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A NOVEL THERAPEUTIC STRATEGY TO CURE THE HOMOZYGOUS FAMILIAL HYPERCHOLESTEROLEMIA WITH RESIDUAL LDL RECEPTOR ACTIVITY

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INTRODUCTION

Hypercholesterolemia

Hypercholesterolemia is a metabolic disorder characterized by high plasma low-density lipoprotein-cholesterol (LDL-C) levels that lead to premature atherosclerosis in the coronary arteries and proximal aorta and to an elevated risk to develop cardiovascular disease (CVD), such as coronary artery disease (CAD) and stroke. It is possible to distinguish an autosomal dominant (ADH) and an autosomal recessive (ARH) hypercholesterolemia.

ADH or classically known as Familial Hypercholesterolemia (FH) is caused by mutations in several genes coding for key proteins involved in low-density lipoprotein receptor (LDLR) pathway (1):

- LDLR gene mutations resulting in a reduced cell uptake and catabolism of plasma LDL (Autosomal dominant familial hypercholesterolemia type 1, ADH-1) (2).
- LDL receptor binding region of apolipoprotein B (APOB) gene defects producing a defective apoB-100 which binds poorly to the LDLR (Familial hypercholesterolemia Defective ApoB-100, FDB or ADH-2) (3).
- Rare gain of function of proprotein convertase subtilisin/kexin type 9 (PCSK9) gene mutations resulting in abnormal function of the proteolytic enzyme PCSK9 (Autosomal dominant familial hypercholesterolemia type 3, ADH-3) (4).

ARH is a less severe form of hypercholesterolemia but with similar clinical features and is caused by defect in the following genes:

- low density lipoprotein receptor adapter protein 1 (LDLRAP1) that encodes an adaptor protein involved in clathrin-mediated internalization of the LDLR/LDL complex. LDLRAP1 mutations prevent the internalization of LDLR and compromise the uptake of LDL-C in the liver (5).
- ABCG5 (Sterolin 1) and ABCG8 (Sterolin 2) genes belong to transmembrane ATP-binding cassette transporters family. Loss of function mutations (LOF) of these genes cause beta-sitosterolaemia characterized by the abnormal intestinal absorption of vegetal sterols (6).
- CYP7A1 gene, coding for enzyme cholesterol-7a-hydroxylase involved in the first step of cholesterol catabolism and bile acids formation (7).

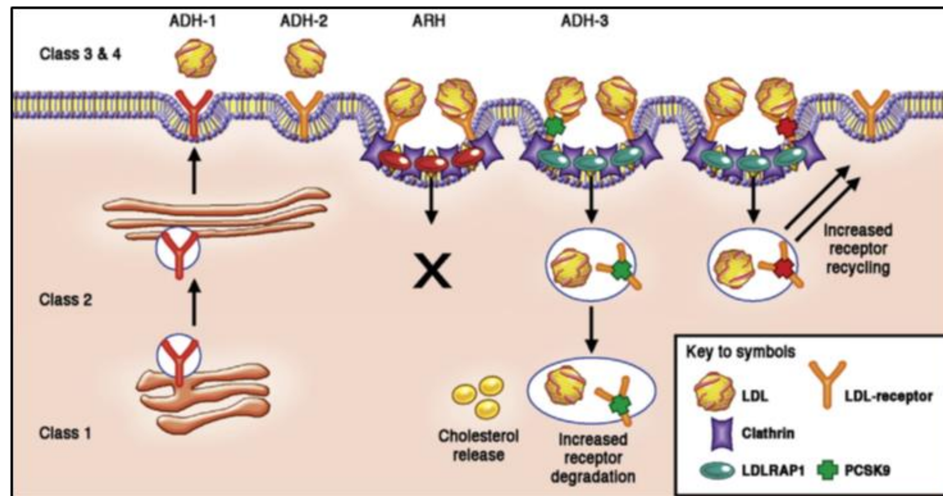


Fig.1 Classification of autosomal dominant (ADH) and autosomal recessive (ARH) form of Hypercholesterolemia (Figure from Sniderman AD et al. The severe hypercholesterolemia phenotype: clinical diagnosis, management, and emerging therapies. J Am Coll Cardiol. 2014 May 20;63(19):1935-47)

Autosomal dominant familial hypercholesterolemia type 1, ADH-1

ADH-1 is the most commonly known monogenic form of inherited metabolic disease with a prevalence of the heterozygous form of 1:250 and homozygous form of 1:300.000 individuals (8-10). FH heterozygotes (HeFH) patients have only one mutated allele of LDLR gene while FH homozygotes (HoFH) have identical mutation in both alleles and compound heterozygotes two different mutation in each allele of LDLR gene. HeFH show total cholesterol (TC) levels ranging from 310 to 580 mg/dL and LDL-C levels ranging from 200 to 400 mg/dL and are characterized by having premature CAD. HoFH reach TC levels between 460 and 1160 mg/dL and presented extremely low or absent plasma clearance of LDL and faster development of CAD (8). HoFH are classified into two groups based on the residual activity of LDLR: patients with < 2% of normal LDLR activity are classified as receptor negative and patients with 2 to 25% of normal LDLR activity as receptor-defective (1).

The residual activity of LDLR is related to the severity of LDL-C increase and to the development of premature CVD. HoFH patients who are receptor-negative have higher LDL-C levels and more severe clinical phenotype than patients with defective LDLR, including extensive xanthomas and an early and progressive atherosclerotic cardiovascular disease (ASCVD) (11).

The LDLR gene

LDLR is a cell surface glycoprotein that regulates intracellular cholesterol metabolism by mediating endocytosis of low-density lipoproteins (LDL).

The human LDLR gene is located on chromosome 19 (p13.1-p13.3), includes 18 exons and 17 introns and encodes a transmembrane protein of 839 amino acids (3). Exon 1 encodes 21 hydrophobic amino-acids of the signal peptide. This sequence is cleaved during translocation in the endoplasmic reticulum (ER). Exons 2-6 encode for ligand-binding domain which contains 7 cysteine-rich repeats requires for the interaction with the ligand. LDLR is able to bind two apolipoproteins: apoB-100 and apo E. Exons 7-14 encode an epidermal growth factor (EGF) precursor homology domain involve in lipoprotein release at endosome level. This region includes two EGF-like domains (EGF-A and EGF-B) of 40 amino-acids cysteine rich and one EGF-like repeat (EGF-C). Exon 15 encodes 58 amino-acids rich in threonine and serine residues of a domain enriched in O-linked oligosaccharides that stabilize the receptor. Exon 16 and part of exon 17 encode 22 hydrophobic amino acids of the transmembrane domain for anchoring LDLR to the cell membrane.

Finally, the remaining portion of exon 17 and the 5 'end of exon 18 encode 50 amino-acid residues of the cytoplasmic domain involved in the formation of clathrin-coated pits and in the endocytosis of the receptor (Fig.2A). In addition, LDLR promoter contains two TATA-like sequences and three 16 bp direct repeats critical for gene transcription. Repeats 1 and 3 are specific for protein 1 (Sp1) binding site and repeat 2 has a sequence of 8 bp, named Sterol response elements (SRE) (12). SRE sequence is a binding site for transcription factors called Sterol Regulatory Element Binding Proteins (SREBPs). In particular, transcription factor Sterol Regulatory Element Binding Protein-2 (SREBP-2) mainly regulates the expression of genes involved in cholesterol synthesis.

LDL receptors are synthesized as a precursor of 860 amino acids (21-residues at the N-terminus are excised during protein translocation) in the ER, matured in Golgi and then reach the cell surface where are placed inside clathrin-coated vesicles (13). The binding between LDLR and LDLs promotes the internalization of receptor-ligand complex, clathrin polymerizes and forms an invagination and subsequently an endosome inside the cell. The acid environment of the endosome causes the dissociation between LDLR and LDL particles. The LDLR is then recycled back to the cell membrane, available for a new cycle of binding and internalization of the LDL, or to the lysosome, where it is degraded by various proteases and lipases. LDL cholesterol esters are hydrolysed by releasing free fatty acids and cholesterol (14,15) (Fig.2B).

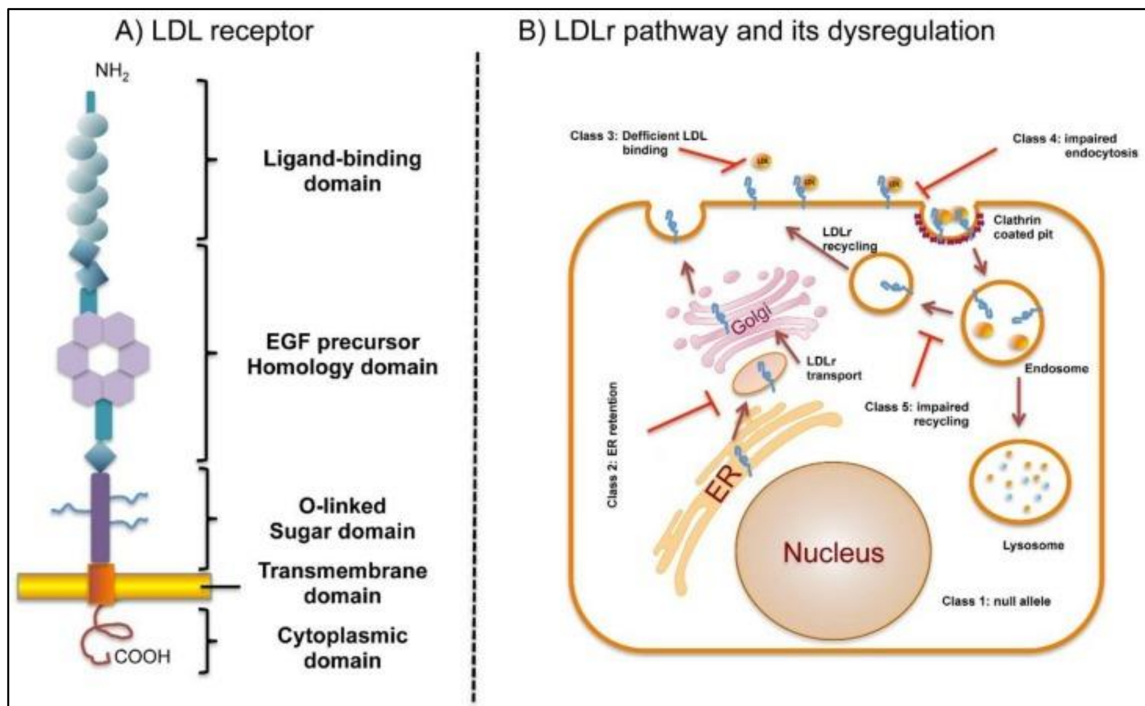


Fig.2 Domain organization of LDLR and LDLR pathway and its dysregulation by defective mutations. (A) Schematic representation of LDLR domains; (B) LDLR cycle. (Figure from Benito-Vicente A et al. Validation of LDLR Activity as a Tool to Improve Genetic Diagnosis of Familial Hypercholesterolemia: A Retrospective on Functional Characterization of LDLR Variants. Int J Mol Sci. 2018 Jun 5;19(6)

Over 1800 mutations of LDLR gene, including large rearrangements, single amino acid substitutions, mutations in the promoter region, have been identified (2,16,17) causing ADH-1. LDLR mutations have been classified into 5 functional classes which affect the lifecycle of the receptor in different way (Fig.2B):

- Class 1: mutations that modify the receptor synthesis known as “null alleles”;
- Class 2: mutations encode proteins defective in LDLR transport from the ER to the cell surface. Due to missense mutations and small in frame deletions these proteins don’t have a correct structure and are retained, completely or partially (2A and 2B, respectively) in the ER;
- Class 3: mutations encode proteins that are synthesized and transported to the cell surface, but defective in the binding LDL particles. Most of these mutations are rearrangements in binding ligand domain or deletions in EGFP-like domain;
- Class 4: mutations encode protein unable to internalize the LDL-LDLR complex. These mutations affect cytoplasmic domain (A) or transmembrane domain (B);

- Class 5: mutations encode proteins that fail in recycling of the LDLR protein to the cell surface. As a consequence of these mutations, LDL particles don't dissociate from LDLR that is degraded in the lysosome instead of being recycled.

Class 1 mutations are called receptor-negative mutations while the mutations from class 2 to 5 are known as receptor-defective mutations (3,4).

Familial hypercholesterolemia Defective ApoB-100, FDB or ADH-2

ADH-2 has a prevalence of the heterozygous form of 1:500/1:700 individuals in the Caucasian population of North America and Europe. The disorder is also known as Familial Defective apo-B100 (FDB) and is associated with levels of TC levels ranging from 250 to 400 mg/dL. Patients with APOB mutations usually have a less severe phenotype than FH patients carrier of LDLR mutations. Four allelic variants of APOB gene were identified (18) and the most common mutation in Europe is Arg3500Gln that causes defective binding with LDLR. 2-5% of patients with hypercholesterolemia are heterozygous for the defective allele (19).

