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Scuola di Dottorato in Scienze Farmacologiche

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XXVI Ciclo

Macrophage expression of PCSK9 influences atherosclerosis development

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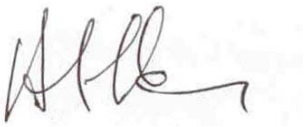

A.A.
2013

To my family

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Part I
Introduction

1. Overview on lipid metabolism

Cholesterol is a fundamental component of animal cell membranes that contributes to the modulation of their fluidity and permeability. In addition, it is the precursor of all steroid hormones, bile acids and vitamin D.

Almost all cells synthesize cholesterol, but the main organ involved in its production is the liver. Cholesterol synthesis occurs in both cytoplasm and endoplasmic reticulum (ER) from the precursor acetyl CoA through the mevalonate pathway. The rate limiting enzyme in cholesterol synthesis is HMG-CoA reductase that converts HMG-CoA to mevalonic acid. After synthesis, cholesterol leaves the ER and is targeted mainly to the plasma membrane but it may also access other sites in the cell, such as endosomes (Baumann, Sullivan et al. 2005). Free cholesterol accumulation is dangerous for cells (Fazio, Major et al. 2001), so it is converted to cholesterol esters by acyl-CoA: acyltransferase (ACAT) and stored in cytoplasmic lipid droplets (Chang, Chang et al. 1997). Cholesterol esters can be hydrolyzed by another enzyme, named cholesterol ester hydrolase, and provide a source of cholesterol to be transferred to other intracellular compartments.

Cholesterol homeostasis is based on a balance between dietary intake and endogenous biosynthesis. Dietary cholesterol is taken up by specific transporters on the enterocyte surface, such Niemann-Pick C1 like 1 (NPC1L1) (Altmann, Davis et al. 2004) and esterified by ACAT2 (Temel, Gebre et al. 2003). Then chylomicrons are assembled from triglycerides, cholesterol, cholesterol esters and apoB48 and secreted into the circulation. Triglycerides are delivered to peripheral tissues to provide fatty acids for energy or fat storage and remnant chylomicrons are internalized by the liver. In the liver, the excess of cholesterol and fatty acids are combined to apoB100 to

originate very low density lipoproteins (VLDL), that reach the circulation (Rustaeus, Lindberg et al. 1999) and deliver triglycerides to peripheral tissues, becoming more enriched in cholesterol (intermediate density lipoproteins, IDL). IDL can be either taken up by the liver or transform into low density lipoproteins (LDL), the major transporters of cholesterol in the circulation.

LDL binds to LDL receptor (LDLR) and is internalized by clathrin-dependent endocytosis. After internalization, the receptor and its ligand detach in the endosomes, due to the lower pH and LDLR is recycled to the cell surface, while cholesterol is hydrolyzed by acid lipase (Brown and Goldstein 1986). To provide some numbers, the process of internalization and recycle takes place every 10 minutes and the lifespan of LDLR is about 20 hours (Brown, Anderson et al. 1983). Considering that one particle of LDL transports about 1600 molecules of cholesterol, this is a clear demonstration that the process of cholesterol delivery is characterized by a very high efficiency.

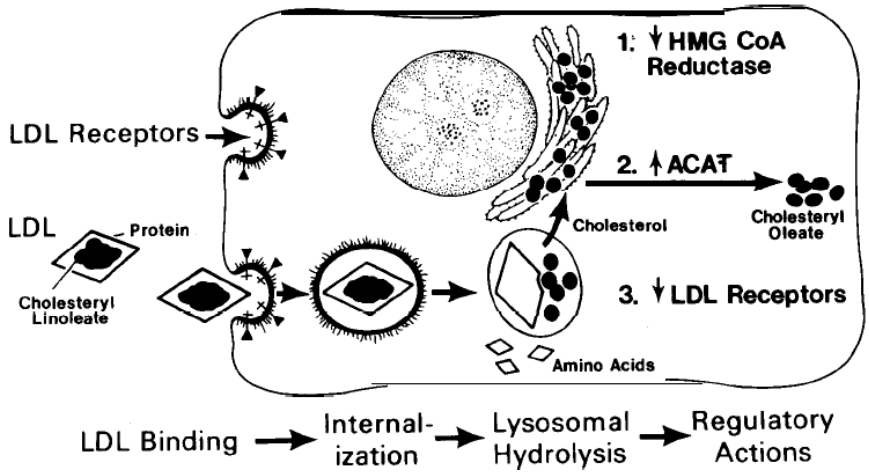


Figure 1. Sequential steps in the LDLR pathway. *from Brown M.S. et al. PNAS, 1979;76:3330-3337.*

