Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
BIOCHIMICA, BIOLOGIA MOLECOLARE
E BIOTECNOLOGIE

CICLO XXII

COORDINATORE Prof. Francesco Bernardi

ABCA1 TRANSPORTER IN MONOGENIC DISORDERS OF CELL CHOLESTEROL METABOLISM: TANGIER DISEASE AND FAMILIAL HYPERCHOLESTEROLEMIA

Settore Scientifico Disciplinare BIO/11

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Anni 2007/2010

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Table of Contents

List of Figures

List of Tables

Abstract

Background: ABCA1 is an ubiquitous plasma membrane transporter that promotes the efflux of cholesterol and phospholipids from the cell membrane to an extracellular acceptor, known as apolipoprotein A-I (apoAI), the main protein constituent of plasma high density lipoprotein (HDL). ABCA1 mediated cholesterol/phospholipids efflux leads to the extracellular formation of HDL. Loss of function mutations of both ABCA1 alleles is the cause of Tangier Disease (TD), a recessive disorder characterized by extremely low levels of plasma HDL, increased risk of cardiovascular disease (CAD) and other clinical manifestations. Heterozygous mutations of ABCA1 gene cause Familial HDL Deficiency (FHD), characterized by half normal level of HDL-C and increased CAD risk. Familial Hypercholesterolemia (FH) is a monogenic disorder due to mutations of LDLR gene. In addition to the striking elevation of plasma LDL, FH patients (especially homozygotes) have greatly reduced plasma HDL levels, suggesting that they may have a defect in ABCA1-mediated cholesterol efflux pathway. Aim: Specific aims of this work were: i) the identification and functional characterization of several new ABCA1 mutations found in TD and FHD; ii) the study of ABCA1 regulation in fibroblasts derived from FH patients. Methods: ABCA1 gene was analyzed in 27 subjects with familial low HDL-C levels, by DHPLC and direct sequencing. Among the novel 22 mutations identified, 3 splice site mutations, 6 missense mutations and 1 frameshift mutation were investigated. The effect of splice site mutations was investigated ex-vivo, using patients' fibroblasts or blood mononuclear cells, or in vitro by looking at transcripts generated by the expression of a mutant ABCA1 minigene in a heterologous cell system. ABCA1 protein expression was studied in eight FH derived fi-

broblast cell lines under different conditions: basal culture conditions, stimulation with free cholesterol (FC), 22-hydroxycholesterol/9cis-Retinoic Acid (22OH/9cRA), or the LXR agonist T0901317. Cholesterol efflux to ApoA-I was also measured in these fibroblasts. Main results: 22 novel ABCA1 mutations were reported. The effect of 3 splice site mutations (c.814-14InsA, c.2961-2A>C, c.4773+1G>A) was investigated with an ex-vivo or with a minigene approach. The c.814-14InsA resulted to be non-pathogenetic, the c.2961-2A>C resulted in the generation of three abnormally spliced mRNA species, the c.4773+1G>A caused the formation of an ABCA1 protein containing an in-frame deletion, which was poorly synthesized by cells. Five additional fibroblast cell lines derived from TD or FHD patients were studied in terms of ABCA1 protein expression and function. Fibroblasts carrier of R587W, A1046D, D1099Y, H1600R/M586Fs.629X, R130K/N1800H showed a null ABCA1-mediated cholesterol efflux. ABCA1 protein expression was studied in eight FH fibroblasts under different stimuli. Under basal culture conditions ABCA1 expression resulted lower in FH cells with respect to control cells. This reduction was observed also after stimulation of cells with a load of FC or with 22OH/9cRA, known to stimulate ABCA1 expression via the transcription factor LXR. Treatment of cells with a non-steroidal LXR agonist (T0901317) was able to restore an ABCA1 expression similar to that of control fibroblasts. Also ABCA1-mediated cholesterol efflux in FH fibroblasts was lower than in control fibroblasts. Conclusions: Among 27 individuals with low HDL-C, 22 novel mutations in ABCA1 gene were identified. Three splice site, 6 missense and 1 frameshift mutation were functionally characterized in TD/FHD derived cells, allowing us to gain insight on genotype-phenotype correlations. Furthermore we demonstrated a defect in ABCA1 protein expression and function in FH, which may be partly responsible for the low plasma HDL-C levels exhibited by FH patients.

Chapter 1

INTRODUCTION

1.1 ABCA1 protein

1.1.1 ABC proteins family

ATP-binding cassette (ABC) transporters constitute one of the largest known protein superfamilies. These evolutionary highly conserved multispan transmembrane molecules use the energy of ATP hydrolysis to translocate a broad spectrum of molecules across the cell membrane. Substrates that are transported by ABC molecules include lipids, peptides, amino acids, carbohydrates, vitamins, ions, glucuronide and glutathione conjugates and xenobiotics. In eukaryotes, ABC transporters are located in the plasma membrane, in the membranes of intracellular compartments such as the Golgi, endosomes, multivesicular bodies, endoplasmic reticulum, peroxisomes and in mitochondria. To date, 59 members of the human family of ABC transporters have been identified which, based on their structural relatedness, are subdivided into 7 families, designated ABC A–G [1,2].

Among these we cite:

- Subfamily ABCA, including 12 transporters, which is distinguished from others for being expressed only by multicellular organisms. In this subfamily, transmembrane domains and ATP binding domains constitute a single polypeptide.

- Subfamily ABCB, including transporters involved in multidrug resis-

tance.

- Subfamily ABCC, including proteins which mediate ions transport, toxins elimination and signal transmission. The most famous is CFTR (Cystic Fibrosis Transport Regulator), which mediate Cl2 export: functional defects in this transporter are responsible of Cystic Fibrosis.

Functional ABC transporters show a common global organization. So-called "full-size ABC transporters" are typically composed of two tandemly linked functional units while "half-size transporters" are usually composed by a single unit; each ABC unit (200-250 aa) contains 2 short, highly-conserved peptide motives directly involved in ATP binding (called Walker A and Walker B) and an "ABC signature" localized between them.

Being membrane transporters, ABC proteins contain one or more transmembrane domains, tipically constituted by 6 helices, which can either be part of a single polypeptide (full-transporters) or be involved in a multiprotein complex bound to the membrane, so that they can form homo- or heterodimers, which consist of two half-size transporters that are encoded by distinct genes.

It is thus conceivable that full-size ABC transporters, as large components of membrane associated multiunit complexes, are not necessarily restricted to serve one defined function but are likely involved in a spectrum of biologic activities depending on the specific assembly of the functional complex and the biologic activities of their interaction partners [3].

1.1.2 ABCA1 protein structure

ABCA1 (formerly known as ABC1) is the prototypic member of ABCA subfamily and was initially identified by Luciani et al. [4] in mouse in 1994; 5 years later it was found to be responsible of two human genetic diseases [5], familial high-density lipoprotein (HDL)-deficiency (FHD) and Tangier disease (TD), which identified ABCA1 as a major regulator of HDL metabolism [6-8].

ABCA1 is an integral membrane full-size transporter formed by two mirror halves each one containing 6 transmembrane domains, one large, highly-glycosylated, extracellular loop and one Nucleotide Binding Domain (NBD)

composed by two conserved peptide motifs known as Walker A and B (thus it binds two ATP molecules). Both amino and carboxi-terminals are intracitoplasmatic [9]. The two large extracellular loops are linked by a disulfide bond.

It is a 2261-amino acid polypeptide with a molecular weight of 220 kDa [10,11].

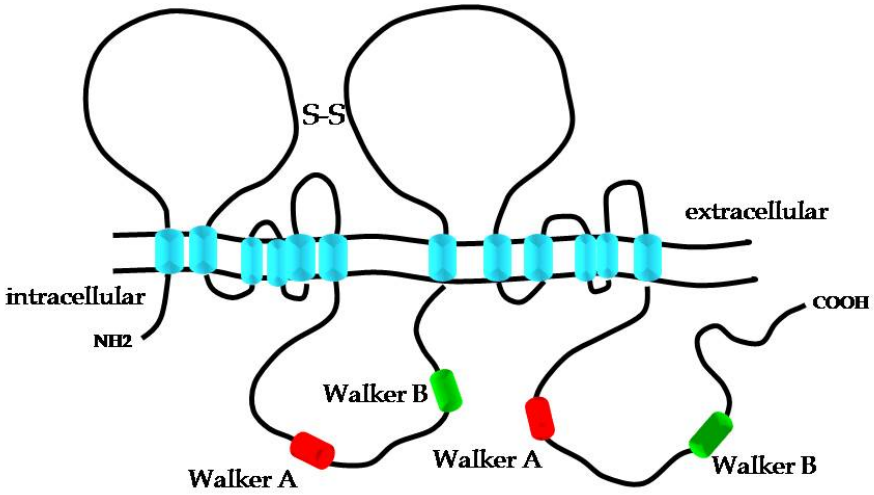

Figure 1.1: **ABCA1 protein structure.**

ABCA1 is a ubiquitous protein and promotes the unidirectional active transport of two major components of the plasma membrane, cholesterol and phospholipids, to an extracellular acceptor represented by some apolipoproteins (the main one is apolipoprotein A-I, apoA-I) [12,13]. This ABCA1-mediated efflux is one of the first steps involved in the Reverse Cholesterol Transport (RCT), a process by which cholesterol is eliminated from peripheral cells and transferred to the liver via the plasma compartment.

A fraction of ABCA1 is located in internal endocytic compartments where it may facilitate cholesterol efflux from early/late endosomes and lysosome [14]; it is also likely that ABCA1 influences vesicles trafficking from Golgi apparatus, maybe through its phospholipid-transferase activity [15].

Recent studies, however, suggested that ABCA1 transports cholesterol to apolipoproteins exclusively at the cell surface [16-18] thus different mechanisms of action are still proposed and have to be considered.

1.1.3 Cell cholesterol efflux

The intracellular cholesterol content is the result of three different metabolic processes: endogenous synthesis, LDL-mediated internalization and elimination (see 1.5.2). The combination of these mechanisms is highly dynamic to meet cellular requirements and it is important that the equilibrium is maintained to avoid a damaging over-accumulation of free, unesterified cholesterol (FC). Even a too large accumulation of esterified cholesterol (EC) is responsible of cellular damage as it happens in macrophages. This is the reason why a redundancy and complementarity of mechanisms exist to manage cell cholesterol efflux.

Four are the main processes involved:

i) the first one is a bidirectional passive diffusion mechanism, where free-cholesterol molecules spontaneously dissociate from the plasma membrane, diffuse through the aqueous phase and are incorporated in high density lipoprotein (HDL) particles by collision [19].

ii) The second mechanism is a bidirectional transport mediated by scavenger receptor class B type 1 (SR-B1): on one side SR-B1 is the receptor for HDL, mediating the transport of cholesterol and cholesteryl esters from HDL particles [20,21]; on the other side it favours the excretion of cholesterol via a facilitate passive transport. It is primarily expressed in liver and nonplacental steroidogenic tissues where SR-B1 directly interacts with HDL particles, releasing cholesterol and phospholipids localized on the plasma membrane [22-24].

iii) The third mechanism is an ABCA1-mediated unidirectional transport: ABCA1 is involved in ATP-dependent cholesterol efflux and its main acceptor is lipid poor apoA-I, the main peptide component of HDL particles [25].

iv) A fourth, recently identified, mechanism proposes the involvement of the ABC transporter G1 (ABCG1), widely expressed in human tissues, in promoting the efflux of cholesterol to mature HDLs. This ABCG1-mediated efflux has not been completely characterized yet but it probably contributes and complements ABCA1 transport.

It is hard to define which of these four mechanisms is the most important

due to the fact that they have different roles in different types of cells. The most efficient seems to be the ABCA1-mediated cholesterol efflux: phospholipids and unesterified cholesterol are transferred to a monomolecular, pre-β-migrating, lipid-poor or lipid-free form of apoA-I. This mononuclear form of apoA-I is quite distinct from the pre-β-migrating discoidal HDL which contain 2 or 3 molecules of apoA-I per particle and which are present as minor components of the HDL fraction in human plasma [26,27]. The cholesterol-phospholipid complex transferred to apoA-I forms or increases its lipid content, creating nascent, pre-β-HDL which increase their lipid content, during their movement in lymphatic and blood circulation. Free cholesterol taken up by HDL is then esterified by lecithin:cholesterol acyltransferase (LCAT) and hydrophobic cholesteryl esters are retained into the core of HDL so that new cholesterol molecules can be translocated on the HDL surface. The enrichment of cholesterol and localization of cholesteryl esters in the core of the particles lead to transformation of discoidal pre-β-HDL into spherical mature HDL.

If ABCA1-mediated cholesterol and phospholipid transport doesn't occur, then apoA-I is not enriched in lipids and is rapidly catabolised by the kidney. The total amount of HDL in the plasma therefore shows a large decrease.

1.1.4 Reverse Cholesterol Transport

Reverse Cholesterol Transport is a process where exceeding cholesterol is removed from peripheral, extra-hepatic tissues and transported to the liver for reuse in enterohepatic cycle where it can be converted in biliary acids or eliminated via biliary excretion [28].

HDL and apoA-I ability to promote Reverse Cholesterol Transport is believed to be one of the main atheroprotective mechanisms.

Atherosclerosis is a progressive disease characterized by the accumulation of cholesterol in the arterial walls. Increased levels of low-density lipoprotein cholesterol (LDL) are causally related to atherosclerosis, supported by substantial evidence from large-scale intervention trials, from animal studies, and from genetic studies of families and of the general population. LDL par-

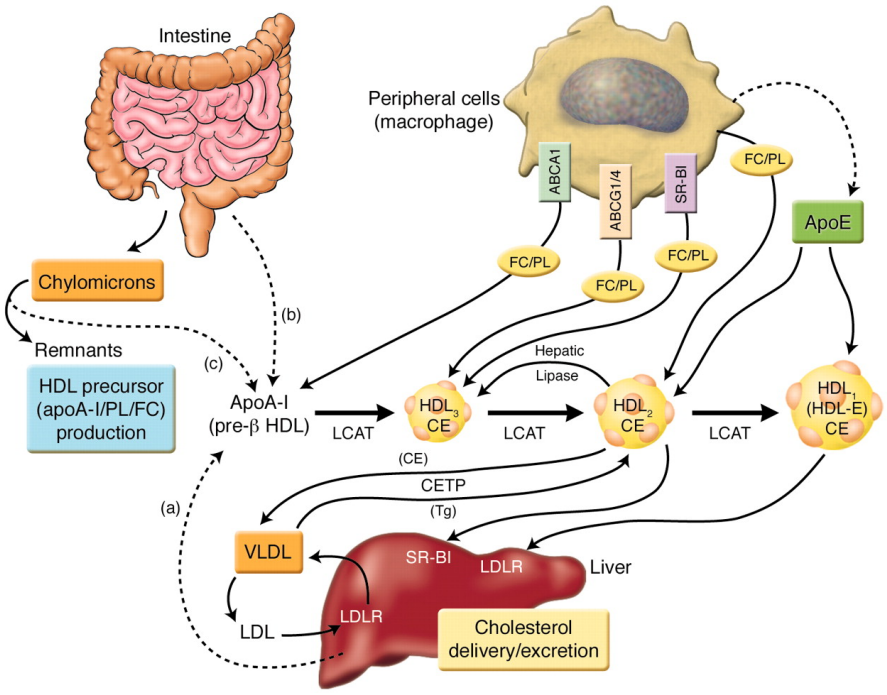

Figure 1.2: **Reverse Cholesterol Transport.**

ticles in plasma enter the arterial wall where they are oxidized. In response to oxidized lipids, arterial-wall cells secrete proteins that attract monocytes into the intima and lead to their locally differentiation into cholesterol-engorged macrophages, the so-called foam cells which continuously accumulate lipids. These foam cells constitute the initial lesions that subsequently develop into more advanced atherosclerotic plaques, characterized by the accumulation of lipid-rich necrotic debris and smooth muscle cells. Plaque rupture and total occlusion due to thrombus formation result in clinical ischemic vascular events.

In this process, the Reverse Cholesterol transport assures mobilization of excess cellular cholesterol from arterial-wall macrophages preventing or reducing foam cell formation [29].

1.1.5 Mechanism of action of ABCA1 protein

Many different models have been proposed so far to explain ABCA1 mechanism of action.

The three main processes are the following:

- In a first model a direct interaction with a small regulatory pool of apoA-I is required in order to transfer lipids outside the cell [30]; ABCA1 recognizes and interacts with a cluster of amphipathic helices in the apolipoprotein structure. This interaction likely involves the first and forth extracellular loop [31,32]. Formation of novel apolipoprotein binding structures protruding from the cell surface results as an intermediate step in the pathway by which apolipoproteins remove excess cholesterol from cells [33]. In this model ABCA1 moves cholesterol and phospholipids to the outer leaflet, but it isn't known if this transfer is either simultaneous or phospholipids are transferred before cholesterol [34] or, in a third option, the translocation is simultaneous unless phospholipid and cholesterol-rich domains are too distant so that the two phases are dissociated.

- In the second model ABCA1 is able to reach and bind the plasma membrane without necessarily interact with apoA-I. The two symmetrical ABCA1 transmembrane bundles come together to form a chamber that scans the inner leaflet of the membrane for substrates, incorporates them into the chamber, and flips them to the outer leaflet for extrusion from the cell. This involves a series of conformational changes in the ABC protein that is driven by ATP hydrolysis in the NBD domains. ABCA1 amino acid motifs in the chamber are likely to bind phospholipids. Again, it is unknown whether inner leaflet cholesterol is co-transported with these phospholipids or phospholipids translocation alone increases the accessibility of cholesterol that flips to the outer leaflet by other processes. The observation that ABCA1 can affect the cholesterol content of lipid rafts, which probably relies on lateral diffusion of cholesterol along the membrane, supports the idea that ABCA1 also translocates cholesterol [35]. When induced

by cholesterol loading of cells, ABCA1 therefore constitutively generates cholesterol and phospholipid-rich membrane domains, even in the absence of apolipoproteins [36] and these domains bend from the plasma membrane to relieve the strain of the densely packed phospholipids, generating curved and disordered lipid surfaces that favour apolipoprotein interactions. During the first several minutes of exposure to apolipoproteins, JAK2 is activated by autophosphorylation, which in turn increases binding of apolipoproteins to ABCA1. This binding facilitates the interaction of apoA-I with the protruding lipid domains, promoting their solubilization and release from the cells.

- The third model involves retroendocytosis; the pioneering study of Takahashi and Smith [37] showed that following internalization, apoA-I is recycled back to the cell surface to be re-secreted. Other studies showed that apoA-I and ABCA1 are co-localized in endosomal compartments, and that ABCA1 rapidly shuttles between intracellular compartments and the plasma membrane [38,39]. These results support the idea that ABCA1-bound apoA-I is delivered from the plasma membrane to early and late endosomal and/or lysosomal compartments, where it forms nascent lipoprotein particles that are subsequently secreted from the cell. However, several groups examining internalization and recycling of apoA-I concluded that the retroendocytosis pathway does not contribute significantly to HDL formation and that the majority of internalized apoA-I is directly transported to late endosomes and lysosomes for degradation while only a small fraction is re-secreted from cells and the majority of re-secreted apoA-I seems to be degraded in the medium. These results suggest that the mass of retroendocytosed apoA-I is not sufficient to account for HDL produced by cholesterol efflux [17].

On the other side, it has been recently demonstrated that apoA-I exhibits saturable association with the plasma membrane and intracellular compartments (ICCs), that apoA-I induces ABCA1 endocytosis and co-localizes with cell-surface derived ABCA1 on endosomal compartments; moreover apoA-I dissociation from ICCs is 4-fold faster than from the plasma membrane

and approximately 30% of endocytosed ABCA1 is recycled to the cell surface giving a critical contribution to HDL formation under conditions where cells have accumulated excess lipoprotein-derived cholesterol in endosomal compartments [39,40].

In conclusion the mechanism of active cellular lipids ABCA1-mediated efflux to apoA-I is not yet clear; it may involve 1) binding of apoA-I either directly to ABCA1 or indirectly to a lipid site created by ABCA1 activity, 2) either simultaneous or sequential release of membrane phospholipid and cholesterol to apoA-I, and 3) assembly of nascent HDL particles either at the cell surface or at intracellular sites during the retroendocytosis of ABCA1 [30].

The precise mechanism of action of ABCA1 in promoting delivery of cellular cholesterol and phospholipids to apoA-I remains to be determined.

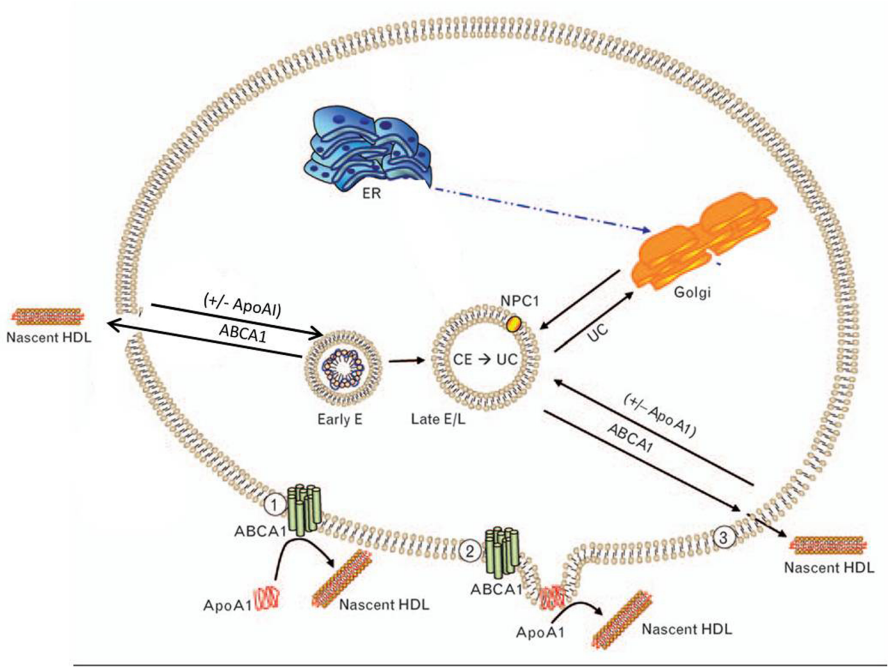

Figure 1.3: **Mechanism of action of ABCA1.**The three mechanisms of action of lipid-efflux ABCA1-mediated. Image adapted from [14].

1.1.6 ABCA1 intracellular traffic

An important intracellular mechanism that seems to characterize ABCA1 pathway is the formation of multiple structures like dimers, tetramers and oligomers.

In 2004 Denis et al. [41] demonstrated that the majority of ABCA1 exists as a tetramer which binds apoA-I, that neither apoA-I nor lipid molecules affect ABCA1 oligomerization and that apoA-I is associated with both dimeric and tetrameric, but not monomeric forms of ABCA1. These findings support the concept that the homotetrameric ABCA1 complex constitutes the minimum functional unit required for the biogenesis of HDL particles.

Trompier et al [42] further highlighted that ABCA1 is associated in dimeric structures that undergo transition into higher order structures, i.e. tetramers, during the ATP catalytic cycle. The assembly in homodimers takes place in the endoplasmic reticulum, which is a rather common rule in the assembly of multimolecular channel structures; the ABCA1 dimers are oriented so that the C-terminal extremities of ABCA1 are in close proximity. Both a putative PDZ binding motif present at the C terminus and the VFVNFA sequence located at positions 2210–2215 have been identified as potential interaction sites; they may be required either to stabilize the dimeric interaction or to introduce additional molecular partners in the complex. These assemblies are transient in nature but can be trapped experimentally by interfering with the ATP catalytic cycles, namely by stabilizing the ATP-bound state; they are tetramers, rather than dimers, undergoing major conformational changes upon ATP binding. Resetting of the transporter in the initial conformation takes place after effluxes of membrane phospholipids.

As explained in 1.1.3, current evidences suggest that ABCA1-mediated cholesterol efflux to apoA-I involves mobilization of cholesterol from plasma membrane, endoplasmic reticulum, trans-Golgi network, late endocytic and lysosomal compartments and cholesteryl ester droplets.

Neufeld et al [38] indeed demonstrated that ABCA1 resides on the surface of both small, mobile vesicles as well as on large, relatively immobile perinuclear vesicles. The small, fast-moving ABCA1-containing vesicles are likely to represent early endosomes, whereas the ABCA1-containing large, static,

perinuclear vesicles are likely to represent late endocytic compartments (late endosomes/lysosomes). The ABCA1-containing early endosomes undergo fusion and fission events: they transiently interact with one another, with the ABCA1-containing late endocytic vesicles, and with the cell surface. They shuttle and tubulate while moving vectorially, consistent with directed movement along cytoskeletal elements such as microtubules or actin filaments. In addition to ABCA1, the membrane components that may be transferred from the ABCA1 early endosomes to other cellular compartments may include substrate lipids for ABCA1 [43,44] and/or acceptor apolipoproteins [38-40]. Cholesteryl Esters (CE) in LDL are hydrolyzed into unesterified cholesterol in late endosomes and lysosomes and this cholesterol may serve as a direct substrate pool for ABCA1-mediated cholesterol efflux to apoA-I, suggesting that late endosome/lysosome cholesterol might represent a significant substrate pool for ABCA1 and HDL formation and this provides the opportunity for the removal of excess endocytosed LDL-derived cholesterol from the cell, prior to its potential distribution to other sites by NPC1.

Moreover, ABCA1 mobilizes endogenously synthesized cholesterol in the Golgi apparatus to lipidate de-novo synthesized apoA-I [44]; it also depletes cholesterol released from cytoplasmic cholesteryl ester droplets but since there is no evidence of ABCA1 localization or association with cholesteryl ester droplets, it is more likely that the unesterified cholesterol released from cholesteryl ester droplets is transported rapidly to the plasma membrane, via the Golgi apparatus, to become part of the ABCA1 substrate pool [45].

1.2 ABCA1 gene

The gene encoding ABCA1 transporter is located on the long arm of chromosome 9 (9q31.1) [46], has a total length of 149 kb and includes 50 exons and 49 introns. Exons dimensions vary between 35 bp (Ex 33) and 244 bp (Ex 49). Introns dimensions are more variable, from a minimum length of 111 bp (IVS 38) to a maximum length of 24000 bp (IVS 1) [47].

Exon 1 encodes for part of the 5' UnTraslated Region (5'UTR). Exon 2 encodes for the remaining part of 5'UTR, for the first methionine and the subsequent 21 aa of ABCA1 amino-terminal region. The putative tran-

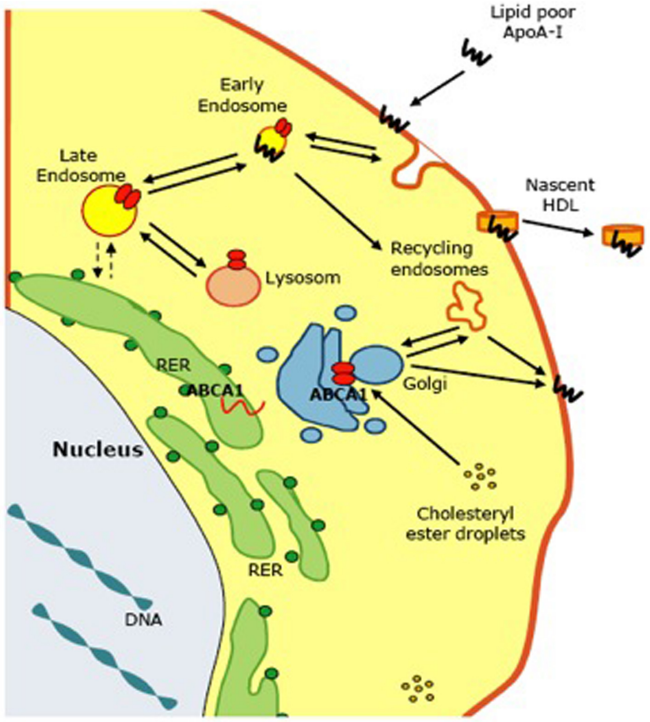

Figure 1.4: **ABCA1 intracellular traffic between early, late endosomes, lysosomes and the plasma membrane.**

scription starting site is localized 315 bases upstream of the ATG in exon 2. The open reading frame length is 6723 nucleotides. The nucleotide sequence around the initial ATG in exon 2 is conformed to the Kozak consensus sequence (RNNATGG), with a purine in position -3 and a G in position +4 [47].

If translation starts at the ATG in exon 2, the protein obtained from the corresponding mRNA contains 2261 aa [6,7,10,48]. The first 45 aa have chemical and physical properties similar to those of the signal peptide of many secreted proteins.

Another possible translation starting point is located in exon 4, where a 2201aa-long protein could originate. This ATG, however, is positioned in a context not related to the Kozak consensus sequence; it is therefore unlikely that this starting point is active [47].

With regard to the end of translation, the first polyadenylation site is about 3000 bp downstream from the stop codon. Thus the 3'UTR of ABCA1

mRNA is quite long [11,46,47].

ABCA1 gene promoter contains many motifs with a relevant role in expression regulation.

Regulation sequences are located for 221 bp upstream of exon 1, as common as in many genes, and for 24 kb inside IVS1.

Between exon 1 and 2 there is an alternative exon, called exon 1a, 136 bp in length and localized 2210 bp upstream of exon 2 [49]. This alternatively spliced transcript encloses the complete coding sequence and is expressed in testis and liver but not in macrophages [50]. Upstream of exon 1a an alternative promoter containing a TATA box and a CAAT box sites is present, together with other potential binding sites for nuclear receptors containing sterol regulatory elements.

1.2.1 Gene expression regulation

ABCA1 gene has ubiquitous expression and in basal conditions is mostly translated in tissues like placenta, lung, liver, kidney, intestine, adrenal gland and some areas of the nervous system. A high expression is present in the same tissues also during the fetal development [11].

Many different factors are responsible of ABCA1 gene expression or inhibition and some of them are tissue-specific.

First of all, ABCA1 expression is markedly induced by overloading cells with cholesterol, especially in macrophages [51,52]. This induction occurs exclusively through activation of the nuclear receptors liver X receptor (LXRα and/or LXRβ) and retinoid X receptor (RXR). LXR and RXR form obligate heterodimers that preferentially bind to response elements within the ABCA1 gene promoter and the first intron. LXRs and RXRs bind to and are activated by oxysterols and retinoic acid, respectively. 9-cis retinoic acid (9-cisRA), either alone or in combination with oxysterols, is the major retinoic acid that upregulates ABCA1 mRNA levels. The LXRα gene promoter in human macrophages contains a LXR responsive element, indicating that LXRα can autoregulate its own expression; this would serve to amplify the effects of oxysterols on the ABCA1 lipid efflux pathway. Because uptake of non-oxidized cholesterol by cells increases ABCA1 expression, cholesterol

must be converted to oxysterols before inducing ABCA1. It is believed that 22-hydroxycholesterol, 24-hydroxycholesterol, and 24(s),25-epoxycholesterol are the major naturally occurring liver LXR ligands. Most oxysterols are generated by cytochrome P-450 enzymes that are particularly prevalent in the liver and play a role in bile acid metabolism. One of these enzymes, sterol 27-hydroxylase (Cyp27), is broadly distributed in various tissues and cell types, including macrophages, suggesting that 27-hydroxycholesterol is the major LXR ligand in macrophages and other peripheral cells.