The APOB gene

The gene APOB is located on chromosome 2, includes 29 exons and 28 introns and encodes for an amphipathic glycoprotein with two isoforms: apo B-100, with 4536 amino acids (550kDa), and apo B-48 (265kDa) (20). The apo B-100 gene is transcribed only for the first 2152 N-terminal amino acids to form apo B-48 that lacks of the C-terminal region of binding to the LDLR.

Apo B-100 is synthesized in hepatocytes and is the major apolipoprotein placed on the surface of LDL and is essential for packaging and secretion of VLDL particles from the liver. The ApoB-100 represents about 95% of the apolipoproteins (ApoB, ApoC and ApoE) present in LDL and is the main ligand of the LDLR. The LDLR-binding domain was localized between residues 3386 and 3396. In addition, the regions between amino acid 3174 and 3184 and 4181 and 4540 are important for the correct folding of APOB and for the binding to the LDLR (21). The production of defective ApoB, unable to bind LDLR, alters the catabolism of LDLs that remain in circulation for a long time cause an increase in plasma cholesterol levels.

The synthesis of ApoB-48 is the result of mRNA editing, a post-transcriptional mechanism in which a cytosine normally found in codon 2153 (CAA) is replaced by an uracil by a deamination reaction catalysed from the cytidine deaminase. This process leads to the formation of a stop codon (UAA) and to the synthesis of a truncated ApoB form (22).
Apo B-48 is synthesized in small intestine cells and is responsible for the formation and secretion of chylomicrons.

Autosomal dominant familial hypercholesterolemia type 3, ADH-3

In 2003, Abifadel et al. studying a cohort of French families affected by severe autosomal dominant hypercholesterolemia but without mutations in candidate genes (LDLR and APOB) have found in these patients gain-of-function (GOF) mutations in PCSK9. This finding led to the identification of another form of autosomal dominant hypercholesterolemia called ADH-3 (23). This form is relatively rare and the estimated frequency is 1:250 of subjects with ADH.

The PCSK9 gene

The human PCSK9 gene is located on chromosome 1(1p33-p34.3), includes 12 exons and encodes for a glycoprotein of 692 amino acids know as Neuronal apoptosis regulated convertase-1 (NARC-1).
NARC-1 is more expressed in the liver, renal mesenchymal cells, small intestinal, central nervous system and belongs to the mammalian subtilisin serine protease family which contains nine members responsible for the proteolytic cleavage of a wide variety of secreted proteins. Convertases have an active site capable of catalyse the enzymatic hydrolysis of peptides (24,25). The NARC-1 protein, commonly called PCSK9 is synthesized as a 75 KDa inactive zymogen precursor composed of a single peptide with an N-terminal pro-domain, a catalytic domain, and a cysteine and histidine-rich C-terminal domain. In the ER, zymogen precursor undergoes a self-catalytic cleavage to form the mature protein of about 60KDa, releasing the N-terminal pro-domain that remains non-covalently bound to the catalytic domain. It works as a catalytic activity inhibitor and as a chaperone for the correct folding of mature PCSK9 that moves from ER to Golgi (Fig.3). This processed form is present in the circulation as a free protein and bound to plasma proteins.

Several experiments showed that PCSK9 is able to bind LDLR both intracellularly and on the surface of the plasma membrane of hepatocytes, by the first EGF-like repeat (EGFA) located in the extracellular portion of the LDLR (26-28). The PCSK9-LDLR complex is internalized by clathrin-dependent endocytosis, PCSK9 bind LDLR through the C-terminal residue stabilizing the binding between the two proteins and promoting the degradation of LDLR by lysosome. The PCSK9 promoter hold a Sp1 site, an HNF1α site (Hepatocyte Nuclear Factor1α) which is an essential positive regulator of PCSK9 transcription and two SRE-1 and SRE-2. In vivo experiments have shown that SREBP-2 lead an increase in the sterol-dependent transcription of PCSK9 binding the sterol-regulatory elements (29).

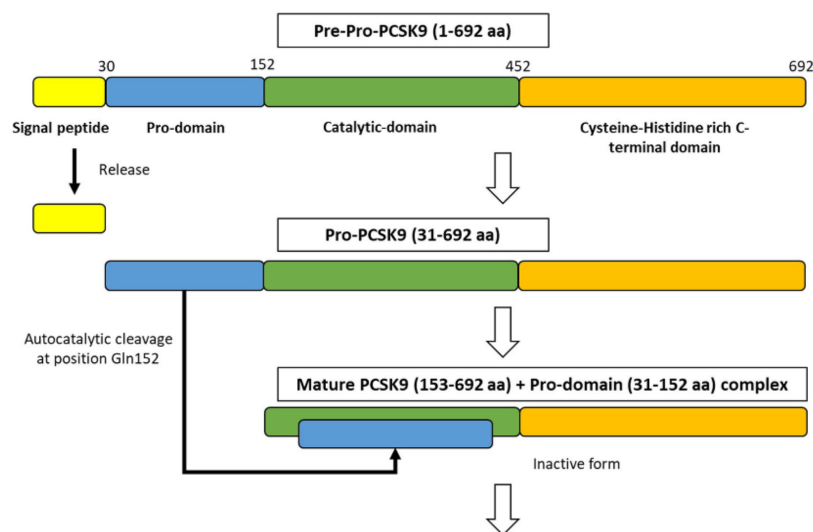


Fig.3 Protein structure of PCSK9 (Figure from Nishikido T. et al. Non-antibody Approaches to Proprotein Convertase Subtilisin Kexin 9 Inhibition: siRNA, Antisense Oligonucleotides, Adnectins, Vaccination, and New Attempts at Small-Molecule Inhibitors Based on New Discoveries. Front Cardiovasc Med. 2019 Jan 29; 5:199)

PCSK9 gene mutations

Based on their effect on protein function, PCSK9 mutations have been divided into two classes:

- Loss of function (LOF) mutations associated with hypocholesterolemia and significant protection against CAD
- Gain of function (GOF) mutations associated with hypercholesterolemia and higher risk of CAD

LOF mutations are responsible for an increase of 16-28% in the expression levels of LDLRs and a 35% increase in the internalization of LDL, while GOF mutations lead to a decrease of 23% in the expression levels of the LDLRs and a 38% decrease in the internalization of LDL (30).

In 2005 Cohen at al. identified two nonsense PCSK9 variants Y142X and C679X responsible for the formation of a premature stop codon and a consequent truncated no function protein. These nonsense mutations are associated with a reduction in plasma LDL-C levels by 40% (31). Another PCSK9 variant, R46L, is associated with a significant increase in the number of LDLRs on the cell surface and in the internalization of LDL compared to wild-type PCSK9 (32). Is also known that R46L mutation is able to protect against myocardial infarction. Besides the LOF mutations, mutations that led to gain of function were also identified. GOF mutations of PCSK9 cause an increase in the level of this protein and elevated LDL-C levels. The mutation D374Y has been shown to increase the interaction between PCSK9 and LDLR by 5-30 folds. PCSK9-D374Y mutant has a higher affinity for LDLR as compared with the wild-type PCSK9 and subject carrier of this mutation are prepared to develop premature CHD and respond less to statin treatment (33). Another GOF mutation, R218S, has been identified in a French family. This mutation lead to a significantly decrease of PCSK9 catabolism and an increase of cholesterol levels (34).

Transcriptional and post-transcriptional regulation of LDLR

Based on the amount of free cholesterol in ER membranes the levels of LDLR are subjected to a control at the transcriptional and post-transcriptional level.

When cholesterol levels are low, LDLR gene expression is activated by the transcription factor SREPB-2. SREBP-2 form a complex with SREBP Cleavage-Activating-Protein (SCAP) e Insulin-induced gene 1 (INSIG-1) that moves from ER to Golgi, where SREBP-2 undergoes a double proteolytic cleavage mediated by proteases. After that, SREBP-2 moves to the nucleus where binds LDLR promoter stimulating its transcription. SREBP-2 also stimulates the expression of genes involved in endogenous cholesterol synthesis such as hydroxy-3-methylglutaryl CoA reductase (HMGCoAR) which leads to increased endogenous synthesis of cholesterol and the expression of PCSK9 gene. PCSK9 induces the degradation of the LDLR, causing a lower availability of receptors on plasma membranes and a lower internalization of plasma LDL (35).

Conversely, when cholesterol levels increase, the transcriptional factor Liver X receptor (LXR) is activated while the activation of SREBP-2 is blocked. LXR is activated by the binding of oxidized cholesterol derivates (oxy-sterols) in high cell cholesterol condition.
LXR stimulates the expression of ATP binding Cassette Transporter 1 (ABCA1) and ATP binding Cassette Transporter G1 (ABCG1), membrane transporter genes involved in cholesterol efflux from the cell and the expression of Inducible Degrader of LDL receptor (IDOL) gene that encodes a homonymous protein responsible for LDLR degradation (36). ABCA1 and ABCG1 eliminate cholesterol from the cell while IDOL increases the degradation of the LDLR, reducing the capture of LDL avoiding a further increase in the level of intracellular cholesterol (Fig.4).

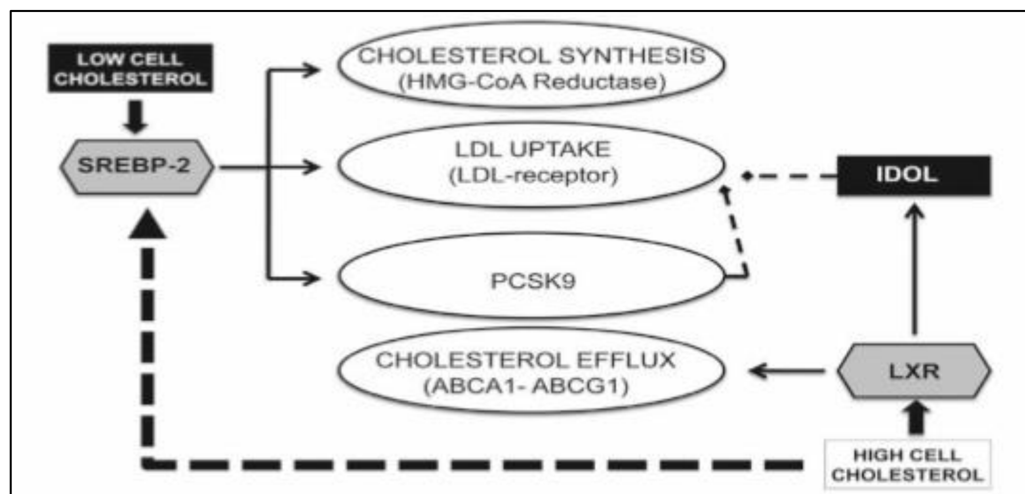


Fig.4 Transcriptional and post-transcriptional regulation of the LDLR activity (Figure from Giornale Italiano dell'Arteriosclerosi 2013; 4 (3): 7-18)

Both PCSK9 and IDOL are regulators that can facilitate the degradation of LDLR protein but the mechanisms used to achieve this target are distinct. PCSK9 binds to the extracellular portion of the LDLR and controls LDLR levels promoting its intracellular degradation, interfering with its recycling after endocytosis while IDOL binds to the cytoplasmic tail of the LDLR and catalyzes the binding of polyubiquitin chains to the LDLR.
In 2011, Scotti at al. demonstrate that in knock-out cell for IDOL, PCSK9 preserves the ability to degrade LDLR (37). Other studies, show that if LDLR missing the intracellular domain, PCSK9-mediated degradation occurs anyway. Therefore, these studies suggest that the IDOL and PCSK9 pathways for degrading the LDLR are distinct and independent

(38,39). In contrast, Sasaki et al. showed that overexpression of IDOL in mice, using an adenoviral vector Ad-IDOL induce a liver-specific reduction in LDLR expression and an increase in LDL-C and PCSK9 plasma levels. The overexpression of IDOL activates the PCSK9/LDLR promoter in a SREBP2-dependent way. Furthermore, hepatic cells of human, rat and hamster treated with statins exhibit an opposite effect on the expression of PCSK9 and IDOL, which modulate LDLR degradation in a similar manner (40).

Inducible Degrader of LDL receptor (IDOL)

The human IDOL gene is located on chromosome 6 (p23-p22.3) and was originally indicated as Myosin regulatory Light chain Interacting Protein (MYLIP) (41).

This gene encodes a protein of 455 amino acids that belong to a class of enzyme called E3 ubiquitin ligases. The ubiquitylation plays a significant role in cholesterol metabolism, cholesterol efflux, synthesis and uptake. IDOL presents two different protein domain: the FERM domain in N-terminal residue and the RING domain in C-terminal residue. The FERM and RING domains are connected by a small linker region. The FERM domain presents three subdomains F1, F2 and F3 that contains a phosphotyrosine-binding (PTB) site (fig.5).