Cholesterol homeostasis is maintained by a feedback regulatory system on the basis of cholesterol content in the membrane (Goldstein and Brown 1990; Brown and Goldstein 1997). Cholesterol synthesis is transcriptionally regulated by sterol response element binding proteins (SREBPs), three proteins of about 1150 amino acids, anchored to the membrane of the ER. When cellular cholesterol is low, SREBPs are relocated from the ER to the Golgi apparatus, where a first protease, called site-1-protease (S1P), cleaves in the region of their luminal loop. A second enzyme, called site-2-protease (S2P), cleaves at the level of the first transmembrane domain releasing N-terminal region that reaches the nucleus and activates the target genes. In the liver, SREBP1c regulates the pathway of lipogenesis, whereas SREBP2 activates genes of cholesterol metabolism (Brown and Goldstein 1997). Statins, the most widely used lipid lowering agents available on the market, bind and inhibit HMG-CoA reductase and reduce cholesterol production. As a direct consequence, cholesterol depletion activates SREBP2, thereby increasing the number of LDLR present on the cell surface. Also HMG-CoA is a target of SREBP2, but since the enzyme is also the target of statins, there is no effect in terms of increased cholesterol synthesis (Goldstein and Brown 2009).

Despite SREBPs, other transcription factors, such as liver X receptor (LXR), are known to contribute to cholesterol homeostasis. In fact, when intracellular cholesterol concentration is high, LXR is activated and regulates the transcription of genes involved in reverse cholesterol transport, such as ATP binding cassette A1 (ABCA1) and ABCG1 (Tontonoz and Mangelsdorf 2003), that mediate cholesterol efflux to apoA-I and HDL, respectively.

Recently, another protagonist involved in LDLR regulation, pro-protein convertase subtilisin/kexin type 9 (PCSK9) has emerged as a valid new target for reduction of LDL cholesterol and consequently cardiovascular risk.

2. Pro-protein convertase subtilisin/kexin type 9 (PCSK9)

PCSK9 has been recently identified as the third gene involved in autosomal dominant hypercholesterolemia (ADH) (Abifadel, Varret et al. 2003).

PCSK9 is a protein that belongs to a superfamily of subtilisin-like serine proteases and is the ninth mammalian member identified in the pro-protein convertase family. Seven members of the family (PC1/PC3, PC2, furin, PC4, PACE4, PC5/PC6, PC7/LPC) cleave single or paired basic amino acids with the motif $(R/K)X_n(R/K)\downarrow$, where X_n corresponds to a 0-, 2-, 4-, or 6-amino acid spacer (Seidah and Chretien 1999). PCSK9 is an atypical member and cleaves non-basic residues at the C-terminal end of the motif $RX(L/V/I)X\downarrow$, where X represents any amino acid except cysteine and proline (Seidah, Benjannet et al. 2003).

It was first cloned and sequenced during biopharmaceutical screenings and called neural apoptosis-regulated convertase 1 (NARC1) because the mRNA was upregulated after induction of apoptosis by serum deprivation in primary cerebellar neurons (Seidah, Benjannet et al. 2003).

In humans, the gene is about 22-kb long and comprises 12 exons. It is located on chromosome 1p32 (figure 2).

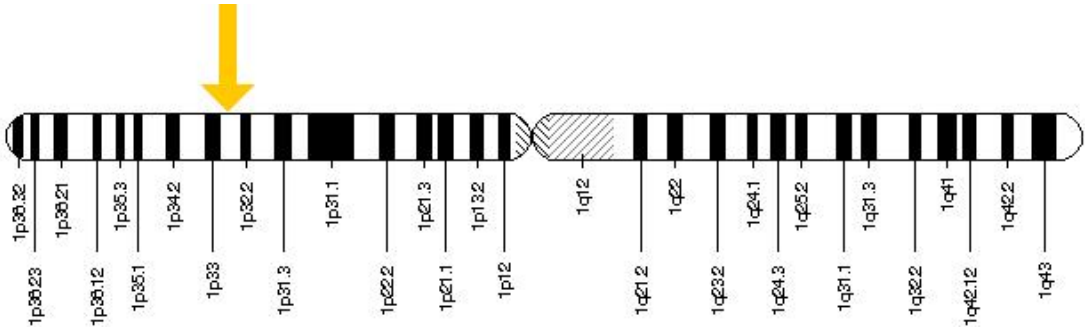


Figure 2. Location of PCSK9 gene. *from http://ghr.nlm.nih.gov/gene/PCSK9.*

Protein sequences homologous to full-length human PCSK9 were identified in many vertebrates, including primates, rats, mice, squirrels and other placental mammals, such as opossums, chickens, frogs, some fish species and lancelets. Interestingly, the Bos Taurus genome was found to contain a region on chromosome 3 with homology to PCSK9 that might be a remnant of a PCSK9 pseudo-gene, but no bovine PCSK9 homologues appear to be functional, though. The simplest explanation can be that cows, with their diet based on grass, might be thriving without PCSK9, as do some human individuals without functional PCSK9 (Cameron, Holla et al. 2008).