A sterol-responsive element has been mapped to an imperfect Direct Repeat (AGGTCA) spaced by four nucleotides (DR-4) which was shown to bind LXR/RXR heterodimers; mutations of the DR4 element strongly reduced oxysterol-responsive ABCA1 gene activation [53,54].

The induction of ABCA1 gene transcription activity by 25-hydroxycholesterol, another important stimulator of ABCA1 expression, is critically dependent on Sp1 elements and an E-box motif present in the proximal promoter region of ABCA1, 147 bp upstream of the originally identified transcriptional start site.

The two ABCA1 promoters, upstream exon1 and inside intron 1 respectively, contain other transcription regulators like a TATA box located 24 bp upstream the transcriptional start site, essential for the promoter activity.

Lipid metabolites other than sterols can modulate ABCA1 expression by the LXR system [49]:

- Polyunsaturated fatty acids act as antagonists to oxysterol binding to response elements in the LXRα gene, potentially interfering with induction of ABCA1 by sterols.

- Geranylgeranyl pyrophosphate (GGPP), a product of the mevalonate pathway which isoprenylates proteins, was shown to suppress LXR-induced ABCA1 synthesis by two mechanisms: as an antagonist of the interaction of LXR with its nuclear co-activator SRC-1 and as an activator of Rho GTP-binding proteins. This second mechanism might alter sterol trafficking in cells, reducing their availability as ligands for LXR.

- Activators of Peroxisome Proliferator Activating nuclear Receptors (PPARs) also enhance ABCA1 transcription in some cells and stimulate cholesterol efflux by activating transcription of the LXRα gene through a complex interaction between PPARα, PPARγ and LXRα, which in turn induces ABCA1 transcription [55,56].

- Thyroid hormone receptor was reported to suppress ABCA1 transcription by forming a complex with RXR that competes for LXR/RXR binding to its response elements.

- ABCA1 transcription is also regulated by factors independent of LXR/RXR: membrane-permeable analogs of cAMP and inhibitors of cAMP-specific phosphodiesterase 4 stimulate ABCA1 transcription in macrophages by unknown mechanisms; cAMP act in these cells by promoting the binding of Sp1 and E-box binding factors to the proximal promoter of ABCA1 thus stimulating ABCA1 gene transcription and surface expression in absence of cholesterol, whereas ABCA1 expression in fibroblasts is critically dependent on cholesterol loading [57,58].

- The calcium channel blocker verapamil can also enhance ABCA1 transcription by an LXR-independent process [59].

The ABCA1 gene promoter contains other transcriptional response elements that are potential sites of regulation, some of which may influence constitutive and tissue-specific expression of ABCA1: cytokines such as interferon-γ (IFN-γ), along with a two-fold increase in the activity of acylcoenzyme A: cholesterol acyltransferase (ACAT) decreases ABCA1 mRNA in macrophages under basal conditions as well as after sterol-loading. This results in a two-fold decrease of cholesterol effux [60,61].

Transforming growth factor β1 (TGF-β) and oncostatin M have been reported to modulate ABCA1 transcription in cultured macrophages and hepatoma cells. Sterol regulatory element binding protein 2 (SREBP2), a transcription factor that regulates sterol synthesis, appears to interact with the ABCA1 promoter in vascular endothelial cells and to suppress ABCA1 transcription, thus providing an additional mechanism for suppressing cholesterol efflux when cells are sterol depleted [13].

It has also been demonstrated a role of LDLR in regulation of ABCA1 expression as illustrated below (see 1.6.2).

Moreover negative control elements binding the transcriptional repressor zinc-finger protein 202 (ZNF202) are thought to counterbalance ABCA1 mRNA expression and may be influenced by the genetic background. ZNF202 requires the presence and of a SCAN domain that mediates selective hetero- and homodimerization and of a functional TATA box to bind to in order to repress ABCA1 expression.

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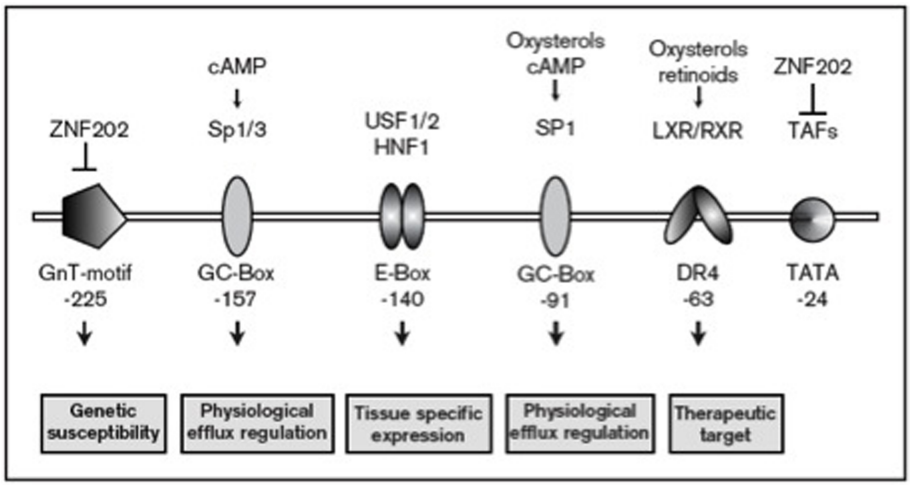


Figure 1.5: **Regulation of the ABCA1 gene promoter.**

Catepsine D (CTSD), a lysosomal proteinase, is another regulator of ABCA1 expression: it increases both ABCA1 mRNA expression and cellular ABCA1 protein, while its inhibition results in ABCA1 retention in lysosomal compartments, reducing its traffic to the plasma membrane [62]. CTSD expression is regulated by NPC1 activity in hepatocytes: NPC1-null hepatocytes show increased CTSD expression, both transcriptionally and post-transcriptionally and this therefore has an effect on ABCA1 expression and protein production [63].

Recently, ORP8, a protein belonging to OSBP (oxysterol-binding protein) and ORP (OSBP-related) gene family, was shown to negatively regulate ABCA1 transcription by a mechanism involving DR4 and E-box elements [64]. Since ORP8 is anchored to the ER by a C-terminal transmembrane do-

main, transcriptional inhibition is probably indirect and related to the sterol binding or metabolic activity of this ORP.

1.2.2 Post-transcriptional regulation

The first proposed mechanism of ABCA1 post-transcriptional regulation occurs by phosphorylation of residues in the regulatory domain by protein kinase A (PKA) following cAMP stimulation.

IFNγ appears to suppress ABCA1 expression by further destabilizing its mRNA: the kinetics of ABCA1 mRNA and protein decrease is consistent with the early IFN-γ-induced changes in Stat1 phosphorylation and nuclear translocation. Therefore, down-regulation of ABCA1 by IFN-γ is a post-transcriptional response that occurs early in the process of IFN-γ-induced macrophage activation [65].

1.2.3 Post-translational regulation

Several processes that modulate ABCA1 protein stability have been described and there is not always a strict correlation between ABCA1 mRNA and protein levels.

When ABCA1 inducers are removed in the absence of apolipoproteins, ABCA1 mRNA and protein are degraded at a fast rate (half-life of 1–2 h) [66,67].The rapid protein turnover is largely due to a sequence at amino acids 1283–1306 in the first intracellular loop that is enriched in proline-glutamate-serine-threonine (PEST motif). Phosphorylation of T1286 and T1305 in this motif promotes ABCA1 proteolysis by an unknown member of the calpain protease family. There are several metabolic factors that modulate the rate of ABCA1 protein degradation by either this calpain system or other processes.

The interaction of apolipoproteins with ABCA1-expressing cells dramatically reduces the rate of ABCA1 protein degradation by inhibiting ABCA1 proteolysis by calpain [66] and activating other signaling events that stabilize the protein [68]. This regulation acts as a feedback mechanism to sustain ABCA1 levels when acceptors for cellular lipids are available. First, the cellular interactions of apolipoproteins interfere with the phosphorylation of the PEST motif. Second, the removal of membrane sphingomyelin by

apolipoproteins activates phosphatidylcholine phospholipase C, which signals phosphorylation of ABCA1 at a site that stabilizes the protein [69]. It is unknown if either or both of these mechanisms require apolipoprotein binding to ABCA1.

Unsaturated free fatty acids directly destabilize ABCA1 protein in cultured cells by a signaling pathway involving activation of phospholipase D2 and protein kinase Cδ and phosphorylation of ABCA1 serines [67,70-72] [71,74-76].

ABCA1 also interacts with β1- and α1-syntrophin, scaffolding proteins that regulate transport through linkage to the cytoskeleton, resulting in stabilization at intracellular sites and the plasma membrane [73,74].

Oxisterol binding protein (OSBP) is implicated in the integration of sterol sensing/transport with sphingomyelin synthesis and cell signaling. OSBP negatively regulates ABCA1 by decreasing its stability in the cytoplasm; this effect is not correlated with OSBP function in sphingomyelin synthesis or interaction of OSBP with the ER or Golgi apparatus but is dependent on OSBP sterol-binding activity [75].

The LXRβ/RXR complex also has a role in regulating ABCA1 activity: this complex binds directly to ABCA1 on the plasma membrane of macrophages and modulates cholesterol secretion; when cholesterol does not accumulate, ABCA1-LXRβ/RXR localizes on the plasma membrane but is inert; when cholesterol accumulates, oxysterols bind to LXRβ and the LXRβ/RXR complex dissociates from ABCA1, restoring ABCA1 activity and allowing apoA-I dependent cholesterol-efflux. Upon binding oxysterols in the cytosol, LXRβ/RXR is translocated to the nucleus and activates the transcription of ABCA1 and other genes [76].

Moreover, recently Singaraja et al [77] highlighted that ABCA1 is robustly palmitoylated by the palmitoyl transferase DHHC8 at cysteines 3, -23, -1110, and -1111 and that abrogation of palmitoylation of ABCA1 by mutation of the cysteines results in a reduction of ABCA1 localization at the plasma membranes and a reduction in the ability of ABCA1 to efflux lipids to apoA-I. Thus, palmitoylation regulates ABCA1 localization at the plasma membrane, and regulates its lipid efflux ability.

1.3 Tangier Disease

1.3.1 Historical Notes

In the history of American literature, the name Tangier is usually associated with the eccentric writer William Borroughs, who conceived some of his novels in this Moroccan town on the Straight of Gibraltar.

At the beginning of 1960s, D. Fredrickson et al. [78] from the National Institute of Health (USA) described a particular syndrome in two siblings characterized by: hypertrophic yellow-grayish tonsils, limph adenopathy, hepathosplenomegaly and sensitive-motor like peripheral neuropathy, in association with almost complete absence of HDL-C in the plasma (Analphalipoproteinemia). These two siblings belonged to a family living in the Tangier Island, a small isle located in Chesapeake Bay, approximately 20 miles west of the eastern shore of Virginia (see Figure 6). The island was discovered by the English explorer Captain John Smith in 1608 and owes its name to the poetic imagination of the sailor, who was reminded by its wide, sandy shores of the white dunes of the port of Tangier in Morocco [79].

After its discovery, the island was without a single inhabitant for the next 78 years, until 1686 when John Crockett together with a few comrades founded a settlement there. Except for a calamitous outbreak of cholera in 1886, nothing particular happened for the next 300 years, and the inhabitants made a modest living from the fishery. Tangier Island was well insulated from the mainland both economically and socially, so when Dr Fredrickson traveled there in the early 1960s, he found only one road, one car and about 900 residents, many of them speaking a unique local dialect and bearing the surname Crockett [79].

Because other three couples of siblings were found affected by the same disease in Tangier Island, Fredrickson proposed the denomination "Tangier Disease" (TD). The presence of many individuals affected in one family without a vertical transmission of the phenotype, suggested a recessive transmission of this syndrome, as explained in the big pedigree originally described by Fredrickson in 1964 [78].

The manifestation of this disease in the Tangier Island is likely the result of a geographic isolation and of endogamy that happened among the in-

habitants during 400 years, all descendents from the small group of English refugees.

After the first description of Tangier Disease in 1964, many others followed in different geographic regions (USA, UK, New Zeeland, Australia, Switzerland, Germany, Poland, Italy, Canada, France, Spain, The Netherland, Pakistan, Japan and Egypt).

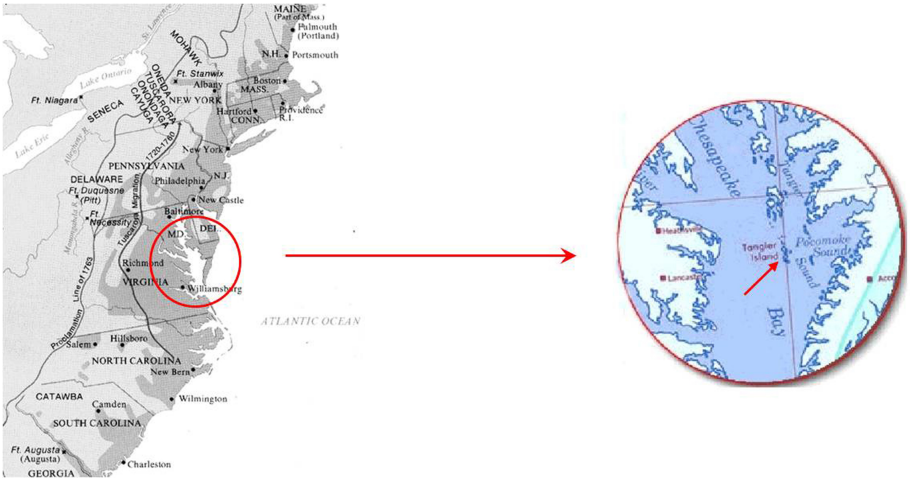


Figure 1.6: **Tangier Island.** North-East coast of United States and Chesapeake Bay with Tangier Island.

1.3.2 Case history of Tangier Disease

The phenotypic, pathognomonic case history that allowed the identification of the first cases of Tangier Disease was the association of almost total absence of HDL-C in the plasma and presence of yellow-grayish tonsils [46].

The clinical phenotype of Tangier Disease has autosomic recessive transmission. However the biochemical phenotype (very low HDL-C and apoA-I levels) has a co-dominant transmission [81], that obligate heterozygotes have HDL-C and apoA-I levels intermediate between normal values and those of Tangier patients, but do not present any clinical feature.

Tangier Disease is characterized by two different biochemical alterations:

- Cholesteryl Esters (CE) accumulation in many cell types, especially in histiocytes and in the reticuloendothelial system (aka: mononuclear phagocyte system);

- Almost total absence of HDL-C in plasma.

Cholesteryl Esters accumulation is particularly evident in tonsils, lymph nodes, bone marrow, liver, intestine and Schwann cells of the peripheral nervous system.

At a morphological level, histiocytic cells (e.g. macrophages) appear like foam cells with the cytoplasm full of lipid droplets, which can be highlighted with Sudan red/black, sometimes with crystal structures. This lipidic material is formed by esterified cholesterol accumulated outside lysosomes.

The serious HDL defect is considered as a predisposition factor for cardiovascular diseases (angina pectoris, myocardial infarction, stroke etc.) on atherosclerotic base [53].

The phenotypic manifestations differ among individuals but symptoms may be hypertrophic tonsils, hepatosplenomegaly, anemia, thrombocytopenia, stomatocytes, peripheral neuropathy and increased incidence of cardiovascular disease, whose clinical penetrance is however extremely variable [48]; the most represented feature is anyway tonsils hypertrophy, with typically yellow-grayish lobulated tonsils, and hepatosplenomegaly [84]. Obviously, tonsillectomy and therefore absence of tonsils can complicate the diagnosis.

Two major reports that dealt with this issue, published prior to the time that the ABCA1 gene was identified, indicated that despite the near absence of HDL, CVD (cardiovascular disease) only represents a significant problem in middle age and elderly TD patients (over 35 years) [84,85]. Taken the epidemiological evidence that a drug-induced increase of 1% in HDL-C concentration may be associated with as much as 3% decrease in death or myocardial infarction incidence, it is nevertheless remarkable that TD patients with HDL-C below the 5th percentile (for age and sex) do not appear to develop CVD until the age of 35-40 and some of these patients live well into their seventies. One factor that has been brought forward to explain this is the very low level of LDL-C found in TD patients but this hypothesis has not yet been confirmed. Anyway the Italian patients with TD, diagnosed up to now by our group ($\approx$ 30 patients), show a marked predisposition in developing premature atherosclerosis.

1.3.3 Familial HDL Deficiency

Familial HDL deficiency (FHD) is a more common disorder of lipid metabolism than Tangier disease, characterized by isolated moderate reductions of HDL-C in absence of the typical characteristics of clinically evident TD. Recently Frikke-Schmidt et al. revealed that i) approximately 10% of individuals with the lowest percentile of HDL cholesterol in the general population are heterozygous for mutations in ABCA1 and ii) the frequency of FHD caused by ABCA1 mutations in the general population is about three in 1000 or considerably higher than previously assumed [87].

1.3.4 Cellular defects in Tangier Disease and Familial HDL deficiency

The Cholesteryl Esters accumulation in many different cell types of the reticuloendothelial system suggested that in TD a defect of cholesterol intracellular traffic is present. This hypothesis was sustained by a series of experimental evidences obtained from dermal fibroblasts and monocyte-macrophages in TD patients. TD fibroblasts when overloaded with cholesterol (after incubation with acetylated LDL or free cholesterol) show a defect in phospholipid and cholesterol efflux to plasma apolipoprotein acceptors (e.g. apoA-I, apoA-II, apoC or apoE) [88-90].

1.3.5 Mutations on ABCA1 gene, Tangier Disease and Familial HDL deficiency

Until genetic bases of TD and FHD were unknown, patients were diagnosed on the basis of their clinical phenotype. The discovery of the cellular defects characterizing TD and FHD patients initiated a virtual race to identify the molecular background responsible for the clinical and biochemical phenotype, and in 1999 three groups independently reported that homozygosity and heterozygosity for mutations in ABCA1 caused Tangier disease and the milder form, Familial HDL deficiency (FHD), respectively [6-8].

Through the usage of genetic markers (wide genomic screening) in many Tangier families and the application of strict criteria for the biochemical

phenotyping of heterozygotes, the "Tangier gene" was localized via linkage studies on the long arm of chromosome (9q31) [51].

The further refinement of linkage studies and the differential analysis of mRNA in wild type and TD macrophages when overloaded with cholesterol, led to the identification of ABCA1 as involved in TD [6-8].

Since this discovery, the diagnosis of TD and FHD has been obtained through analysis of genotype. Currently, the phenotypic variability of TD is evident, with some TD patients showing very low HDL levels ($<1\%$) and others with HDL levels $>10\%$.

Knowing the mutations characterizing affected patients, it is now possible to determine functional deficits for a certain phenotypic spectrum associated both with heterozygotes and homozygotes.

Nowadays ABCA1 genetic analysis is a key element in TD and FHD diagnosis and can represent an important instrument for a wider comprehension of ABCA1 role in many pathways.

The investigation technique mainly used is the systematic sequencing of all exons: the spread of mutations identified along ABCA1 gene up to now forbids the restriction of the analysis area. The presence of 50 exons and 49 introns, for a total of 149 kb, transforms the complete sequencing in a long procedure that requires on average one month for the diagnosis of a single proband. For these reasons it is important to create a rapid, automated and standardized pre-screening system in order to reduce the sequencing phase to a low group of exons.

Some years ago we set up a technology that resembles these requirements through the usage of Denaturing High Performance Liquid Chromatography (DHPLC). This method allows the selection of those amplicons which show abnormal elution profiles (on average 10-15 amplicons in ABCA1 case) and thus the restriction of the following sequencing procedure [80].

1.3.6 ABCA1, Ischemic Heart Disease and atherosclerosis

Numerous population studies have shown an inverse relationship between plasma HDL levels and CVD risk, implying that HDL protects against atherosclerosis [81]. It is therefore clear that the relative activity of ABCA1

plays a role in this atheroprotective mechanism. The low plasma HDL levels identified in individuals with impaired ABCA1 pathway would also promote accumulation of cholesterol in tissue macrophages and perhaps increase local inflammation. It has been also demonstrated that ABCA1 over-expression protects against atherosclerosis in ApoE-/- mice, a strain that spontaneously develop atherosclerotic lesions [28].

Subjects with loss-of-function homozygous or compound heterozygous mutations in ABCA1 (Tangier disease) have a 4–6-fold higher than normal incidence of CVD, which applies equally to both men and women. This moderately high risk for atherosclerosis is not as dramatic as one would expect for individuals with a virtual absence of HDL. A thorough review of all known Tangier disease patients worldwide reported that only 20% of patients had atherosclerotic manifestations, thus emphasizing a huge discrepancy between the virtual absence of plasma HDL cholesterol in these homozygotes and the lack of the expected large increase in risk of cardiovascular disease, predicted from epidemiological studies [82].

Their low levels of LDL (40–60% normal) may partially protect these subjects from atherogenesis. Some subjects with common ABCA1 variants have been reported to have premature CVD. However, the number of identified Tangier disease patients is very low and not all individuals with the same ABCA1 variant show signs of CVD. Therefore, it is difficult to draw firm conclusions about the cardio-protective role of ABCA1 so far.

Studies of ABCA1 heterozygotes, which tend to have more normal levels of LDL, have produced ambiguous results. Analyses of a small number of FHD patients showed significant increases in CVD and carotid artery intima media thickness when compared to control subjects and these increases were associated with impaired ABCA1-dependent cholesterol efflux from their cultured fibroblasts. These studies strongly support the idea that impaired ABCA1 increases atherosclerosis in humans, but not in all cases and by mechanisms that may not reflect reduced HDL levels. A huge study with total of 41,961 participants from the Danish population including 109 ABCA1 heterozygotes and 6666 IHD (Ischemic heart Disease) cases, were ascertained and it was showed that heterozygotes for four loss-of-function mutations had lower levels of HDL cholesterol and apoA-I than non-carriers

but plasma triglycerides and remnant cholesterol did not differ; low plasma levels of HDL cholesterol due to heterozygosity for these four mutations were not associated with an increased risk of IHD [81].

There are several possible reasons for these variable responses to ABCA1 mutations. First, many ABCA1 heterozygotes have only modest reductions in plasma HDL levels and ABCA1 cholesterol export activities, thus the defects may not be significant enough to accelerate CVD in all individuals. Second, most of the HDL is produced by liver ABCA1, which may not reflect the activity of ABCA1 in arterial macrophages. Third, impairment of other ABCA1 functions, such as anti-inflammation, could have bigger impacts on atherogenesis than reduced cholesterol export, especially in individuals prone to enhanced inflammatory responses [34].

Recently Frikke-Schmidt highlighted that because data from observational studies of the inverse relationship between low HDL-C levels and risk of IHD is confounded by high triglycerides, marking the presence of atherogenic remnant lipoproteins, and because genetically low HDL-C per se does not translate into the expected increase in risk, low levels of HDL cholesterol as a primary causal factor in the pathogenesis of IHD needs re-evaluation [82].

1.4 ABCA1 gene screening

1.4.1 Mutations identified up to the present in ABCA1 gene

Since 1999, when ABCA1 was identified as the main gene involved in TD (see 1.3), over than 112 different mutations have been described `http://www.uniprot.org/uniprot/O95477` both in TD and FHD patients.

Among these variants, 76 are responsible of non conservative aminoacid substitutions (missense mutations), 10 introduce a stop codon (nonsense mutations), 20 are small insertions or deletions and 6 are splice-site variations.

There is therefore a heterogeneity of mutant alleles that requires sequencing of the entire ABCA1 gene when a new case of TD or FHD is found, in order to identify the causative mutation. All variants that cannot be referred as frequent polymorphisms (SNPs) usually lead to a reduction of lipid efflux

from peripheral cells.

Even if ABCA1 mutations are distributed along the entire gene, their localization is not random. Most of variants functionally studied so far are localized on the two large extracellular loops.

1.4.2 Biological impact of ABCA1 mutations

Due to the complexity of ABCA1 gene, the protein size and the high allelic heterogeneity observed, it is really hard to define the biological meaning of each mutation identified.

Despite these limitations, some hypothesis have been proposed to explain the functional defect depending on the position of a certain mutation.

Mutations in the extracellular loops: approximately half of missense mutations in ABCA1 gene associated with TD and FHD fall within the 2 extracellular loops. Mutations in the first and second extracellular loops might be expected to result in a lack of lipid efflux caused by dysfunctional interaction of ABCA1 with apoA-I; the lipid-poor pre-β-HDL particles would require either direct interaction or close proximity to ABCA1 for the lipid transfer to occur. It is also possible that mutations in the extracellular domains will result in a disruption of the tertiary structure of ABCA1, preventing its function at the plasma membrane where it is normally localized.

Mutations in the transmembrane domains: mutations in the transmembrane domain region have been proven to disrupt the integration of ABCA1 into membranes and therefore prevent it from exiting the endoplasmic reticulum and the Golgi and its integration into the plasma membrane. This could result in the rapid turnover of the mutant ABCA1 protein.

Mutations in the Nuclear Binding Domains: it has been demonstrated that mutations at this level avoid ATP binding and hydrolysis and therefore ABCA1 ability to mediate cholesterol efflux but this doesn't mean that ABCA1 is unable to reach the cell surface. Moreover, mutations in the NBDs lead to an impairment of ABCA1 oligomerization as explained before [42].

Mutations at the C-terminus: ABCA1 C-terminus is critical because containing a PDZ binding domain [83,84] that, if mutated, leads to protein mislocalization. Both the PDZ binding motif and the VFVNFA sequence

located at positions 2210–2215 have been identified as potential interaction sites for homodimers creation, the predominant ABCA1 organization [73,84,85]. They may be required either to stabilize the dimeric interaction or to introduce additional molecular partners in the complex. It is therefore possible that an alteration of the C-terminus blocks ABCA1 oligomerization [42]. Only few mutations in this domain have been identified and/or characterized so far.

1.4.2.1 Polymorphisms and their biological value

Due to its dimensions, it is not surprising that ABCA1 gene presents many polymorphisms, the most common shown in Appendix B. SNPs analyses have identified over 20 common polymorphisms (>1% allele frequency) in the coding, promoter and 5'UTR regions of ABCA1 and several of these are associated with either low of high HDL-C plasma levels [34]. It is likely that other polymorphisms are present in ABCA1 introns. The functional meaning of polymorphisms positioned in the promoter, especially in the proximity of the transcription start site, is unknown.

Many exonic polymorphisms lead to conservative aminoacid substitutions. The functional role of these common substitutions is not clear; some of them are related to changes in HDL plasma levels. For instance, homozygotes for I883M variant have HDL plasma levels significantly increased compared to normal population [86,87]. Heterozygotes for R219K variant (46% of North-central European subjects) have lower triglyceride levels and higher HDL levels than controls. They also seem to show fewer vascular atherosclerotic alterations and a lower number of coronary events.

Polymorphisms make the genetic investigation for causative mutations more difficult. Moreover, intronic variants which meaning is unknown (possible splice-site variations etc.) are quite common.

The study of extreme phenotypic groups from a general population sample has increased the chance of identifying genetic variants with effects on a specific phenotype. Frikke-Schmidt et al. showed that common variants on ABCA1 gene may be differentially distributed in the extreme tails of HDL levels and that these SNPs are most likely to be associated with the level of

this specific trait in the general population. From this screening approach it was evident that common variants are present in both extreme groups but tend to differ in frequency if they affect the specific phenotype, whereas a rare variant with a strong phenotypic effect is more likely to be observed in only one extreme [88-91]

1.4.2.2 Biological effect of mutations: genotype to phenotype correlation

An investigation on the relationship between discrete mutation and phenotype is instrumental in understanding what governs the variable penetrance of clinical signs in Tangier pedigrees.

As direct genotype/phenotype correlations are impossible due to the rarity of the disease, alternative approaches such as the generation of appropriate animal models exclusively expressing informative ABCA1 mutants or ex vivo and in vitro studies have to be envisioned. Finally, as ABCA1 can be considered one of the most clearly identified therapeutic targets in the prevention of cardiovascular disease, several efforts have arisen in the aim of predicting how and whether genetic polymorphism may contribute to differences in phenotypic traits.

Not always aminoacid variants on ABCA1 protein lead to a pathogenetic effect. In order to clarify the repercussion that a certain variant has on ABCA1 function, intracellular traffic and localization, it is necessary to functionally characterize it.

Knockout mice demonstrated that ABCA1 dysfunction is sufficient to generate hypolipidemic profiles [90,92-94]: they faithfully reproduce the human syndrome; however, a few traits appear limited to the mouse models. First, the engulfment of cells dying by apoptosis is clearly impaired during embryonic development in the mice. This defect, which is devoid of major developmental consequences, has obviously not been investigated in humans. In addition, ABCA1$-/-$ females are infertile due to impaired placental development, a phenotype related to the high expression level of ABCA1 in the pregnant uterus. A similar sign is lacking in human Tangier patients though an increased incidence of spontaneous abortions has been informally

recorded. Although the alterations in metabolic profile and most phenotypes related to ABCA1 loss of function appear robust and background-insensitive, exquisite differences in the ABCA1 expression indeed exist between the various mouse strains. The invalidation of ABCA1 gene in mice leads also to clinically relevant phenotypes unrelated to lipid metabolism; these span from aberrant responses to malaria infection to increased development of Alzheimer's related degenerative lesions.

Another method involves patients' derived cells, either fibroblasts or monocytes-macrophages or immortalized lymphocytes.

Immortalized lymphocytes show the advantage of a long-lasting maintenance but show a weak ABCA1 expression and are hard to immortalize and maintain in culture [95,96].

Fibroblasts are the most investigated patient-derived cells and show a good ABCA1 expression, but they are not always available, collection requires an invasive approach and their culture is complex [97-100]. The usage of dermal fibroblasts may produce some other limitations, especially if the proband is a simple heterozygote, thus carrying a wild type allele, or a compound heterozygote, thereby with two different mutant alleles.

Monocyte-macrophages instead are isolated from blood without invasive approaches and easily cultured [99,101,102].

Another way to investigate the effect of a novel ABCA1 variant on the protein function is that of using in vitro studies via transfection of heterologous cells with mutant cDNA.

Transfection of mammal cells with mutant cDNA allows overcoming all the problems listed above, leading to the analysis of the only mutant desired.

The main issue in choosing the transfection method is to obtain a specific mutant cDNA containing the only mutation of interest and able to create a mutant protein identifiable inside the cells. This problem arises in particular for ABCA1, due to the gene dimensions, a discouraging factor for the analysis of missense mutations. However the new mutagenesis technologies overcome this matter so that more and more researchers are applying in vitro studies for the functional analysis of novel ABCA1 missense mutations in order to clarify the biological role of specific ABCA1 protein domains (Walker domains, extracellular loops, apoA-I binding sites, carboxi-terminal region

etc.) [30,73,97,103-109].

It should be remarked anyway that transfection technologies induce an over-expression of ABCA1 gene in the host cells, which does not resemble the most physiological situation since it could generate some aspecific responses like ERAD (ER-Associated Degradation) and UPR (Unfolded Protein Response). Whenever possible, ex-vivo studies should be therefore preferred or at least performed in parallel with in vitro studies [110-113].

