed the nucleotide change by using a more direct technique (RFLP-PCR or ASO-PCR[21])

In exons where there was no polymorphism, an electrophoretic profile is specific for a given mutation. However, very tiny migration differences were sometimes hardly visible so that DGGE patterns may sometimes appear falsely similar. Therefore, we decided to sequence all abnormal patterns when discovered in a new family. Of course, once a mutation has been identified in an index patient, we can use DGGE to screen other members of the family. In an exon where one (or more) polymorphisms is (are) present, a same mutation may give different patterns (example: a mutation in exon 10 that has one polymorphism may give two possible patterns; in exon 12 that has 2 polymorphisms, it may give 4 different patterns).

2.3. The search of large rearrangements (deletions and duplications)

The search for rearrangement was first performed by sending in other labs (in the laboratory of Fred Van Leuven[22] in Leuven and laboratory of Steve Humphries in London[23] which used mostly Southern blotting), the samples for which there was a high suspicion of FH but where we did not find any significant point mutation by DGGE. Since 2005, however, we used the recently developed multiplex ligation-dependent probe amplification (MLPA) (Holla 2005, Damgaard 2005, Wang 2005). The work to characterize molecularly the rearrangements is ongoing.

2.4. Functionality of the identified mutations

For nonsense mutations, mutations causing frameshift and large rearrangements, there is no doubt that the LDL receptor is profoundly altered (incomplete protein or truncation). For missense mutations or mutations in splice site, the incrimination of the sequence alterations as cause of FH was examined with the following criteria (Cotton 1998, Lombardi 1997) before being classified as pathogenic.

1) ***The mutation changes a known functional or species-conserved amino acid.*** In the coding sequence, we compared the amino acid position between 7 species (human, hamster, rat, house mouse, rabbit, shark and xenopus; this covers 450.000.000 years of evolution) and, when the aminoacid belonged to repeat motives, we compared the relative homologous position within the other repeats. Of course, an amino acid substitution in a critical region of the protein with hypercholesterolemia is not an absolute proof. For example, the A370T variation (or "Stul polymorphism") (Kotze 1989) is not causing FH. To estimate the likelihood that a DNA variation affected a splicing site, we used the "splice-site predictor" (SSP) software as well as the "SLP" software[24] (the two gives concordant results so that we presented only the score of the first software)[25].

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[21] RFLP : Restriction Fragment Length Polymorphism site ; ASO: Allele specific Oligonucleotide.

[22] Fred Van Leuven: Experimental Genetics Group, Center for Human Genetics (CME), Flemish Institute for Biotechnology, K.U.Leuven-Campus Gasthuisberg O&N 06, B-3000, Leuven, Belgium.

[23] Steve Humphries, UCL Medical School, Rayne Institute, London, UK.

[24] These are freely available in http://www.fruitffly.org and http://genomic.sanger.ac.uk).

[25] It is possible to verify the predicted abnormal mRNA structure using lymphoblastoid cell line obtained and grown from the patient. However, we did not perform such interesting but quite laborious work.

2) ***Common polymorphisms are excluded after testing 50 normal individuals.*** Such criteria is also an insufficient proof as illustrated by "T705I" (initially called FH-Paris 9 by Hobbs), that was shown later to be non-pathogenic (Lombardi 1997, Heath 2003). As a matter of fact, to exclude the possibility that a mutation is a common polymorphism with a fair probability of 95%, we should test a sample of size respectively of 150, 300 or 600 patients, given a prevalence of polymorphism at 5%, 2%, 1% (1% is the cut off prevalence in the usual discrimination between "mutation" and "polymorphisms, see box 1-1.). In our DGGE analysis, we currently run control samples that are regularly changed so that we have analysed so far more than 800 control alleles for possible non pathogenic sequences.
3) ***No further mutation was found after scanning the remainder of the gene.***
4) ***The same mutation is observed in two unrelated probands***
5) ***The mutation co-segregates with HC in the family.*** We performed such segregation analysis as much as possible in at least 4 members of the family (at least two with HC and at least 2 without).
6) ***Functional studies at the cellular level.*** A two-colour fluorescence flow cytometry assay (FACS analysis) has been developed and used by us in 1995-1996 (Verhoeye 1996) and was later adapted by other investigators (Chan 1998, Raungaard 1999, Martinez-Botas 1999). FACS analysis offered some simplification compared to previous techniques. However, it did not allow confirming specifically the culprit of a discovered mutation, was very time consuming, very expensive, not suitable for great series of samples and needs freshly sampled blood (Descamps 1997c). Therefore, we did not pursue our lymphocyte assays.