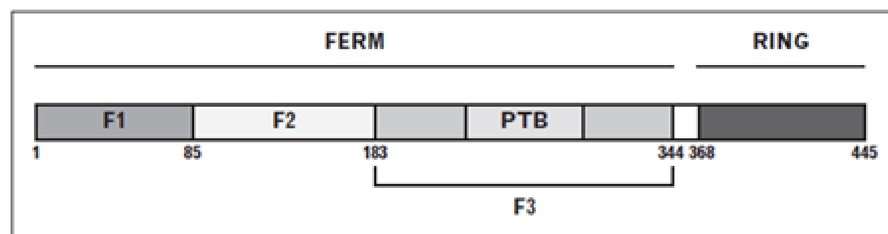


Fig.5 The IDOL protein structure (Figure from Sorrentino et al. Current opinion in lipidology. June 2012, p 213-219)

The FERM domain, through PTB, connects cytoplasmatic tail of LDLR to IDOL on the plasma membrane. The RING domain is responsible for E3 ubiquitin ligases function to bind polyubiquitin chains to conserved residues in the intracellular tail of the LDLR. The ubiquitin binding directs LDLR-ubiquitin complex to lysosomal degradation. IDOL allows the transfer of ubiquitin to LDLR acting in cooperation with the ubiquitin-conjugating enzyme 2 D (UBE2D). To help the lysosomal degradation of LDLR, IDOL also recruit ubiquitin specific peptidase 8 (USP8) and endosomal-sorting complex required for transport

(ESCRT) complexes (42,43). ESCRT complexes after the identification of ubiquitin tag directs the protein to luminal vesicles in the multivesicular body (MVB) protein-sorting pathway (Fig.6). IDOL also mediates its own degradation in the proteasome.

Several studies have demonstrated that the over-expression of IDOL in cultured cells is associated with greater ubiquitination and lysosomal degradation of LDLR. The mutation C387A on RING domain lead to inactivation of E3 ubiquitin ligase function of IDOL avoiding LDLR degradation (37,44). The mutation Arg266X lead to the formation of a premature stop codon and a consequent truncated protein avoid of function. *In vitro* experiments have demonstrated that this truncated protein is unable to interact with LDLR and induce its ubiquitination and subsequent degradation in lysosomes. IDOL LOF mutation, as well as the LOF variants of PCSK9, are associated with lower LDLR degradation, higher LDL capture and reduction in plasma LDL levels (45).

IDOL not only regulates LDLR but also interacts with the very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (APOER2) using the same mechanism to target LDLR which is the ubiquitination of a conserved lysine in the intracellular tail of these receptors (46).

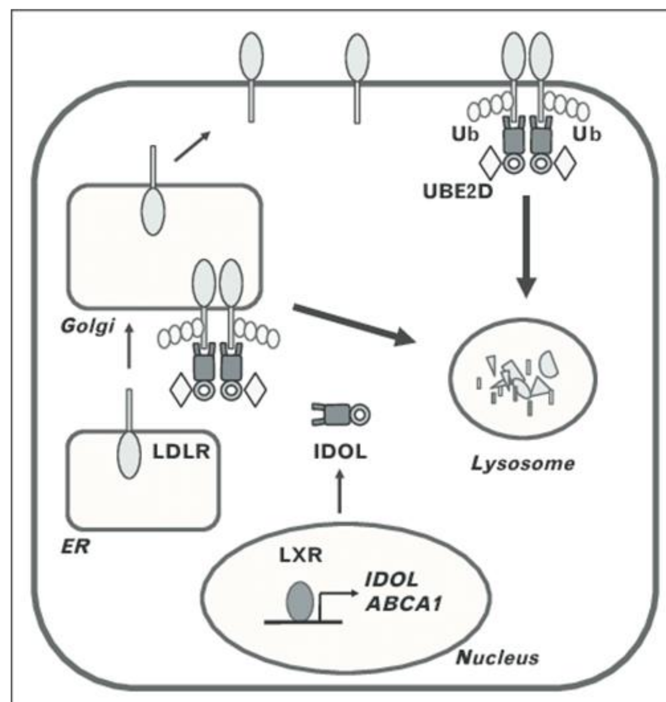


Fig.6 Molecular mechanism of IDOL protein (Figure from Sorrentino et al. Current opinion in lipidology. June 2012, p 213-219)

The microRNA regulation of LDLR activity

In addition to classical transcription factors, the post-transcriptional regulation of LDLR has been reported to be controlled by microRNAs (miRNAs), a small (~22 nucleotides) non-coding RNA molecules that bind to 3'UTR of complementary mRNA and repress gene expression through RNA interference (RNAi) pathway. The miRNAs are transcribed from intronic regions of genes encoding proteins or intergenic regions. A long primary miRNA transcript (pri-miRNA) is generated by the RNA Polymerase II enzyme, after that is processed by the Drosha/DGCG8 complex that generate a precursor miRNA transcript (pre-miRNA) of about 70 nucleotides. Pre-miRNA binds RNA-exporter proteins and is translocating to the cytoplasm where it is cleavage by DICER that generate a mature miRNA duplex of 20-23 nucleotides. MicroRNA strands are separated by Argonaute proteins and incorporated into the RNA-induced silencing complex (RISC). MicroRNA assembles into RISC bind to 3'UTR of complementary target sites obtaining a translational repression and/or a destabilization of mRNA (Fig.7) (47-50). If mRNA and miRNA have a perfect match this causes the degradation of the target mRNA, but if one or more bases are poorly paired occurs the repression of the translation. Therefore, miRNAs are able to regulate a large number of target mRNAs inducing both the down-regulation and the up-regulation in expression of specific genes.

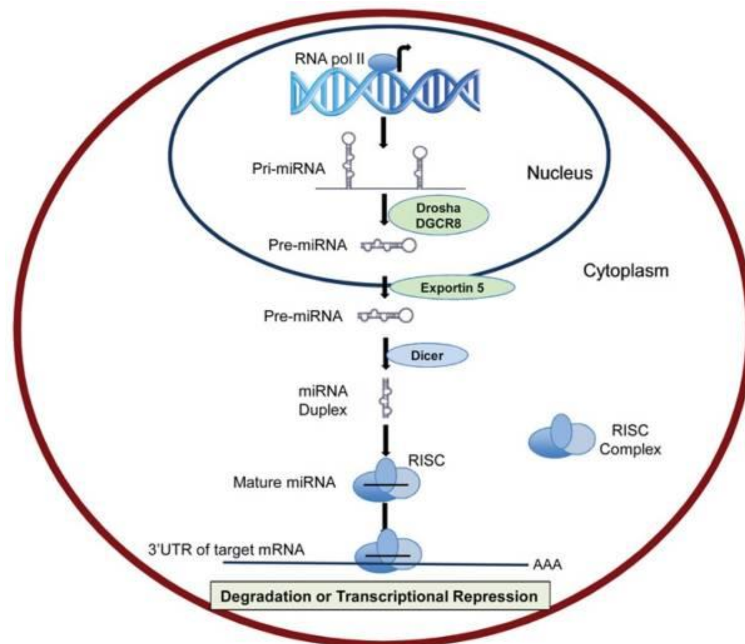


Fig.7 Schematic representation of miRNA biogenesis (Figure from Vickers KC et al. MicroRNAs in the onset and development of cardiovascular disease. Clin Sci (Lond). 2014 Feb;126(3):183-94)

In the last few years several miRNAs involved in the regulation of lipid metabolism were identified. In particular, miR-122, miR-27a and miR-148a has been linked to the regulation of cholesterol. Mir-122, an abundant liver-specific miRNA, was the first reported miRNA that modulate the cholesterol pathway and the lipid metabolism *in vivo*, regulating the expression of a large number of target mRNAs in the liver. Several studies demonstrate that the pharmacologic inhibition of miR-122 in mice and nonhuman primates and genetic knockout of miR-122 in mice lead to a significant decrease in plasma cholesterol levels. MiR-122 plays also an important role in the fatty acid synthesis (51,52).
Recently, Alvarez et al., found that miR-27a, a member of the miR-23a~27a~24-2 cluster mapping on chromosome 19 (19p13.13), is able to decrease the levels of the LDLR on liver cell surface and also targets other LDLR pathway members. The human LDLR 3' UTR has one predicted conserved miR-27a binding site.
Experiments in HepG2 cells have shown that the overexpression of miR-27a leads to a decrease in the levels of LDLR, LRP6, and LDLRAP1 important key players of LDLR pathway. LDLR activity decreased about 50% in HepG2 cells transfected with miR-27a compared to the control. Conversely, has been observed an increase in the level of PCSK9 mRNA in HepG2 cells compared to the control. However, miR-27a doesn't own a putative binding site in the 3'UTR of PCSK9, but have a potential binding site inside the promoter region of gene which may be responsible for the up-regulation of PCSK9 (Fig.8) (53).

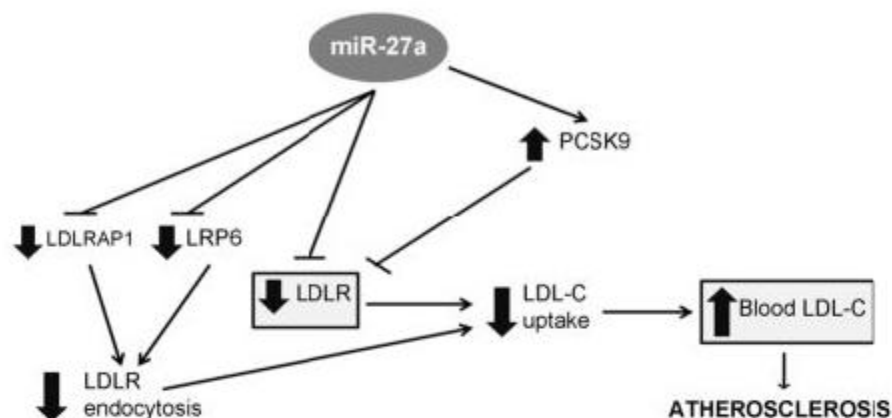


Fig.8 Mechanism of action of miR-27a (Figure from Alvarez ML et al. MicroRNA-27a decreases the level and efficiency of the LDL receptor and contributes to the dysregulation of cholesterol homeostasis. Atherosclerosis. 2015 Oct;242(2):595-604)

Shirasaki et al., shown that ABCA1, a lipid transporter and the transcription factors SREBP1 and SREBP2, responsible for the activation of LDLR and PCSK9 transcription, are also targets of miR-27a that directly control their expression in HuH7 cells experiments. Conversely, miR-27a is able to repress apolipoproteins ApoA1, ApoB100, and ApoE3 expression (54).

From genome-wide miRNA screening, miR-148a was identified as directly regulator of LDLR metabolism and it is transcriptionally activated by SREBP1c *in vitro* and *in vivo.*

The miR-148a is located within an intergenic region of chromosome 7, it is highly expressed in liver and is regulated by hepatic lipid content. This miRNA has two predicted binding sites in the 3' UTR of the LDLR. Goedeke at al. found that the overexpression of miR-148a in HuH7 cells significantly decreased LDLR at mRNA and protein levels (55).

MiR-148a has also a conserved predicted binding site in the 3' UTR of ABCA1. Several studies shown that the overexpression of miR-148a reduce ABCA1 expression demonstrating that this miRNA contribute to the post-transcriptional regulation of ABCA1 and cellular cholesterol efflux to ApoA1 (55).

Diagnosis of FH

The clinical diagnosis of FH is frequently performed by a diagnostic tool: the Dutch Lipid Clinic Network (DLCN) score and is based on several criteria: personal and family history of premature CAD, physical examination and lipid phenotype include high plasma levels of total and LDL-C (56). It is essential to know the family history of patient, make an observation of cardiovascular events in first-degree relatives, evaluate the age of onset and perform a physical examination, finding the physical signs typical of the different forms of dyslipidemia (tendon xanthomas, xanthelasmas, corneal arcus, etc.). The DLCN score is based on a points system that allows a "definite" diagnosis if the subject scores is > 8 points, a "probable" diagnosis if the subject scores is from 6 to 8 points, a "possible" diagnosis if the subject scores is from 3 to 5 points and finally an "unlikely" diagnosis can be made if the subject scores <3 points (Fig.9).

The diagnosis of FH is "definite" (DLCN >8) in the presence of LDL cholesterol over the 90th percentile, presence of vertical transmission in the family and tendon xanthomas, while the diagnosis is "possible" (DLCN 3-5) in the presence of high cholesterol and premature coronary event in the proband or in first degree relatives (57).