It is anyway difficult to generalize the relation between the position of a certain variant on ABCA1 protein domains and its biological effect on ABCA1 structure and function.

1.5 Cell cholesterol homeostasis

Mammalian cells require cholesterol for maintenance of membrane integrity and multiple cellular functions [114]. Cholesterol is vital to several cellular processes, including cell growth, gene regulation, and synthesis of lipoproteins, steroid hormones, and bile acids [115]. However, an excess of intracellular free cholesterol could be toxic for the cell. Moreover, cholesterol is important in the pathogenesis of cardiac and brain vascular diseases, in dementias, diabetes and cancer as well as in several rare monogenic disorders. For these reasons the amount and distribution of intracellular and whole-body cholesterol are tightly controlled processes. Since lipids are not genetically encoded, genome and transcriptome changes can only reflect cholesterol homeostasis indirectly; many of the proteins that preserve cellular cholesterol homeostasis exhibit considerable functional redundancy [116] thus leading to the conclusion that the organism needs a huge regulatory apparatus to finely tune cholesterol balance.

1.5.1 De novo cholesterol synthesis

The major sources of cellular cholesterol are represented by: i) de novo synthesis and ii) uptake of cholesterol from circulating lipoproteins.

All cells of the body can synthesize cholesterol; synthesis occurs in the Endoplasmic Reticulum (ER) compartment where the synthesis apparatus

is located. The rate-limiting step of the synthetic pathway is the conversion of HMG-CoA to mevalonate mediated by the HMG-CoA Reductase enzyme (Fig. 1.7). HMG-CoAR, similarly to other enzymes that function in the later steps of cholesterol synthesis, is an integral ER membrane protein [116]; it is composed of 8 TM domains and it contains (between TM and 6) a "Sterol Sensing Domain" (SSD), a conserved motif common also to several key proteins involved in cellular cholesterol metabolism regulation like SCAP, NPC1, SREBPs, Insigs. This domain allows to "sense" the cholesterol content of the ER membrane and to consequently regulate the synthetic rate of cholesterol.

HMG-CoAR is regulated, at the transcriptional level, by SREBPs (a group of proteins which mainly regulate cholesterol synthesis and uptake, as illustrated in 1.5.2.2); gene transcription is suppressed by intracellular cholesterol accumulation. At the post-transcriptional level the reductase is regulated by sterol-accelerated degradation: in particular in sterol-deprived cells HMG-CoAR is slowly degraded, while when sterols accumulate, the enzyme is rapidly ubiquitinated and degraded with an half-life of less than 1 hour. Both regulation processes are mediated by Insigs as described in section 1.5.2.2 [115].

1.5.2 Cholesterol uptake (influx) and endosomal cholesterol traffic

The alternative and preferred source for cellular cholesterol acquisition is the uptake of lipoproteins from the circulation through a receptor-mediated mechanism. The process is mediated by the cell surface LDL Receptor (LDLR) which binds plasma lipoproteins containing ApoB or ApoE (mainly LDL but also VLDL and chylomicron remnants). Lipoprotein particles, once bound to the receptor, are internalized via clathrin-coated pits; these vesicles then fuse with early endosomes where the lower pH allows the dissociation of LDL from LDLR. The LDLR is then localized to a recycling endocytic compartment, which transfers back the receptor to the cell surface. Cholesteryl esters contained in the internalized LDLs are hydrolyzed in late endosomes and lysosomes by an acid activated enzyme, namely LAL (lysosomal acidic

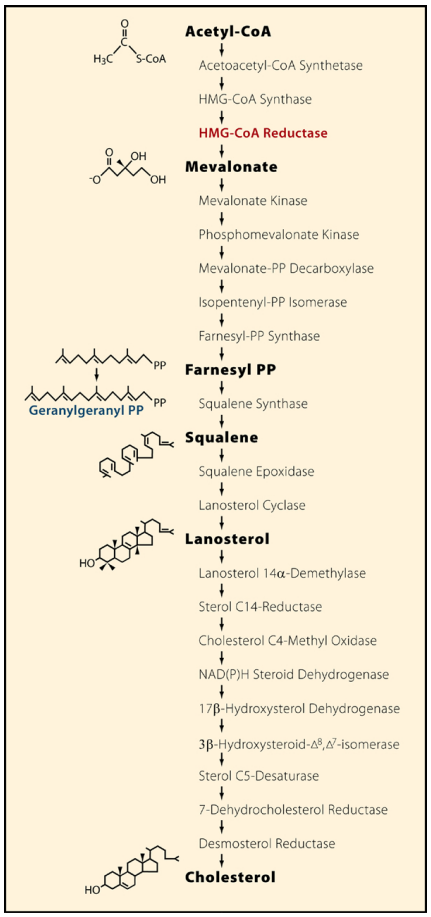


Figure 1.7: **Cholesterol synthesis.**

lipase), which generates free cholesterol.

1.5.2.1 LDLR structure

LDLR molecule is a glycoprotein of 839 aa, including 5 functional domains: i) a ligand binding domain at the NH2 side; ii) an Epidermal Growth Factor (EGF) homology region; iii) an "O-linked" oligosaccharide-rich region; iv) a TM domain; v) a cytoplasmatic tail at the COOH side (Fig. 1.8).

1. The ligand binding domain is organized in 7 repeated motifs, each one containing a cysteine-rich region important for the maintenance of the proper 3D folding. The conformation of the ligand binding domain ensures the exposure of negatively charged groups which allow

the interaction with the positively charged groups of the ApoE and ApoB100 proteins.

2. The EGF precursor homology region includes three regions (A, B and C); it is involved in the acid-dependant conformational change needed for the dissociation of the receptor to LDLs, which occurs in endosomes. Moreover it has been recently reported that a post-transcriptional regulator of LDLR, PCSK9, is able to bind to LDLR in this region [117] and to drive it to lysosomal degradation.

3. The "O-linked" oligosaccharide-rich region is a domain rich in threonine and serine, but its function is still unknown; it might be involved in the overall stabilization of the molecule.

4. The TM domain is necessary for the anchorage of the receptor to the cell surface since any mutant devoid of this domain results in a secreted form of the protein.

5. The cytoplasmatic tail of the receptor is highly conserved among different species and its role is mainly related to the positioning of the receptor on the "coated pits" on the cell surface. The proper localization of the receptor allows the binding and the subsequent internalization of LDLs since natural or artificial mutants of this domain are able to bind LDLs but not to mediate their endocytosis.

Lipoprotein uptake occurs in all cells of the organisms but has its main significance in hepatocytes where lipids and TG can be used for VLDL synthesis or driven for excretion with the bile.

1.5.2.2 The SREBP-SCAP system

Both cholesterol synthesis and uptake are transcriptionally regulated by SREBP transcription factors. SREBPs proteins are a group of transcription factors, which are present in three different isoforms in mammalian cells: SREBP-1a, SREBP-1c, and SREBP-2. All three SREBPs are ER transmembrane proteins, shaped like hairpins with both the NH2-terminal transcription factor domain and the COOH-terminal regulatory domain facing the

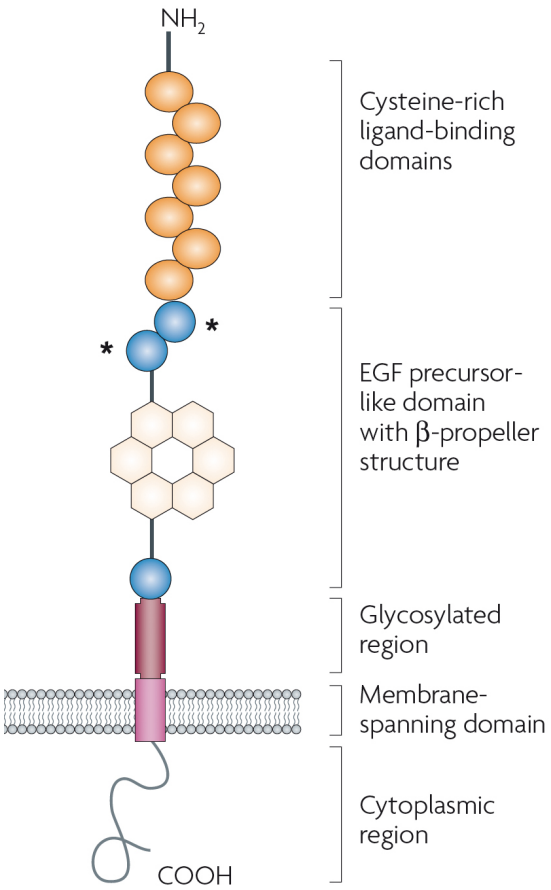


Figure 1.8: **LDLR structure.** Schematic representation of LDLR structure.

cytosol. Immediately after their synthesis on ER membranes the SREBPs bind to another transmembrane protein called SCAP (SREBP Cleavage Activating Protein). SCAP is an SSD-containing protein, embedded in ER membranes through its 8 TM domains.

When cholesterol is abundant, building up also in ER membranes, the sterol binds to SCAP, inducing a conformational change that causes SCAP to bind to a third protein called Insig (Insulin Induced Gene protein 1), an ER retention protein. In this way SCAP remains in the ER along with its attached SREBP [115,118].

In sterol-depleted cells the SCAP/SREBP complex exits the ER in vesicles that bud from ER membranes, thanks to a conformational change in the sterol-sensing domain of SCAP, dissociating Insig and allowing SREBP-

SCAP to reach the Golgi. Two proteases in the Golgi release the active form of SREBP, which translocates to the nucleus to activate transcription of the target genes [118]. SREBP bind to a highly conserved sequence, called SRE (Sterol Responsive Element) located in the promoter region of both LDLR and HMG-CoAR genes thus inducing their transcription.

With respect to the post-trancriptional regulation of HMG-CoAR, accelerated degradation begins when lanosterol (a cholesterol precursor) accumulates in ER membranes. This accumulation induces the reductase to bind to Insig-1, which is pre-associated with a complex of proteins, including an ubiquitin ligase. These events trigger the ubiquitination and subsequent proteasome degradation of HMG-CoAR.

Thus in regulating cellular sterol concentrations, nature has engrafted a specific control mechanism upon two general processes; in both processes the central regulator is the same protein, Insig [115] (Fig. 1.9).

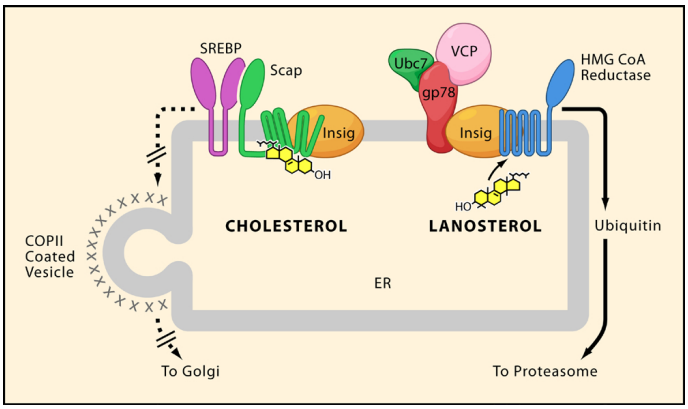


Figure 1.9: **Two Actions of Insigs in Cholesterol Homeostasis.** Insigs regulate ER-to Golgi transport of Scap/SREBP in a process that is inhibited by cholesterol. Insigs also regulate the ubiquitin-mediated proteasomal degradation of HMG CoA reductase in a process that is stimulated by lanosterol.

1.5.2.3 LDLR regulation

The SREBP/SCAP system ensures a tight regulation of the transcription of LDLR, but LDLR is also regulated at a post-transcriptional level by other mechanisms:

1. PCSK9 is a pro-protein convertase, recently discovered, whose role is

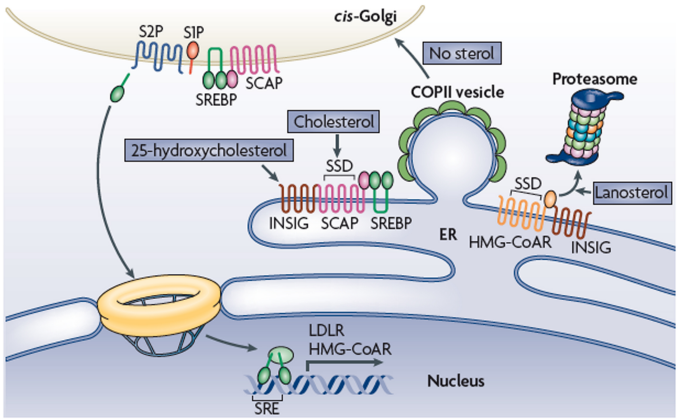


Figure 1.10: **SREBP regulation of cholesterol metabolism.**

to regulate LDLR number on the cell surface of hepatocytes. PCSK9 is secreted by the hepatocytes, binds to the extracellular EGF-A segment of the LDLR, is internalized through clathrin coated vesicles and targets the LDLR towards the endosomal/lysosomal degradative pathway [119]. Both the LDLR and PCSK9 are transcriptionally regulated by SREBP-2. Thus, as more LDLR protein is produced, more PCSK9 is made, ultimately leading to the degradation of the LDLR protein in hepatocytes. One possible explanation for this seemingly futile regulatory cycle is that PCSK9 might act as a 'brake' to slow the uptake of LDL-derived cholesterol by degrading LDLRs. PCSK9 can potentially avert excessive cholesterol accumulation within the cell by preventing the recycling of LDLRs to the cell surface [120]. Its principal site of action is the hepatocyte where PCSK9 molecule is mainly produced, but its action is going to be elucidated also in extra-hepatic tissues.

2. The sterol sensitive nuclear receptor LXR regulates LDLR dependent cholesterol uptake through a pathway independent of the SREBPs. LXR induces the expression of Idol (a newly discovered protein named Inducible degrader of the LDLR), which in turn catalyzes the ubiquitination of the LDLR, thereby targeting it for degradation. Identification of the Idol-LDLR pathway fills a gap in our understanding of how LXRs control cholesterol homeostasis. The ability of LXRs to respond to excess cellular cholesterol by promoting efflux through ABC

transporters has been extensively documented. The LXR-Idol-LDLR pathway provides a mechanism to simultaneously limit LDL cholesterol uptake. Idol and ABCA1 are coordinately regulated by LXR in a cell-type specific manner [121].

1.5.3 Intracellular cholesterol transport

Cholesterol derived from endogenous synthesis or uptake is differentially distributed in subcellular compartments. The ER is the crucial regulatory compartment in cholesterol homeostasis, despite being a cholesterol-poor organelle. It has been estimated that less than 0,5-1% of total cellular cholesterol is located in the ER membranes, but ER cholesterol levels can fluctuate widely; perturbations resulting in modest changes in PM cholesterol, rapidly translate into large changes in ER cholesterol pool, thus demonstrating that cholesterol exchange between PM and ER is highly dynamic. For instance when total cholesterol was increased two fold, the ER cholesterol content rose to 5%; at these levels SREBP cleavage is suppressed, and both cholesterol synthesis and uptake are blocked. Through ER cholesterol pool fluctuations, cells are able to respond with great precision to changes in membrane cholesterol and thereby to finely tune their cholesterol content [122,123].

Furthermore ER cholesterol pool is considered the main regulatory pool because also cholesterol esterification, mediated by ACAT (Acyl-coenzyme A:cholesterol acyltransferase) enzyme occurs in the ER. ACAT-mediated esterification of cholesterol limits its solubility in the cell membrane lipids and thus promotes accumulation of cholesterol ester in the fat droplets within cytoplasm; this process is important because it prevents the intracellular accumulation of free cholesterol which is toxic for the cell. [116,118].

There are two general ways that cholesterol can move intracellularly, vesicular and non-vesicular. Vesicular traffic typically requires an intact cytoskeleton, along which vesicles move, while non-vesicular transport can be mediated by diffusible carrier proteins, with hydrophobic cavities to bind cholesterol and transport it across the aqueous cytosol. The de novo synthesized cholesterol leaves the ER rapidly thereby helping to maintain low ER sterol content. The main destination of this “new” cholesterol is represented

by the plasma membrane, which accounts for the 65-80% of total cellular cholesterol. This movement of cholesterol is likely non-vesicular and sites of close physical membrane apposition between ER and PM could facilitate this transport [118].

The FC originated in late endosomes/ lysosomes from LDL-derived cholesteryl esters, is distributed to other subcellular compartments through the coordinated action of two important proteins, NPC1 and NPC2 (Niemann Pick type C, 1 and 2). Both proteins can bind sterol but if NPC1 is a large glycoprotein, comprising 13 TM domains (5 of which constitute a SSD), located in endosomal membranes, NPC2 is a small soluble cholesterol-binding protein located in the lumen of late endosomes and lysosomes. Their way of transferring and move cholesterol has not been fully elucidated but a model suggests that NPC2 could transfer cholesterol from the inner endosomal membranes to the outer endosomal membrane, where cholesterol efflux must occur, or where NPC1 can "sense" it and mediate its transfer [118].

Despite their low cholesterol content, mitochondria are the site of some important cholesterol-metabolizing enzyme, such as sterol 27-hydroxylase (Cyp27). This enzyme converts cholesterol to 27-hydroxycholesterol, the most abundant oxysterol in plasma. This oxysterol is: i) the first intermediate in the alternative pathway of bile acid synthesis from cholesterol; ii) a more soluble transport form of cholesterol in plasma; iii) a potent repressor of SREBP processing and iv) a partial LXR agonist. It is not clear how cholesterol reaches the mitochondria, probably it could either derive from the PM, either from endosomes, either from cytosolic lipid droplets [118].

1.5.4 Oxysterols

Oxysterols are oxygenated metabolites of cholesterol, present in low concentrations in mammalian cells, since they are more potent than cholesterol itself in affecting critical genes in cholesterol turnover. Perhaps the best support for this model is the existence of nuclear receptors that bind these compounds with high affinity and the fact that oxysterols potently regulate the expression of sterol-sensitive genes in vitro. Furthermore formation of an oxysterol is a mechanism by which some cells may eliminate excess

cholesterol, since oxysterols are more soluble than cholesterol itself.

Besides 27-hydroxycholesterol, which is formed in the mitochondria by Cyp27, other oxysterols like 25-hydroxycholesterol and 24,25- epoxycholesterol are formed during cholesterol synthesis in the ER. Although cholesterol synthesis in isolated cultured cells can be suppressed by either exogenous cholesterol or oxygenated cholesterol derivatives, several studies demonstrated that oxysterols, rather than cholesterol itself, downregulate cholesterol synthesis and uptake by the inhibition of SREBP cleavage. In particular, 25-hydroxycholesterol has been found to be one of the most potent suppressors of sterol synthesis in cultured cells [124].

Moreover oxysterols are generally believed to be the most important physiological activators for Liver X Receptors, the nuclear receptors which regulate ABCA1 synthesis (see 1.2.1).

So oxysterols behave as important regulators of cholesterol homeostasis, inhibiting cholesterol synthesis and uptake through their action on SREBP processing, and promoting cholesterol efflux as LXR ligands.

1.5.5 Cholesterol efflux

The process of FC efflux has been extensively described above (see 1.1.3) anyway in terms of cellular cholesterol homeostasis, it should be remarked that: i) the formation of oxysterols is one of the crucial step in promoting cell cholesterol efflux; ii) the availability of the substrate for ABCA1-mediated efflux, represented by free cholesterol (FC) is also a limiting factor; iii) given the first two issues, the third limiting concept is the accessibility of the FC pool to ABCA1. Any perturbation in cell cholesterol homeostasis, resulting in decreased oxysterol formation, reduced availability of FC or reduced accessibility of the FC pool to ABCA1 may result in an efflux defect, and lead to intracellular cholesterol accumulation. This is the case of some monogenic disorders of cholesterol metabolism, in which the primary defect is not in ABCA1 gene, but the clinical phenotype is often worsened by an efflux defect, consequently leading to intracellular cholesterol accumulation and HDL-C levels reduction in plasma.

1.6 Monogenic disorders of cholesterol metabolism and low HDL-C

Perturbations of CH homeostasis occur in several monogenic disorders such as Niemann-Pick type C disease (NPC), Cerebrotendinous Xanthomatosis (CTX), Familial Hypercholesterolemia (FH). Despite different biochemical defects affecting different intracellular cholesterol pathways, these disorders share a common phenotype that is low levels of plasma HDL. This HDL deficiency may suggest that the mechanisms of HDL formation via ABCA1/ABCG1 mediated FC efflux is defective.

1.6.1 The NPC and CTX example: a systemic defect in HDL levels due to a cellular defect in IC cholesterol transport

Niemann Pick Type C (NPC) disease is a severe autosomal recessive lipidosis characterized by the accumulation of unesterified cholesterol in the endosomal/lysosomal system. Affected individuals show progressive neurodegeneration and hepatosplenomegaly, and the disease is frequently fatal in the first or second decade. The most prominent cellular feature is the accumulation of unesterified cholesterol and glycosphingolipids in the late endosome/lysosome compartment, as well as the trans-Golgi cisternae, and a delay in the transfer of unesterified cholesterol to other intracellular destinations. NPC disease is caused by mutations in two genes: NPC1, which accounts for 95% of NPC patients, and NPC2, which accounts for the remaining 5% of patients [125].

In NPC patients, in addition to the severe phenotypic manifestations listed above, plasma HDL-C levels were low (below 1 mmol/l or 40 mg/dl) in 17 of 21 (81%) NPC disease patient fasting plasma samples studied [126]. It was demonstrated that these reduced levels of HDL-C, observed in NPC patients, are the consequence of impaired ABCA1 regulation, rather than mutation.

The defects in either the two proteins NPC1 and NPC2 cause the entrapment of FC in lysosomes, which is responsible for several downstream

alterations (i.e. loss of feedback inhibition of cholesterol synthesis, lack of down regulation of LDL-receptor expression, reduced formation of oxysterols etc.), due to the inability of FC to down regulate the expression of SREBPs. In addition the conversion of FC to oxysterols is impaired in NPC cells, reducing LXR ligand availability and activation of LXR-dependent genes. In NPC1 cells, expression of the ABCA1 transporter is reduced, resulting in decreased FC efflux [126,127]. A recent study demonstrated that NPC1 has an atheroprotective role in mice through regulation of LXR-dependent FC efflux pathways, specifically mediated by ABCA1 and ABCG1 [128].

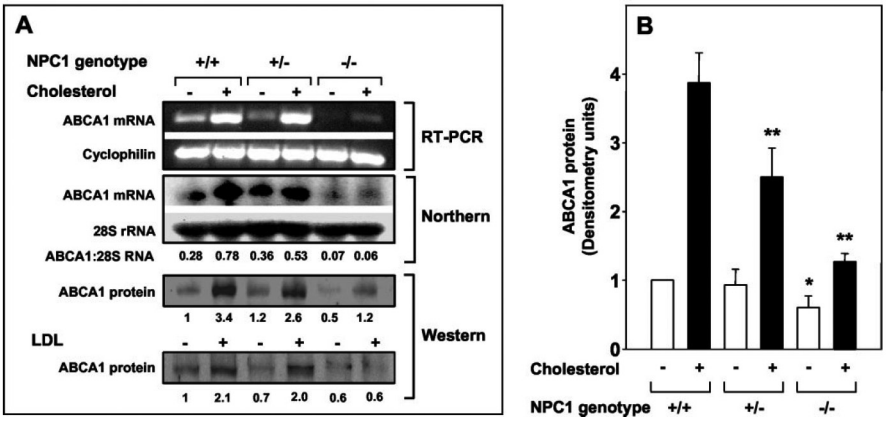
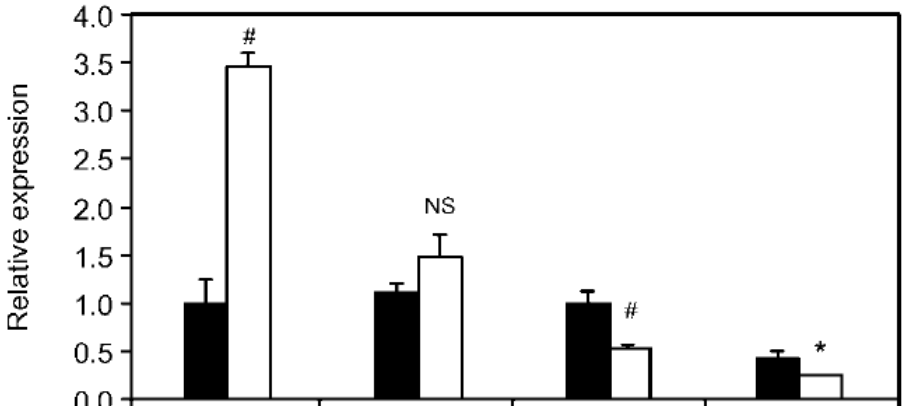


Figure 1.11: **Expression of ABCA1 in human NPC1-deficient fibroblasts.** A, ABCA1 mRNA and protein levels; B, average ABCA1 protein levels in cells incubated in absence or presence of free cholesterol [126].

Cerebrotendinous Xanthomatosis (CTX) is a neurodegenerative disease due to the accumulation of cholesterol and cholestanol in the central nervous system; the molecular defect resides in the lack of activity of sterol 27-hydroxylase (CYP27) which converts cholesterol to 27-hydroxycholesterol. In the liver this enzyme is involved in the conversion of FC to bile acids, while in other tissues the formation of 27-hydroxycholesterol plays a key role in i) the activation of LXR, as this oxysterol is one of LXR ligands, and LXR dependent gene expression (i.e. ABCA1 and ABCG1) [129]; ii) providing a way of facilitating sterol efflux via the formation of a more hydrophilic compound than cholesterol. In macrophages RAW264.7 ABCA1-mediated FC efflux to apoA-I is induced by overexpression of CYP27A1 gene [130].

CTX patients have a complex phenotype, partly reflecting the impor-

tance of CYP27 for bile acid synthesis. Thus, high levels of toxic bile acid intermediates circulate in these patients. There are, however, other features of CTX that are not readily explained by the bile acid biosynthetic defect but are compatible with the notion of a defective formation of endogenous LXR ligands. For example, intracellular cholesterol is elevated in CTX patients, and premature atherosclerosis is relatively common despite low to normal LDL cholesterol levels. A single study [131], reported very low HDL levels in CTX patients. This would be expected if the absence of CYP27 caused a low intracellular LXR tone leading to low ABCA1-mediated high density lipoprotein formation.



	ABCA1 normal	ABCA1 CTX	SREBP-1c normal	SREBP-1c CTX
■ -chol	1.0	1.1	1.0	0.4
□ +chol	3.5	1.5	0.5	0.2
□ Ratio	3.5	1.3	0.5	0.6

Figure 1.12: **ABCA1 and SREBP-1c expression in CYP27 deficient fibroblasts** ABCA1 and SREBP-1c gene expression in normal and CYP27-deficient human fibroblasts: response to cholesterol loading [129].

1.6.2 Familial Hypercholesterolemia: cholesterol influx defect and intracellular defects

Familial Hypercholesterolemia (FH) is an autosomal codominant disease caused by mutations in LDLR gene. FH heterozygosity is retrieved in the general population at a frequency of about 1:500; these patients

show high levels of total cholesterol (from 350 to 450 mg/dl) and LDL-C in plasma, tendon xanthomas, premature atherosclerosis. FH homozygotes (1:1.000.000) have extremely high levels of total (600-1000 mg/dl) and LDL cholesterol in plasma, xanthomas and a severe, accelerated and premature atherosclerosis. Low levels of HDL-C are frequently observed in FH (HDL-C 30,7 ± 9,5 mg/dL, [132]) but the molecular mechanism underlying this defect has not been fully characterized.

In FH cell the defect in LDL-R prevents the uptake of plasma LDL and the internalization of LDL-derived cholesterol. While the accumulation of plasma LDL represents the major pathogenic factor in FH, the defective receptor mediated uptake of LDL derived cholesterol causes cell free-cholesterol deprivation. This is responsible for the activation of SREBP transcription factors which induce the expression of cholesterol related genes (i.e. HMG-CoA reductase, LDL-receptor). SREBP has recently been described as a negative regulator of ABCA1 expression and function in LDL-R-/- murine macrophages. In particular SRPEB1a inhibits ABCA1 promoter activity in an E-box independent and LXRE-dependent manner and ABCA1 expression in animal tissues has been demonstrated to be inversely correlated to active SREBP1. Oxysterols usually inactivate SREBP1 in macrophages, thus strengthening ABCA1 activity but, if LDLR is absent, it is not able to insure inhibition of SREBP1 proteolysis and this leads to an abnormal response of SREBP1 processing of sterol [133]. This mechanism suggests that the low HDL levels identified in FH patients, could be caused by a defect in ABCA1 regulation and function. Moreover it has been recently reported that monocytes derived from FH homozygotes have a decreased expression of ABCA1 protein accompanied by a reduced level of ABCA1 mRNA [134]. These recent findings and the availability of FH derived fibroblasts encouraged us to elucidate if the lack of a functioning LDLR impact on cholesterol efflux and ABCA1 regulation: thus a new pathway connecting cellular lipids uptake and cholesterol efflux could contribute to intracellular cholesterol homeostasis.

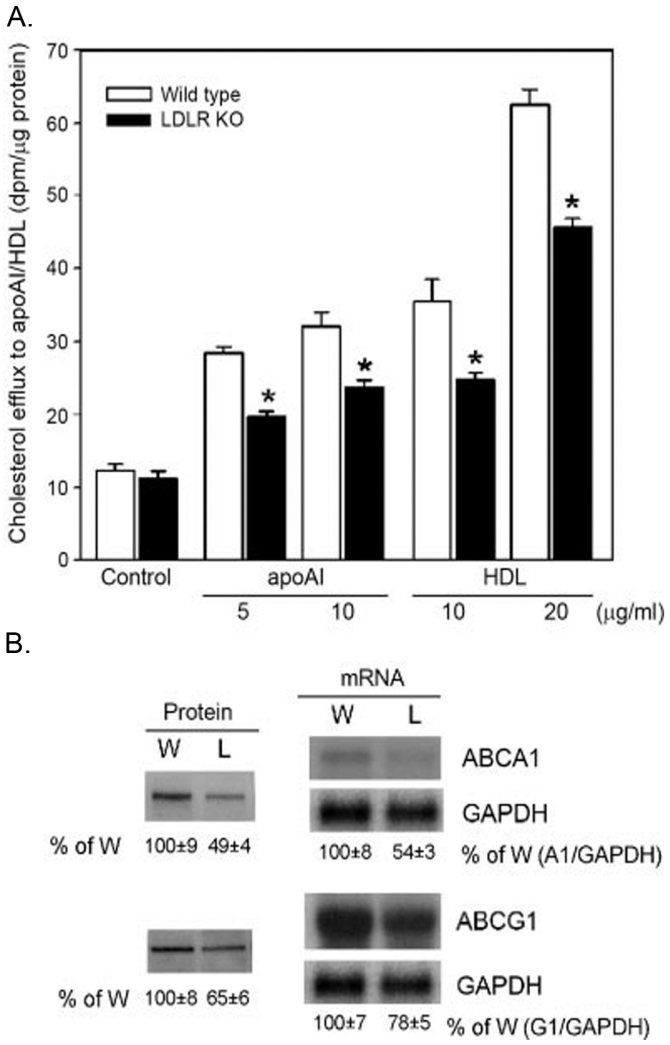


Figure 1.13: **ABCA1 expression and function in LDLR_/_ macrophages**A, LDLR_/_ reduces cholesterol efflux and expression of ABCA1 and ABCG1 in macrophages. Wild type and LDLR_/_ macrophages were used to conduct free cholesterol efflux. B, mRNA and protein levels of ABCA1 and ABCG1 in LDLR_/_ macrophages [133]

Chapter 2

AIM OF THE STUDY

Aim of this study was the investigation of ABCA1 transporter regulation and function in monogenic disorders of cholesterol metabolism as Tangier Disease (TD) and Familial Hypercholesterolemia (FH).