3. Ethical disposition

I summarised here the debates with our local ethical committee and the final decisions about the practical attitude in our strategy to screen and identify FH.

3.1. The adult patients

First, it was important to distinguish two situations in these projects:
1. The patient of our lipid clinics. In this case, the causal evaluation of his HC is part of the diagnostic procedure requested by the visit of the patient. It is important however to examine whether the patient is willing to know the genetic nature of his lipid disorder and to be prepared to questions that people may ask when they are told to be "a carrier of FH". These questions may be different than in other genetic diseases. As FH is treatable, the patient is not in the situation to cope with dramatic future and with decision on family planning (marriage, reproduction, amniocentesis) like it is the case for genetic diseases such as Huntington's disease or muscular dystrophy. Most of the questioning that may face up the patients undergoing test for FH have been carefully examined and reviewed beforehand with the ethical committee.

If the test is positive, the patients may feel to be part of a ***"very high risk group"*** associated with the "FH label" (especially since the patients have accessed to the information on the web, which are too often displayed without nuance) and a change

of ***perception of controllability*** which may induce reactions such as fatalism or anxiety, sometimes unnecessary (patients with positive test but without greatly elevated cholesterol or with HC easily controlled by diet would feel very worried despite they do not have (any more) phenotypic expression. He may be confronted also to ***an inappropriate partner's reaction*** (If young and without children yet), the problem to ***deal with the risk for the health of his (future) children and the need (obligation?) to inform their brothers and sisters*** about their carrier status (knowing that this gives them a 50'% chance of being a carrier too). In contrast, if the test is negative, people without genetic condition could think that they may not request any cardiovascular prevention. We usually discussed these questions before asking a genetic test for the patients in routine practice. They are, of course, discussed again when the result is announced to the patient. This has to be explained case by case with the nuance associated with the phenotypic expression of the disease, other risk factors, the special family circumstances ….

As for all clinical studies performed in our Hospital, an informed consent for the realisation of a genetic test and for the use of their data for our studies was requested. We should also mention that all examinations (ultrasonography, stress test, CT scan) performed in all our studies were part of the good clinical practice applied for CVD prevention in "very high risk" patients.

2. The relative of a patient diagnosed as FH carrier. Compared to the realisation of a genetic test in a patient of our lipid clinics, the approach of relatives was the subject of more intense and passionate discussion with the ethical committee especially because, in this circumstance, the relative does not initiate themselves the medical investigation and has *a priori* no personal reason for concerns about a health problem and because, unless he comes to visit us, he does not have a direct contact with us, so that we could not explain him all the issues concerning FH (see above). Furthemore, like for the patients, the realisation of genetic analysis for inherited disease might bring all sorts of possible negative human feelings and psychological stress in people unaware of their genetic condition, of their lipid profile, of their family history and of their potentiality to transmit a "disease" to their descent. Box 4.1 summarises the arguments, rights and duties in such circumstances. Many of these rights and duties are in conflict each other. For example, for the doctors, there is clearly a potential conflict between his duty to maintain patient confidentiality and his duty to inform at-risk family members. The debate of these considerations in the ethical committee have brought the necessity to have clear attitude regarding some practical issues that (Box 4.2) and the decision of the following principles to adopt in our strategy.

1. The proband is asked to contact his relatives, to discuss with them about the problem of cholesterol they may carry and to advise them to consult his doctor.
2. At a next visit, the proband provides us with the address of the GP of his relatives.
3. We contact each GP of the relatives to advise him to check blood cholesterol in his patient. At this moment, we provide also a complete explanation about the possibility, the purpose and the interest of a genetic test.
4. Finally, a genetic analysis is proposed only if the cholesterol is found elevated and if the relatives and his GP agree.
5. If done, we sent the result of the genetic test to the GP, along with explanation (often by phone) about the significance of the genetic test.