Family history	Score
1. First-degree relative with premature coronary heart disease	1
or	
2. First-degree relative with LDL cholesterol >95th percentile by age and gender for country	1
3. First-degree relative with xanthoma and/or arcus cornealis	2
or	
4. Children <18 years with LDL cholesterol >95th percentile by age and gender for country	2
Clinical history	
1. Premature coronary heart disease	2
2. Premature cerebral or peripheral vascular disease	1
Physical examination	
1. Tendon xanthoma	6
2. Arcus cornealis <45 years	4
LDL cholesterol	
1. >8.5 mmol/l	8
2. 6.5–8.4 mmol/l	5
3. 5.0–6.4 mmol/l	3
4. 4.0–4.9 mmol/l	1
DNA analysis	
1. Causative mutation in *LDLR*, *APOB* or *PCSK9*	8
Clinical diagnosis	
Definite	>8
Probable	6–8
Possible	3–5
Unlikely	<3

Fig.9 Dutch Lipid Clinic Network criteria for diagnosing familial hypercholesterolemia (Figure from Alonso R. et al. Familial Hypercholesterolaemia Diagnosis and Management. Eur Cardiol. 2018 Aug;13(1):14-20)

The genetic-molecular testing must be performed after an accurate clinical-anamnestic evaluation and gives information for family cascade screening. This genetic diagnosis is strongly recommended in subjects with a definite or probable diagnosis of FH (DLCN score >5) and particularly in presence of xanthoma and high cholesterol plus a family history of premature CHD. However, a large proportion of patients with a clinical diagnosis remain devoid of genetic-molecular diagnosis because a genetic mutation is not identified. These subjects may have a mutation in other unknown genes without the contribution of the classical genes involved in the pathogenesis of FH or a polygenic condition.

In addition to DCLN score, the UK Simon-Broome Register Group criteria and the USA MedPed (Make Early Diagnosis to Prevent Early Death) are also widely used.

The DCLN score and other criteria set are used in order to diagnose HeFH. Conversely, the diagnosis of HoFH can be done on the basis of genetic confirmation of biallelic pathogenic variants in one of these genes LDLR, APOB, PCKS9, LDLRAP1 or on clinical criteria. These criteria take into account the levels of untreated plasma LDL-C >13 mmol/L (>500 mg/dL), or levels of treated LDL-C ≥8 mmol/L (≥300 mg/dL) together with cutaneous or tendon xanthomas before 10 years old, or the presence of untreated elevated LDL-C levels in presence of both parents affected by HeFH (11).

In HoFH receptor-negative subjects cutaneous and tendon xanthomas occur earlier than in receptor-defective. In HoFH the first cardiovascular events often occur during adolescence especially in subjects receptor-negative who, if untreated, die in the first decade of life. While, HoFH patients who are receptor-defective develop clinically significant cardiovascular events and consequent accelerated atherosclerosis by age of 30 (58). HoFH patients should be treated as soon as possible after the diagnosis.

Therapeutic approach for FH patients

Cardiovascular disease (CVD) is the main cause of morbidity and mortality in the world. There is a close association between high levels of plasma LDL-C and the likelihood of developing CVD, such as coronary artery disease (CAD) and stroke (59). All individuals affected by FH are considered at "high risk" for developing CAD and should be treated promptly with a therapeutic approach aimed at decreasing LDL-C levels. Current pharmacological strategies for lower cholesterol levels include statins, ezetimibe, bile acid-binding resins, PCSK9 inhibitor, lipoprotein apheresis and liver transplantation. Furthermore, recently for the treatment of HoFH has been introduced novel potent LDL-C reducing agents such as lomitapide and mipomersen (60,61).

Statins

Statins, introduced in the 1980s, represent the most effective pharmacological treatment widely used for hypercholesterolemia and they act inhibiting HMG-CoA reductase (62). This enzyme is able to convert the 3-hydroxy-3-methylglutaryl-CoA molecule to mevalonic acid, a cholesterol precursor and its inhibition results in endogenous cholesterol synthesis block. The consequence of this reduced cholesterol synthesis is the translocation of the SREBP-2 protein from the ER to the Golgi and subsequently to the nucleus where it activates the transcription of different genes including HMG-CoA reductase and LDLR. The increased expression of LDLR leads to a greater number of LDL receptors on the plasma membrane of hepatocytes and a higher clearance of plasma LDL-C (Fig.10) (63). Statin therapy is powerful in patients with heterozygous FH but in homozygous patients carriers of mutation that completely abolish the LDLR activity is not very effective (64-66).
Several clinical trials have demonstrated the efficacy of statins in preventing cardiovascular events and in the reduction of mortality after CHD events (67). There are different statins approved for lowering cholesterol levels and the best reductions obtained is of approximately 60% using rosuvastatin 40mg. Furthermore, statins also decrease plasma triglyceride and VLDL levels. Statins not only act on lipid metabolism but also have anti-inflammatory, antioxidant, anti-proliferative and anti-thrombotic action (68,69).
These drugs are generally well tolerated from patients, but a small percentage could develop high hepatic transaminase levels, myopathy and gastrointestinal disorders (70). For a large

part of patients, the target levels of LDL are not reached using statins treatment alone, so it's possible to co-administered another cholesterol-lowering drug such as ezetimibe.

Ezetimibe

Ezetimibe belongs to a class of cholesterol absorption inhibitors that reduces the intestinal absorption of cholesterol coming from the diet and the bile without interfering the absorption of triglycerides, bile acids and vitamins A, D and E (71).
Ezetimibe is able to selectively inhibit the activity of the Niemann-Pick C1-Like1 (NPC1L1), a sterol transporter protein which is expressed in the brush border of intestinal epithelial cells and in the canalicular membrane of liver cells. The binding between ezetimibe and NPC1L1 leads to a reduction in cholesterol absorption that cause a decrease of the cholesterol supply of the liver and an increase of hepatic LDL receptors on hepatocyte cell surface, due to the activation of SREBP-2 (Fig.10).
Clinical studies have underlined that administering ezetimibe at 10 mg/d to patients with primary hypercholesterolemia, a 10-30% of LDL-C levels reduction is obtained after 12 weeks of therapy (72). Furthermore, it has been demonstrated that the co-administration of ezetimibe with statins such as simvastatin or atorvastatin, cause a more effective reduction of LDL-C levels by 70% (73,74). Ezetimibe does not appear to have significant side effects.

Bile acid-binding resins

Bile acid-binding resins are polymeric compounds with anionic exchange that have some positive charges used for the exchange with other anions (such as the chloride ion) of bile acids, sequestering them from the enterohepatic circulation. These resins bind bile acids in the intestine and increase their fecal excretion. The amount of bile acids returning to the liver is reduced and this forces the liver to produce more bile acids to replace those excreted (75).
Because the reuptake process of bile acids is blocked, the liver converts cholesterol into bile acids. The synthesis of bile acids decreases hepatic cholesterol levels, activates SREBP-2 and increases the number of LDL receptors on cell surface causing a consequent decrease in circulating plasma LDL-C (Fig.10). Furthermore, there is an up-regulation of HMG-CoA reductase for a compensatory increase in cholesterol synthesis.
Bile acid sequestrant is particularly effective in patients with heterozygous FH. They are generally used in co-administration with statins resulting in a 60% reduction of LDL

cholesterol levels when high doses of both drugs are used (76). Unlike ezetimibe, bile acid resins reduce the absorption of vitamin A, D, E, and K. They cause gastrointestinal synthoms such as abdominal bloating and discomfort but have no effect on liver or muscle.

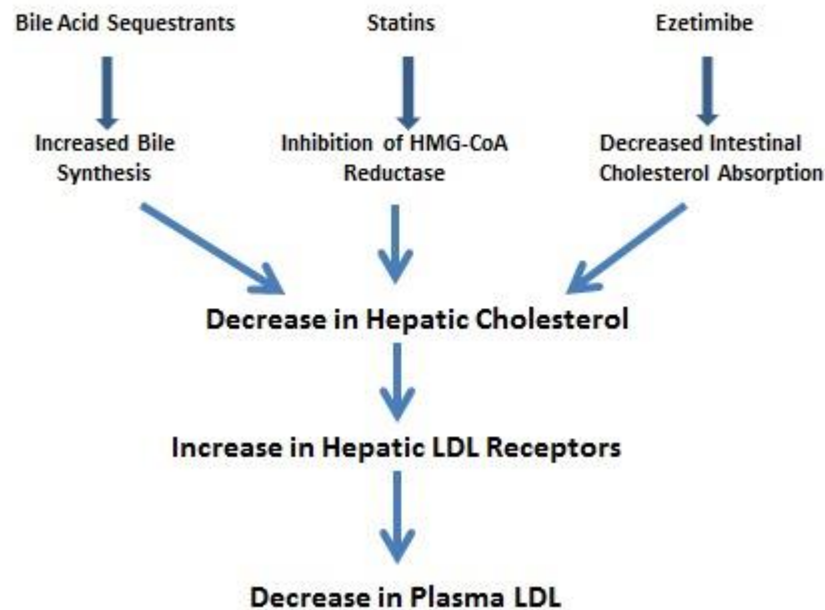


Fig.10 Mechanism for lower cholesterol levels (Figure from Kenneth R Feingold and Carl Grunfeld, Cholesterol Lowering Drugs, Endotext 2018)

PCSK9 inhibitors

PCSK9 inhibitors as well as statins, ezetimibe and bile acid-binding resins are able to regulate LDLR levels but they act by decreasing the degradation of LDLR, while statins, ezetimibe and bile acid-binding resins work by stimulating the production of LDLR.
PCSK9 inhibitors are human monoclonal antibodies (mAbs) that bind to PCSK9 inhibiting the formation of a complex between PCSK9 and LDLR and thus preventing the degradation of LDLR. Because the degradation of LDLR is reduced, there is an increase in receptors number on the plasma membrane of hepatocytes which leads to a greater clearance of LDL and therefore plasma LDL-C levels are reduced (77,78).
Cholesterol-lowering drugs lead to the activation of SREBP-2 that stimulate the expression of PCSK9 increasing its plasma levels. Therefore, the treatment with PCSK9 monoclonal antibodies is more effective in patients under statin therapy because these subjects have a higher levels of plasma PCSK9.

Clinical trials have showed that the effect of PCSK9 inhibitors is related to the residual activity of the LDLR. HoFH patient receptor defective have a decrease in plasma LDL-C levels by 26% while patients carrying homozygous mutations that almost completely abolished receptor function showed a negative response to anti-PCSK9 treatment (79,80). Conversely, in heterozygous patients, PCSK9 inhibitors lead to a reduction of plasma LDL-C levels by 65% in co-administration of other lipid-lowering therapy (81).
The major adverse effect of PCSK9 monoclonal antibodies has been reactions in the injection site including erythema, swelling, pain, and tenderness.

Lipoprotein apheresis

Lipoprotein apheresis is an extracorporeal method for remove the apoB-containing lipoproteins from plasma or whole blood using different methods such as plasmapheresis, immunoadsorption, direct adsorption, dextran sulphate adsorption, and heparin extracorporeal LDL apheresis. Plasmapheresis was the initial method used to remove LDL from serum but was unselective method because removed also plasma protein including HDL and immunoglobulins. Dextran sulphate adsorption is the most efficient method to remove atherogenic particles from circulation and it is specific to apolipoprotein-B containing lipoprotein (82).
Apheresis process can lower LDL-C plasma levels by 55-70% after a single treatment but after 24-48 hours the cholesterol level rises again and reaches similar values to those pre-apheresis after 2 weeks. Apheresis may cause different side effects such as hypotension, abdominal pain, nausea, hypocalcaemia, iron-deficiency anaemia, and allergic reactions.
This therapeutic approach is proposed in addition to statin therapy for patients with homozygous FH or patients who are resistant to the pharmacological treatment or intolerant to statins and is normally performed every 2 weeks (83). Long-term apheresis is also able to block the progression of atherosclerotic lesions and in some cases induce the regression or the stabilization of atherosclerotic plaques.

Liver transplantation

In patients with homozygous FH, that cannot reach optimal LDL levels after drugs treatments and for those do not tolerate lipoprotein apheresis, the only definitive strategy to

restore a normal liver cholesterol metabolism is the liver transplantation because it corrects the molecular defect in the organ involved in the clearance of LDL (84). This transplant, often performed in combination with a heart transplant, ensures a reduction in plasma LDL-C of 80% that induce a rapid regression of cutaneous and tendinous xanthomas. The major disadvantages about liver transplant are represented by surgical complications, rejection and the need for life-long treatment with immunosuppressive therapy (85).

Therapeutic approach for HoFH patients

In HoFH patients, high doses of statins (simvastatin, atorvastatin or rosuvastatin) in combination with ezetimibe are able to reduce LDL-C by 22-25% (65,72). Recent clinical trials have demonstrated that the inhibition of PCSK9 by monoclonal antibodies may be useful in HoFH subjects to lower LDL-C but the effectiveness is related to the residual LDLR function. In fact, HoFH carriers of mutations with a residual activity of LDLR have a decrease in plasma LDL-C levels by 26% while patients carrying mutations that almost completely abolished receptor function showed a low response to anti-PCSK9 treatment. The mechanism responsible for the reduction of LDL-C can be explained by the up-regulation of residual LDLR function, as demonstrated by the absence of response in those patients with no LDLR activity (79). These lipid lowering treatments are often insufficient to achieve target LDL-C levels and this requires more aggressive treatment such as lipoprotein apheresis and even liver transplant.
Recently, two new drugs named lomitapide (approved by FDA and EMa) and mipomersen (approved by FDA) are available for the treatment of homozygous familial hypercholesterolaemia.