A first objective was the identification of new ABCA1 gene mutations in subjects with low HDL-C levels.

A second aim was the characterization of the novel identified mutations, preferentially employing an "ex-vivo" approach, using Tangier Disease or FHD patients' derived fibroblasts. If this approach was not possible due to lack of patient derived cells, in vitro studies were performed on transfected cells.

A third purpose of this work was the study of ABCA1 expression and function in FH patients' derived fibroblasts, in order to verify the existence of a regulatory pathway connecting lipoprotein uptake and cholesterol efflux in a physiological human cell model.

Chapter 3

PATIENTS, MATERIALS AND METHODS

3.1 Subjects analyzed between 2005 and 2009

Subjects with almost complete HDL-C deficiency (HDL-C below the 5th percentile of the distribution in the population: <0.77 mmol L^{-1} in males and <0.93 mmol L^{-1} in females), suspected to have primary hypoalphalipoproteinemia or with clinical signs of Tangier Disease were identified by an international network of Lipid Clinics and referred to our laboratory for genetic analysis. They were investigated in our laboratory in collaboration with the laboratory of prof. S. Bertolini from the department of Internal Medicine, University of Genoa.

In the last 5 years, 27 subjects with low HDL were investigated. On the basis of biochemical (nearly absent HDL-C) and clinical data, 12 of these patients were expected to be affected by Tangier Disease and 15 by FHD, since they didn't show any clinical feature except reduced HDL-C levels (approximately half of normal value).

Informed consent was obtained from all subjects. The study protocol was approved by the relevant institutional human investigation committees as appropriate.

Plasma cholesterol and triglycerides were measured enzymatically and apoA-I, apoA-II, and apoB by immunoturbidimetry (Roche Diagnostics GmbH,

Mannheim, Germany) using an automated analyzer (Hitachi model 912, Hitachi, Ltd., Tokyo). HDL-C levels were measured in plasma supernatant after precipitation of apoB-containing lipoproteins by phosphotungstate-MgCl2. Plasma lipoproteins were separated by density gradient ultracentrifugation. Two-dimensional gel electrophoresis of apoA-I was performed.

3.2 Materials

3.2.1 Buffers and media

Medium/buffer	**Composition**
DNA loading buffer III 6X	0.25% Bromophenol Blue 0.25% Xylene cyanol 30% Glycerol
TAE 50X buffer	242 g/L Tris 57.1 ml Glacial Acetic acid 100 ml of 0.5M EDTA pH 8
LB broth	10g/L Bacto-Tryptone 5g/L Bacto-Yeast extract 10g/L NaCl taken to pH 7 with NAOH 5N
LB agar	Obtained through resuspension of LB broth with final concentration 1.5% Agar powder, then solubilized via autoclavation
SOC medium	20g/L Bacto-Tryptone 5g/L Bacto-Yeast extract 0.5g/L NaCl final 2.5mM KCl added with final 20mM Glucose and 10mM MgCl2
P1 buffer for Miniprep	3.722 g/L EDTA, 6.055 g/L Tris, to pH 8 with HCl plus 100 µg/ml RNAse

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Medium/buffer	Composition
P2 buffer for Miniprep	20% SDS
	8 g/L NaOH
P3 buffer for Miniprep	294.45 g/L Potassium Acetate to pH 5.5 with Glacial Acetic Acid.
TE buffer	100 mM Tris-HCl
	1 mM EDTA pH 8
DMEM-F12 Glutamax-I	From Gibco
	10% FBS
	100 U/ml Penicillin
	100 μg/ml Streptomycin
PBS 1X buffer	137 mM NaCl
	2.7 mM KCl
	10.1 mM Na2HPO4
	1.8 mM KH2PO4
Protein extraction Buffer B	50 mM Hepes
	100 mM NaCl, pH 7.4
Protein extraction Buffer C	1 mM PMSF
	10 mM EDTA
	10 mM EGTA
	2.2% DMSO, to volume with buffer B
Lysing Buffer	1% Triton X100
	0.5 mM Leupeptin, to vol. with buffer C.
Laemmli sample Buffer 1X	62.5 mM Tris-HCl pH 6.8
	2% SDS
	25% Glycerol
	0.01% Bromophenol blue

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Medium/buffer	**Composition**
SDS-PAGE Resolving gel 8%	4.6 ml ddH2O
	2.7 ml of 30% Acrilamide mix
	2.5 ml of 1.5M Tris pH 8.8
	0.1 ml of 10% SDS
	0.1 ml of 10% Ammonium Persulfate
	0.006 ml TEMED for a total vol. of 8 ml.
SDS-PAGE Stacking gel	2.7 ml H2O
	0.67 ml of 30% Acrilamide
	0.5 ml of 1M Tris pH 6.8
	0.04 ml of 10% Ammonium Persulfate
	0.04 ml of 10% SDS
	0.004 ml of TEMED for a total vol. of 4 ml.
Western Solution 10X	30.3 gr Tris
	144 gr Glycin in 1L of water
SDS-PAGE Running buffer	10% Western Solution
	1% SDS
Western Blot Transfer buffer	10% Western Solution
	20% Methanol
TBS 10X	0.1 M Tris pH 7.5
	1M NaCl
TBS-T 1X	10% TBS 10X
	0.1% Tween 20

Table 3.1: Buffers and Media

3.2.2 Instruments and softwares

In the following table a list of all instruments and softwares used is provided:

Web-based tools and softwares

Software	**Type**	**URL**
PolyPhen	In silico prediction	http://genetics.bwh.harvard.edu/pph
Panther	In silico prediction	http://www.pantherdb.org/
Automated Splice Site Analysis Software	In silico prediction	https://splice.uwo.ca
WebCutter	DNA restriction map	http://rna.lundberg.gu.se/cutter2/
BLAST	Basic Local Alignment Search Tool	http://blast.ncbi.nlm.nih.gov/
The QuickChange® Primer Design Program	Mutagenesis Primer Design	http://www.stratagene.com/
Mutation Discovery	PCR Protocol Writer	http://www.mutationdiscovery.com/
WAVEMAKER Software	DHPLC NAVIGATOR software	-
Sequence Scanner, Applied Biosystems	Sequence Analysis Software	https://products.appliedbiosystems.com/
Instrument	**Type**	
DHPLC	WAVE® DHPLC Instrument (Transgenomic Inc.)	
Thermal Cycler	Biometra T3 Thermalcycler, Dasit.	
DNA sequencer	ABI Prism®3100 genetic Analyzer.	
FACS	Epics XL cell sorter, Coulter.	
Confocal microscope	Leica DM IRE2; Leica Microsystems GmbH, Wetzlar, Germany	

Table 3.2: Instruments and softwares.

3.3 Methods

3.3.1 Isolation of DNA from peripheral blood

Genomic DNA was extracted from peripheral blood leucocytes by Invisorb® Spin Blood Maxi Kit (Invitek, GmbH, Berlin, Germany) according to manufacturer's instructions.

3.3.2 Polymerase Chain Reaction (PCR)

The molecular analysis of an heterogeneous sample like DNA demands a former selective amplification of the sequence of interest.

The general protocol for DNA amplification requires a final volume of 50 µl, containing:

• Genomic DNA	(≅ 0.1 µg)	2 µl
• dNTPs	(final 10 mM of each one)	1 µl
• MgCl2/MgSO4	(2 mM final)	4 µl
• Buffer	(final 1X)	5 µl
• Primer	(75 pm of each one)	1 µl
• Taq	(2.62 U/sample)	0.7µl
• ddH2O	until final volume	

In our laboratory we employed two different polymerases namely:

- *DNA polymerase Expand™ High Fidelity PCR System* (Roche Diagnostics Gmbh, Mannheim, Germany) obtained through a combination of a heat-stable polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus* (Taq), and a Pwo DNA polymerase with a 3'-5' exonuclease proof-reading activity which gives a higher fidelity, yield and specificity in DNA synthesis. This polymerase was used for standard PCR amplification.

- *Optimase Polymerase* (Transgenomic, Inc., Omaha, NE, USA), with a 3'-5' exonuclease activity was used for amplification prior to DHPLC analysis, drastically reducing mis-incorporations during amplification and the interference with the stationary phase of the chromatographic column.

Two different amplification programs were used for the 50 exons of ABCA1 gene, due to the different annealing temperatures required by the primer-couples:

Amplification program 3 steps 60°-72°C:

- 3 minutes at 95°C
- 40 seconds at 95°C

- 40 seconds at 60°C
- 2 minutes at 72°C

] 29 cycles

Used for exons: 2→8, 10→15, 17→24, 26, 27, 29, 32, 33, 35, 38, 40.

Amplification program 3 steps 63°-72°C:

- 3 minutes at 95°C
- 40 seconds at 95°C
- 40 seconds at 63°C
- 2 minutes at 72°C

] 29 cycles

Used for exons: 9, 16, 25→28, 30, 31, 34, 36, 37, 39, 41→47, 49.

Three different programs have been used for exons 1, 48 and 50 under advice of the DHPLC Company, Transgenomic (`www.mutationdiscovery.com`).

Amplification program 3 steps 61.4°-72°C for Exon 1

- 2 minutes at 95°C
- 30 seconds at 95°C
- 30 seconds at 61.4°C
- 50 seconds at 72°C

] 29 cycles

- 5 minutes at 72°C

Amplification program 3 steps 58.7°-72°C for Exon 48:

- 2 minutes at 95°C
- 30 seconds at 95°C
- 30 seconds at 58.7°C
- 30 seconds at 72°C

] 29 cycles

- 5 minutes at 72°C

Amplification program 3 steps 61°-72°C for Exon 50:

- 3 minutes at 95°C
- 40 seconds at 95°C
- 40 seconds at 61°C
- 2 minutes at 72°C

] 29 cycles

3.3.3 DHPLC

The DHPLC (Denaturing High Performance Liquid Chromatography) is a sensitive, fast and specific automated technique for a large-scale screening of

sequence variations (both rare pathogenic mutations and common nucleotide polymorphisms).

DHPLC appears to be a reliable method mainly for the analysis of large genes known to be highly polymorphic and with a large variety of pathogenic mutations. Numerous reports in the last years documented the high accuracy and excellent sensitivity of DHPLC (96–100%) in detecting mutations in more than 250 genes [109]. Thus, due to ABCA1 noteworthy dimension, it is sensible to perform a pre-screening through DHPLC that might limit the following Sanger's sequencing step just to a short number of positive PCR samples. The sequencing reaction is still a necessary step for a complete characterization of the identified variants, being the DHPLC only a qualitative method [80,135].

3.3.3.1 Basic Principles

DHPLC is a technology based on the detection of heteroduplexes in PCR products through ion-pair reversed-phase liquid chromatography under partial denaturing conditions within an acetonitrile gradient. Heteroduplexes and homoduplexes are the result of chance re-association of DNA strands after a denaturation cycle in a heterozygous sample. In detail, denatured and re-natured wild type PCR products lead to a homogenous population of DNA duplexes, perfectly matched, known as homoduplexes. PCR products containing a heterozygosity and undergoing a cycle of denaturation and re-naturation, will lead to four different duplex populations: two perfectly matched homoduplexes and two mismatched heteroduplexes. The presence of a sequence mismatch leads to a reduced column-retention time compared with their homoduplex counterparts. The software transforms this different elution-time in a graphic with a unique peak if the analyzed sample is homozygote, while a multi-peak profile will refer to a heterozygous sample (where heterozygosis may be for one or more sequence variants). With the purpose of identifying a homozygous variation, it is required to mix the PCR product with a WT sample in order to artificially reproduce the heterozygous condition.

3.3.4 Electrophoresis on Agarose gel

A control of the DNA amplification was performed through an electrophoresis on Agarose gel at 1-1.5%, stained with Ethidium Bromide; 5 µl of each amplification product are mixed with 1 µl of Loading Buffer 6X and loaded on a gel together with a molecular weight marker, in order to qualitatively and quantitatively verify the specificity of the PCR fragment. After electrophoresis it is possible to detect the amplicon as a neat, single band using a transilluminator. If the intensity of the band was weak, it was necessary to increase the DHPLC injection volume or the volume used for direct sequencing, with the aim to obtain highly comparable samples.

3.3.5 DHPLC sample preparation

Before loading in the DHPLC system, PCR products underwent a denaturation and renaturation cycle by heating for 5 minutes at 95°C and gradually decreasing temperature to room temperature; this step leads to DNA duplexes formation.

Afterwards, PCR products were loaded in a 96-wells platform, automatically injected in the DHPLC system, analysed and eluted again after nearly 8 minutes.

The analysis report appeared as a chromatogram, where peaks were detected by an UV transilluminator at a wavelength of 260 nm.

Our approach was based on the DHPLC analysis as pre-screening step of all 50 ABCA1 amplicons; only the DHPLC positive amplicons were subjected to direct sequencing.

3.3.6 Purification of PCR products

Before performing direct sequencing it was necessary to purify the amplification products in order to eliminate primers, dNTPs, and genomic DNA, which were not incorporated during the PCR reaction. On this purpose we used the High Pure PCR Product Purification Kit (Roche Diagnostics S.p.A. Roche Applied Science, Monza (MI), Italy

3.3.7 Sequence reaction

In order to sequence ABCA1 gene we performed a "cycle sequencing" using the chain-terminator method (or Sanger method). The key principle of the Sanger method is the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators. In detail, we used the Dye-terminator sequencing, where the four ddNTPs chain terminators are labeled with fluorescent dyes, each of which has different wavelengths of fluorescence and emission.

The sequencing reaction is performed as follows:

- Big-Dye Terminator Ready Reaction Mix 2 µl
- Purified PCR product 5-7 µl
- Forward or Reverse Primer 1 µl
- ddH2O until a volume of 10 µl.

For each exon it was necessary to perform two different reactions, either with a Forward primer or a Reverse primer so that it could be possible to compare the two complementary sequences in order to have a more reliable result.

The reaction was carried out in a Biometra T3 Thermalcycler with the following conditions: 96°C for 10 sec, 50°C for 15 sec, 60°C for 4 min for 25 cycles.

3.3.8 Purification of sequence reactions

With the aim to eliminate unused fluorochromes, a purification step was accomplished as follows:

- Addition of 2 µl of 3M Sodium Acetate (pH 4.6) and 50 µl of Ethanol 100% to the sequence reaction
- Incubation on ice for 10 minutes.
- Centrifugation at 13200 rpm for 30 minutes.
- Discard of the supernatant.
- Washing with 250 µl of ethanol 70%.
- Centrifugation at 13200 rpm for 5 minutes.
- Discard of the supernatant.
- Drying of the pellet for 5 minutes at room temperature.

Samples were maintained at -20°C before loading in the Genetic Analyzer (ABI Prism®3100, Applied Biosystems, Inc, Foster City, CA, USA).

The instrument used in our laboratory was an automatic DNA sequencer formed by 16 capillaries. Each purified sample was resuspended in 6 µl of ddH2O and 2 µl of this sample were loaded in a 96-wells platform with 10 µl of Formamide.

After running, the raw data were analyzed by a Sequencing Analysis Software and an electropherogram was elaborated.

3.3.9 Screening of ABCA1 gene mutations

Our diagnostics approach allowed us, in collaboration with Prof. Bertolini's lab at the Department of Internal Medicine, University of Genoa, to identify 22 ABCA1 mutations. Each new mutation was screened in a control population (50-100 alleles) mainly by DHPLC or enzymatic methods. The mutation screening work was mainly performed by prof. Bertolini's lab.

3.3.10 In silico prediction

We performed computational analysis of the missense mutations of ABCA1 using Poly-Phen (http://genetics.bwh.harvard.edu/pph/), PANTHER (`http://www.pantherdb.org/`) [136]. Splice site mutations on ABCA1 gene were analysed through the Automated Splice Site Analysis Software (`https://splice.uwo.ca/`) [137].

3.3.11 Minigene construction of ABCA1 Ex19-23

To investigate the effects of the splice site mutation in the acceptor splice site of intron 20 (c.2961 –2 A>C) we adopted first an in vitro strategy. We constructed a wild type ABCA1 minigene from genomic DNA of a control subject using the following primers: 5'-GGA TCC TTC TGT GGG TTC ATT TCT GTC TTC-3 (forward primer, complementary to intron 18, which includes a Bam HI site at its 5' end, highlighted by underlined nucleotides) and 5'-GCG GCC GCA GAA ATC ATT CAC AGC CAG CAA GT-3' (reverse primer, complementary to intron 23, which includes a Not I site at its 5' end, highlighted by underlined nucleotides). The amplification conditions were the following: 95°C for 2 min, 95°C for 30 sec/60°C for 30 sec/72°C for 6 min for 30 cycles, followed by an extension at 72°C for 5 min. This

minigene, encompassing a genomic region spanning from the 3' end of intron 18 to the 5' half of intron 23 (4129 bp), was cloned in the pTargeTTM (Promega, Madison, WI, USA) expression vector and subjected to site directed mutagenesis to introduce the A>C transversion in the acceptor splice site of intron 20 (c.2961 –2 A>C).

3.3.12 Creation of the pcDNA3.1-ABCA1-GFP vector

3.3.12.1 pcDNA3.1 and pEGFP-C3

pcDNA™ 3.1(+) is a 5.4 kb vector (Invitrogen, Carlsbad, CA, USA) derived from pcDNA™3 and designed for high-level stable and transient expression in mammalian hosts.

pEGFP-C3 (Clontech Laboratories, Inc., Mountain View, CA, USA) is a 4.7 kb vector encoding for a variant of WT GFP with emission in red and containing a Multiple Cloning Site (MCS) in 3' position with respect to EGFP cDNA; this means that it is suitable for cloning of proteins at the ending of EGFP itself.

We did not use this feature but just utilized this vector as a template for a PCR containing EGFP cDNA.

3.3.12.2 pcDNA3.1-ABCA1-GFP

A pcDNA3.1-ABCA1-GFP vector was created in our laboratory through an overlapping PCR strategy few years ago, using as templates a pcDNA1-ABCA1 vector (a kindly gift from Dr. Mason W. Freeman, Harvard Medical School, Massachussets General Hospital, Boston) and the pEGFP-C3 vector showed before.

The procedure involved:

i) Two separate PCR of the 3' part of ABCA1 cDNA and the 5' part of GFP cDNA;

ii) An overlapping PCR, which allowed the fusion of the two amplicons previously obtained [96];

iii) A subsequent cloning step by using restriction enzymes.

First the C-terminal part of ABCA1 cDNA (Ex 47-50) and the GFP cDNA were independently amplified with specific primers (ABCA1 CtermF:

5'-GGG CTG GAA AAT CAT CAA CTT TCA A- 3'; ABCA1 CtermR: 5'-CTT GCT CAC CAT TAC ATA GCT TTC-3'; GFP PrF: 5'-GAA AGC TAT GTA ATG GTG AGC AAG-3'

GFP PrR: 5'-TCT TTG TCG CGG CCG CTT TAC TTG TAC AGC TCG TCC ATG CC-3'), leading to an amplicon of 950 bp and one of 749 bp respectively.

Secondly, an overlapping PCR was conducted in order to join these two fragments (Primers: ABCA1 CtermF: 5'-GGG CTG GAA AAT CAT CAA CTT TCA A- 3' and GFP PrR: 5'-TCT TTG TCG CGG CCG CTT TAC TTG TAC AGC TCG TCC ATG CC-3'), resulting in an amplicon of 1699 bp, containing a BamHI restriction site in ABCA1 (c.6225) and a NotI restriction site artificially inserted in GFP cDNA.

Moreover, a specific double digestion of pcDNA1-ABCA1, pcDNA3.1 and of the overlapping PCR product was performed with BamHI and NotI (Fermentas International Inc., Burlington, Ontario, Canada).

Among all, the following fragments were selected:

- A 6281 bp fragment containing Ex 2-47 of ABCA1 cDNA (BamHI-BamHI cut)
- A 5378 bp pcDNA3.1 fragment (NotI-BamHI cut)
- A 1699 bp fragment obtained from the overlapping PCR and containing the ABCA1 C-term GFP sequence (BamHI-NotI cut).

The final pcDNA3.1-ABCA1-GFP vector was achieved after purification of the digested fragments controlled on a 1-1.5% Agarose gel; a following ligation between fragments pcDNA3.1 (BamHI-NotI) and ABCA1 Cterm-GFP (BamHI-NotI) in order to obtain a close circular plasmid with just one BamHI-site; a digestion of this circular plasmid with BamHI, dephosphorylation of this one and a final ligation with the fragment ABCA1 Ex2-47 (BamHI-BamHI).

3.3.12.3 pcDNA3.1-ABCA1-GFP Δ11-33

This vector was created together with pcDNA3.1-ABCA1-GFP vector in order to have a negative control for all the studies performed. This mutant

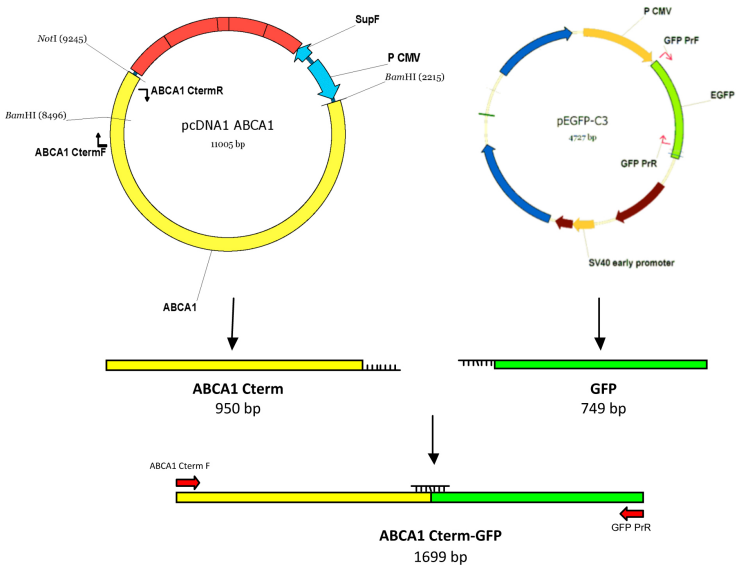

Figure 3.1: **Overlapping PCR strategy.**

encodes an ABCA1 protein with an in-frame deletion of 1102 amino acids (del p.K422_K1524) and, in view of its structural alteration, is expected to be retained in the endoplasmic reticulum.

This deleted vector was obtained via digestion with the restriction enzyme BglII (Fermentas International Inc., Burlington, Ontario, Canada), which has 3 restriction sites on ABCA1 cDNA in position 1265 bp, 2327 bp and 4571 bp. The digestion of pcDNA3.1 ABCA1-GFP led therefore to 3 fragments which were isolated via purification on an Agarose gel; the two central fragments of 1062 bp and 2244 bp were eliminated and the vector closed up by ligation, maintaining ABCA1 sequence in frame.

3.3.13 Transformation chemically competent E.coli

Tranformation of chemically competent E.coli was performed for the autonomous auto-replication of the vectors and isolation of a large amount of plasmid DNA, necessary for the following experiments. We employed TOP10F chemically competent E. coli (Invitrogen, Carlsbad, CA, USA) for the transformation of pcDNA3.1 ABCA1-GFP and pcDNA3.1-ABCA1-GFP Δ11-33 while JM109 competent cells (Promega Corp., Madison, WI, USA)

were employed for the transformation of the pTARGET vector encoding for the minigene ABCA1 Ex19-23.

Since all vectors carried an ampicillin-resistance gene, ampicillin (50 µg/ml) was added to LB agar plates and LB broths for selection of desired colonies.

Transformation was performed according to manufacturers' inctructions.

3.3.14 Amplification of plasmidic cDNA in prokaryotic cells

3.3.14.1 Small scale isolation of plasmid DNA

Miniprep is a method that provides a simple and rapid isolation of plasmid DNA from transformed bacterial cultures.

First of all it is necessary to prepare a number of 15 ml-tubes corresponding to the number of colonies to test. Three ml of LB broth added of 50 µg/ml ampicillin are aliquoted in each tube; with the help of a tip a single colony is picked up and put inside the tube together with the tip and cells are grown at 37°C over night at 225 rpm in a shaking incubator.

The following day bacterial cultures are processed in this way:

1. Collect 1.5 ml of each grown culture in a 2 ml-tube and centrifugation for 5 minutes at 13200 rpm.
2. Pour off the surnatant and wash the pellet resuspending it with 1 ml of 0.15M NaCl.
3. Centrifuge at 13200 rpm for 5 minutes and pour off the surnatant again.
4. Resuspend the bacterial pellet in 300 µl of P1 buffer (see 3.2.2), mix and vortex until thorough resuspension.
5. Add 300 µl of P2 buffer (see 3.2.2) and mix by inverting the tube 4-6 times.
6. Incubate for 5 minutes at room-temperature in order to obtain cell lysis and DNA denaturation (both chromosomal and plasmid DNA).
7. Add 300 µl of P3 buffer (see 3.2.2) and invert the tubes up and down 4-6 times in order to obtain plasmid DNA renaturation.
8. Centrifuge for 15 minutes at 4°C at 13200 rpm to separate cell debris from nucleic acids.
9. Transfer swiftly the surnatant in a new reservoir.
10. Treat each sample with 0.6 volumes of Isopropyl alcohol (˜ 540 µl).
11. Incubate at -80°C for 20 minutes.
12. Centrifuge at 4°C for 20 minutes at 13200 rpm.
13. Pour off the surnatant being careful of not touching the pellet.

14. Wash with 1 ml of 70% ethanol and centrifuge at 4°C for 15 minutes at 13200 rpm.
15. Pour off the surnatant and air-dry the pellet.
16. Elute the plasmid DNA in 21 µl of ddH2O and store at -20°C.

The part of culture that is not processed can be stored at -20°C for long periods after addition of Glycerol in a quantity equal to 30% of the total volume and brief vortexing.

3.3.14.2 DNA selection via sequencing

It was possible to have a confirmation of the specificity of the plasmid DNA obtained through Miniprep via sequencing.

A number of exonic primers designed for ABCA1 cDNA were used to detect the presence of each mutation inserted in pcDNA3.1-ABCA1-GFP vector. For every mutation it was used the primer that, when elongated, covers the region containing the specific variation.

These primers altogether cover, when amplified, the whole length of ABCA1-GFP cDNA.

11F TGTTCTCAGATGCTCGGAGGCTT
15F GATTCAACTTGGTGACCAAGAAG
1,4F TGGCACTGAGGAAGATGCTGAA
1,9F TTTGGGAACAGCAGTTGGATGGCTT
2F CTCAGTGGCTGTGATCATCAAGG
17F TTGCACCAAGTCCTACTGGTTTGG
2,4F GGATGTTGGTTTGCCATCAAG
2,8F TCCTGCAGAAACAGTAGTAGCACTG
3,1F GGCTGGAAACTTACACAGCAACAG
3,2F ATCAAGAAGATGCTGCCTGTGTGTCC
3,3F GCATGCAATCAGCTCTTTCCTGAAT
3,4F TCCCTGCCACACTGGTCATTATCA
4,1F TCTTCATCAGGCCCAGACCTGTAA
4,2F TTGGCAAGGTTGGTGAGTGGGCGATT
BGH TAGAAGGCACAGTCGAGG

3.3.14.3 Large scale isolation of plasmid DNA

This procedure leads to a large amount of plasmid DNA, indispensable for the following steps of this study. For this reason we used the QIAGEN® Plasmid Maxi Kit (QIAGEN, Hilden, Germany): the protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. RNA, proteins, dyes and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and concentrated and desalted by isopropanol precipitation.

Before applying this procedure, a starter culture of 5 ml LB medium (added of 50 µg/ml ampicillin) was inoculated with 200 µl of glycerolized culture stored from Miniprep and incubated over night at 37°C in shaking incubator at 225 rpm.

This culture was then diluted 1/500 into selective LB medium (500 µl of starter culture in 250 ml of medium) and grown over night at 37°C in shaking incubator at 225 rpm. Every following step was performed following the manufacturer's protocol.

The final pellet, consisting in plasmid DNA, was air-dried for 5 minutes and re-dissolved in 300 µl of TE buffer pH 8.

It was possible to determinate the DNA concentration for each sample measuring the absorbance at 260 nm with a spectrophotometer: at this wavelength, 1U of Optic Density (O.D.) corresponds to a concentration of 50 µg/ml of DNA. The DNA quality is defined by the ratio between absorbance at 260 nm and 280 nm, respectively related to absorbance peaks of nucleic acids and proteins. Samples with a ratio ≥ 1.7 were considered acceptable.

3.3.14.4 Control of plasmid DNA through sequence reaction

All samples were analyzed via sequencing with all the primers listed above (see 3.3.14.2) in order to ascertain that no mutation was introduced during the whole procedure and culturing except for the ones desired. 100 ng of plasmid DNA were sequenced using 10 pm/µl of each primer.

3.3.15 Cells

3.3.15.1 HEK293 cell line

Human Embryonic Kidney 293 cells, also often referred to as HEK293 or less precisely as HEK cells, are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture.

We mainly used these cells for functional studies of ABCA1 missense mutations.

They were cultured in T75 flasks using a Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA) with 10% FBS, penicillin and streptomycin (see 3.2.2) at 37°C in a humidified 5% CO2 incubator.

Every 3-4 days of culture the cell monolayer was trypsinized with a solution of 0.05% Trypsin in PBS and 0.02% EDTA. Cells were counted with a Neubauer chamber and plated with a 1:7 dilution.

3.3.15.2 COS-1 cell line

COS is a cell line used to transfect cells to produce recombinant proteins for molecular biology, biochemistry, and cell biology experiments. Two forms of COS cell lines commonly used are COS-1 and COS-7.

The COS cell line was obtained by immortalizing a CV-1 cell line derived from kidney cells of the African green monkey with a version of the SV40 genome that can produce large T antigen but has a defect in genomic replication.

When an expression construct with an SV40 promoter is introduced into COS cells, the vector can be replicated substantially by the large T antigen.

The word COS is an acronym, derived from the cells being CV-1 (simian) in Origin, and carrying the SV40 genetic material. We used COS-1 cells for the in vitro analysis of the c.2961-2A>C mutation. They were cultured in T75 flasks using a Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA) with 10% FBS, penicillin and streptomycin (see 3.2.2) at 37°C in a humidified 5% CO2 incubator. Every 3-4 days of culture the cell monolayer was trypsinized with a solution of 0.05% Trypsin in PBS. Cells were counted with a Neubauer chamber and plated with a

proper dilution. Wild type and mutant minigenes were authenticated by sequencing before transfection into COS-1 cells. Transfection was performed 24 hours after seeding of cells in 60 mm Petri dishes.