This strategy involves the active participation of the patient, his relative and the GP of his relative (patient-relative-GP approach). However, if the relative has no GP, it was decided that he could eventually contact us directly. Later on, after the beginning of our project, much literature has debated the subject (Newson 2005).

Box 4.1. Arguments, rights and duties in contacting a relative of a FH patient.

1. FH is a serious condition, is dominantly inherited but can be controlled by adequate medications.

2. What the relatives knows a priori. The dramatic occurrence of very early heart attack deaths in FH families is usually well known by relatives, and many relatives in these families already worry about having a heart attack death, often without knowing their cholesterol levels.

3. The ***"right to know"*** for the relatives. The fact that the occurrence of heart attack in the family is directly related to cholesterol levels may be an important information that may relieve the anxiety of the relatives: those who are found to have normal cholesterol may be reassured and those with very elevated cholesterol levels may be also reassured by the fact that early death can be prevented.

4. The ***"right not to know"*** for the relatives. One may also wonder if it is moral to expose to genetic screening somebody who has not initiate himself the medical investigation. Some people may not want to know their genetic predispositions, perhaps because of concerns about insurance and employment discrimination, social stigmatization, or increased anxiety.

5. The ***"moral obligation" for the index patient*** to inform his relatives about their genetic risk is in the order of "duty to rescue" for which a party can be held liable for failing to come to the rescue of another party in peril.

6. The ***"right for confidentiality" of the index patient***. To inform a relative about its potency to share a genetic health problem involves taking the risk to inform another person about his own health problem.

7. The ***"moral (professional) obligation" of the doctors*** of an index patient to inform the relatives about his genetic risk. Do the professionals have a moral duty to inform relatives at risk of FH if the index patient is unwilling to do so?

8. The ***professional's duty to maintain patient confidentiality***. Such duty is founded on the premise that it fosters trust in physicians, which encourages patients to seek medical care and to discuss sensitive medical concerns. Confidentiality is based on respect for patient autonomy and for the fact that the patient controls the access to their personal information

9. A concept that is emerging not only in clinical genetics but in many fields in medicine, is that ***"the patient is the family".*** According to this view, genetic information is the property of the family, which justifies screening relatives.

3.2. The children

We decided to give the parents who were suspected or genetically confirmed for FH the information that it is better to test cholesterol in children. This has the advantage

to exclude the possibility of homozygous FH (especially if both parents have FH or simply HC). If we had identified the causal mutation in the parent(s), we informed them about the possibility to precise the causal diagnosis in their HC children by the same genetic test, especially if the HC was not clearly discriminative.

Box 4.2. Four practical issues that we debated

1. "In the case of FH which is a preventable disease, should relatives be alerted?" The answer was yes at an ethical point of view. But, as a matter of fact, in common law, there is no general duty to come to the rescue of another and a person cannot be prosecuted for doing nothing while another person is in peril. In approaching relatives, it is thus important to clearly discern situations where informing relatives can be morally justified or morally obliged.

2. ***"Should the proband be invited to contact his or her family members ("Family contact") or should we contact family members directly ('direct contacting')?"*** The second method appears of course far more effective in term of recruitment but the discussion in our ethical committee turned around the fact that such "aggressive" strategy of screening relatives could bring useless angst and confusion. The method by which index patient initiates contact with relatives ("Family contact") represents the standard practice in clinical genetics.

3. "What to do if the patient declines to share genetic information with relatives?" May a doctor breach the confidentiality of genetic information? According to the Belgian law, health-care professionals have been required to breach confidentiality where the public interest is at stake. For example, it became a "legal" duty to warn identifiable third parties of a threat of violence or dangerous contamination (AIDS). Whether health-care professionals have an ethical or legal duty to inform family members who are at risk for genetic diseases, regardless of a patient's wishes is a real dilemma. Clinicians may need to decide when or whether an ethical duty to inform at-risk family members about an increased genetic risk overrides the duty to maintain the confidentiality required by the patient.

4. "Should the relative contact us before and after the realization of the test?" For those of whom DNA was mailed by GP, without the possibility for us to encounter the patient, we were concerned by the fact that this would sidestep meaningful face to face genetic and general counselling about the disease. It is however a problem to interfere in the established relationship between the GP and his patients.