Lomitapide

Lomitapide is an inhibitor of microsomal triglyceride transfer protein (MTP) that is located in the ER of hepatocytes and enterocytes and acts by transferring triglycerides and phospholipids to ApoB for VLDL and chylomicrons assembly. The inhibition of MTP reduces the secretion of VLDL in the liver and chylomicrons in the intestine leading to a decrease in LDL, apolipoprotein B and triglycerides (86).
Data from a clinical trial on HoFH patients shown that lomitapide treatment in combination with statins and LDL apheresis lead to a reduction of plasma LDL-C and apoB levels by 40-

50%. After 26 weeks LDL-c level was reduced by 50% and by 44% at 56 weeks. At 78 weeks patients achieved a 38% of reduction and some patients have reduced or eliminated apheresis (87). Lomitapide causes gastrointestinal problems such as diarrhoea, nausea, vomiting and hepatic steatosis and reduces the absorption of fat-soluble vitamins and essential fatty acids. Furthermore, patients may develop fatty liver with elevated transaminase because the mechanism of lomitapide action that block the formation of VLDL in the liver.

Mipomersen

Mipomersen is an apolipoprotein B antisense oligonucleotide that was approved for the treatment of patients with Homozygous FH. The mechanism of action of this drug is to targets the apolipoprotein B mRNA and reduce its translation (60). In particular, mipomersen binds to apoB-100 mRNA and creates a double-stranded RNA complex that is cleaved by RNase H, preventing formation of apoB-100. This decrease in apo B protein synthesis results in a reduction of hepatic VLDL secretion leading to a decrease in LDL-C levels (Fig.11).

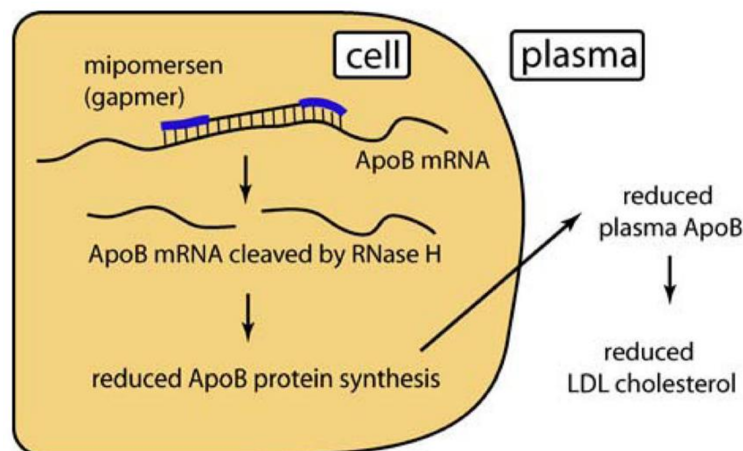


Fig.11 Mechanism of action of Mipomersen (Figure from Watts, J. K. & Corey, D. R. Gene silencing by siRNAs and antisense oligonucleotides in the laboratory and the clinic. J. Pathol. 226, 365–79)

In a clinical trial carried out on HoFH patients, mipomersen in addition to standard therapy is able to reduce LDL-C plasma levels by 25%, apoB by 27%, and Lp(a) by 31% at 26 weeks. Adverse events in 76% of patients are injection site reactions such as erythema, pruritus and

local swelling and flu-like symptoms, which usually appear two days after drugs administration (88). However, the most worrying side effect when using mipomersen is related to liver toxicity. Have been reported elevations in transaminase levels in 15% of patients and presence of fatty liver in 5-20% of patients under treatment with mipomersen but these adverse events disappear when treatment is suspended (89). Because the utilize of both mipomersen and lomitapide can lead to a high risk of developing liver disease, these therapies must be reserved for a limited number of patients if the standard therapies to lower LDL cholesterol are not enough.

Therapeutic approach based on oligonucleotides

The therapeutic approach based on oligonucleotides is already used to treat different diseases such as cancers, infections and cardiovascular diseases and is based on the introduction into the target cells of a synthetic oligonucleotide to suppress the expression of a specific mRNA causing a gene silencing. Therapeutic oligonucleotides can be classified in two classes.
The first class is represented by Short Interfering RNAs (siRNAs), a double stranded structure that have a filament complementary to target miRNA and another filament useful to include siRNA into RISC complex. The second category is represented by synthetic single-stranded antisense oligonucleotides (ASOs) complementary to endogenous miRNA. Regardless of the mechanism of action the result is a protein synthesis inhibition (90).

Short Interfering RNAs (siRNAs)

SiRNAs are double-stranded RNA usually 19–22 bp long that trigger the RNAi process just like miRNAs. These oligonucleotides are formed by a guide strand (antisense strand) that is complementary to the target RNA sequence and a passenger strand (sense strand) that is complementary to the guide strand. The duplex was recognized by RISC complex that removed the passenger strand of the duplex and the guide strand leads the RISC complex to the target RNA (91). The mRNA was cleaved by Argonaute if the duplex siRNA is complimentary to the target mRNA strand while translation is repressed if the siRNA contains mismatches compared to the target mRNA (Fig.12). The siRNA-based therapeutic approaches are based on the release of a synthetic siRNA into target cells to suppress the expression of a specific mRNA and obtain gene silencing.

Single-stranded antisense oligonucleotides (ASOs)

ASOs are short single-stranded sequence of DNA or RNA (12-25 bp) that can be used to activate or inhibit the expression of a target gene through Watson-Crick base pairing.
In 1978 Zamecnik and Stephenson showed, for the first time, that inhibition of viral replication in Rous sarcoma virus was achieved by using a specific complementary antisense oligonucleotide (92,93). The use of ASOs as a therapy has allowed to create of new drugs for the treatment of various diseases. The therapeutic approach is to introduce into the cells a single-stranded antisense oligonucleotide that is complementary to its target mRNA, within the nucleus or cytoplasm. An ASO can inhibit gene expression using two different mechanisms: a mechanism RNase H-dependent and a mechanism of steric blocking (Fig.12) (89).

RNase H-dependent ASOs

RNase H is an endonuclease that is able to cleave the RNA strand of an RNA/DNA hybrid duplex. It is an enzyme present in the nucleus and in the cytoplasm of eukaryotic cells.
The ASO most used are those called "Gapmers" because they have a central DNA gap, flanked by locked nucleic acid (LNA). LNA are a class of nucleic analogues in which the ribose ring is "locked" by a methylene bridge between the 2'-O atom and the 4'-C atoms. Locking the molecule with the methylene bridge lead to an ideal conformation for Watson-Crick binding. As a result, LNA have a high affinity to complementary nucleic acid, a superior binding power and a higher resistance to endonucleases and exonucleases when used for antisense inhibition. LNA oligonucleotides are a mix of LNA nucleosides and DNA or RNA nucleotides. Antisense LNA gapmers are single-stranded antisense oligonucleotides (16 nucleotides long) which catalyze RNase H-dependent degradation of complementary RNA targets. When the gapmer binds RNA target, the central DNA gap activates RNase H cleavage of the opposing RNA strand (90-94).
To increase the enzymatic stability of gapmers many chemical modifications were developed such as the substitution of sulfur atom with oxygen atom in the phosphate backbone turning to phosphorothioate (PS) backbone that confer higher resistance to nuclease degradation.

Steric blocker ASOs

Unlike the previous one, these type of ASOs have a different way of action which consist in the blocking of ribosome from translating the target mRNA into protein and not include the degradation of the RNA (94). These oligonucleotides bind the RNA target with high affinity and prevent the interaction with protein or nucleic acids. Steric blocker oligonucleotides are also able to modify splicing, inhibit miRNAs and regulate long non-coding RNA.
Some oligonucleotides can be construct to target splice sites or exon sequence enhancer in order to modulate splicing and form alternatively splice variants of the mature mRNA.

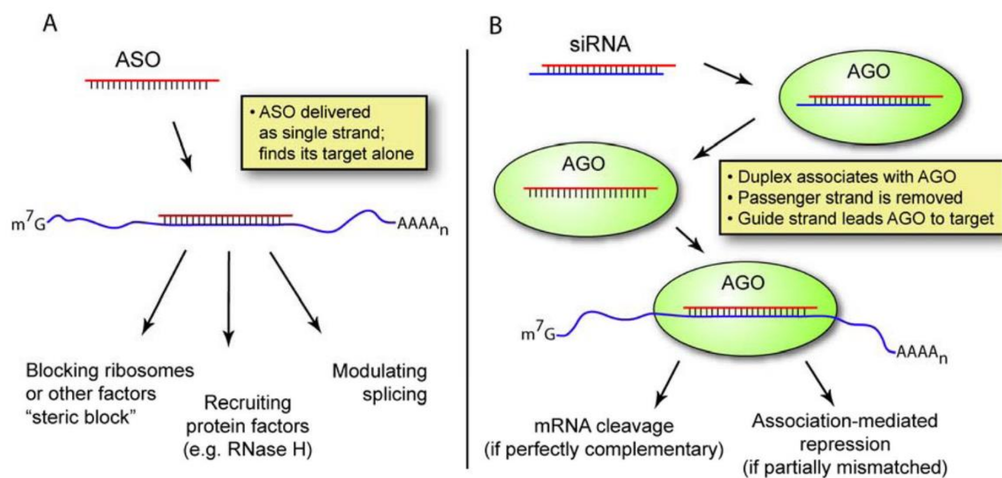


Fig.12 Comparison of the ASO and siRNA mechanisms. (A) ASOs mechanism. (B) siRNAs mechanism. (Figure from Watts, J. K. & Corey, D. R. Gene silencing by siRNAs and antisense oligonucleotides in the laboratory and the clinic. J. Pathol. 226, 365–79).

AntagomiRs (anti-miRs)

The mechanism of RNAi and the ASO technologies can also be used to suppress the action of endogenous miRNAs by using synthetic single-stranded RNAs with a sequence complementary to the miRNA termed as AntagomiRs. The antagomiRs are a novel class of chemically engineered miR antagonists, which are specific silencers of endogenous miRNAs. They are complementary to their mature target miRNA and inactivate their

function. These miRs antagonist strongly sequester specific endogenous miRNA competing with cellular target mRNAs. This complex between anti-miR-miRNA can lead to a wrong configuration of the miRNA which does not allow the incorporation into RISC complex or lead to the degradation of endogenous miRNA.

Several chemical modifications have been developed to improve specificity, stability and delivery of anti-miRs and to increase the binding affinity to a specific miRNA.

The first antagomiR containing 2′-O-methyl modified RNA bases and phosphorothioate modifications was engineered by Krützfeldt et al. in 2005 to target miR-122. The anti-miR formed a duplex with endogenous miR-122 causing the degradation of the targeted miRNA and a consequent reduction of 40% in plasma cholesterol levels compared to controls (95). Similar observations were reported by Esau et al in 2006, performing experiments in mice, have shown that the inhibition of miR-122 through a 2′-O-methoxyethyl-phosphorothioate-modified ASO led to a decreasing in circulating cholesterol by 25–35%, a reducing hepatic lipid synthesis and an increasing hepatic fatty acids oxidation (51).

OBJECTIVES

The treatments for FH patients can reduce LDL-C level and the lifetime risk of coronary heart disease (CHD) with a long-term drug therapy. Statins represent the pharmacological therapy more effective to reduce selectively LDL-C levels in heterozygous patients because statins increase the expression of LDL receptors on cell surface by reducing HMG-CoA reductase, the rate-limiting step in cholesterol synthesis. In homozygous patients (HoFH) the efficacy of such treatments is very low because HoFH are resistant or poor responders to standard lipid-lowering treatments including statins and required other procedures more invasive such as lipoprotein apheresis.

Recent clinical trials have demonstrated that PCSK9 monoclonal antibody (mAbs) may be useful to lower LDL-C in HoFH carriers of mutations with a residual activity of LDLR (77,78) while lomitapide is effective also in carriers of mutations that completely abolish the LDLR activity but it is associated with potentially severe intestinal and hepatic side effects (87). The effect of PCSK9 mAbs is related to the up-regulation of the residual LDLR activity leading to LDL-C levels reduction. Based on these considerations, a therapeutic approach for the treatment of HoFH carrying mutations that preserve a residual LDLR activity might be directed to restore the lacking LDLR activity by the inactivation of its physiological degradation pathways and the inhibition of the physiological pathways which down-regulate LDLR gene expression.