3.3.15.3 Fibroblasts

The Cell Bank of the Department of Biomedical Sciences at the University of Modena and Reggio Emilia was created during the last two decades by collecting many different pathological and control cellular samples. It is mainly composed of fibroblasts derived from cutaneous biopsies of healthy subjects as well as patients affected by differential monogenic disorders. Among this collection, monogenic disorders of cholesterol metabolism are well represented; thus we took advantage of the availability of Tangier Disease fibroblasts for a complete "ex-vivo" characterization of the ABCA1 mutations responsible of the defect. Moreover a huge collection of FH fibroblasts gave us the possibility to study the ABCA1 expression and function in these cells.

In the table 3.3 a complete list of the fibroblasts cells employed in this work was listed:

Cells were cultured in T75 flasks in Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA), 100 IU/ml of penicillin, and 50μg/ml of streptomycin, 2 mM L-glutamine, 10% FBS, and 95% air- 5% CO2. Every 4-5 days or when confluence was reached, the cell monolayer was trypsinized with a solution of 0.05% Trypsin in PBS. Cells were counted with a Neubauer chamber and plated with a proper dilution (1.5-2*10^6/T75 flask).

3.3.15.4 Monocyte isolation from whole blood

10-15 ml of heparinised blood were diluted twice in RPMI 1640 (Gibco, Invitrogen, Carlsbad, California); the mixture was layered on 1.077 g/ml of Ficolle Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), centrifuged at 400g for 30 min. The mononuclear cell layer at the interphase was collected, washed twice with a solution of PBS and centrifuged at 200g for 15 min until depletion of thrombocytes. The whole blood collection and monocyte isolation was performed at the CNR Institute of Physiology (Pisa,

N°	Phenotype	Mutated Gene	Nucleotide change	Mutation	State
TANGIER/FHD Fibroblasts					
1	Tangier	ABCA1	c.1759 C>T	R587W	Ho
2	Tangier	ABCA1	c.66 +5 G>C / c.844 C>T	-- / R282X	C-He
3	Tangier	ABCA1	c.3137 C>A	A1046D	Ho
4	Tangier	ABCA1	c.4773 +1 G>A / c.814 -14 InsA	Del D1567_K1591 / No effect	Ho
5	FHD	ABCA1	c.389 G>A / c.5398 A>C	R130K / N1800H	He
6	Tangier	ABCA1	c.1758 InsG / c.4799 A>G	M586FsX629 H1600R	C-He
7	Tangier	ABCA1	c.3295 G>T	D1099Y	He
FH Fibroblasts					
1	FH	LDLR	c.1946G>A	G528D	Ho
2	FH	LDLR	c.97 C>T	Q12X	Ho
3	FH	LDLR	Del. Ex13-18	--	Ho
4	FH	LDLR	IVS15+1G>A	Truncated in frame Ins	Ho
5	FH	LDLR	c.2054 C>T	P664L	Ho
6	FH	LDLR	c.1056 C>G	C331W	Ho
7	FH	LDLR	Dupl.Ex16-17		Ho
8	FH	LDLR	Del. Ex13-15 / c.1946G>A	-- /G528D	C-He
9	FH	LDLR	Del. Ex2-12	--	He

Table 3.3: FH fibroblasts list.

Italy) by the group headed by Prof. T. Sampietro.

3.3.16 Cells treatment

Cultured fibroblasts from healthy and affected individuals were subjected to multiple treatments prior to further analysis in order to stimulate either ABCA1 or LDLR expression.

ABCA1 expression is regulated by the LXR pathway, thus stimulation of cells with i) a load of Free Cholesterol (FC), ii) oxysterols, or iii) a non-steroidal agonist of LXR as T0901317, resulted in ABCA1 up-regulation.

On the contrary LDLR expression is favored by cholesterol depletion, thus treatment of cells with iv) Lipoprotein Deprived Serum (LPDS), is a condition, which maximize LDLR exposure on cell surface.

1. FC loading: the cell monolayer confluent monolayers were washed twice with phosphate-buffered saline (PBS) and incubated with 30 μg/ml of FC (SIGMA-Aldrich, St. Louis, MO, USA) for 48 h in DMEM containing 1% FBS, 0,2% BSA.

2. Oxysterols: the cell monolayer was treated with the association between 9-cis-retinoic acid (5 μM) and 22-R-hydroxycholesterol (SIGMA-Aldrich, St. Louis, MO, USA) for 18-20 hours in DMEM containing 10% FBS.

3. Non-steroidal LXR agonists: the cell monolayer was treated with T0901317 1-5 μM for 18-20 hours in DMEM containing 10% FBS.

4. LPDS: the cell monolayer was treated with DMEM supplemented with 10% LPDS (SIGMA-Aldrich, St. Louis, MO, USA).

3.3.17 Transfection with LipofecTAMINE 2000

Transfection is the process of introducing nucleic acids into eukaryotic cells by non-viral methods. In our study we used this technique in order to produce the transcript encoded by the minigene ABCA1 Ex19-23 for the study of c.2961-2A>C, or to produce the ABCA1-GFP WT and mutant, by exploiting the translational and transcriptional apparatus of the host cell. For

this purpose we used Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. It is a cationic liposome based reagent able to assemble absorption-complexes with the negatively charged plasmid DNA. An electrostatic reaction is thus created and the complexes are able to enter plasma membranes.

If different format than 24-well are used, it is necessary to scale up and down all the amounts, following manufacturer's table.

3.3.18 RNA extraction from cells

RNA was extracted from COS-1 cells or fibroblasts using Eurozol (Euro-Clone; Celbio, Milan, Italy) and reverse- transcribed to cDNA using Super-Script III (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

Total RNA was extracted from monocytes using RNeasy Plus Micro Kit (Qiagen GmbH, Hilden, Germany), resuspended in 14 μL RNase-free water, and quantified by measuring absorbance at 260 nm. Total RNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) in a final volume of 20 μL, according to manufacturer's instructions. RNA extraction from monocytes reverse-transcription was performed at the CNR Institute of Clinical Physiology (Pisa, Italy) in the lab of Dr. T. Sampietro.

We extracted and analyzed RNA for:

a) ex vivo study of the c.814 -14 InsA mutation (human fibroblasts);

b) in vitro study of the c.2961 -2 A>C mutation (transfected COS-1 cells);

c) ex vivo study of the c.2961 -2 A>C mutation (human monocytes);

d) ex vivo analysis of the c.4773 +1 G>A mutation (human fibroblasts).

3.3.19 ABCA1 cDNA analysis

a) To study the effect of the intronic variant c.814 -14 InsA on ABCA1 pre-mRNA splicing, cDNA was amplified using a forward primer complementary to exon 5 (5' TGT TCT CAG ATG CTC GGA GGC T-3') and a reverse primer complementary to exon 13 (5' TTC CAG TGA ACA CAA TAC

CAG CC-3'). The incubation conditions were 95°C for 2 min, 95°C for 30 sec/65.1°C for 30 sec/72°C for 140 sec for 15 cycles with a reduction of 0.5°C per cycle, followed by 95°C for 30 sec/58.1°C for 30 sec/ 72°C for 140 sec for 20 cycles and a final extension at 72°C for 5 min. The expected size of the wild type RT-PCR product was 1308 bp. The RT-PCR products were separated on 1% agarose gel electrophoresis and sequenced.

b) To study the effect of the c.2961 -2 A>C mutation in vitro, following transfection of the minigene ABCA1 Ex19-23 in COS-1 cells, RNA extraction and reverse-transcription, the following primers were used: i) 5'-AAT GGA GCG GGG AAG ATG ACC ACC ATG TCA ATC–3' (forward primer complementary to exon 19/exon 20 junction); ii) 5'-GAG TAA GGG TCC ACA CCA GC–3' (reverse primer complementary to exon 22). The amplification conditions were the following: 95°C for 2 min, 95°C for 30 sec/67.3°C for 30 sec/72°C for 40 sec for 15 cycles with a reduction of 0.5°C per cycle; 95°C for 30 sec/60.3° C for 30 sec/ 72°C for 40 sec for 20 cycles, followed by an extension at 72°C for 5 min. The RT-PCR products were separated on 1% agarose gel electrophoresis and sequenced. The expected size of the transcript of the wild type minigene was 398 bp.

c) To study the effect of the c.2961 -2 A>C mutation in patients' monocytes, ABCA1 cDNA of the proband and his father were amplified with the following primers: i) 5'-GGCCTGGCACTGAATTTTTA-3' (forward primer complementary to exon 19); ii) 5'-AGATGATGGCAATCCTGTCC-3' (reverse primer complementary to exon 23). The amplification conditions were the following: 95°C for 2 min, 95°C for 30 sec/56,2°C for 30 sec/72°C for 60 sec for 30 cycles, 72°C for 5 min. The expected size of the PCR product from control cDNA was 562 bp. The RT-PCR products were separated on 1% agarose gel electrophoresis and sequenced.

d) To study the effect of the mutation in the donor splice site of intron 35 (c.4773 +1 g>a) on ABCA1 pre-mRNA splicing, cDNA was amplified using the following primers: 5'-ATC AAG AAG ATG CTG CCT GTG TGT CC-3' (forward primer complementary to exon 31) and 5'-TTT CAC CAT GTC GAT GAG CCC T-3' (reverse primer complementary to exon 40). The amplification conditions were the following: 95°C for 2 min, 15 cycles at 95°C for 30 sec/ 67.1°C (– 0,5°C/cycle) for 30 sec/ 72°C for 110 sec, followed

by 20 cycles at 95°C for 30 sec/ 60.1°C for 30 sec/ 72°C for 110 sec and a final extension at 72°C for 5 min. The expected size of the PCR product from control cDNA was 1065 bp. The PCR products were separated on 1% agarose gel electrophoresis and sequenced.

3.3.20 Cells Staining and Confocal Microscopy

In order to perform confocal microscopy analysis of mutant ABCA1 protein sub-localization, HEK293 cells were transfected on a variable number of Lab-tek, depending on the number of constructs contemporary transfected. 100000 cells were seeded in each well, value set up on the base of HEK293 cells' ability to reach 40-60% of confluence the day after.

We used non-transfected cells as negative control and cells transfected with pcDNA3.1-ABCA1-GFP as positive control for plasma membrane localization. HEK293 cells transfected with pcDNA3.1-ABCA1-GFP Δ11-33 were used as controls for Endoplasmic Reticulum retention.

3.3.20.1 Fixation of HEK293 cells

Forty-eight hours after transfection, HEK293 cells were fixed on the glass-surface of the Lab-tek.

The steps applied are:

1. Remove medium and wash twice with 1-1.5 ml of PBS.
2. Cover the cell monolayer with 1 ml of fixing solution, consisting in 4% Paraformaldehyde and 2% Sucrose.
3. Incubate at room-temperature for 25 minutes.
4. Remove fixing solution and wash twice with 1-1.5 ml of PBS.
5. Cover the cell monolayer with 1-2 ml of PBS and keep in the dark at 4°C.

3.3.20.2 Labeling with anti-Calnexin antibody

With the aim to evaluate in a specific way the intracellular localization of WT and mutant ABCA1 proteins produced by HEK293 cells after transfection, Endoplasmic Reticulum was labeled with an anti-Calnexin antibody (Rabbit anti-Calnexin antibody, SIGMA-Aldrich, St. Louis, MO, USA) after plasma membrane permeabilization.

This procedure allowed us to label the ER in order to obtain some coordinates on intracellular protein localization of ABCA1. Infact calnexin (CNX) is a 90 kDa, non-glycosylated chaperone protein, abundant in the transmembrane region of Endoplasmic Reticulum; it is a type I, integral membrane protein, Calcium-binding.

Our purpose was to obtain through confocal microscopy images where ABCA1-GFP chimera was labeled with green fluorescence (emitted by GFP), while calnexin was labeled with a red fluorescence (achieved through the use of a secondary antibody TRITC conjugated).

Treatment for permeabilization requires:

- Wash of the cell monolayer with 1.5 ml of PBS
- Permeabilization in ice with 1-1.5 ml of 0.1% Triton X100 for 5 minutes.
- Two-three washes of the cell monolayer with 1 ml of PBS, with 5 minutes-incubation between the washes.
- Block of permeabilization with 1.5 ml of 1% BSA in PBS for 1 hour.
- Wash of the monolayer with 1.5 ml PBS and addition of the primary antibody diluted 1:100 in 0.1% BSA at room-temperature for 1 hour.
- Two washes of the cell monolayer in 1.5 ml of 0.1% BSA with 5 minutes-incubation each time.
- Addition of the Anti-Rabbit IgG (whole molecule) TRITC conjugated antibody produced in goat diluted 1:100 in 0.1% BSA at room-temperature for 1 hour.
- Two washes with 1.5 ml of 0.1% BSA with 5 minutes-incubation each time.
- Two washes with 1.5 ml of PBS and cover of the monolayer with 2 ml of PBS.

After washing again the monolayer with PBS, images were acquired using a Leica TCS-4D Confocal Microscope with an immersion 63X objective and laser lines at 488 nm for GFP and 575 nm for the anti-rabbit IgG TRITC-conjugated antibody (SIGMA-Aldrich, St. Louis, MO, USA).

3.3.21 Protein extraction from cells

Cultured skin fibroblasts were washed twice with Buffer B, scraped from flasks in Buffer C, centrifuged at 16 100 g for 5 min and resuspended in lysing buffer. Pellets were homogenized in a 1 mL syringe, centrifuged for 5 min at 16 100 g and supernatants were collected.

Proteins extracts were quantified through Bradford Assay, performed in parallel with a Bovine Serum Albumine standard curve. The Bradford

reagent was purchased from SIGMA-Aldrich (St. Louis, MO, USA); 2 μl of proteins extracts were added to 1 mL of Bradford reagent and quantified by reading absorbance at 595 nm. Protein extracts were either loaded on SDS-PAGE either maintained at -20°C for a short period of time, since they are very susceptible to degradation.

3.3.22 SDS-PAGE and Western Blot

The Western blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3D structure of the protein (native/non-denaturing conditions). The proteins are then transferred to a membrane, either nitrocellulose or PVDF (Polyvinylidene Fluoride), where they are probed and thus detected using antibodies specific to the target protein [138,139].

SDS-PAGE (SDS polyacrylamide gel electrophoresis): Separation of proteins happens in our case in denaturing conditions, thus by molecular weight.

Each of our samples obtained from protein extraction was analyzed via SDS-PAGE using a 1.5mm-thick, 8% SDS-PAGE gel (see 3.2.2 for preparation of Resolving gel and Stacking gel). One lane was reserved for a molecular weight marker (Bio-Rad Laboratories Inc., S.r.l., Segrate, Milano).

To preserve the integrity of ABCA1, samples were not heated prior to loading on polyacrylamide gel. For LDLR protein detection samples were heated and added with 2-4% of β-mercaptoethanol prior to loading on the gel, in order to fully denaturate LDLR protein.

SDS-PAGE was carried out loading 20-40 μg of sample added of Laemmli buffer (finale volume of 40 μl) in the cast gel and running it immediately at 15 mA and then (when samples crossed the Stacking gel) at 30 mA for around 1 hour in a Mini–P Tetra Cell (Bio-Rad Laboratories S.r.l., Segrate, Milano).

Transfer (Western Blot): In order to make the proteins accessible to antibody detection, they were moved from within the gel onto a membrane made of nitrocellulose.

The membrane was placed on top of the gel, and a stack of filter paper

and sponge are placed on top and at the bottom of that. The entire "sandwich" was placed in a buffer solution (for Transfer buffer recipe see 3.2.2), which helps transferring the proteins via electro-blotting. Protein binding is based upon hydrophobic interactions as well as charged interactions between the membrane and protein. We transferred our samples to a nitrocellulose membrane in a Mini-P Tetra Cell in cold room for 2 hours at 200 mA.

Blocking: since the membrane has been chosen for its ability to bind proteins, and since both antibodies and targets are proteins, steps must be taken to prevent unspecific interactions with the antibody. Blocking of non-specific binding was achieved by placing the membrane in a diluted solution of protein, TBS containing 5% non-fat dry milk, with a minute percentage of detergent such as Tween 20. The proteins in the diluted solution attache to the membrane in every place where the target proteins have not attached. Thus, when the antibody is added, it can attach only on the binding sites of the specific target protein. This reduces background in the final product of the Western blot, leading to clearer results, and eliminates false positives.

Incubation with primary antibody: after blocking, a diluted solution of primary antibody (TBS-T containing 2.5% non-fat dry milk) was incubated for 1 hour at room temperature with the membrane under gentle agitation.

For ABCA1 protein detection a rabbit polyclonal primary antibody against human ABCA1 (Novus Biologicals, Littleton, CO, USA; 1:750) was employed; for LDLR protein detection rabbit polyclonal IgG anti-LDLR (PROGEN Biotechnik Gmbh, Heidelberg, Germany; 1:2000) was employed [140]. In both cases the loading control was represented by β-actin protein levels, which were detected with a mouse monoclonal anti- β-actin antibody (SIGMA-Aldrich, St. Louis, MO, USA; 1:2500).

Incubation with secondary antibody: after rinsing the membrane with 3 washes in 1X TBS-T to remove unbound primary antibody, the membrane was exposed to another antibody directed at the species-specific portion of the primary antibody. A donkey anti-rabbit or a sheep anti-mouse (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; 1:5000) HRP-conjugated secondary antibodies were employed. Incubation was applied as for the primary antibody and then 3 other washes in 1X TBS-T were performed.

Detection: the secondary antibodies we used were linked to a reporter

enzyme known as horseradish peroxidase (HRP). HRP is used to cleave a chemiluminescent agent and the reaction product gives luminescence in proportion to the amount of protein detected. A sensitive sheet of photographic film was placed against the membrane and exposure to the light from the reaction created an image of the antibodies bound to the blot. This type of detection is called Electro-Chemi-Luminescence (ECL).

On this purpose we used the SuperSignal Chemiluminescent Substrate (Pierce), an ECL system which permits low picograms detection limits to be achieved.

The protocol requires to:

- Mix the two substrate components at a 1:1 ratio to prepare the substrate Working Solution.
- Incubate blot 5 minutes in SuperSignal West Substrate Working Solution.
- Drain excess reagent. Cover blot with clear plastic wrap.
- Expose blot to autoradiographic film into a hypercassette for 20 minutes.

All the autoradiographic films (Hyperfilm ECL 18x24 cm, GE Healthcare, Uppsala, Sweden) were processed by bathe in a developing reagent for 15 seconds and then in a fixing reagent as long as the film cleared.

3.3.23 Efflux studies on transfected HEK293 cells

Efflux studies are the best way to evaluate mutant ABCA1 ability to mediate cholesterol efflux to ApoA-I. This type of experiment was performed on HEK293 after plating these cells in 12-well plates.

Day 1: SEEDING

Cells were plated at a confluency of 5x105 cells/well in DMEM + 10% FBS without Penicillin-Streptomycin (P/S)

Day 2: TRANSFECTION

(following Lipofectamine 2000 protocol by Invitrogen)

Day 3: MEDIUM CHANGE

Morning: DMEMbase (480 μl/well) for 5 hours of incubation. Afternoon: DMEM + 10% FBS + double concentration of P/S (600 μl/well to be added without removing the previous one).

Day 4: LABELING

Wash with PBS 1X and incubate for 24 hours in DMEM + 1% FBS+ 3 μCi/ml ^{3}H-cholesterol + 2 µg/µl ACAT inhibitor (in order to inhibit cholesterol esterification).

Day 5: CHASE/UP-REGULATION OF ABCA1

Wash with PBS 2X and incubate over night in DMEM + 0.2% BSA (in order to equilibrate cholesterol pool) + 2 µg/µl ACAT inhibitor.

Day 6: EFFLUX

Wash with PBS 2X and incubate for 4-6 hours in either DMEM or DMEM ± ApoAI 10 μg/ml.

EFFLUX DETERMINATION

Radioactivity in the medium and cells was determined by scintillation counting and the fractional cholesterol efflux was calculated as the percentage of CPMmed/(CPMmed+CPMcell). For each construct, efflux to ApoA-I was measured in triplicate in 3 independent experiments. Non transfected cells were used as negative control.

3.3.24 Efflux studies on fibroblasts

Skin fibroblasts from TD and FH patients and healthy controls, grown and maintained as described above, were used for the assay of ABCA1-mediated cholesterol efflux.

Briefly, cell monolayers were incubated for 24 h in medium containing [1,2-^{3}H]-cholesterol (2 μCi/mL) and 1% fetal bovine Serum (FBS). Following the 24 h labelling period, cells were washed and incubated overnight in media containing 0.2% bovine serum albumin (BSA), in the presence or absence of 9-cis-retinoic acid (5 μmol/L) and 22-hydroxycholesterol (10 μmol/L). [1,2-^{3}H]-cholesterol-labelled monolayers were incubated for 6 h (efflux time) in the presence and in the absence of human Apo A-I (25 μg/mL). Cholesterol efflux was quantified by measuring the radioactivity of the incubation medium after the removal of floating cells by centrifugation, using a time zero (To) set of cells to calculate total [^{3}H]-cholesterol content in the monolayer. Fractional efflux was calculated as cpm [3H] in the medium / [^{3}H] at To $\cdot$ 100. All the efflux assays were performed in triplicate.

3.3.25 Oxidase treatment and cholestenone measurement

To quantify the enrichment of cholesterol in plasma membrane, fibroblasts were treated with cholesterol oxidase. Briefly, cells were labelled with 2 μCi/mL [^{3}H]-cholesterol for 24 h in DMEM supplemented with 1% FBS. Monolayers were then incubated for 18 h with 0.2% BSA with 5 μmol/L 9-cis-retinoic acid and 2.5 μg/mL of 22-hydroxycholesterol. Cholesterol oxidase (0.5 U/mL) was added, and cells were incubated for 4 h. Lipids were extracted with isopropanol, and radioactive cholesterol and cholestenone were separated using thin-layer chromatography and quantified [141].

Chapter 4

RESULTS

SECTION A: DIAGNOSTICS

4.1 ABCA1 gene analysis

Subjects with HDL-C levels below the 5^{th} percentile, suspected to have primary hypoalphalipoproteinemia or with clinical signs of Tangier Disease were identified by an international network of Lipid Clinics and referred to our laboratory for genetic analysis. In the last 5 years 27 subjects with low HDL were investigated. On the basis of biochemical (nearly absent HDL-C) and clinical data, 12 of these patients were affected by Tangier Disease and 15 by FHD, since they didn't show any clinical feature except reduced HDL-C levels (approximately half of normal value).

If the patient had clinical manifestations of TD besides extremely low levels of HDL the genetic analysis was immediately centred on ABCA1 gene, since we expected to find loss of function of two alleles in ABCA1 gene. If the subject arrived to our attention with the unique diagnosis of low HDL-C levels in the absence of any clinical manifestation we first analyzed other candidate genes like Apo-AI and LCAT and subsequently ABCA1 gene.

Analysis of ABCA1 gene was carried out by an integrated approach DHPLC/direct sequencing [80], which was developed and validated five years ago in order to speed up the analysis of an huge gene like ABCA1. This approach consists of a pre-screening step performed with DHPLC and a subsequent

sequencing step of the DHPLC positive amplicons. This semi-automated approach allowed us to identify 22 ABCA1 gene mutations (about 1/5 of all ABCA1 mutations reported so far):

- 6 identified in homozygous carriers;
- 6 identified in compound heterozygous carriers;
- 10 in heterozygous carriers.

So we were able to accomplish the genetic diagnosis to 14 patients out of 27; in the remaining 13 subjects we have not found any mutation in ABCA1, ApoAI or LCAT genes. From recent data it is expected a rate of 3 heterozygotes for ABCA1 mutations out of 1000 individuals in the general population [82]; consequently in the Italian population we could estimate the presence of 120-150 Tangier patients. We have identified 12 Tangier disease patients (9 Italian, 1 English, 1 Australian, 1 Pakistani) during this study; even if less than expected, they represent, so far, all the Italian cases arrived to our attention with a clinical diagnosis of Tangier disease.

4.1.1 List of the identified mutations

The list of mutations that we found in ABCA1 gene is reported below; besides the nucleotide and amino acid substitution, we also reported whether the mutations were in homozygous or heterozygous state (Ho: homozygous, C-He: compound heterozygous, He: heterozygous) and the phenotype of the carriers (TD: Tangier Disease, FHD: Familial HDL Deficiency).

These mutations were either reported or studied for the first time by our group [80,99,142-144] with the exception of the mutations W590L, N1800H and D1099Y [88,145,146].

Mutations listed in table 4.1 can be divided in:

- 2 nonsense mutations (R282X, R557X);
- 2 small insertions leading to frameshift and premature stop codon formation (I74YFs.X76, M586Fs.629X);
- 4 splice site mutations (c.66 +5G>C, c.814-14 insA, c.2961 –2 A>C, c.4773 + 1 G>A);

EXON/INTRON	NUCLEOTIDE SUBSTITUTION	AMINOACIDIC SUBSTITUTION	STATE	PHENOTYPE
IVS 2	c.66 +5G>C		C-He	TD
Ex 4	c.219 insT	I74YFs.X76	Ho	TD
Ex 5	c.389 G>A	R130K	He	FHD
IVS 8	c.814-14 insA		He/Ho	FHD
Ex 9	c.844 C>T	R282X	C-He	TD
Ex 9	c.850 G>A	E284K	He	FHD
Ex 12	c.1445 A>G	Y482C	He	FHD
Ex 13	c.1669 C>T	R557X	He	FHD
Ex 14	c.1758 InsG	M586Fs.629X	C-He	TD
Ex 14	c.1759 C>T	R587W	Ho/C-He	TD
Ex 14	c.1769 G>T	W590L	He	FHD
Ex 19	c.2819 C>T	T940M	He	FHD
Ex 22	c.3137 C>A	A1046D	Ho	TD
IVS 20	c.2961 –2 A>C		Ho	TD
Ex 23	c.3295 G>T	D1099Y	He	FHD
IVS 35	c.4773 + 1 G>A		Ho	TD
Ex 36	c.4799 A>G	H1600R	C-He	TD
Ex 37	c.5097 G>T	W1699C	C-He	TD
Ex 40	c.5398 A>C	N1800H	He	FHD
Ex 42	c.5689 C>T	R1897W	He	FHD
Ex 42	c.5703 A>C	R1901S	C-He	FHD
Ex 49	c.6588 G>C	Q2196H	He	FHD

Table 4.1: List of mutations identified in TD/FHD patients.

- 14 missense mutations (R130K, E284K, Y482C, R587W, W590L, T940M, A1046D, D1099Y, H1600R, W1699C, N1800H, R1897W, R1901S, Q2196H)

If the 2 nonsense mutations and the 2 small insertions are certainly pathogenetic, since they lead to a truncated ABCA1 transporter, little is known about the biological impact of the splice site and missense mutations. One of the purposes of this work was the investigation of the *in vitro* effect of the splice site mutations and of some of the missense mutations.

SECTION B: FUNCTIONAL CHARACTERIZATION OF ABCA1 MUTANTS

4.2 Functional characterization of mutations

In order to accomplish this task we first selected a set of mutations considered eligible for further characterization. The selection was guided by the following criteria:

- type of mutation;
- absence of any previous characterization;
- position of the mutation along the gene and position of the amino acid in the protein;
- phylogenetic conservation of the mutated amino acid;
- in silico prediction of the effect of the mutation on the protein function;
- availability of skin fibroblasts from the patients

The table 4.2 reports, for each missense variant, the position of the substituted aminoacid on the protein and the in silico prediction output. We used two different predictive softwares, namely Polyphen (`ttp://genetics.bw`.harvard.edu/pph/) and PANTHER (`ttp://www.pant`erdb.org/), to obtain a preliminary idea of mutation effect. PoplyPhen assigns to each mutation a score, based on physical and comparative considerations, which is then translated into a classification consisting of 3 categories of damage: “Benign”, “Possibly Damaging”, “Probably Damaging”. PANTHER database otherwise works on the basis of the phylogenetic conservation of the mu-

MISSENSE MUTATION	POSITION	In silico PREDICTION	
		POLYPHEN PREDICTION	subPSEC score
R130K	1° EC loop	Possibly damaging	/
E284K	1° EC loop	Benign	/
Y482C	1° EC loop	Probably damaging	/
R587W	1° EC loop	Probably Damaging	-6.41899
W590L	1° EC loop	Probably Damaging	-3.04551
T940M	1° Walker A motif	Probably Damaging	-8.26504
A1046D	IC	Possibly Damaging	-8.13301
D1099Y	IC	Probably Damaging	-8.79834
H1600R	4° EC loop	Probably Damaging	-6.9287
W1699C	IC	Probably Damaging	-8.90189
N1800H	6° EC loop	Possibly damaging	-3.6634
R1897W	IC	Probably Damaging	-6.56875
R1901S	IC	Probably Damaging	-4.90587
Q2196H	C-terminal IC tail	Benign	-4.33333

Table 4.2: Position and in silico prediction of the missense mutations.

tated residue and assigns a score (called subPSEC score) to each mutation. The probability that a given variant causes a deleterious effect on protein function is estimated by subPSEC score, such that a subPSEC score of -3 corresponds to a Pdeleterious of 0.5; in other words the lower the subPSEC score the higher is the probability of damage of the protein [136]. When a subPSEC score is missing, it means that the residue is not highly conserved in evolution.

W590L, N1800H and D1099Y were not novel mutations; the N1800H was fully characterized and resulted to be causative since it showed a complete lack of function of ABCA1 protein in terms of cholesterol efflux [147]. W590L was never studied but there is another known ABCA1 variant affecting the same position, the W590S, which was functionally investigated [147]. W590S localized normally to the plasma membrane and was able to bind Apo-AI as the wild type ABCA1, but it showed a reduced cholesterol efflux. We

assumed that the W590L had a similar behaviour or even a lower impact than the W590S on the protein function, since the multiple alignment sometimes shows a leucine residue in this position. The R587W was reported for the first time by Lawn et al. in 1999 [57] and then by a group of our collaborators in 2001 [142] but the molecular diagnosis of the mutation wasn't followed by a functional analysis of the same. The A1046D was reported by Wang J. et al. in 2000 [86] and it was characterized together with the R587W by Singaraja R.et al. in 2006 [147] using a transfection approach. Singaraja R. concluded that R587W clearly showed major biochemical defects (virtually absent cholesterol efflux and highly reduced Apo-AI binding) and that A1046D caused a reduced localization of ABCA1 to the plasma membrane, a reduced Apo-AI binding and probably it influenced the folding of the protein or its phosphorylation. Since we obtained the cells of the patients carrying these variants, we had the possibility to better understand the biological effect of the R587W and A1046D without inducing an overexpression of mutated proteins (as it is expected in "in vitro" transfection) but using a more physiological "ex-vivo" approach.

The novel mutations E284K, Y482C, T940M, W1699C, R1897W were selected for further analysis. For their characterization we used a transfection approach because unfortunately we didn't obtain the dermal fibroblasts of carriers, and also because all of them, except the W1699C mutation (found in a compound heterozygote) were found in heterozygous state. Even if this part of the work is not the main focus of the present thesis, an overview of the approach employed is given in section 4.4.

R130K, D1099Y and H1600R were identified only recently; we obtained fibroblasts from the heterozygote carrier of both R130K and N1800H on the same allele, the heterozygote carrier of D1099Y and the compound heterozygote for H1600R and for a frameshift mutation (M586Fs.629X). On these cells we could only perform efflux studies up to now; in the next future they could be employed for further characterization. Finally R1901S and Q2196H were not selected for further studies.