3.3. DNA Banking

The information given to the patient before performing a genetic test included the fact that his DNA sample is used primarily for FH diagnosis, with a provision that, after this primary analysis, it will be stored for future researches related only to lipid metabolism. We mentioned to the patient that he (and his doctor) will be informed of any future finding, provided that this is helpful for lipid management. The DNAs were stored according to the following procedure. When blood sample arrives in our laboratory, it receives a code with the correspondence only known by 2 persons (the medical (OD) and one technical responsible of the laboratory) whereas other technicians proceed their analysis only on the basis of the codes. The DNA samples were systematically destroyed with the completion of all meaningful analysis related

to lipid metabolism or after a delay of 5-7 years, as the quality of the DNA became too low after this period.

4. The recruitment of the patients

Since 1995, the recruitment has proceeded according to 3 strategies differing in term of geographical expansion and recruiters. We started from our own patients and their relatives but we extended the recruitment to distant patients in 1996 for the general practitioners participating to the GHHainaut project and in 2003, for all Belgian doctors asking us a DNA service in the perspective of statin reimbursement.

4.1. Patients of our lipid clinics

Most of these patients lived around the hospital and were referred to us by their GP for very high levels of cholesterol, family history or early CVD or inefficacy of usual hypolipemic treatment. The geographical area where live these patients includes the regions of La Louvière, Soignies, Le Roeulx, Binche, Trazegnies, plus two other regions around the hospital of Nivelles and Lobbes where we also consult (Fig. 4-1; about 250000 inhabitants). The data (lipid, risk factors, cardiovascular status and (for many of them) subclinical atherosclerosis) were used in the papers described in this thesis. The strength and interest of these data resulted from the facts that standardized procedures were used systematically by the same clinical, biological and radiological examinators and were repeated over the following years.

Table 4-3. Various types of recruitment of patients

	Our lipid clinics	**GHHainaut project**	**DNA service**
Period	Since 1992	1996-1997	Since 2001
Recruiters	Ourselves	GP of Hainaut	All doctors
Geographical origin	Our area (see map)	Hainaut	Hainaut, Brabant, Namur +Flanders
Inclusion upon strict criteria	Yes	Yes	Out of control *
Clincal an family Information	Complete	Variable	Not always available

* First criteria for the 100 first patients followed by extended criteria. ** although criteria requested

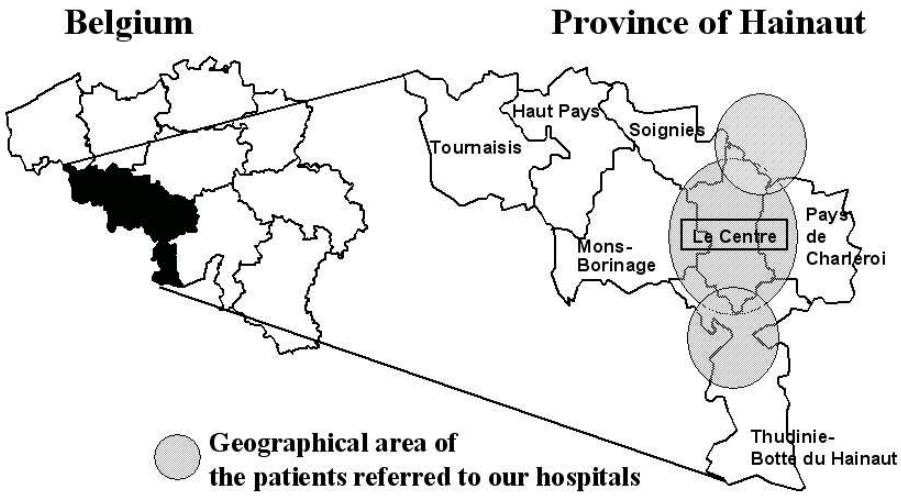
4.2. Patients from the GHHainaut project

The GHHainaut study (Genetic Hypercholesterolemia in Hainaut, Chapter 7) was limited to the sub-regions of “Pays de Charleroi”, “Tournaisis” and “Mons-Borinage” and did not include our regions where we had already developed a proactive recruitment of the suspected patients (see 4.1.).

4.3. DNA service for statin reimbursement after 2003

Following the change of rule of reimbursement in 2003 (Chapter 14), many Belgian doctors sent us blood samples to confirm the diagnosis of FH by genetic test. To interested doctors, we sent formulary informing about the best indications of prescribing the test, in order to limit the number of useless prescriptions.