The objective of my work was to provide a novel strategy *in vitro* to rescue LDLR activity and to lower plasma LDL-C levels applicable in HoFH patients with a residual LDLR activity > 2%. This strategy is based on the inactivation of PCSK9 and IDOL by using an antisense-specific RNA to reduce LDLR degradation and the inhibition of repression of LDLR expression by using specific anti-miRs in order to stop mRNA degradation with an increase of LDLR on cell surface. The inactivation of PCSK9 and/or IDOL and the inhibition of LDLR repression may act synergistically with statins to increase the number of LDLR, reduce plasma cholesterol levels and improve the severity of the clinical phenotype.

METHODS

HuH7 Cell cultures

HuH7 (human hepatoma cells) were cultivated in 25 cm^2 flasks in Dulbecco's modified Eagle's medium (D-MEM-glutamax, Gibco) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (MM NEAA), penicillin and streptomycin 100 UI/ml. Cells were cultured at 37°C with 5% CO2.

LNA antisense oligonucleotides design

LNA antisense oligonucleotides (LNA ASO) complementary to the human PCSK9 and IDOL mRNAs were designed using the online tool available at www.exiqon.com/gapmer. Exiqon LNA GapmeRs are 16 nucleotides long enriched with LNA (Locked Nucleic Acid) in the flanking regions and DNA in an LNA free central gap. The LNA containing flanking regions confer nuclease resistance to the antisense oligo while, at the same time, increases target binding affinity regardless of the GC content. The central DNA "gap" activates RNase H cleavage of the target RNA upon binding.

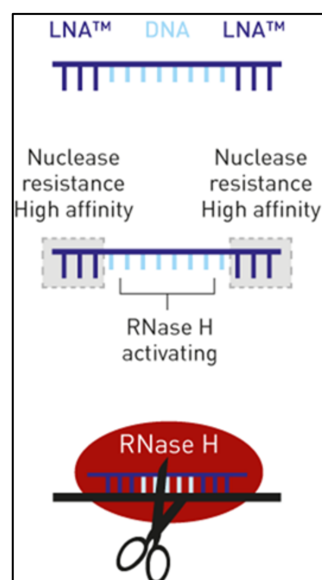


Fig.13 LNA Gapmers and heteroduplex RNA/DNA degradation (www.exiqon.com)

LNA are a class of high-affinity RNA analogues in which the ribose ring is locked in the ideal conformation for Watson-Crick binding. For this reason, LNA oligonucleotides have a greater thermal stability when hybridized to a complementary DNA or RNA strand. For each incorporated LNA monomer, the melting temperature (Tm) of the duplex increases by 2-8ºC.

It was submitted a specific RNA target on Exiqon and the online tool using an empirically derived design algorithm has designed different LNA GapmeRs for PCSK9 and IDOL. For each RNA target the tool evaluates thousands of possible LNA GapmeR designs against >30 design parameters and identify LNA GapmeRs most likely to give potent and specific target knockdown. The LNAs with the highest score have a GC content between 30 and 60% and are described so as to avoid self-complementarity between the neighboring LNA strands. LNA GapmeRs were purified with HPLC (High Performance Liquid Chromatography) ion exchange (Na +) because they contain phosphorothioate modifications which cause impurities.

Sequences of ASO used in this study:

LNA GapmeRs anti-PCSK9 5'→3'	LNA GapmeRs anti-IDOL 5'→3'
GCATAGAGCAGAGTAA	**TTAACATCGCATGGCA**

Before use, the lyophilized oligonucleotide was resuspended in nuclease-free water at a concentration of 50µM and aliquoted according to the manufacturer's instructions.

Anti-miRs selection

Two principal miRNA databases are available on line: miRBase (microRNA database, release 21) and TargetscanHuman (prediction of microRNA targets, release 7.0). MiRBase is a database of published miRNA sequences and annotation; each entry in the miRBase sequence database represents a predicted hairpin portion of a miRNA transcript (termed mir in the database), with information on the location and sequence of the mature miRNA sequence (termed miR). TargetScan predicts biological targets of miRNAs by searching for the presence of 8mer, 7mer, and 6mer sites that match the seed region (positions 2-7 of a mature miRNA) of each miRNA. After an accurate screening of miRNA involved in lipid

metabolism and with a documented effect on LDLR, hsa-miR-27a-3p and hsa-miR-148a-3p miRCURY LNA miRNA Power Inhibitor (Qiagen) were selected.
The miRCURY LNA miRNA Power Inhibitors are antisense oligonucleotides with perfect sequence complementary to their targets. When introduced into cells, they sequester the target miRNA in highly stable heteroduplexes, effectively preventing the miRNA from hybridizing with its normal cellular interaction partners. Power inhibitors, as well as LNA Gapmers, have a phosphorothioate modified backbone, which makes them highly resistant to enzymatic degradation.

Sequences of miRCURY LNA used in this study:

miRCURY LNA anti-hsa-mir-27a-3p 5'→3'	miRCURY LNA anti-hsa-mir-148a-3p 5'→3'
CGGAACTTAGCCACTGTGA	**CAAAGTTCTGTAGTGCACT**

Before use, the lyophilized oligonucleotide was resuspended in nuclease-free water at a concentration of 50µM and aliquoted according to the manufacturer's instructions.

HuH7 transfection with LNA Gapmers

HuH7 cells were seeded one day before transfection at a density of 3 x10^5 cells per well in 6-well plates. At 60–70% confluence, cells were transfected with specifics Antisense LNA GapmeRs (Exiqon) against PCSK9 and IDOL, using Lipofectamine 3000 or Lipofectamine RNAiMAX Reagent (Invitrogen), according to manufacturer instruction at different concentrations (10nM, 25nM, 50nM, 75nM and 100nM). The lipofectamine method is based on cationic lipid structure consists of a phospholipid bilayer, with a positively charged head group and one or two hydrocarbon tails. The polar head creates electrostatic interactions with the phosphate backbone of the nucleic acid. The positive surface charge of the liposomes allows their endocytosis through the negatively charged cell membrane. Lipofectamine RNAiMAX is a RNAi-specific cationic lipid formulation designed specifically for the delivery of siRNA and miRNA into all cell types.
In control HuH7 cells were added water without oligonucleotide. After transfection, cells were incubated for 48 hours at 37°C, 5% CO2 in D-MEM complete medium. After incubation, cells were washed twice with phosphate-buffered saline (PBS) and they were

treated in order to extract protein and RNA. All experiments were performed in biological duplicates.

Reverse transfection with LNA Gapmers

In reverse transfections, the LNA ASO-lipid complexes are prepared inside the wells, after which cells and medium are added. 7.5 $x10^5$ HuH7 cells were seeded per well in a 6-well plate containing Lipofectamine RNAiMAX (Invitrogen) supplemented with specifics Antisense LNA GapmeRs (Exiqon) against IDOL or water for control cells, according to manufacturer instruction at different concentrations (50nM and 75nM). After incubation of 48 h, cells were washed twice with phosphate-buffered saline (PBS) and they were treated in order to extract protein and RNA. All experiments were performed in biological duplicates.

HuH7 transfection of anti-miR-27a and anti-miR-148a inhibitors

HuH7 cells were seeded one day before transfection at a density of 3 $x10^5$ cells per well in 6-well plates. At 60–70% confluence, cells were transfected with hsa-miR-27a-3p and hsa-miR-148a-3p miRCURY LNA miRNA Power Inhibitor (Qiagen) using Lipofectamine RNAiMAX Reagent (Invitrogen), according to manufacturer instruction at different concentrations (25nM and 50nM). In control HuH7 cells were added water without anti-miR. Cells were incubated for 48 hours at 37°C, 5% CO2 in D-MEM complete medium. After incubation, cells were washed twice with phosphate-buffered saline (PBS) and they were treated in order to extract protein and RNA. All experiments were performed in biological duplicates.

Total RNA isolation from cell line

HuH7 cells were washed two times with PBS and incubated with TRIzol reagent (Invitrogen) for 5 min in order to extract total RNA. TRIzol Reagent is a monophasic solution of phenol, guanidine isothiocyanate and other proprietary components. It is added directly to culture well to disrupting cells and dissolving cell components during sample homogenization

maintaining the integrity of the RNA due to highly effective inhibition of RNase activity. After that, chloroform is added for 10 min to eliminate membranes, proteins and DNA. Samples are centrifuged at 12000 x g for 15 min. at 4 °C.

The mixture separates into a lower red phenol-chloroform, and interphase, and a colourless upper aqueous phase. The aqueous phase containing the RNA, the interphase containing the DNA and the lower phase containing protein. The aqueous phase containing the RNA is transferred to a new tube without disturbing the other phases and isopropanol is added for 10 min to precipitate RNA. Samples are centrifuged at 12000 x g for 10 min. at 4°C.

Total RNA precipitate forms a white gel-like pellet at the bottom of the tube and it is washed with 75% ethanol. After another centrifugation at 7500 x g for 5 min a 4°C, RNA is resuspended in 20-50 µl of RNase-DNase free water and quantified by Qubit RNA Assay Kits (Life Technologies).

Reverse transcription (RT)

1 µg of total RNA per reaction was used for cDNA synthesis using High Capacity cDNA Reverse Transcription Kits (ThermoFisher scientifics). The Reverse Trascriptase is an RNA-dependent DNA polymerase, which allows to obtain a cDNA molecule from an RNA molecule, used as a mold. An RT master mix containing 10X RT Buffer, 25X dNTP Mix (100 mM), 10X RT Random Primers, Multiscribe Reverse Transcriptase and Nuclease-free water is prepared in a final volume of 10 µl on ice. Total RNA is added to master mix pipetting up and down two times to mix. The reaction is incubated according to the following thermal profile:

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Real-Time PCR (qPCR)

Real-Time PCR was performed using TaqMan Universal Master Mix (Thermofisher scientific) in a Viia 7 DX instrument (Life Technologies). For each sample, was prepared a PCR reaction mix of cDNA, TaqMan Universal Master Mix 2X, TaqMan Assay 20X and nuclease-free water in a final volume of 20 µl. The qPCR was performed as follows: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min x 40 cycles.

For quantification of PCSK9, IDOL and LDLR mRNAs, TaqMan Gene Expression Assays (Thermofisher scientific) were used. TaqMan Gene Expression Assay consist of a pair of unlabeled PCR primers and a TaqMan probe with a FAM or VIC dye label on the 5'end and minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3'end (Fig.14). The minor groove binder acts as a probe Tm enhancer. Quantification was performed using the ΔΔCt method and the housekeeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used for normalization. The ΔΔCt method is used to calculate fold changes of target gene expression in a target sample relative to a reference sample, normalized to a reference gene. The threshold cycle (*Ct*) is the cycle at which the fluorescence level reaches a certain amount (the threshold). This method directly uses the *Ct* information to calculate relative gene expression in target and reference samples, using a reference gene as the normalizer. First, ΔCt (Ct value for the target minus Ct value for the reference gene) was calculated for each sample. The ΔΔCt is the difference in ΔCt between the target and reference samples. Data were analyzed using Applied Biosystems Viia7 Software.

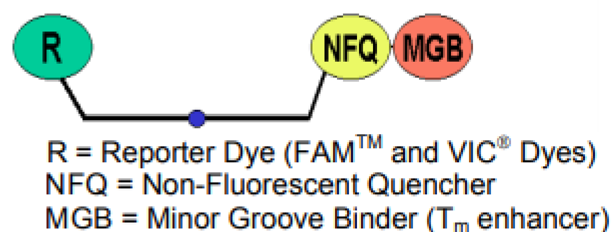


Fig.14 TaqMan gene expression probe structure

RESULTS AND DISCUSSION

Several studies have demonstrated the correlation of loss-of-function (LOF) mutations of PCSK9 with low cholesterol levels and a consequent marked reduction in the risk of developing coronary heart disease (CHD) (32). Experiments on PCSK9 knock-out mice have shown a decrease in the levels of circulating LDL-C due to an elevated concentration on cell surface of hepatic LDLRs (96). Genome-Wide Association Studies (GWAS) identify an association between genetic variation in the IDOL locus and circulating levels of LDL-C. IDOL LOF mutation, as well as the LOF variants of PCSK9, are associated with lower LDLR degradation, higher LDL capture and reduction in plasma LDL levels (45). These evidences suggested that the inhibition of these two regulators of LDLR degradation may be a useful tool to lower plasma LDL-C levels and to develop a novel therapeutic approach to reduce PCSK9/IDOL-mediated LDLR degradation and restore the residual activity of LDLR in HoFH receptor-defective subjects.
To prove this hypothesis, different LNA antisense oligonucleotides (ASO) against IDOL and PCSK9 were tested at different concentration in order to obtain the maximum effect on LDLR with the minimal concentration of antisense.
The first set of experiments was performed on HuH7 cells using Lipofectamine 3000 at three different concentration of IDOL antisense: 10nM, 25nM e 50nM, to test the effect of LNA ASO on mRNA levels of IDOL. Cells were harvested for 48 h and total RNA was extracted. Real-Time PCR (qPCR) was performed to assess the decrease in IDOL mRNA expression. In mock control cells, water instead of LNA ASO was added. Each experiment was performed in duplicate and data were normalized to GAPDH.