4.3 Mutations affecting splice sites

Surprisingly for a large gene such as ABCA1, only few mutations affecting splice sites have been reported so far in TD or FHD patients; among the 125 known mutations of ABCA1 gene only 9 variants affect splice sites. Four of these were identified and studied for the first time by our group:

- the c.66 +5 G>C (IVS2+5 G>C) was studied in 2003. In patients' fibroblasts three different species of mRNA were found: devoid of exon 2 (Ex2-/mRNA), exon 4 (Ex4-/mRNA) or both these exons (Ex2-/Ex4- mRNA) [143].

- the c.814-14 insA (IVS8-14 insA) was identified both in heterozygote and in homozygote state in patients with a milder phenotype. It was also identified in 10 alleles out of 190 in the general population with low HDL-C [81], so probably it is a relatively common variant of the population with low HDL-C. We obtained the fibroblasts of the homozygote carrier and studied its effect "ex vivo" (see 4.3.1).

- the c.2961 –2 A>C (IVS20-2 A>C) was recently identified in a patient with clinical manifestations of Tangier Disease, and severely progressive coronary and peripheral artery disease [148]. We had the opportunity to investigate the effect of this mutation with a combined approach, since on one hand we obtained the monocyte-derived mRNA of the carrier and of an heterozygote relative; on the other hand we created a minigene harbouring the mutation (see 4.3.3) [149].

- the c.4773 + 1 G>A (IVS35+1 G>A) was found in a Pakistani boy of 7 years old with clinical signs of Tangier Disease associated with developmental delay and mild facial dysmorphism. We obtained his fibroblasts so that we could analyse mutation effects "ex vivo" (see 4.3.2).

A more detailed study of the splice site mutations and their effects is described in the following paragraphs.

4.3.1 Ex-vivo investigation of the c.814-14 insA

We described this variant in 2005 when we found it in heterozygous state in a proband with low HDL-C [80]. Since then, we found again the same variant in other two probands: i) in heterozygous state in a proband carrying also the R130K and the N1800H mutations; ii) in homozygous state in a proband carrying also the c.4773 +1 G>A. Frikke-Schmidt R. et al. reported the same variant in 2004 which was found in 10 out of 190 alleles in not-affected individuals, selected from the general population only for their low HDL-C levels [81].

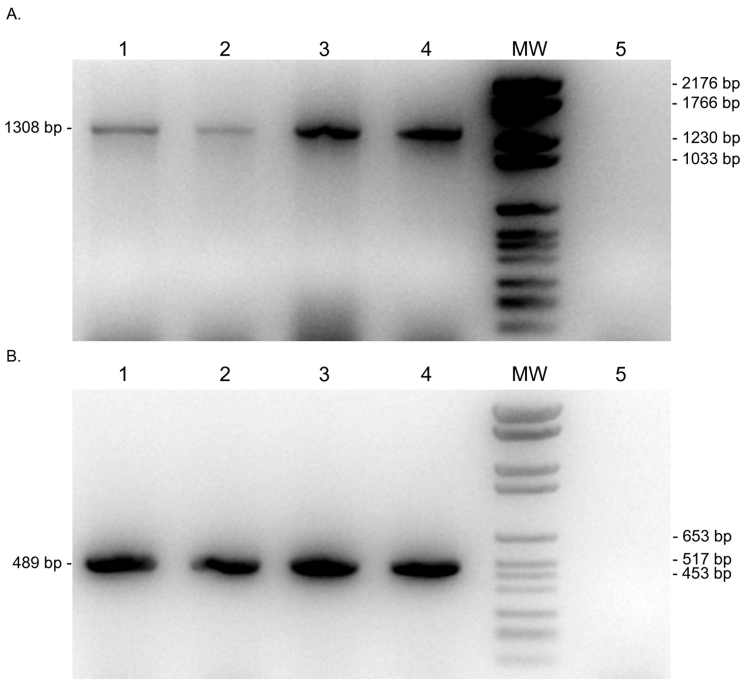

Figure 4.1: **PCR products for c.814 -14 ins A analysis.** Agarose gel electrophoresis of ABCA1 cDNA isolated from cultured skin fibroblasts from a subject homozygous for a variant in intron 8 (c.814 -14 ins A). RNA was isolated from cells before and after incubation with 22-hydroxycholesterol and 9-cis retinoic acid. Lane 1: ABCA1 cDNA in unstimulated wild type cells; lane 2: cDNA in unstimulated cells from c.814 -14 ins A homozygous carrier; lane 3: cDNA in wilt type cells incubated with 22-hydroxycholesterol and 9 cis-retinoic acid; lane 4: cDNA in c.814 -14 ins A cells incubated with 22-hydroxycholesterol and 9 cis-retinoic acid. MW= molecular weight marker. The 489 bp band represents the transcript of the 18s subunit of human rRNA.

From these observations we thought that the IVS8 c.814-14 insA could be a recurrent variant in the population with low HDL-C levels which might

contribute to the phenotype of low HDL, by interfering with ABCA1 function in some way.

This hypothesis was also supported by the in silico prediction of mutation effect (Automated Splice Site Analysis https://splice.uwo.ca) which suggested: a nearly 30 fold decrease in the strength of the canonic acceptor site of intron 8; the abolishment of a criptic acceptor splice site placed at -12 with respect to the canonic one; the formation of a new splice acceptor site at -13 and the possible creation of a new branchpoint.

In our cell bank we had the fibroblasts of the homozygous carrier of both c.4773 +1 G>A and c.814-14 insA; even if it was a model complicated by the presence of the 2 variants we tried to investigate the effects of the IVS8 mutation. We extracted the total mRNA from fibroblasts, reverse-transcribed it, and amplified the cDNA in the region spanning from exon 5 to exon 13 (amplicon size 1308 bp). PCR amplification from total cDNA showed the presence in mutant fibroblasts (before and after stimulation of ABCA1 expression with LXR agonists) of a single PCR product, whose size was similar to that found in control fibroblasts. The relative abundance of this transcript, after stimulation of ABCA1 expression by LXR agonists, was super-imposable to that found in control fibroblasts. The sequence of this transcript was identical to that of its wild type counterpart (Fig. 4.1 and 4.2).

We concluded that c.814-14 insA did not apparently alter ABCA1 splicing and so we classified it as non-pathogenetic. Anyway the frequency in the low HDL-C population, the in silico prediction and the limitations of our model (carrier also of a highly pathogenetic mutation, which might have masked the effect of the milder one) can not exclude a possible undetected effect of this variant on ABCA1 function.

4.3.2 Ex-vivo investigation of the c.4773 + 1 G>A

The c.4773 +1 G>A was identified in a 7 years old Pakistani boy with clinical manifestations of Tangier Disease. The in silico prediction (Automated Splice Site Analysis `https://splice.uwo.ca`) suggested that the mutation abolished the function of the donor splice site of intron 35. To confirm this

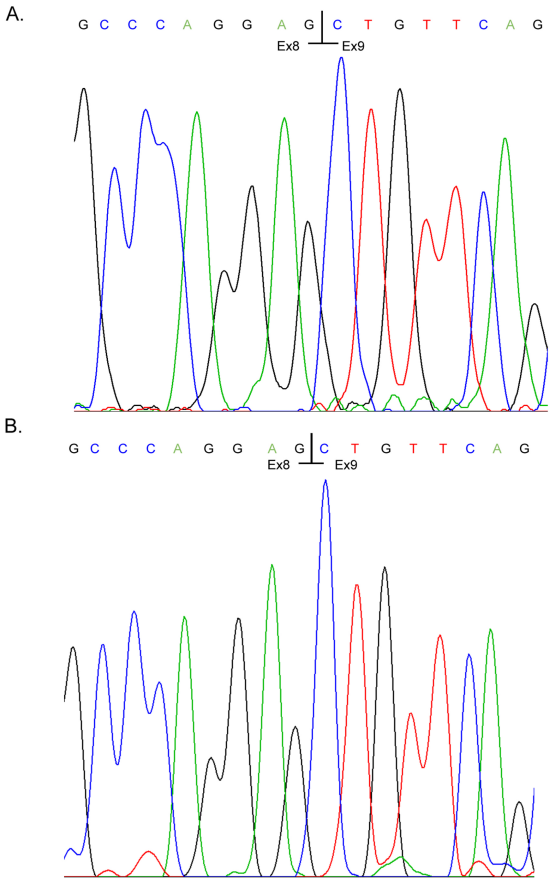

Figure 4.2: **Sequence of exon 8/exon 9 junction in ABCA1 cDNA.** Sequence of exon 8/exon 9 junction in ABCA1 cDNA in human skin fibroblasts. Panel A: ABCA1 cDNA in wild type fibroblasts. Panel B: ABCA1 cDNA in fibroblasts of a subject homozygous for the c.814 -14 ins A variant.

hypothesis we extracted total mRNA from fibroblasts of the homozygous carrier, performed the reverse-transcription and the PCR from cDNA. The region spanning from exon 31 to exon 40 was amplified and products were analyzed by agarose gel electrophoresis and direct sequencing. The PCR product from proband's fibroblasts consisted of a single fragment of 988 bp, as opposed to a fragment of 1065 bp observed in control fibroblasts (Fig. 4.3A). The sequence of the 988 bp fragment showed that exon 34 joined directly to exon 36, with the complete skipping of exon 35 (Fig. 4.3B). This abnormal mRNA is predicted to encode a mutant ABCA1 protein with an in-frame deletion of 25 amino acids (del D1567_K1591) in the second

extracellular loop.

This aberrant ABCA1 protein was produced in a very little amount as demonstrated by western blotting (see 4.5.1). Moreover the little quantity of ABCA1 produced was not able to promote any cholesterol efflux from cells to ApoA-I (see 4.5.2) and we hypothesized that it could not reach the plasma membrane of cells (see 4.5.3).

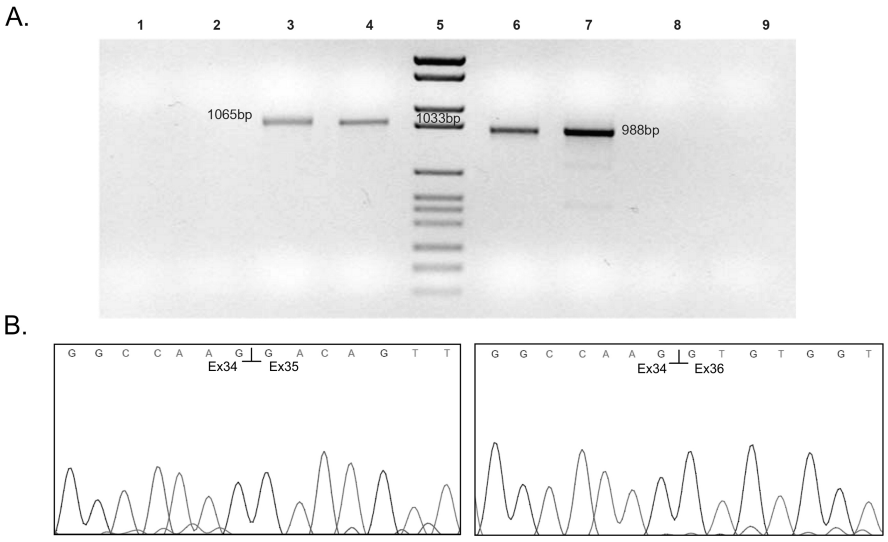

Figure 4.3: **Analysis of transcripts generated by c.4773+1G>A mutation** Analysis of ABCA1 mRNA in cultured skin fibroblasts of proband homozygous for the mutation in the donor splice site of intron 35 (c.4773 + 1G>A). A, Agarose gel electrophoresis of the PCR products encompassing the exon 31–exon 40 region of ABCA1 cDNA. Lanes 3 and 4: PCR products obtained from a control cDNA. Lane 5: molecular weight markers. Lanes 6 and 7: PCR products obtained from the cDNA of the proband. B Partial nucleotide sequence of the junction between exon 34 and exon 35 (in control cDNA, on the left) and between exon 34 and exon 36 (in proband's cDNA, on the right).

4.3.3 Analysis of the splice site mutation c.2961 –2 A>C

Recently we identified a new splice site mutation, affecting the acceptor splice site of intron 20. The variant was found in a 37 years old man with large, orange tonsils, hepatosplenomegaly and severely progressive coronary and peripheral artery disease [148]. In silico mutation analysis (Automated Splice Site Analysis https://splice.uwo.ca) predicted the inactivation of the acceptor canonic splice site of intron 20.

4.3.3.1 Ex vivo analysis of c.2961 -2A>C

We obtained the monocyte- derived mRNA of the proband and of the heterozygote proband's father (kindly provided by Dr. Sampietro T, CNR Institute of Clinical Physiology, Pisa) [148]. We reverse transcribed total mRNA of the proband, proband's father and a control sample and amplified the cDNA in the region spanning from exon 19 and exon 23. The control transcript gave a single PCR product of 562 bp (Fig. 4.4, lane 1). The sequence of the wild type transcript showed that exon 20 was followed by exon 21 as expected. The proband's transcripts gave two amplification products, one band of 623 bp and the other of 548 bp (Fig. MonocytesGel, lane 3); as shown by Fig. MonocytesGel, lane 3, the longer transcript was more abundant than the shorter one. The sequence of the upper band showed that in the 623 bp transcript exon 20 was followed by the last 61 nucleotides of intron 20, and the entire exon 21. This transcript appeared to be the result of the activation of a cryptic acceptor splice site in the 3' end of intron 20. The product of this transcript was predicted to be a protein of 1024 amino acids containing a tail of 37 novel amino acids (M987M_FS1025X) (ins c.2965 -61_-1). In the 548 bp transcript exon 20 was followed by exon 21, in which the first 14 nucleotides were deleted. This transcript was due to the activation of a cryptic acceptor splice site within exon 21; its product was predicted to be a protein of 999 amino acids containing a tail of 13 novel amino acids (M987I_FS1000X (del c.2964_2977).

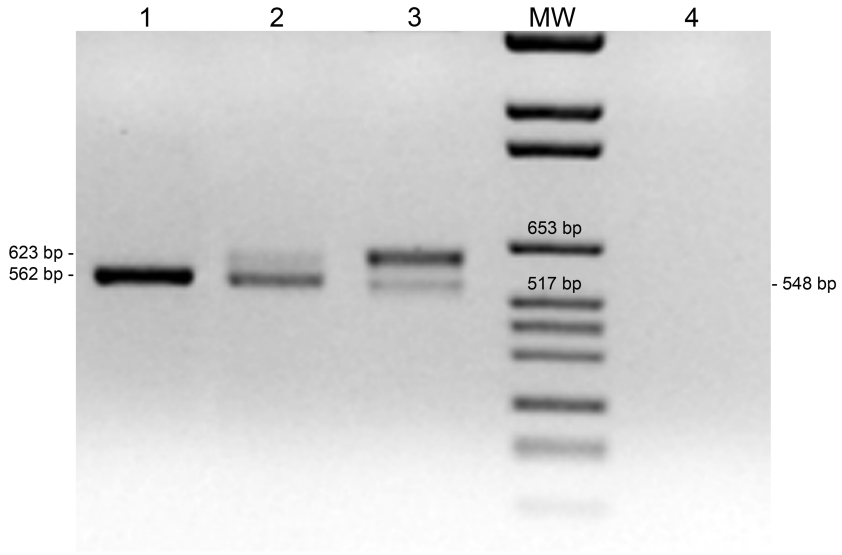

Figure 4.4: **Analysis of transcripts found in monocytes of c.2961-2 A>C proband.** Agarose gel electrophoresis of the ABCA1 transcripts obtained from amplification of the cDNA derived from human monocytes. The ABCA1 cDNA region spanning from exon 19 to exon 23 was amplified by PCR. Lane 1: control subject; lane 2: proband's father; lane 3: TD proband; lane 4: mock; MW = molecular size markers.

The PCR amplification of proband's father sample (heterozygote for c.2961 –2 A>C) gave two products too, of apparently the same size observed in proband's sample(Fig. MonocytesGel, lane 2), but of different intensity; the most abundant product was the lower one, which, as revealed by direct sequencing, resulted by the co-migration of two different transcripts very close in size (the 562 bp control transcript and the 548 bp mutant transcript). The upper band corresponded, as demonstrated by direct sequencing, to the ins c.2965 -61_-1 transcript already found in the proband [149].

4.3.3.2 In vitro analysis of c.2961 –2 A>C

We also created a mutated minigene in order to study mutation effects in vitro. We amplified the whole genomic region spanning from intron 18 to intron 23 and cloned it into pTARGET vector. The mutation was inserted by site-directed mutagenesis and the wild type and mutant minigenes were then transfected in COS-1 cell line. Through mRNA extraction, reverse transcription and cDNA amplification we looked at the transcripts expressed

by the wild type and mutant minigenes. The wild type minigene generated a single transcript of the expected size (398 bp. Fig. 4.5, lanes 2 and 3). The mutant minigene generated three transcripts of 459 bp, 384 bp and 255 bp, respectively (Fig. 4.5, lane 4) instead of the two expected on the basis of the previous analysis on proband's cDNA. The sequence of the wild type transcript showed that exon 20 was followed by exon 21 as expected (Fig. 4.6A). The sequence of the mutant transcripts showed that: i) in the 459 bp transcript exon 20 was followed by the last 61 nucleotides of intron 20, which, in turn, were followed by the whole exon 21 (Fig. 4.6B) resulting from the activation of the cryptic acceptor splice site in the 3' end of intron 20. This transcript corresponded to the longer transcript already found from patient's cDNA amplification. ii) In the 384 bp transcript exon 20 was followed by exon 21, in which the first 14 nucleotides were deleted (Fig. 4.6C). This transcript corresponded to the lower band already obtained from patient's cDNA amplification. iii) In the 255 bp transcript exon 20 was followed by exon 22, with the complete skipping of exon 21 (Fig. 4.6D). This transcript was predicted to encode a protein of 1003 amino acids with a tail of 16 novel amino acids (M987M_FS1004X (del c.2964_3103) (Fig. 4.7). This shortest transcript was not obtained during proband's cDNA amplification and in the gel it appeared the less abundant one.

Figure 4.8 also shows that the endogenous ABCA1 transcript (corresponding to the region spanning from exon 19/exon20 junction to exon 22) was detectable in untransfected COS-1 cells (lane 1). In agarose gel, the band corresponding to this transcript migrates close to that of wild type human minigene transcript; we sequenced the endogenous transcript (including the exon 20/exon 21) and found that it corresponded to the homologous of ABCA1 gene in rhesus monkey (Genbank XM_001106713) from where COS-1 cell line is derived (Fig. 4.8) [149].

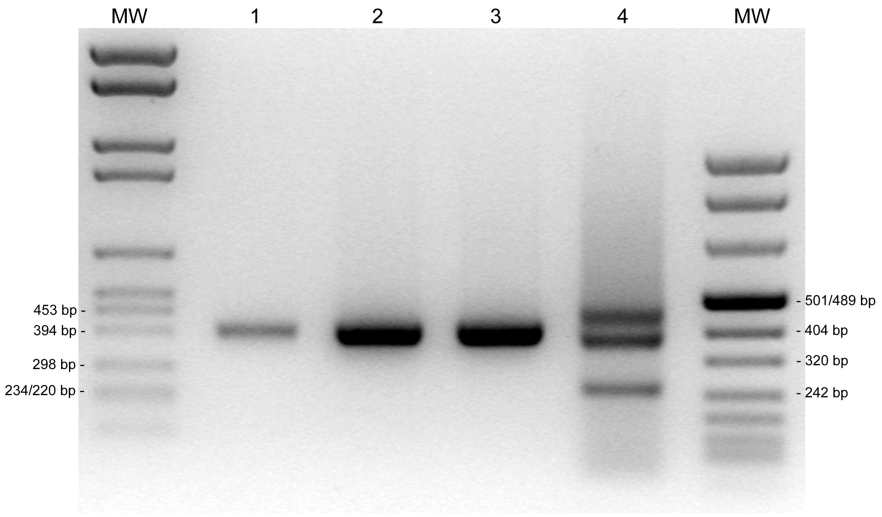

Figure 4.5: **Analysis of transcripts generated by c.2961-2 A>C minigene.** Agarose gel electrophoresis of the transcripts of the mutant ABCA1 minigene (c.2961 –2 A>C) in COS-1 cells. RT-PCR of RNA isolated from COS-1 cells. A cDNA region spanning from exon 19 to exon 22 was amplified by PCR. Lane 1: endogenous transcript in non transfected cells; lanes 2 and 3: transcripts generated by wild type minigene; lane 4: transcripts generated by the mutant minigene; MW = molecular size markers.

4.4 "In vitro" functional characterization: design and validation of the approach.

A mutation in ABCA1 gene could affect ABCA1 protein maturation and function at multiple levels; for example, since ABCA1 is a transporter, functioning mainly on the plasma membrane, its trafficking to the cell membrane could be hampered by a mutation. With the purpose to monitor the intracellular trafficking of ABCA1 protein, in all the cases in which we couldn't obtain patients' cells, we designed a strategy based on the creation of a GFP tagged plasmid. Briefly, starting from two different plasmids, one containing ABCA1 cDNA and the other including the cDNA of GFP, we created an expression vector in which GFP cDNA was fused in frame with ABCA1 cDNA at the 3' end of ABCA1. This task was accomplished through: i) two indepedent PCR of the 3' part of ABCA1 cDNA and the 5' part of GFP cDNA; ii) an overlapping PCR, which allowed the fusion of the two amplicons previously obtained [96] and iii) a subsequent cloning step by using restriction

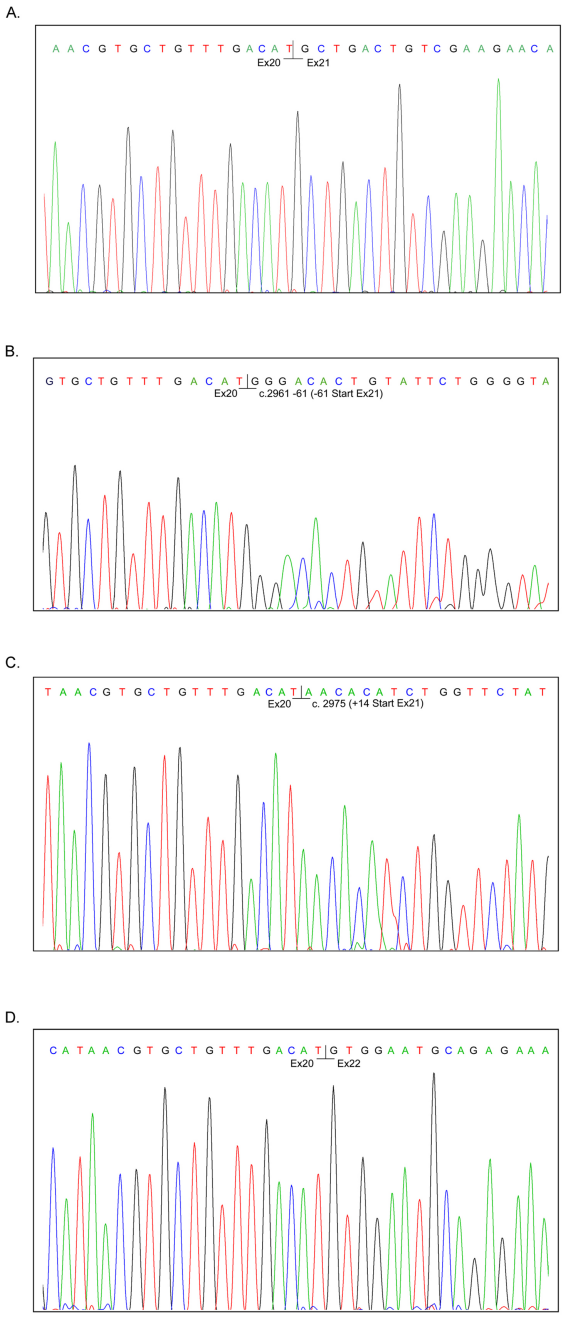

Figure 4.6: **Partial sequence of transcripts generated by c.2961-2 A>C minigene.** Nucleotide sequence of exon 20/exon 21 junction in transcripts of ABCA1 minigenes expressed in COS-1 cells. Panel A: wild type transcript; Panels B-D: transcripts generated by the mutant minigene in the following order: 459bp (B), 384bp (C), 255bp (D).

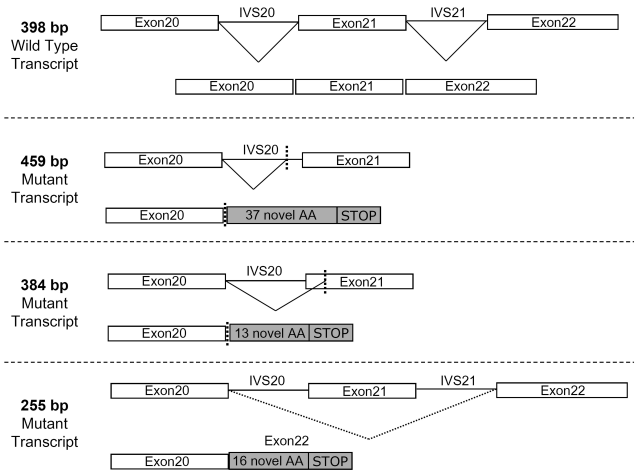

Figure 4.7: **Scheme of abnormal mRNA splicing.** Schematic representation of the abnormal mRNA splicing caused by the mutation in the acceptor splice site of intron 20 of ABCA1 minigene (c.2961 –2 A>C).

enzymes. This ABCA1-GFP plasmid was then subjected to site-directed mutagenesis in order to introduce the specific mutations selected for study. The constructs were transfected in HEK293 cells in which the intracellular destination of ABCA1-GFP chimeric protein was followed by confocal microscopy (green fluorescence) [99]. Transfected cells were co-stained with anti-calnexin antibody, as calnexin was taken as marker of the endoplasmic reticulum (red fluorescence).

In order to validate this approach, we compared the intracellular localization of the wild type ABCA1-GFP protein with that of an artificial mutant, consisting of a large deletion spanning from Ex11 and Ex33 (Del p.K422_K1524). As demonstrated by figure 4.9, the ABCA1-GFP wild type protein was localized at the plasma membrane as well as in the cytoplasm in minute discrete granules, as expected. The overlay panel shows little co-localization with calnexin, as indicated by a clear separation between red and green fluorescence. The artificial mutant ABCA1-GFP del p.K422_K1524 was completely retained in the endoplasmic reticulum, as shown by the complete overlay of ABCA1-GFP and calnexin fluorescence. For this reason the artificial mutant was regarded as a positive control for ER retention.

Another important requirement for system validation was the functional

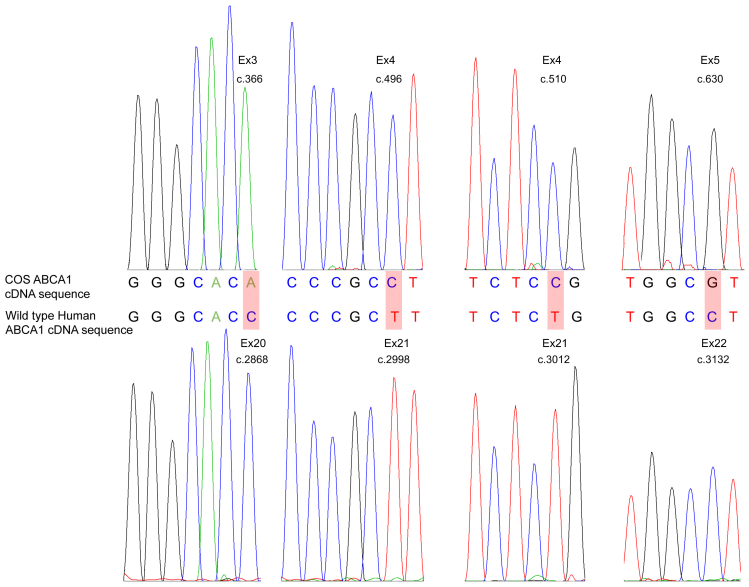

Figure 4.8: **Partial sequence of endogenous ABCA1 cDNA from COS-1cells.** Partial nucleotide sequence of some exons of endogenous ABCA1 cDNA from COS-1 cells. The nucleotide changes with respect to human ABCA1 cDNA are indicated by pink columns.

assay of ABCA1-GFP chimera: cholesterol efflux was measured in transfected HEK293 cells by the group headed by Prof. Bernini, from University of Parma. As shown in figure 4.10 wild type ABCA1-GFP protein had a significant efflux of ^{3}H-cholesterol to Apo-AI, while the artificial mutant showed an efflux to ApoA-I equal to the one observed in basal condition, that is not an ABCA1 specific efflux. Thus we validated an experimental system in which the two controls were represented on one side by the wild type ABCA1-GFP and on the other side by the del p.K422_K1524 ABCA1-GFP. This experimental setting was employed to characterize E284K, Y482C, T940M, W1699C, R1897W but the complete description of results is not a purpose of this work of thesis.

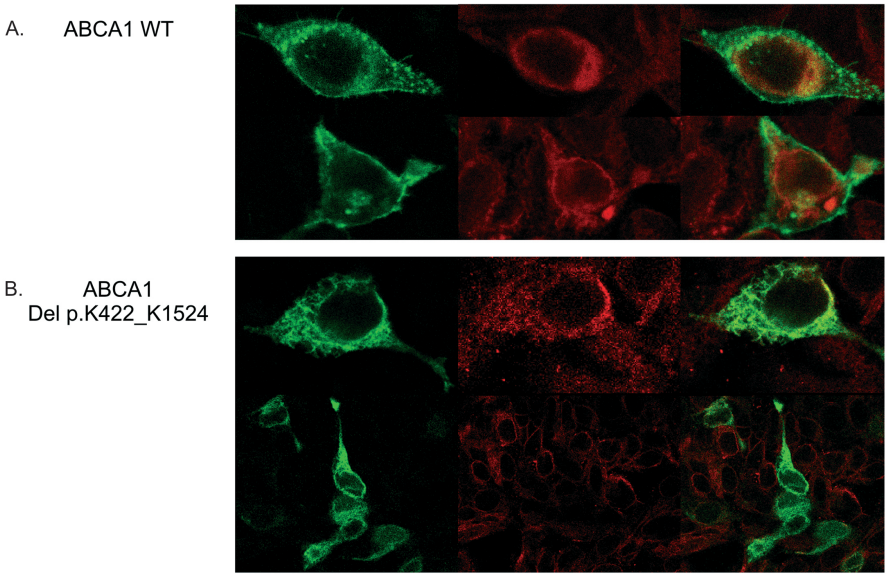

Figure 4.9: **Confocal microscopy image of transfected HEK293.** Measure of cholesterol efflux from HEK293 transfected with ABCA1-GFP wild type (A) and Del p.K422_K1524 (B).