Figure 4-1. Geographical location of the recruitment for patients suspected of FH.



4.4. Collaborative works with Flanders

We also performed collaborative works with Jean-Paul Deslypere (80 index patients in 1996) in Gent (Descamps 1997a and 1997d), Paul Van Combrugge in Aalst, Guy de Backer of Gent (6 index patients in 1997), Fred Van Leuven and Eric Muls in Leuven (12 index patients in 2000) (Van Leuven 2001).

5. Information and awareness-raising amongst GP about FH

Because in the early 90's, very few GP appeared to be aware of the disease, it was crucial to diffuse useful and updated information about FH through conferences in GP groups (dodecagroupe & Glem) and Belgian medical journals widely distributed across the nation and usually read by the GP (Box 4-3). We emphasized the idea that FH needs to be recognised and informed about our current projects of research.

6. Funding for the project

The projects were supported by our hospital (scientific count), la region Wallone, "la Ligue Cardiologique Belge", the Belgian Lipid Club as well as personal funding. The scientific count of our department of internal medicine was fed through the financial supports obtained from pharmacolagical companies (Astra-Zeneca, Bristol Myer Squibb, Merck-Sharp-Dohme, Pfizer) for the participation to international studies or to educational conferences.

Box 4.3. Papers in Belgian medical journals published in the two national languages (French/Flemish)or in englsh(*). All published by Descamps OS.

- L'hypercholestérolémie familiale. Revue Pratique de Médecine Générale 1994. 5, 35-39.
- L'hypercholestérolémie familiale en Belgique. Lipid Letter 1997. 8, 2-7.
- Diagnostic moléculaire de l'hypercholestérolémie familiale. Lipitrends 1997. 8 (2), 3-4.
- L'hypercholestérolémie familiale. Entrevue 1997. 4, 9-13.
- Génétique de maladies cardio-vasculaires. Journal du médecin 1998. 5, 15-19.
- L'hypercholestérolémie familiale homozygote. Lipitrends 1998. 2, 5-6.
- L'hypercholestérolémie familiale. Notre Coeur, nos artères 2000. 3, 6-10.
- A propos du bon et du mauvais cholestérol. Lipid letter 2001. 12, 5-14.
- Patients avec une hypercholestérolémie sévère et une histoire familiale. Lipid Letter 2002. 13(2), 5-8.
- Diagnostic moléculaire de l'hypercholestérolémie familiale. Lipid Letter 2003. 14(2), 14-16.
- Diagnostic moléculaire de l'hypercholestérolémie familiale. INAMI/RIZIV 29 mai 2002 (Question 2/1)

  http://inami.fgov.be/drug/fr/pharmanet/consensus/2002%2D05%2D28/pdf/lv.pdf;p 30-32
- Familial hypercholesterolemia in a Belgian community. Acta Clinica Belgica 2000. 55(6):327-333.
- Hypercholestérolémie chez l'enfant ? Guide de Poche du Belgian Lipid Club. 2004. Ducobu Jean, Heller FR, Van Gaal L. 4th Edition, 2004.
- Les tests génétiques dans l'hypercholestérolémie familiale. Lipid Letter. 2005. 17(2), 5-9.
- L'hypercholestérolémie familiale. Louvain Médical. 2005. 124 (4), 153-157
- L'hypercholestérolémie familiale. Vaisseaux-Cœur-Poumon. 2005. 10(8) ; 217-221.
- L'hypercholestérolémie familiale. Diagnostic et traitement. Médisphère 2005. 260 ; 26-30.
- L'hypercholestérolémie familiale chez l'enfant. Lipid Letter. 2005. 17(2), 9-11.
- European Network of Genetic Dysipdemia (ENID). Journal du Médecin 2005.
- Faut-il traiter l'hypercholestérolémie chez l'enfant ? Agenda pédiatrie 2006. 47; 4-5.
- Infarctus avant l'âge de la retraite et cholestérol très élevé. Notre Cœur, nos artères* 2006, 4: 6-10.
- From hypercholesterolemia to hypocholesterolemia. Lipid Letter 2006. 18(4), 5-9.
- Histoire d'un nouveau gène. Vaisseaux-Cœur-Poumon. 2007. in press.