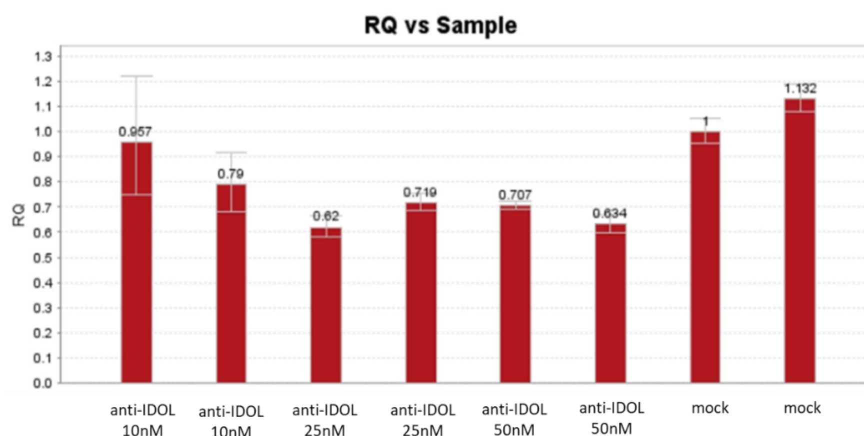


Fig.15 Effect of LNA anti-IDOL on the expression of IDOL mRNA using Lipofectamine 3000

As shown in Figure 15, in presence of 10 nM of LNA antisense has been observed a decrease of 12,5% in IDOL mRNA expression while at higher concentration of 25nM and 50nM a decrease of 33% compared to the control cell (mock).

To optimize the transfection conditions and increase the knockdown of the RNA transcript of interest, the same experiments were repeated using another lipofectamine: Lipofectamine RNAiMAX. Lipofectamine RNAiMAX is a RNAi-specific cationic lipid formulation designed specifically for the delivery of siRNA and miRNA into all cell types. With Lipofectamine RNAiMAX Transfection Reagent it is possible to get a superior transfection efficiency requiring lower RNAi concentrations, leading to more effective gene knockdown with minimal non-specific effects. IDOL antisense was tested in a dose-response set up using three oligonucleotide concentrations: 10nM, 25nM and 50 nM. Data were normalized to GAPDH.

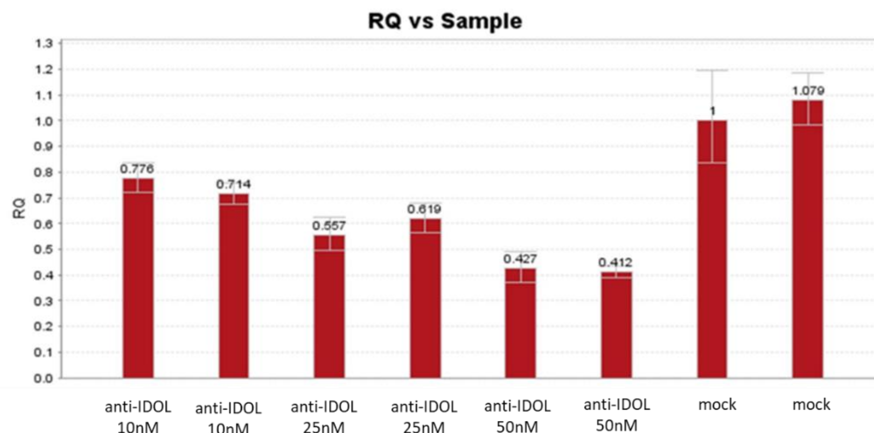


Fig.16 Effect of LNA anti-IDOL on the expression of IDOL mRNA using Lipofectamine RNAiMAX

The chart shows the reduction rate of IDOL mRNA expression. In presence of 10nM of LNA antisense has been observed a decrease of 26% while at higher concentration of 25nM and 50nM a decrease of 41% and 58% respectively.

Thereafter, a reverse transfection was performed using Lipofectamine RNAiMAX. In reverse transfections, the complexes are prepared inside the wells, after which cells and medium are added. Reverse transfections are faster to perform than forward transfections and are the method of choice for high-throughput transfection. Because the cells and ASO

complexes are prepared on the same day, it's recommended using 2,5x more cells that for a regular transfection method. IDOL antisense was tested in a dose-response set up using two oligonucleotide concentrations: 50 nM and 75nM.

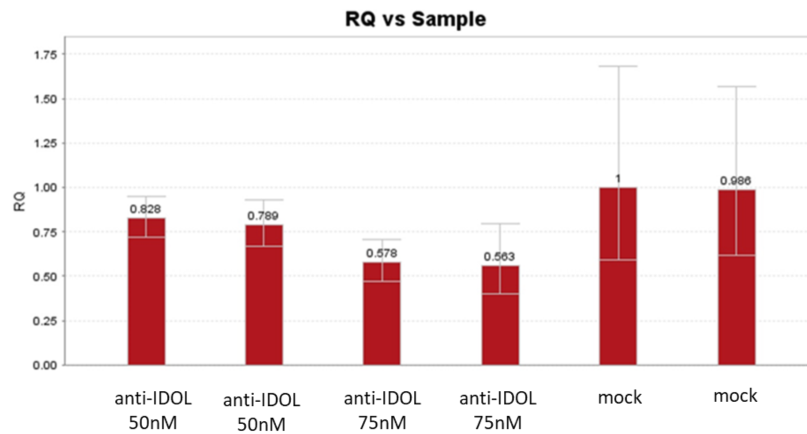
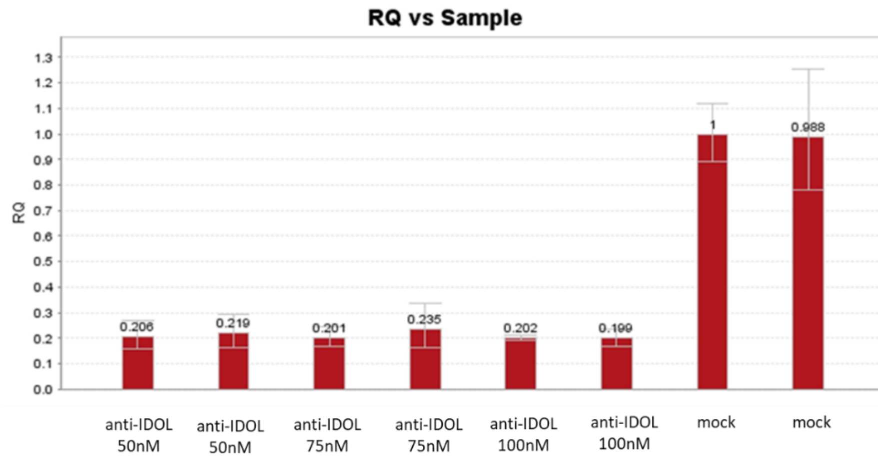


Fig.17 Effect of LNA anti-IDOL on the expression of IDOL mRNA performing reverse transfection

As shown in Figure 17, in presence of 50 nM of LNA antisense has been observed a decrease of 19% while at concentration of 75nM a reduction of 43% compared to the control (mock). As a result, IDOL antisense can be delivered into HuH7 cells more efficiently by Lipofectamine RNAiMAX compared with the other transfection reagent and highly efficient knockdown of target gene was obtained even at a low concentration of antisense. Lipofectamine RNAiMAX seems to be the best methods for these experiments performing the forward transfection.

Finally, IDOL antisense was tested at higher concentration: 75nm and 100nM to verify if the plateau was reached or if can get a greater silencing. In presence of 50nM, 75nM and 100 nM of LNA antisense has been observed a decrease of about 70-80%, then seems that the silencing has the same efficacy (Fig.18A). Has been also measured the effects of IDOL ASO on the levels of PCSK9 transcript which showed a decrease about 30% in the mRNA expression of PCSK9 (Fig.18B). The reduction of PCSK9 expression could be due to the presence of lipids and sterols contained in the lipofectamine reagent that led to a down-regulation of SREBP activity, as previously described (97).

A



B

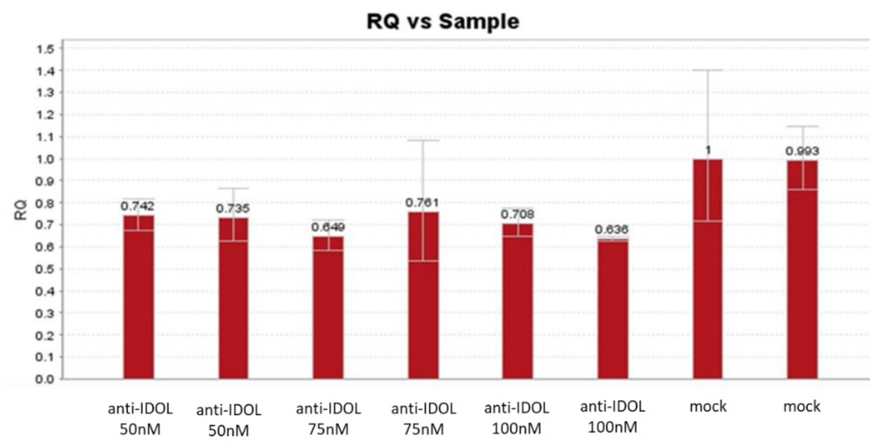


Fig.18 Effect of LNA anti-IDOL on the expression of IDOL and PCSK9 mRNA. (A) mRNA expression of IDOL, (B) mRNA expression of PCSK9.

For PCSK9, were performed the same experiments made for IDOL. The Figure 19 shows the reduction rate of PCSK9 mRNA expression. In the experiment using Lipofectamine 3000 at 3 different concentration: 10nM, 25nM e 50nM has been observed that in presence of 10 nM of LNA antisense there is no a decrease, while at higher concentration of 25nM and 50nM a reduction of 5% and 16% respectively. Each experiment was performed in duplicate and as control, mock transfections were done with water instead of LNA ASO. Data were normalized to GAPDH.

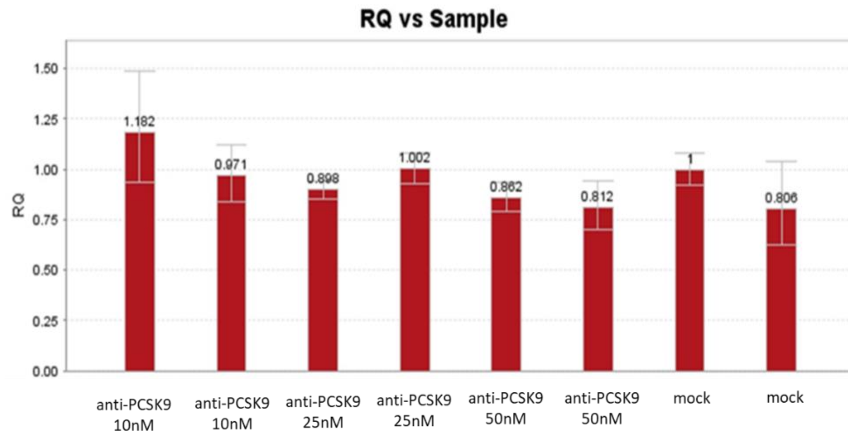
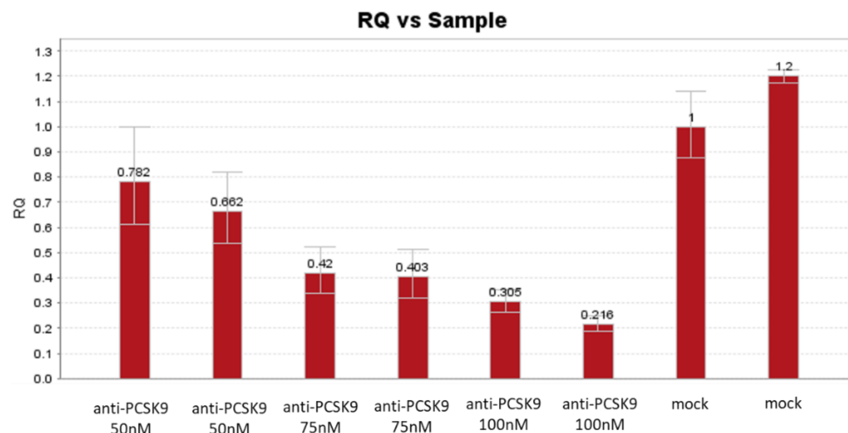


Fig.19 Effect of LNA anti-PCSK9 on the expression of PCSK9 mRNA using Lipofectamine 3000

The experiments performed using Lipofectamine RNAiMAX have led to a greater increase in the knockdown of the PCSK9 mRNA as well as for IDOL. In presence of 50 nM of LNA antisense has been observed a decrease of 28%, while at higher concentration of 75nM and 100nM a decrease of 59% and 74% respectively (Fig.20A).