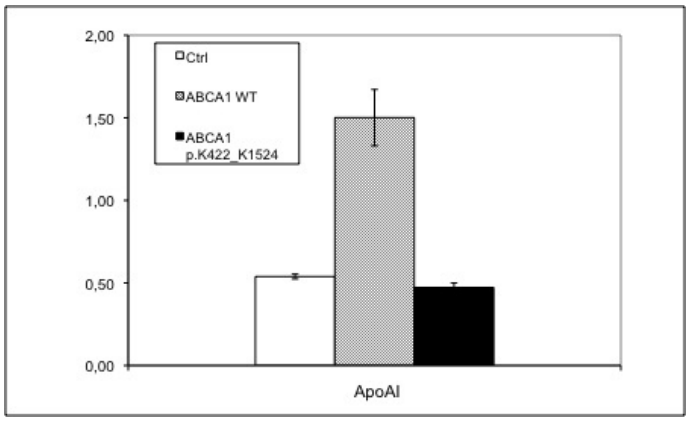

Figure 4.10: **Cholesterol efflux from transfected HEK293.** Measure of cholesterol efflux from HEK293 transfected with ABCA1-GFP wild type and Del p.K422_K1524.

4.5 Ex vivo studies for R587W, A1046D and IVS35+1G>A

4.5.1 Protein extraction from fibroblasts and Western blot for ABCA1 protein

As mentioned above, in our cell-bank we had the dermal fibroblasts of:

- a patient homozygous for R587W;

- a compound heterozygote for IVS2 c.66 +5G>C and for R282X;
- a patient homozygous for A1046D;
- a patient homozygous for IVS35 c.4773 + 1 G>A;
- a patient heterozygous for R130K and N1800H (on the same allele);
- a patient heterozygous for D1099Y;
- a compound heterozygote for M586Fs.629X and H1600R.

We first concentrated on ABCA1 protein expression so, even if the R587W and the A1046D were already studied with an over-expression approach in an heterologous cell line, we wanted to know if the mutated ABCA1 proteins were normally expressed in their physiological cell system. We stimulated control and mutant fibroblasts with 22-OH-cholesterol and 9-cis Retinoic Acid in order to induce LXR stimulation and consequently ABCA1 expression and extracted the total cellular proteins. Stimulation resulted in a 5-fold increase in ABCA1 protein expression in control fibroblasts as shown in lanes 1 and 2 of fig. 4.11, (corresponding to the western blot analysis of control and mutant proteins respectively). R587W ABCA1 protein, after stimulation, was produced but its concentration was clearly lower than that seen in control fibroblasts; we estimated that its content was about the 60% of that observed in control cells (fig. 4.11, lane3). In stimulated fibroblasts from the proband carrying the IVS2 c.66 +5G>C and R282X, ABCA1 was not produced at all, as expected in view of the type of the two mutations, and demonstrated by the lane 4 of fig. 4.11. In stimulated fibroblasts, carrying the A1046D, the ABCA1 protein was detectable but in lower amount (about $1/5^{\text{th}}$ of control cells). Finally, in fibroblasts from the proband homozygous for c.4773 + 1 G>A, ABCA1 protein was hardly detectable (about 8% of that found in control cells; lane 6, fig. 4.11) [99].

Fibroblasts derived from the compound heterozygote for c.66 +5G>C and for R282X were regarded as negative control of ABCA1 expression, since these mutations resulted in two ABCA1 null alleles [144]. As expected these fibroblasts resulted completely devoid of ABCA1 protein expression (fig. 4.11, lane 4).

Western blot analysis has not been performed yet on the fibroblasts carrier of R130K and N1800H, D1099Y and M586Fs.629X / H1600R since we

obtained these cells only recently.

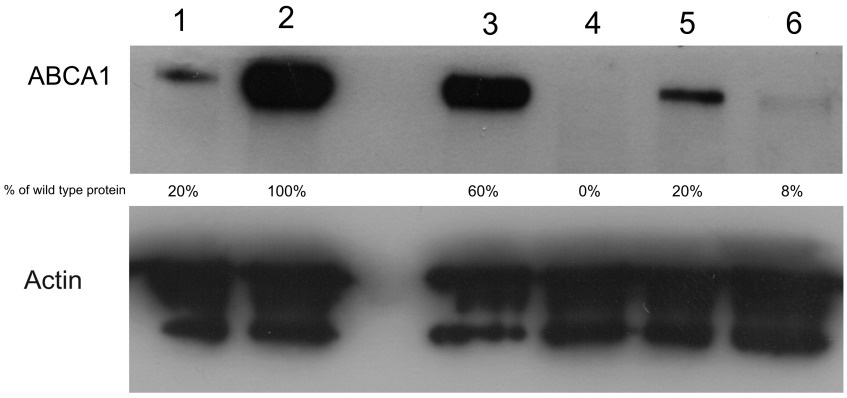

Figure 4.11: **Immunoblot of ABCA1 protein from cultured skin fibroblasts.** Lanes 1 and 2: control fibroblasts before (lane 1) and after (lane 2) stimulation of ABCA1 gene expression with $22OH/cRA$; lane 3: fibroblasts from a homozygous patient for p.R578W mutation; lane 4: fibroblasts of a compound heterozygote for ABCA1 null alleles; lane 5: p.A1046D fibroblasts; lane 6: del $p.D1567_K1591$ fibroblasts. ABCA1 protein shown in lanes 3˘6 was from fibroblasts stimulated with $22OH/cRA$. The per cent values refer to the reduction in the intensity of the band with respect to the intensity of the band of wild type ABCA1 (lane 2) in stimulated control fibroblasts.

4.5.2 Efflux studies

The same control and mutant fibroblasts were subjected to efflux assays in order to verify the ability of ABCA1 protein to promote cholesterol efflux from cells. These assays were performed in collaboration with the group headed by Prof. F. Bernini at the Department of Pharmacological and Biological Sciences and Applied Chemistries, at the University of Parma. The treatment of fibroblasts with 9-cis-retinoic acid and 22-hydroxycholesterol (to induce ABCA1 gene expression) resulted in a nine-fold increase in cholesterol efflux to ApoA-I in control fibroblasts but had no effect in fibroblasts carrying the R587W, the A1046D and the c.4773 + 1 G>A mutation (Fig. 4.12). All three mutants resulted loss of function in terms of cholesterol efflux, since they completely impair the cholesterol efflux to ApoA-I from cells.

Recently, cholesterol efflux to ApoA-I was measured also in fibroblasts carrier of R130K and N1800H, D1099Y and M586Fs.629X / H1600R. The 22OH/9cRA stimulation resulted in a five-fold increase in cholesterol efflux

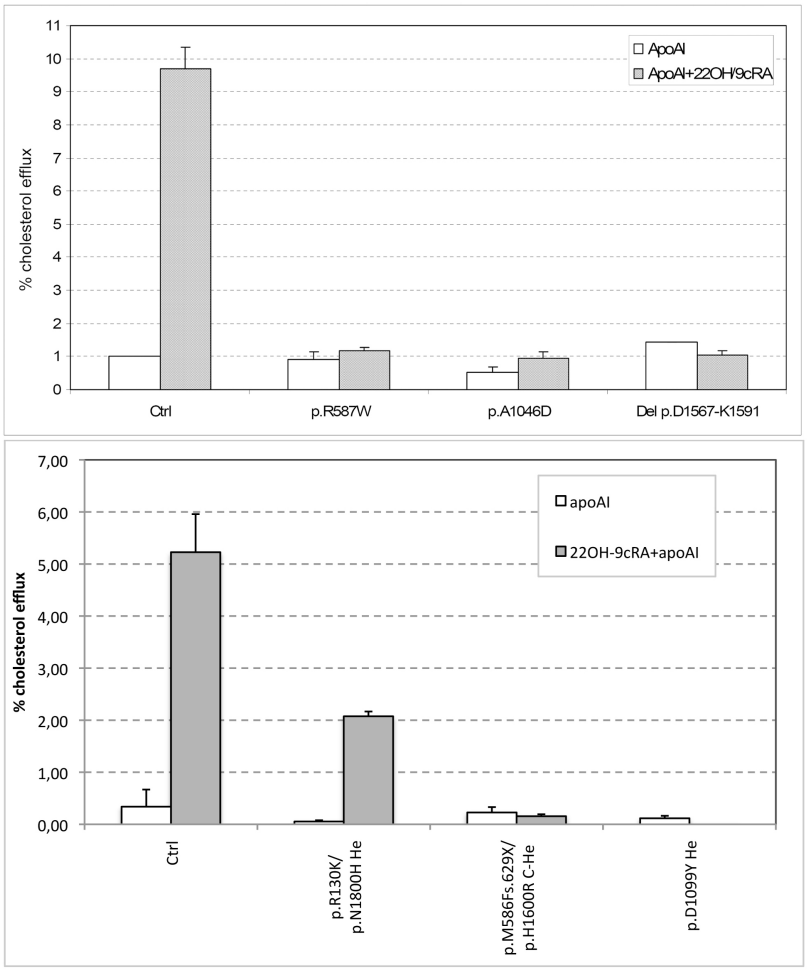

Figure 4.12: **Cholesterol efflux to Apo A−I in skin fibroblasts.** ^{3}H-cholesterol efflux to Apo A-I in skin fibroblasts of TD/FHD patients under basal conditions and following stimulation of ABCA1 gene expression with 22OH and cRA).

to ApoA-I, in control fibroblasts. Fibroblasts heterozygote for R130K and N1800H, gave a cholesterol efflux approximately half of the one observed in control cells, while fibroblasts carrier of D1099Y and carrier of M586Fs.629X / H1600R gave no efflux to ApoA-I, leading us to classify them as Tangier Disease fibroblasts (Fig. 4.12).

4.5.3 Quantification of membrane cholestenone content

Previous studies had shown that the expression of ABCA1 in normal skin fibroblasts treated with LXR-RXR agonists produces an increase in the size of membrane free cholesterol pool, an effect not observed in cells from patients with TD [35,150]. This amount of FC can be converted to cholestenone by treatment with an oxidase, since being on the plasma membrane is accessible to the enzyme.

Figure 4.13 shows that, following the ABCA1 induction by incubation with LXR-RXR agonists, the membrane cholesterol increased 2.5-fold in control fibroblasts. In probands' fibroblasts the increase in membrane cholesterol varied considerably. In the A1046D and R587W cells this increase was significant, although much lower than that seen in control cells (2.2 and 1.3 fold respectively), whilst in c.4773 +1G>A mutant cells no increase in membrane cholesterol was observed. These results suggested that ABCA1 mutations present in patients' fibroblasts show a different ability to drive the cholesterol to the plasma membrane, even if the efflux process to Apo A-I is reduced to approximately the same extent (Fig. 4.12). From these measure we obtained also an indirect indication about the presence of ABCA1 on the plasma membrane; it's likely that in c.4773 + 1G>A cells the little amount of ABCA1 protein expressed (see fig. 4.11 lane 6), was not able to reach the cell surface.

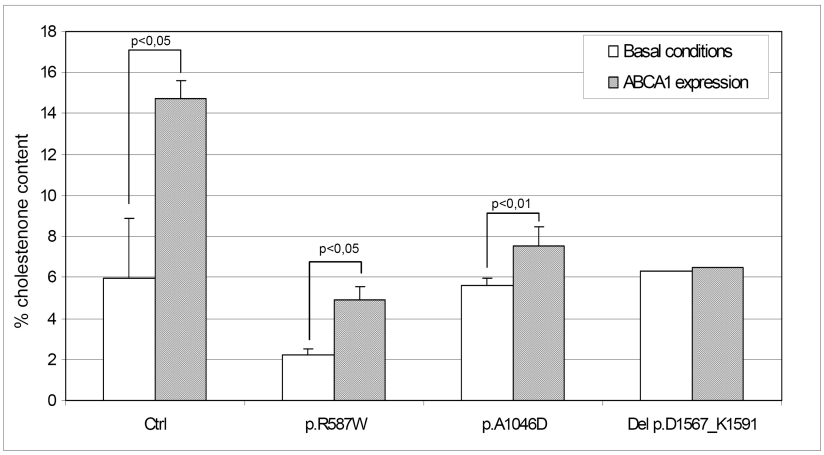

Figure 4.13: **Quantification of membrane cholestenone content** Enrichment of cholesterol in fibroblast plasma membrane following stimulation of ABCA1 expression by 22OH and cRA. This figure shows the ^{3}H-cholestenone content in plasma membrane before (white bars) and after stimulation of ABCA1 gene expression (grey bars).

SECTION C: ABCA1 EXPRESSION AND FUNCTION IN FH FIBROBLASTS

4.6 Study of ABCA1 protein in FH fibroblasts

Several studies in the last three decades, often reported low levels of HDL-C levels in FH patients in association with the other plasma lipid abnormalities typical of FH patients. The correlation between the low HDL trait and FH has never been investigated until last year, when a study reported that macrophages from LDLR-/- mice had a reduced expression and function of ABCA1 transporter, suggesting a possible link between LDLR function and ABCA1 mediated cholesterol efflux [133].

We took advantage of the availability of several FH fibroblasts cell lines, collected in our Cell Bank during the last two decades, to assess whether these cells showed a defect in ABCA1 protein expression and function. We selected 9 fibroblasts cell lines (listed in Table 4.3 from our cell bank derived from FH patients (seven homozygotes, one compound heterozygote and one simple heterozygote) with an average of HDL-C levels of 32,5 $\pm$ 10,3 mg/dL; they carried different mutations on the LDLR gene as specified in table 4.3.

	LDLR mutation	State	Class/Effect	HDL-C (mg/dL)
1	G528D	Ho	2A	15,8
2	Q12X	Ho	1	41,7
3	Del. Ex13-18	Ho	1	37,8
4	IVS15+1G>A	Ho	2, 5, ?	18,6
5	P664L	Ho	2B	37,8
6	C331W	Ho	2B, 5	40,9
7	Dupl. Ex16-17	Ho	6	42,8
8	Del. Ex13-15 / G528D	C-He	1/ 2A	24,7
9	Del. Ex2-12	He	1	--
			MEAN	**32,5 ± 10,3**

Table 4.3: FH fibroblasts cell lines.

4.6.1 Western blot for LDLR

In order to ascertain the differential defect in LDLR protein we performed a Western Blot for LDLR in all FH and in control fibroblasts.

We extracted total cellular proteins before (one control and one FH) and after incubation of fibroblasts in a medium containing LPDS (LipoProtein Deprived Serum), which is known to induce LDLR expression leading to an increase in LDLR on the cell surface.

As expected, before LPDS treatment, LDLR was mainly present in its immature form in control cells (Fig. 4.14 upper panel, lane 1). Treatment with LPDS induced the appearance of the mature form of LDLR in control cells, while the precursor was not detectable anymore (Fig. 4.14 upper panel, lane3). In G528D fibroblasts (Fig. 4.14 upper panel, lane 2) only the precursor of LDLR was detectable in western blot, both before and after treatment with LPDS (Fig. 4.14 upper panel, lane 2 and 4); this mutation is classified as 2A so a complete block of receptor trafficking in the endoplasmic reticulum was expected.

Fibroblasts carriers of the Q12X (class 1 mutation) were completely lacking LDLR protein (Fig. 4.14 upper panel, lane 5). Otherwise, cells carriers

of the Del. Ex13-18 showed the expression of an LDLR of reduced size (Fig. 4.14 upper panel, lane 6), due to the premature truncation caused by the large deletion; probably this protein was either retained or secreted but it was completely devoid of function.

Cells derived from the compound heterozygote, carrier of Del. Ex13-15 and G528D, showed only one band in the blot probably corresponding to the G528D allele, so to the immature form of the receptor (Fig. 4.14 upper panel, lane 7).

Cells carrying the Del. Ex2-12 in heterozygous state showed the expression of only the normal allele, resulting in a pattern super-imposable to the one of control cells (Fig. 4.14 lower panel, lane 1 and 2). C331W and P664L mutations caused the expression of the mature form of LDLR but also a certain amount of the protein remained in the immature form as expected since these were classified as 2A mutations (Fig. 4.14 lower panel, lane 3 and 4). Duplication of Exon16 and 17 had a migration pattern super-imposable to the one of control cells (Fig. 4.14 lower panel, lane 5), probably because this mutation causes a difference in size of only five amino acids [151]. Finally the splice site mutation IVS15+1G>A exhibited the presence of both the mature and the precursor form; this mutation caused the formation of three different mRNAs, one truncated, one with an in frame insertion and one with an in frame deletion [152].

4.6.2 ABCA1 protein expression in FH cells

Once confirmed the defect in LDLR protein expression in all FH fibroblasts selected, we pointed our attention to the ABCA1 transporter expression, which was investigated by western blot. ABCA1 expression was first studied in basal culture conditions, namely in presence of a 10% of FBS in the culture medium. Under these conditions ABCA1 expression resulted always lower in FH fibroblasts than in control fibroblasts (Fig. 4.15). There was a high degree of variability in ABCA1 expression among the different FH cases, but all of them resulted lower in intensity than controls. The presence of serum and of its lipoproteins probably caused a differential uptake of lipoproteins from the medium between FH and control fibroblasts, which could account

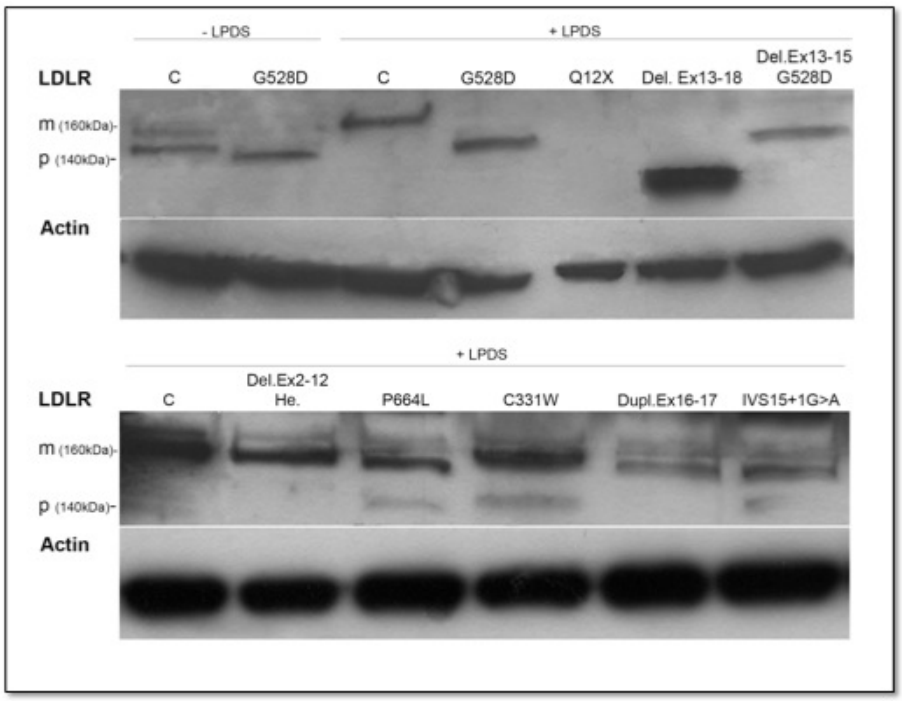

Figure 4.14: **Immunoblot of LDLR protein from cultured skin FH fibroblasts.** Upper panel; Lanes 1 and 2: control and *G*528*D* FH fibroblasts before LPDS treatment, lanes 3˘7: control and FH fibroblasts after LPDS tratment. Lower panel: control and FH fibroblasts after LPDS tratment.

for the observed difference in ABCA1 expression.

We then loaded control and FH fibroblasts with free cholesterol in absence of serum in the culture medium [14]. Our first issue was to verify if control and FH cells had a similar uptake of FC from the culture medium, so the intracellular mass of FC was measured before and after loading (Prof. Bernini's group, University of Parma). It was demonstrated that cholesterol loading resulted in a significant increase in intracellular cholesterol mass, both in control and FH cells. There was a slight reduction in the uptake of FC in FH cells (about 10%) but was not statistically significant (Fig. 4.16). As a control for decreased intracellular cholesterol mass was taken a Tangier Disease fibroblasts cell line.

Cholesterol loading was reported by several studies to induce ABCA1 expression through the generation of oxysterols [14,53,153]. The western blot analysis demonstrated that upon cholesterol loading ABCA1 protein

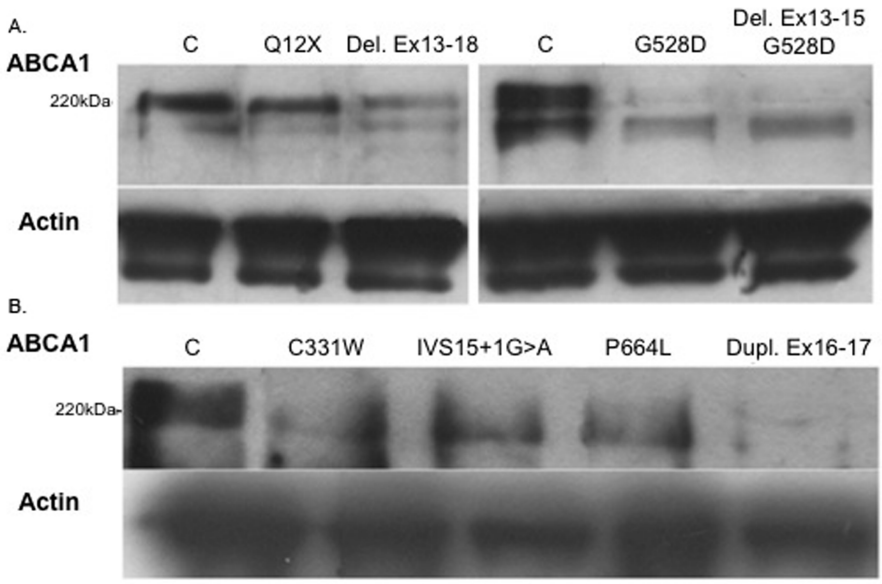

Figure 4.15: **Immunoblot of ABCA1 protein from cultured skin FH fibroblasts. Basal conditions.** A, B: ABCA1 protein expression in control and FH fibroblasts under basal culture conditions (i.e. in presence of FBS 10%.

was induced both in control and FH cells but in FH it did not reach the level found in controls (Fig. 4.17).

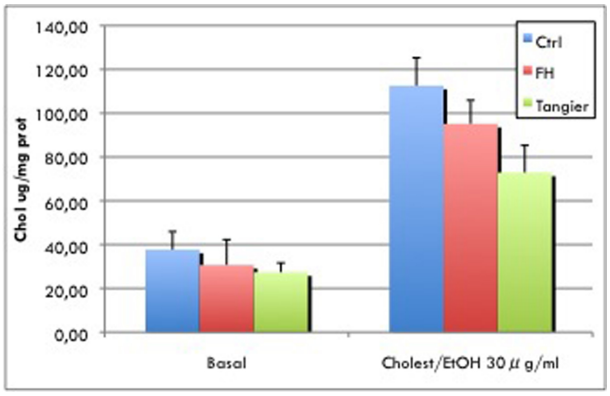

Figure 4.16: **Measure of intracellular cholesterol mass in control, FH and Tangier fibroblasts.** Measure of intracellular cholesterol mass in control, FH and Tangier fibroblasts before and after loading with 30 μg/ml of non-lipoprotein cholesterol.

Since ABCA1 expression is induced by the activation of the nuclear receptor LXR we treated control and FH fibroblasts with agonists of LXR in order to up-regulate ABCA1 protein. Cells were incubated with 22-

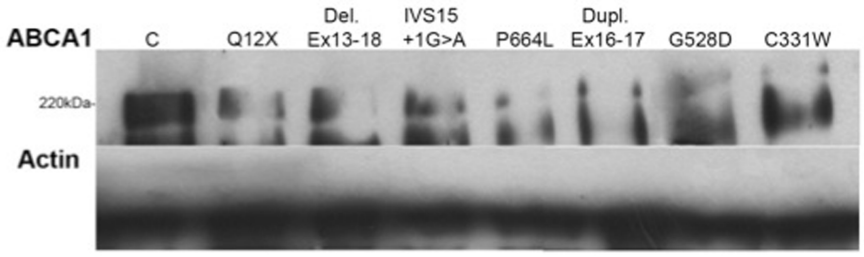

Figure 4.17: **Immunoblot of ABCA1 protein from cultured skin FH fibroblasts. Cholesterol loading.** ABCA1 protein expression in control and FH fibroblasts upon loading with 30 μg/ml of non-lipoprotein cholesterol.

hydroxycholesterol and 9-cis-Retinoic Acid, which acted as LXR-RXR ligands [99]; after treatment, ABCA1 expression was markedly increased as demonstrated by the western blot shown in figure 4.18. In control cells we always obtained a strong up-regulation of ABCA1 protein expression (Fig. 4.18 A, B lane 1) while Tangier fibroblasts (with ABCA1 null alleles) were regarded as negative controls (Fig. 4.18 A, lane 6, B lane 8). A greater variability was observed in ABCA1 expression in FH cells, but again it resulted always lower than the one exhibited by controls (Fig. 4.18 A, lanes 2-7, B lanes 2-5, C lanes 2-6).

Finally we incubated and four FH fibroblasts with a non-steroid agonist as T0901317. T0901317 is known to induce an over-expression of ABCA1 gene, through its action on LXR. We obtained a great up-regulation of ABCA1 protein, as shown by Fig. 4.19 A and B, with a similar degree of expression in FH and control fibroblasts.

Our findings were further corroborated by the work of Mosig et al., published during the course of our experimental work, reporting a reduced ABCA1 protein expression and mRNA levels in monocytes derived from homozygous FH patients [134].

4.6.3 Efflux studies

In order to confirm the findings on reduced ABCA1 protein expression in FH fibroblasts, efflux studies were performed in collaboration with prof. Bernini's group at the University of Parma. The cholesterol efflux from

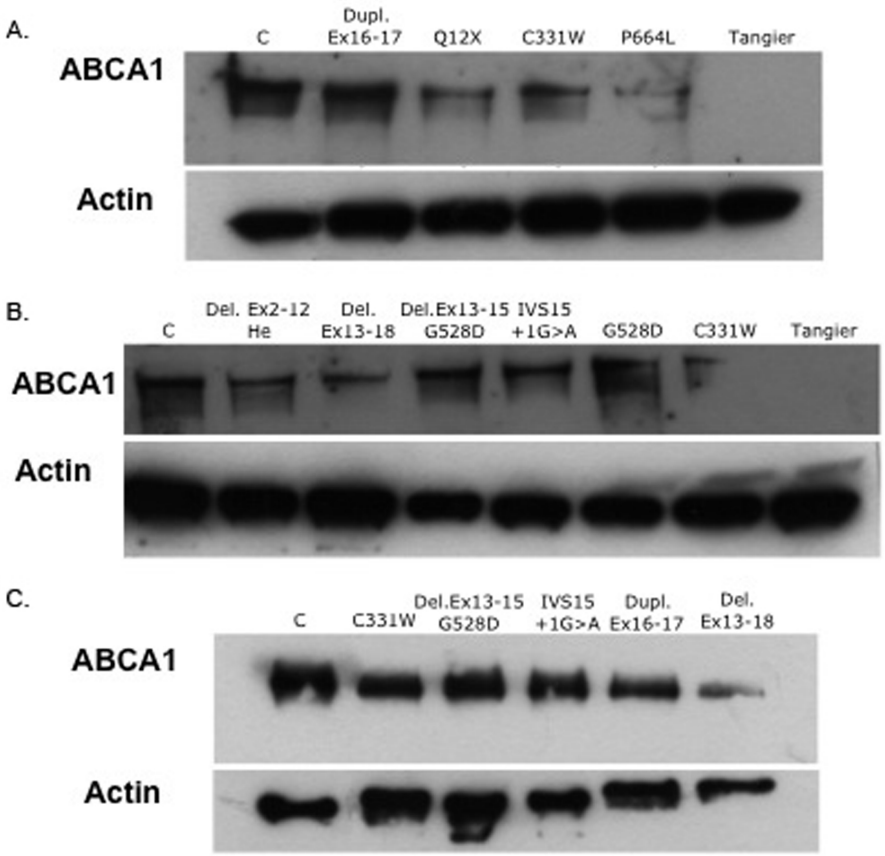

Figure 4.18: **Immunoblot of ABCA1 protein from cultured skin FH fibroblasts. LXR stimulation.** ABCA1 protein expression in control and FH fibroblasts upon stimulation of LXR pathway with 22-hydroxycholesterol and 9-cis-Retinoic Acid. A,B, C; lane 1: control cells. A lane 6 and B lane 8: Tangier Disease fibroblasts.

FH and control fibroblasts was measured after stimulation of cells with 22-OH-cholesterol and 9-cis-Retinoic Acid as described above (see 3.3.16). In FH cells the efflux of ^{3}H-cholesterol to ApoA-I resulted significantly reduced in all cases, except one, with respect to control cells (Fig. 4.20).

We tried to verify if there was any linear correlation between the amount of ABCA1 protein measured from at least three independent experiments of Western blot (stimulation with 22OH/9cRA) and cholesterol efflux to ApoAI. We found a trend towards a linear correlation (R^2= 0,3), even if not highly significant (Fig. 4.21), because of the low number of cases studied up to now.

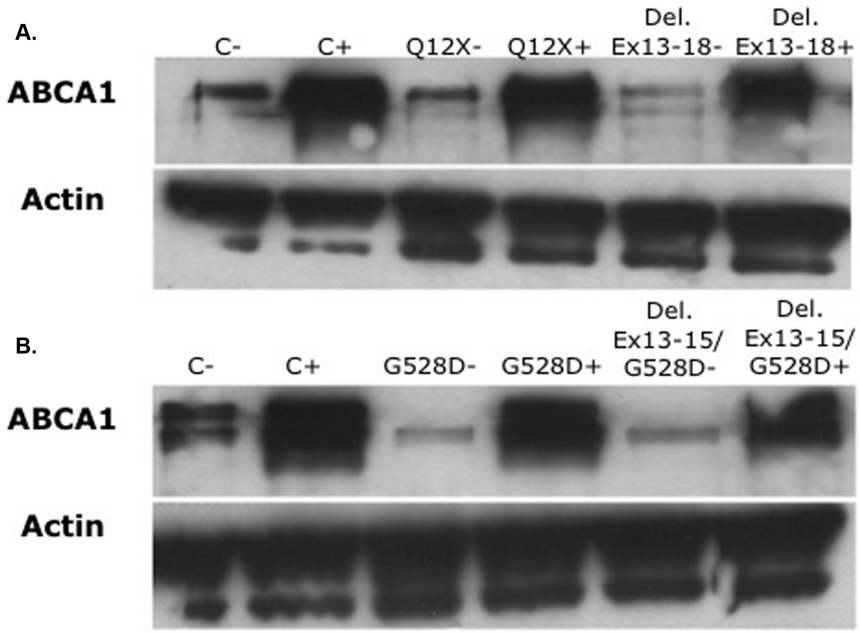

Figure 4.19: **Immunoblot of ABCA1 protein from cultured skin FH fibroblasts. LXR stimulation with T0901317.** ABCA1 protein expression in control and FH fibroblasts before and after stimulation of LXR pathway with T0901317. (−) Not treated cells; (+) Treated cells.

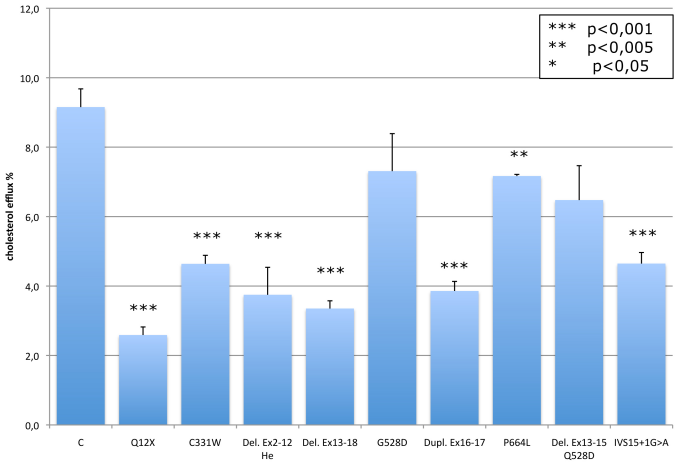

Figure 4.20: **Cholesterol efflux from FH fibroblasts.** Measure of ^{3}H-cholesterol to ApoAI after treatment of cells with 22 hydroxycholesterol and 9 cisRetinoic Acid. p-value was calculated with T-test algortihm.

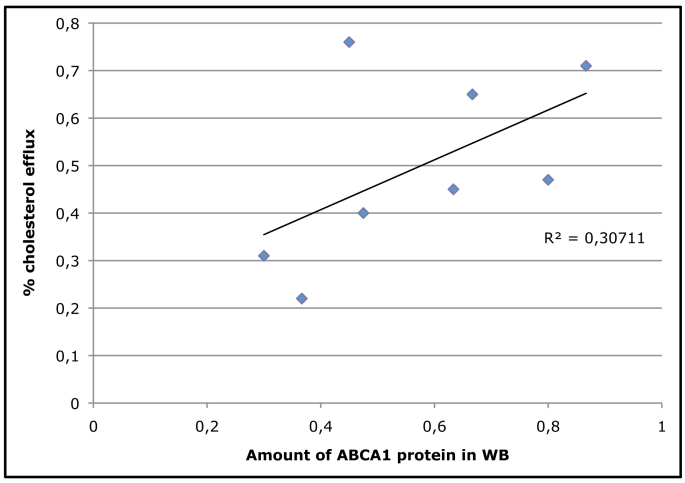

Figure 4.21: **Linear correlation ABCA1 protein - cholesterol efflux.** Linear correlation between the amount of ABCA1 protein in FH fibroblasts and cholesterol efflux, both measured upon treatment of cells with 22OH/9cRA. The amount of ABCA1 protein was measured with Gel-doc (Bio-Rad Laboratories Inc., S.r.l., Segrate, Milano) software and the average value between 3 independent Western Blot experiments was calculated.

Chapter 5

DISCUSSION

SECTION A: DIAGNOSTICS

First aim of this study was the identification of ABCA1 mutations in subjects with low plasma levels of HDL-C. We analysed ABCA1 gene with a combined approach (DHPLC/direct sequencing) in 27 individuals. We were able to accomplish a molecular diagnosis in 14 patients, 12 of which resulted affected by Tangier Disease, (i.e. with complete loss of function of both ABCA1 alleles). These patients represent all the Italian cases of Tangier Disease, reported up to now. In the 14 carriers of ABCA1 gene mutations, we identified 22 mutations (about 1/6 of all ABCA1 gene variants reported so far), 19 of which are novel mutations. We performed an in silico prediction for all missense and splice site variants in order to obtain some indications about the biological impact of mutations.

We tried to characterize first all novel splice site mutations. To accomplish this goal we performed, whenever possible, an "ex-vivo" analysis, using patients' derived fibroblasts or monocytes; in some cases (e,g, the splice site variant c.2961-2A>C) the study was carried out using patients' derived cells as well as in cells transfected with a minigene harbouring the mutation.

The c.2961-2A>C mutation resulted in the formation of two aberrant mRNAs, detected in peripheral blood mononuclear cells derived from the proband and his father. By contrast three aberrant mRNAs were found in COS-1 cells transfected with a mutant minigene carrying the mutation. While the two longer transcripts were found in patients' cells as well as in

transfected cells, the shortest transcript was detected only in transfected cells over-expressing the mutant minigene. Probably this short transcript was not produced at all in vivo or was produced in minute amounts and was rapidly degraded. Indeed even in over-expression conditions (transfected COS-1) this transcript was less abundant than the two longer mRNAs. This shortest transcript was the result of the complete skipping of exon 22, while the longer transcripts were generated by activation of two cryptic acceptor splice sites, located close to the canonic acceptor site. These findings highlighted that the functional assessment of intronic mutations should be performed, whenever possible, with two parallel approaches, the ex-vivo and the in-vitro strategy, since these approaches complement each other thus strengthening the final result [149].

The c.4773+1G>A mutation generated an ABCA1 protein with an in-frame deletion of 25 amino acids in the second extracellular loop. The ABCA1-mediated cholesterol efflux in fibroblasts of this patient was negligible, suggesting that the abnormal ABCA1 protein is either retained in the endoplasmic reticulum or, if it reaches the plasma membrane, is functionally inactive. Immunoblot analysis showed that in patient's fibroblasts the level of mutant ABCA1 protein was extremely low compared with the level found in control fibroblasts. This finding indicates that this structurally abnormal protein is probably degraded in the endoplasmic reticulum and does not reach the plasma membrane. Indirect evidence, suggesting that the mutant protein does not reach the plasma membrane, is given by the quantification of membrane cholesterol content, which indicates that the enrichment of cholesterol in the plasma membrane, was absent in fibroblasts of this proband. Even if the mutant protein reaches the plasma membrane, it is probably incapable of binding to Apo A-I, as the 25 amino acid deletion (del D1567–K1591) involves the second large extracellular loop of ABCA1 (1371–1650 amino acid residues), thought to be one of the Apo A-I binding sites. Previous studies have shown that two missense ABCA1 mutants (C1477R and S1506L) located in the second extracellular loop, when expressed in transfected cells, exhibited a dramatic reduction in the interaction capacity with Apo A-I [30]. It is also possible that the 25 amino acid deletion could generate structural changes which prevent the oligomerization of the ABCA1 protein [41,42,99].

SECTION B: FUNCTIONAL CHARACTERIZATION OF ABCA1 MUTANTS

We also fully or partially characterized also the missense mutations: R587W, A1046D, D1099Y, H1600R, R130K/N1800H. The R587W mutation was first reported by Lawn et al. [57] in a compound heterozygote with HDL deficiency, in whom the second allele failed to produce detectable mRNA. Subsequently, we reported a patient with severe HDL deficiency and premature CAD homozygous for this mutation [142], which abolished ABCA1-mediated cholesterol efflux in cultured fibroblasts. Previous in vitro experiments in transfected cells expressing the R587W mutant (designed to define the alteration in the ABCA1 pathway) were somewhat inconsistent. Fitzgerald et al. [30] showed that the R587W mutant reached the cell surface just like its wild type counterpart, but showed a 50% reduction in the cross-linking efficiency to Apo A-I. Singaraja et al. [147] showed that the R587W mutation prevents the migration of the protein to the plasma membrane, causing a 75% decrease in ABCA1-mediated cholesterol efflux. In our patient homozygous for the R587W mutation the ABCA1 protein content was lower than that observed in control fibroblasts (Fig. 4.11 lane 3), possibly suggesting an increased rate of intracellular degradation (an event expected if the mutant protein is retained, at least in part, in the endoplasmic reticulum). The reduced ABCA1 protein content in these cells is consistent with the strong reduced ability of promoting cholesterol efflux and plasma membrane cholesterol enrichment [99].

The ABCA1 A1046D was previously reported by Wang et al. [86] in a kindred with HDL deficiency, which included three compound heterozygotes and two heterozygotes for this mutation. Previous in vitro experiments have shown that the A1046D mutation, which occurs in the intracellular domain between the first Walker A and B motifs, severely impairs cholesterol efflux from transfected cells, even if it does not abolish the localization of ABCA1 in the plasma membrane, where it retains a residual capacity to bind Apo A-I [147]. Because ABCA1-mediated cholesterol efflux was completely abolished in the proband's fibroblasts (Fig. 4.12) we assumed that, even if this mutant maintains a residual capacity to reach the cell surface and to bind Apo A-I (as shown in transfected cells), it has an impaired transfer activity.

This consideration is also consistent with the observed defect in cholesterol enrichment in plasma membrane (Fig. 4.13). Immunoblot analysis showed that the content of mutant ABCA1 was greatly reduced in the proband's fibroblasts (Fig. 4.11 lane 5), strongly suggesting that the amino acid change causes an increased intracellular degradation of the ABCA1 protein [99].

In conclusion previous in vitro experiments were somewhat inconsistent [86,147] about mutant proteins expression since they were performed in transfected cells, forced to over-express the mutant protein; otherwise in our patients' cells the observed ABCA1 protein content was always lower than that of control fibroblasts possibly suggesting an increased rate of intracellular degradation (an event expected if the mutant protein is retained, at least in part, in the endoplasmic reticulum).

The D1099Y was found in an heterozygous patient with strongly reduced HDL-C levels and clinical manifestations of TD. Even if we expected to find a complete loss of function of both ABCA1 alleles, we were able to identify only this variant in heterozygous state. We demonstrated that in fibroblasts derived from D1099Y carrier, cholesterol efflux to ApoA-I was completely absent, thus confirming our assumption that the proband was a "Tangier Disease like" individual. We could not exclude the presence of an additional mutation in introns, outside the coding regions or the exon-intron boundaries usually analysed with direct sequencing; we could not also exclude the presence of a huge molecular rearrangement on the apparently normal allele, which might be responsible for the obliteration of the second allele. Anyway, since in our hands, this proband appears as a simple ABCA1 heterozygote we hypothesized a dominant negative effect of this variant. The D1099Y mutation was previously reported by Ho Hong et al. in 2002 [146] in a compound heterozygote. This mutation occurs in a highly conserved hydrophobic linker region between the sixth and the seventh helix (34 aa downstream the Walker B motif) of each transmembrane domain of ABCA1 protein, suggesting that it may impair the specificity of the transported molecules, ABCA1 protein oligomerization [42], or the ATP hydrolysis cycle. Indeed the only other dominant negative mutant, reported so far in ABCA1, was the M1091T variant [85,147], which is quite close to the 1099 residue. So we assumed that this region of ABCA1 protein is involved in oligomerization,

thus a single mutant allele could interfere with the normal allele by impairing the assembly of multimeric protein complexes, important for the proper transporter functioning.

We demonstrated also that fibroblasts carrier of H1600R and M586Fs.629X showed a null cholesterol efflux to ApoA-I, as expected by the severe phenotype of the patient, and that fibroblasts heterozygous for both R130K/N1800H showed a capacity to promote cholesterol efflux intermediate between control and TD cells. The latter finding was expected, since the proband was carrier of both mutation in heterozygous state on the same allele and had HDL values typical of an FHD subject.

The investigation of other novel missense mutations of ABCA1 gene, for which cells of the carriers were not available, was performed using an in vitro approach. Even if beyond the purposes of this work, we provided a rational for the strategy used and a formal demonstration of the effectiveness of our model. Indeed we constructed a plasmid carrying ABCA1 cDNA fused in frame with GFP cDNA at the 3' terminus. This vector was transfected in HEK293 cells, ABCA1-GFP distribution in transfected cells was assayed with confocal microscopy and cholesterol efflux was measured. As a negative control we created an artificial ABCA1 mutant, carrier of a large in-frame deletion, thus coding for a half transporter fused in frame with the GFP cDNA. In transfected HEK293 cells we demonstrated that wild type ABCA1 was mainly localized on the PM and in minute intracellular deposits inside the ER, while the mutant ABCA1 was completely retained in the ER. Moreover the wild type protein was able to promote a significant cholesterol efflux from transfected cells, while the mutant one did not. On the basis of these results we inserted in ABCA1-GFP cDNA the desired missense mutations and studied them with this validated approach.

SECTION C: ABCA1 EXPRESSION AND FUNCTION IN FH FIBROBLASTS

Finally we studied ABCA1 protein regulation in fibroblasts derived from FH patients. Our purpose was to try to assess a link between the two pathways of cholesterol influx and efflux from cells. The rationale for this study was based on two observations: i) the low levels of HDL-C often reported in FH patients; ii) the down regulation of ABCA1 protein and mRNA in

murine macrophages derived from LDLR -/- mice as the result of a persistent activation of SREBP1 transcription factor [133]. We demonstrated the decreased ABCA1 protein content in FH fibroblasts with respect to control fibroblasts incubated under different conditions: i) basal culture conditions: ii) following an ABCA1 transcriptional stimulation (22OH/9cRA), and iii) in presence of a non-lipoprotein derived cholesterol loading. The only stimulus, which in FH cells restored a level of ABCA1 expression similar to that found in control cells was represented by T0901317, a non-steroidal agonist of LXR. However this latter finding should be taken with caution, since the observed effect is most likely due to the over-expression of ABCA1 gene induced by the high dose of T0901317 used in the experiment.

The reduced ABCA1 protein level of FH fibroblasts was confirmed also by cholesterol efflux assays, (performed with the transcriptional stimulation of 22OH/9CRA) which showed that in seven out of eight FH cell lines ABCA1-mediated efflux was greatly reduced.

As suggested Zhou et al. we put forward the hypothesis that the altered ABCA1 regulation in FH cells was consequent to an impaired intracellular cholesterol homeostasis. Oxysterols are generated during cholesterol synthesis or when intracellular cholesterol levels rise. Zhou et al. demonstrated that in murine macrophages the response to oxysterols is different in cells lacking the LDLR. We demonstrated that in FH fibroblasts, oxysterols were produced because if we loaded cells with FC, ABCA1 protein is upregulated, but the extent of the upregulation is lower than in control cells. Thus we concluded that the oxysterols generated by FH fibroblasts were not able to completely shut down SREBPs transcription factors; a persistent activation of SREBPs proteins could act in the nucleus as a brake on LXR activation and so on ABCA1 expression. SREBPs transcription factors show an inverse pattern of expression with ABCA1 protein and the activation of the LXRE present in ABCA1 promoter was indirectly impaired by activated nuclear SREBP [133].

We assumed that this different behaviour between control and FH cells could be a consequence of an impaired accessibility of oxysterols to LXR and SREBPs or of free cholesterol to ABCA1. The distribution of intracellular sterols depends upon the cholesterol regulatory pool; while in control cells

the majority of cholesterol derives from lipoprotein uptake, in FH cells it derives from intracellular cholesterol synthesis. The LDL-derived cholesterol and the newly synthesized cholesterol belong to two different pools, which probably have different intracellular destinations and regulations. Further studies will be required to clarify this issue; we certainly demonstrated the existence of a connection between lipoprotein uptake and cholesterol efflux, and we provided a possible explanation for the low HDL-C levels found in FH patients, giving further elements to consider for the treatment of these patients.

Chapter 6

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Appendix A

Database of mutations identified in ABCA1 gene

Exon/ Intron	**Position and nt change**	**Effect on mRNA or DNA**	**Effect on protein**	**Phenotype**	**State**
IVS 2	c.66 +5 G>C	Skipping Ex 2, Ex 4		T	Che
4	c.219 ins T		I74YFs>Term76	T	Ho
4	c.254 C/T	CCG>CTG	Pro85>Leu	FHD	He
5	c.389 G>A	AGA>AAA	Arg130>Lys	FHD	He
6	c.479 del A		His160Fs>Term173	FHD	Che
7	c.593 C/A	TCA>TAA	Ser198>Term	FHD	He
7	c.688 C/T	CGT>TGT	Arg230>Cys	FHD	He
IVS7	c.720 +6 C>T	Skipping Ex 7	Del Val 182_Leu 240	T	Che
8	c.742 C/A	CCC>ACC	Pro248>Thr	FHD	He
8	c.763 G/A	GCC>ACC	Ala255>Thr	T,FHD	Ho
9	Del 8bp (c.815-822)	(TGTTCAGC)	Fs>5aa>Term276	T	Che
9	c.844 C/T	CGA>TGA	Arg282>Term	T	Che

9	c.850 G/A	GAG>AAG	Glu284>Lys	FHD	He
9	c.917 G/A	CGT>CAT	Arg306>His	FHD	He
9/IVS 9	c.1052-54 +1,2 dupl	CAAgt dupl	Thr351Fs>Term360	T	Ho
10	c.1091 C/G	TCT>TGT	Ser364>Cys	FHD(pol)	He
11	c.1196 T/C	GTG>GCG	Val399>Ala	T	Che
11	c.1201 A/C	AAG>CAG	Lys401>Gln	FHD	He
12	c.1375 A/C	ACA>CCA	Thr479>Pro	FHD	He
12	c.1445 A/G	TAC>TGC	Tyr482>Cys	FHD	Che
12	c.1486 C/T	CGG>TGG	Arg496>Trp	Ipera	He
IVS12-IVS14	Del 1221nts	Del Ex 13, 14	Del Ex 13, 14	T	Che
13	Del 14bp (c1584-1597); ins 110bp		Del 6aa; ins 38aa	T	Ho
13	c.1651 C/G	CAT>GAT	His551>Asp	FHD	He
13	c.1664 A/C	AAG>ACG	Lys555>Thr	Ipera	He
13	c.1669 C/T	CGA>TGA	Arg557>Term	FHD	He
14	c.1719 C/A	TAC>TAA	Tyr573>Term	T	Ho
14	c.1758 ins G		Met586>Fs>Term629	T	Che
14	c.1759 C/T	CGG>TGG	Arg587>Trp	T	Ho,Che
14	c.1769 G/C	TGG>TCG	Trp590>Ser	T	Che
14	c.1769 G/T	TGG>TTG	Trp590>Leu	FHD	He
14	c.1790 A/G	CAG>CGG	Gln597>Arg	T	Ho
14	Del G c.1824	CT(G) 608	Fs>27aa>Term635	T	Ho
14	c.1847 G/T	GGT>GTT	Gly616>Val	FHD	He
14	c.1881 C/G	TAC>TAG	Tyr627>Term	T	Che
15	c.1913 G/A	CGG>CAG	Arg638>Gln	FHD	He
15	c.1974 A/G	ATC>GTC	Ile659>Val	FHD	He
15	Del 3bp c.2077-2079	(CTT) 693	Del Leu693	FHD	He

16	c.2320 A/T	ACA>TCA	Thr774>Ser	FHD	He
16	c.2327 G>C	AAG>AAC	Lys776>Asn	FHD(pol)	He
IVS16-IVS31	Del 19.9kb	Del Ex 17-31	Del Ex 17-31	T	Che
17	c.2444 A/G	GAG>GGG	Glu815>Gly	FHD	He
17	c.2518 T/A	TGG>AGG	Trp840>Arg	FHD	He
18	c.2602 G/A	GAG>AAG	Glu868>Lys	FHD	He
19	Del C c.2725	(C)GA	Fs>3aa>Term912	T	Che
19	c.2725 C/T	CGA>TGA	Arg909>Term	FHD,T	He, Ho
19	c.2737 A/T	AAG>TAG	Lys913>Term	T	Ho
19	c.2786 C/T	ACC>ATC	Thr929>Ile	T	Che
19	c.2803 A/C	AAT>CAT	Asn935>His	T	Ho, Che
19	c.2804 A/G	AAT>AGT	Asn935>Ser	T	Ho
19	c.2810 C/T	GCG>GTG	Ala937>Val	T	Che
20	c.2893 C/T	CGC>TGC	Arg965>Cys	FHD	He
IVS20	c.2961 -2 A>C			T	Ho
21	c.3077 T/C	CTG>CCG	Leu1026>Pro	FHD	He
22	c.3137 C/A	GCC>GAC	Ala1046>Asp	T	Che,Ho
22	c.3193 C/T	CCT>TCT	Pro1065>Ser	FHD	He
22	c.3202 C/T	CGC>TGC	Arg1068>Cys	FHD	He
22	c.3203 G>A	CGC>CAC	Arg1068>His	T	Ho
23	c.3272 T/C	ATG>ACG	Met1091>Thr	FHD	He
23	c.3295 G/T	GAC>TAC	Asp1099>Tyr	FHD	Che,He
23	Ins G c.3286	After codon 1095	Fs>50aa>Term1146	T	Che
23	Del 2bp (c.3340-3341)	(TC)C 1114	Fs>30aa>Term1145	T	Ho
25	c.3542 C/T	TCT>TTT	Ser1181>Phe	FHD	He
25	c.3647 G/T	GGA>GTA	Gly1216>Val	FHD	He

IVS 25	c.3738 +1 G>C			T	Che
27	c.3808 C>T	CGA>TGA	Arg1270>Term	TD	Ho
27	c.3835 C>A	CAG>AAG	Gln1279>Lys	FHD	He
27	Del CGCC c.3847-50	(CGC C)CG	Fs>14aa>Term1297	FHD	Ho
27	c.3865 G/A	GAT>AAT	Asp1289>Asn	T	Ho, Che
28	c.3966 G/A	TGG>TGA	Trp1322>Term	FHD	He
28	c.4022 G/C	AGA>ACA	Arg1341>Thr	FHD	He
29	c.4126 A/G	AGC>GGC	Ser1376>Gly	FHD	He
29	c.4135 C/T	CTT>TTT	Leu1379>Phe	T	Che
29	c.4156 G/C	GAA>CAA	Glu1386>Gln	FHD	He
30	c.4261 G/T	GGA>TGA	Gly1421>Term	FHD	He
31	c.4429 T/C	TGT>CGT	Cys1477>Arg	T	Che
31	c.4430	TGT>TTT	Cys1477>Phe	FHD	He
IVS31	c.4465 -1 G>C	Skipping Ex 32	Gln1488>Fs> Term1497	T	Che
32	c.4517 C/T	TCG>TTG	Ser1506>Leu	T	Che
34	c.4595 A>G	TAT>TGT	Tyr1532>Cys	T	Che
34	Ins A c.4630	After codon 1543	Fs>8aa>Term1552	T	Che
IVS35	c.4773 +1 G>A		Del Asp1567_Lys1591	T	Ho
36	c.4799 A>G	CAT>CGT	His1600>Arg	T	Che
36	c.4831 A/G	AAT>GAT	Asn1611>Asp	FHD	Ho
36	c.4844 G/A	CGG>CAG	Arg1615>Gln	FHD	He
36	c.4844 G>C	CGG>CCG	Arg1615>Pro	FHD	He
36	Del GG c.4882-3, ins C	After codon 1627	Fs>8aa>Term1636	T	Che

37	c.4978 T>C	TGT>CGT	Cys1660>Arg	T	Ho
37	c.5008 G/A	GCC>ACC	Ala1670>Thr	FHD	He
37	c.5038 C/T	CGG>TGG	Arg1680>Trp	T	Ho
37	c.5039 G/A	CGG>CAG	Arg1680>Gln	Iperα	He
37	c.5097 G/T	TGG>TGT	Trp1699>Cys	T	Che
37	c.5111 T/A	GTC>GAC	Val1704>Asp	T	Che
37	c.5116 G/A	GAT>AAT	Asp1706>Asn	FHD	He
38	Ins 138nt after c.5122	In codon 1708	+ 46aa	T	Che
38	c.5192 C/G	TCC>TGC	Ser1731>Cys	FHD	He
40	c.5398 A/C	AAT>CAT	Asn1800>His	T	Ho, Che, He
40	Del 3' Ex40→Ex50		Del 427 terminal aa	T	Ho
41	c.5520 del T	Codon 1640	Fs1840>Term1869	FHD	He
41	c.5551 C/T	CGA>TGA	Arg1851>Term	T	Ho
41	c.5552 G/A	CGA>CAA	Arg1851>Gln	T	Che
42	Del 6bp (c.5677-5682)	(GAAGAT)	Del Glu1893, Asp1894	FHD	He
42	c.5689 T/C	CGG>TGG	Arg1897>Trp	FHD	He
42	C,5703 A/C	AGA>AGC	Arg1901>Ser	FHD	Che
42	Ins 14nt after c.5757	After codon 1919	Fs>44aa>Term1964	T	Che
43	c.5774 G>A	CGG>CAG	Arg1925>Gln	Scott Syndrome	He
45	c.6010 G>A	AGA>AAA	Arg2004>Lys	FHD	He
45	c.6026 T/C	TTC>TCC	Phe2009>Ser	FHD	Che,He
46	c.6082 C/T	GCG>GTG	Ala2028>Val	FHD	He
IVS 46	c.6205 -39....-46 del T	Skipping Ex 47, Term2073		T	Che

47	c.6217 A/G	ACA>GCA	Thr2073>Ala	FHD	He
47	c.6241 C/T	CGG>TGG	Arg2081>Trp	T	Che
49	Del C c.6430	Codon 2144 (C)GA	Fs>1aa>Term2145	T	?
49	c.6430 C/T	CGA>TGA	Arg2144>Term	FHD	He
49	c.6449 C/T	CCG>CTG	Pro2150>Leu	FHD	He
49	c.6488 T/C	TTT>TCT	Phe2163>Ser	T	Che
49	c.6532 T/C	TAC>CAC	Tyr2178>His	FHD	He
49	Del TT c.6546-6547	TC(T T)CA	Fs>20aa>Term2203	T	?
49	Ins 4bp after c.6573	Ins CATT	Fs>8aa>Term2200	T	Che
49	c.6588 G/C	CAG>CAC	Gln2196>His	FHD	He
49	Del c.6601-3 ins	Del CTC ins TT	Fs>2aa>Term2203	T	Ho
49	c.6616 T>G	TAC>GAC	Tyr2206>Asp	FHD	He
49	c.6630 G/C	CAG>CAC	Gln2210>His	T	Che, He
50	c.6729 C/G	GAC>GAG	Asp2243>Glu	FHD	He
50	c.6730 G/A	GTT>ATT	Val2244>Ile	T	Che

Appendix B

Database of polymorphisms identified in ABCA1 gene

Promoter	-878 C/T (-564)	↑ Aci I	
Promoter	-820 A/C (-506)		
Promoter	-721 G/C (-407)		
Promoter	-592 G/C (-278)	↓ Hga I	
Promoter	-328 C/T (-14)	↓ Bsm AI	
Exon 2	-76 GGG/GG (237)	↓ Bsl I	
Exon 2	-18 C/G (297)		
Exon 6	474 G/A	CTG>CTA	Leu158
Exon 7	656 G/A	AGG>AAG	Arg219>Lys
Exon 8	765 C/T	GCC>GCT	Ala255
Exon 9	936 C/T	CCC>CCT	Pro312
Exon 9	948 G/A	GGG>GGA	Gly316
Exon 11	1196 T/C	GTG>GCG	Val399>Ala

Exon 15	2040 C/A	ATC>ATA	Ile680
Exon 16	2311 G/A	GTG>ATG	Val771>Met
Exon 16	2320 A/C	ACA>CCA	Thr774>Pro
Exon 16	2328 G/C	AAG>AAC	Lys776>Asn
Exon 17	2473 G/A	GTC>ATC	Val825>Ile
Exon 18	2649 A/G	ATA>ATG	Ile883>Met
Exon 20	2880 G/A	CTG>CTA	Leu960
Exon 22	3159 T/G	GTT>GTG	Val1053
Exon 24	3516 G/C	GAG>GAC	Glu1172>Asp
Exon 31	4281 G/A	ACG>ACA	Thr1427
Exon 34	4664 C/T	ACC>>ATC	Thr1555>Ile
Exon 35	4760 G/A	AGA>AAA	Arg1587>Lys
Exon 36	4943 C/T	CCG>CTG	Pro1648>Leu
Exon 38	5192 C/G	TCC>TGC	Ser1731>Cys
Exon 44	5921 G/A	AGA>AAA	Arg1974>Lys
Exon 49	6447 C/T	AAC>AAT	Asn2149
Exon 49	6503 C/T	CCA>CTA	Pro2168>Leu

RINGRAZIAMENTI

La storia personale e professionale che mi porta a concludere il Dottorato di Ricerca può apparire non molto lineare e forse un pò bizzarra, in quanto costruita su decisioni pesate e ripensate più volte. Nonostante ciò il traguardo che mi trovo ora a raggiungere è esattamente quello che ho auspicato e desiderato con forza per gli ultimi 3 anni. Forse ora il mio futuro seguirà strade diverse da quelle sperate e si discosterà dal mondo accademico, ma spero di aver acquisito una formazione che possa rivelarsi utile in ogni ambito della mia vita. Gli sforzi che mi hanno portato a concludere il percorso sono più collettivi che personali, pertanto desidero dedicare un pensiero di ringraziamento a coloro che sono sempre stati in prima linea a guidarmi e sostenermi.

Un vivo ringraziamento al Prof. Sebastiano Calandra, da cui non finisco mai di imparare, per avermi guidato e formato passo passo, nel corso di questi 6 anni di collaborazione. Lo ringrazio in particolar modo per il suo spessore umano, per la sua vastissima cultura (non solo scientifica!) e per avermi trasmesso la passione per lo studio, la curiosità per il mondo e la passione per il sapere.

Un ringraziamento sentito al Prof. Francesco Bernardi che ha accettato di essere mio supervisore; pur avendolo incontrato poche volte mi ha sempre trasmesso grande entusiasmo e passione per il mondo della ricerca scientifica.

Ringrazio sentitamente tutti i colleghi che mi hanno accompagnato del tutto o in parte negli ultimi 3 anni e che si sono anche rivelati dei veri amici: Tommaso, Chiara, Claudio P., Claudio R., Alessia, Paolo, Margherita, miei compagni di laboratorio. Ringrazio anche i colleghi di altri laboratori che mi hanno insegnato moltissimo: Chiara, Federica, Giulia, Antonietta,

Clara, Giovanna, Rita e molti molti altri. Tommaso merita forse un pò più di un grazie per essere non solo un bravo medico e un brillante scienziato ma anche per essersi sempre rivelato un amico sincero, acuto e fedele. Chiara (Pasqui) merita un ringraziamento speciale non solo per la sua pazienza nell'insegnarmi a coltivare le cellule, ma soprattutto per essere un'amica leale, sincera, simpatica e una mamma premurosa che mi ha insegnato tantissimo! Ringrazio Chiara perchè anche con lei si è sviluppato un rapporto che va molto oltre il rapporto professionale... tant'è vero che ho fatto di tutto perchè potesse essere nuovamente mia collega! Ci sono tantissimi amici e amiche che vorrei ringraziare, tra questi ricordo solamente Chiara G. che ormai è parte della mia famiglia e che è sempre fonte di ammirazione per me per le sue idee, la sua intraprendenza e per l'amicizia che condividiamo.

La mia famiglia più che ringraziata va abbracciata tutta intera per riaffermare quanto sono loro grata per l'affetto e il sostegno che continuano a prestarmi. Prima di tutto ringrazio la mia mamma e il mio papà, che nel frattempo sono diventati nonni, che non si scordano mai di me e sono sempre vigili sulla mia felicità e la mia realizzazione personale; i miei cognati Lorenzo e Serena, amici, compagni di avventure, consiglieri fidati, zii premurosi. Infine un grazie a Carla che ha reso possibile gran parte di questo lavoro, prendendosi cura nel migliore dei modi della sua "nipotina acquisita" Anna.

I più importanti li nomino qui in fondo: Alain mio compagno di vita, per l'amore che mi dona ogni giorno in ogni gesto che compie e in ogni parola che mi dice e Anna per essere arrivata a "scombussolarci" e a riempirci di gioia e di vita.