Has been also measured the effects of PCSK9 ASO on the levels of IDOL transcript which showed an increase of 28% in mRNA expression in presence of 50nM, of 39% at concentration of 75nM and 88% at concentration of 100nM (Fig.20B). It can be assumed that the silencing of PCSK9 results in an increase of hepatic LDLR expression and LDL uptake with a consequent rise in cellular cholesterol content in the liver. High levels of intracellular cholesterol lead to LXR activation which in turn increase IDOL transcription and secretion.

A



B

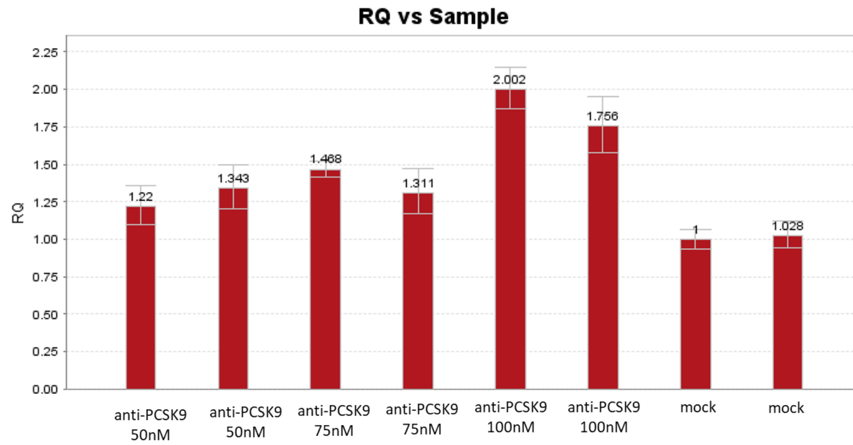
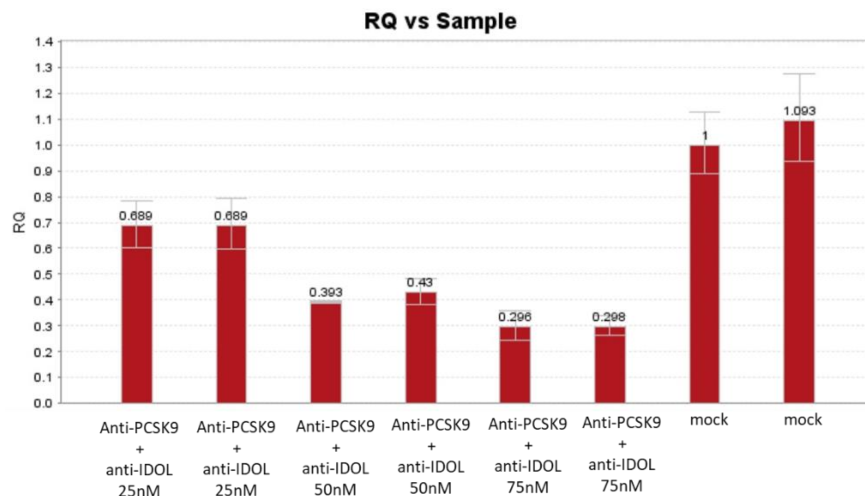


Fig.20 Effect of LNA anti-PCSK9 on the expression of PCSK9 and IDOL mRNA. (A) mRNA expression of PCSK9, (B) mRNA expression of IDOL.

Based on these results, another experiment was performed to test the combined effects of PCSK9 and IDOL antisense inactivation. HuH7 cells were transfected using Lipofectamine RNAiMAX at 3 different concentration of PCSK9 antisense: 25nM, 50nM and 75nM. Cells were incubated for 1 hour in transfected medium; after that, IDOL antisense was added and cells were incubated, harvested for 48 h and total RNA was extracted. Real-Time PCR (qPCR) was then performed to assess the decrease in PCSK9 and IDOL mRNA expression. Each experiment was performed in duplicate and as control, mock transfections were done with water instead of LNA ASO. Data were normalized to GAPDH.

A



B

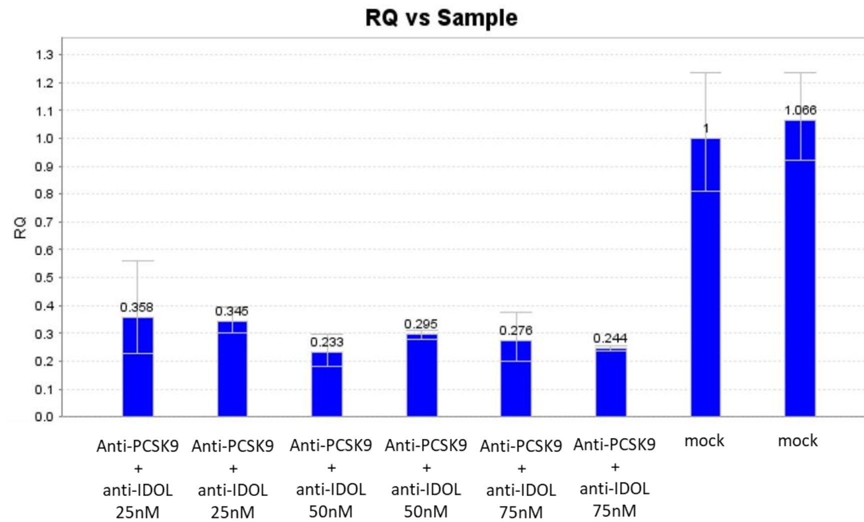


Fig.21 Effect of LNA anti-PCSK9 and LNA anti-IDOL in combination using RNAiMAX. (A) mRNA expression of PCSK9, (B) mRNA expression of IDOL

As shown in Figure 21A, has been observed a greater reduction in PCSK9 mRNA expression level when co-transfected with IDOL LNA ASO than when transfected alone. In fact, it possible to observe a decrease of 31% at 25nM, 59% at 50nM e 70% at 75nM of LNA ASO transfection. Conversely, for IDOL mRNA expression seems that the transfection in combination with PCSK9 LNA ASO does not significantly modify the reduction rate.
Figure 21B show a decrease of 65% in presence of 25 nM of LNA antisense while at higher concentration that are 75 and 100nM of LNA antisense a decrease of 74%.

The LDLR activity is not only regulated by PCSK9 and IDOL but also by microRNAs acting at post-transcriptional level. The post-transcriptional regulation of LDLR has been reported to be controlled by the 3'untranslated region (3'UTR)-mediated modulation of mRNA stability. Therefore, different microRNAs involved in cholesterol metabolism and in particular in the regulation of LDLR and other key players of LDLR pathway have been selected to target. Experimental evidence has shown that mir-27a and mir-148a have several predicted binding sites in the 3' UTR of the LDLR and are able to modulate its gene expression. To increase LDLR mRNA level by preventing its degradation has been planned to use specific antagomirs against mir-27a and mir-148a by inhibiting the binding of these miRNA to LDLR mRNA. The miRCURY LNA anti-hsa-mir-27a-3p and the miRCURY LNA anti-hsa-mir-148a-3p were tested in HuH7 cells using Lipofectamine RNAiMAX at two different concentration: 25nM and 50nM in order to identified the optimum balance

between dose of antagomir and the effect on LDLR expression. Cells were harvested for 48 h and total RNA was extracted. Real-Time PCR (qPCR) was performed to assess the reduction rate of LDLR mRNA expression. In mock control cells, water instead of miRCURY LNA was added. Each experiment was performed in duplicate and data were normalized to GAPDH.

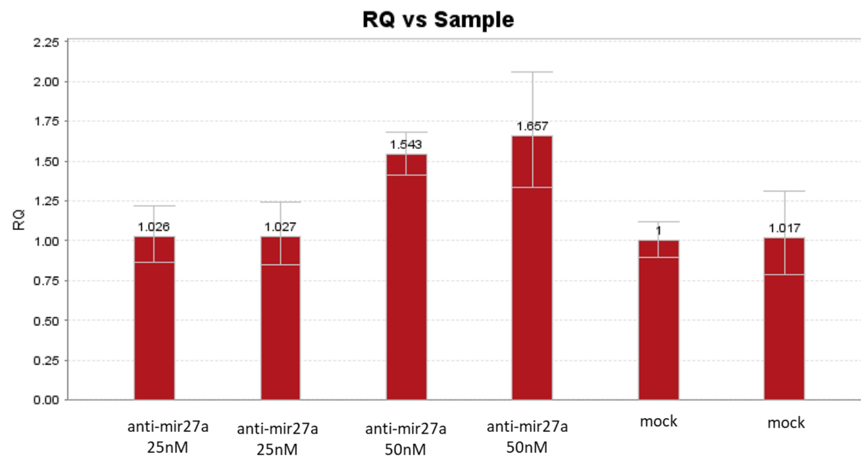


Fig.22 Effect of miRCURY LNA anti-hsa-mir-27a-3p on the expression of LDLR mRNA

As shown in Figure 22, in presence of 25nM of miRCURY LNA anti-hsa-mir-27a-3p there is no an increase in LDLR mRNA levels but at the highest concentration of 50nM has been observed an increase of 60% compared to the control cell (mock) (Fig.22).

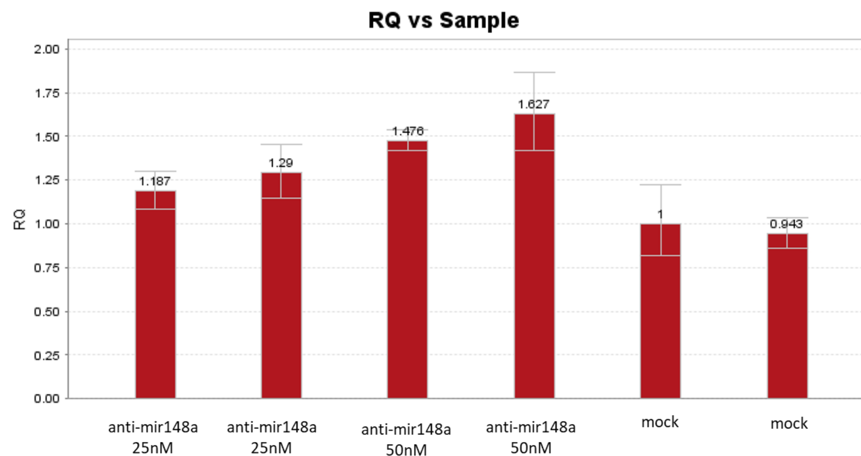


Fig.23 Effect of miRCURY LNA anti-hsa-mir-148a-3p on the expression of LDLR mRNA

Has been found that also the inhibition of miR-148a using miRCURY LNA anti-hsa-mir-148a-3p induced an increase in LDLR mRNA levels. In presence of 25nM of LNA has been observed an increase of 24% while at the highest concentration of 50nM an increase of 55% (Fig.23).

CONCLUSION

In the present study, have been designed and used different LNA antisense oligonucleotide (ASO) in order to lower PCSK9 and IDOL gene expression, and thus inhibit the physiological pathways of LDLR degradation. Different concentrations of PCSK9 and IDOL ASOs alone or in combination, were tested *in vitro* (HuH7 cells) in order to get the maximum effect on mRNA expression with the minimal concentration of antisense. Have been compared different transfection methods (forward and reverse transfection) and different transfection reagents (Lipofectamine 3000 and Lipofectamine RNAiMAX) in order to find the best methods for these experiments.
From these comparisons, has been concluded that our LNA ASO oligonucleotides against PCSK9 and IDOL can be delivered into HuH7 cells more efficiently by Lipofectamine RNAiMAX even at a lower concentration of antisense compared with Lipofectamine 3000 performing the forward transfection. A decrease of 80% in mRNA expression level of IDOL and a decrease of 74% in mRNA expression level of PCSK9 was observed at the maximum concentration of antisense. Also, has been found that when PCSK9 and IDOL ASO were used in combination the reduction rate of PCSK9 mRNA was higher.

Subsequently, further experiments using specific antagomirs against mir-27a and mir-148a were performed to increase LDLR mRNA level by preventing its degradation. In fact, the activity of LDLR is not only regulated by PCSK9 and IDOL but also by microRNAs, involved in cholesterol metabolism, acting at post-transcriptional level. Has been observed that miR-27a and miR-148a inhibition using specific miRCURY LNA ASOs in HuH7 cells induced an increase about 50-60% in the levels of LDLR mRNA at the highest concentration of anti-miR.

These results demonstrate the feasibility and efficacy of using LNA ASO technology to down-regulate mRNA and miRNA targets of interest. *In vitro* experiments presented in this study support the use of LNA ASO for the development of new therapeutic treatments to increase the number of LDLR on cell surface, reduce LDL-C levels and ameliorate the severity of the clinical phenotype of HoFH patients.

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