2.1 Synthesis, mechanism of action and metabolism

PCSK9 is synthesized primarily by the liver, intestine and kidney as 75 kDa precursor composed of 692 amino acids (Seidah, Benjannet et al. 2003). It contains a signal sequence (1–30 amino acids), a pro-domain (amino acids 31–152), a catalytic domain (amino acids 153–425) and a C-terminal domain (amino acids 426–694) (Benjannet, Rhainds et al. 2004). Pro-PCSK9 undergoes autocatalytic cleavage between residues 152 and 153 in the ER, to originate the mature form of PCSK9 of about 62 kDa (Benjannet, Rhainds et al. 2004). The pro-domain remains tightly associated to the mature protein, forming a PCSK9-pro-domain complex that reaches the Golgi, where undergoes

sulfation and is then secreted (Seidah, Benjannet et al. 2003; Benjannet, Rhainds et al. 2004). The presence of the pro-domain is strictly required for its secretion from the cell and prevents any substrate access the catalytic site (Cunningham, Danley et al. 2007). In fact no other substrates except for PCSK9 itself have been since now identified.

Despite some limitations in the methods used for measuring PCSK9 levels, it has been found a positive correlation (r= 0.15–0.58) between PCSK9 and LDL cholesterol levels in the circulation (Lambert, Ancellin et al. 2008; Lakoski, Lagace et al. 2009). More importantly, statin treatment completely disrupts this correlation, reducing the possibility of using PCSK9 as a marker of altered lipid metabolism and risk of coronary diseases (Welder, Zineh et al. 2010).

Circulating PCSK9 is present in different molecular forms: the mature 62 kDa form, the furin-cleaved 55 kDa form and higher molecular weight forms, mainly dimers and trimers. It was recently demonstrated by different groups that around 40% of PCSK9 is associated with LDL in plasma (Kosenko, Golder et al. 2013; Tavori, Fan et al. 2013) and that the 62 kDa form is only present on LDL and directly interacts with apoB (Tavori, Fan et al. 2013). The rest of PCSK9 is mainly found in the apoB-deficient fraction in dimeric and trimeric forms (Tavori, Fan et al. 2013). Moreover, the LDL-bound 62 kDa form is less active than the higher molecular weight forms, suggesting a direct influence of LDL on PCSK9 activity in the circulation (Fan, Yancey et al. 2008).

Once released in the circulation, it acts modulating the expression of LDLR in different tissues, including liver, intestine, kidney, lung, pancreatic islets and adipose tissue (Lalanne, Lambert et al. 2005; Zaid, Roubtsova et al. 2008; Poirier, Mayer et al. 2009; Tavori, Fan et al. 2013). The ability of PCSK9

to promote LDLR degradation is not related to its catalytic activity, suggesting a role for PCSK9 as a chaperone, involved in the targeting of LDLR toward intracellular degradation (McNutt, Lagace et al. 2007). Although the adrenals express very high levels of LDLR, they are not sensitive to the effects of circulating PCSK9 (Tavori, Fan et al. 2013). Other authors showed that PCSK9 is secreted by vascular smooth muscle cells (SMCs) and enhances the degradation of LDLR in macrophages, thus suggesting a possible role for PCSK9 in modulating LDLR levels in atherosclerotic plaques (Ferri, Tibolla et al. 2011).

PCSK9 binds to the extracellular domain of the LDLR located in the first epidermal growth factor-like repeat homology domain (EGF-A) (Zhang, Lagace et al. 2007). The binding is calcium-dependent and occurs at neutral pH (Fisher, Lo Surdo et al. 2007; Lo Surdo, Bottomley et al. 2011). At neutral pH, such as at the plasma membrane, only the catalytic domain of PCSK9 interacts with the EGF-A domain (Kwon, Lagace et al. 2008; Lo Surdo, Bottomley et al. 2011). In contrast, at acidic pH, such as in the endosomes, the affinity of PCSK9 for the receptor is much higher and the pro-domain of PCSK9 forms salt bridges with the β-propeller domain of the receptor; moreover, the positively charged C-terminal domain of PCSK9 binds to the negatively charged ligand-binding domain of LDLR (Tveten, Holla et al.; Lo Surdo, Bottomley et al. 2011). This conformation completely abolishes the normal recycling of LDLR, thus targeting LDLR toward the lysosomes, where it is degraded (Lo Surdo, Bottomley et al. 2011).

Although PCSK9 mainly acts on LDLR as a secreted factor, it has been proposed a possible interaction of PCSK9 and LDLR early in the secretory pathway. In fact, blocking intracellular trafficking from the trans-Golgi

network to the lysosomes by knocking down of clathrin light chains, rapidly increased LDLR levels in a PCSK9-dependent fashion, suggesting that either PCSK9-LDLR complex or PCSK9 alone could be targeted directly from the Golgi to late endosomes/lysosomes without cycling through the cell (Poirier, Mayer et al. 2009).

2.2 Animal studies

Several animal studies confirmed the relationship between PCSK9 and its target LDLR. Parabiosis experiments in transgenic mice expressing human PCSK9 (hPCSK9) in the liver established that PCSK9 is secreted into the bloodstream, binds to LDLR and is internalized by hepatocytes through LDLR, thereby promoting its intracellular degradation (Lagace, Curtis et al. 2006). Also transgenic overexpression of hPCSK9 in kidney increased plasma PCSK9 levels in the circulation and caused a reduction of LDLR in the liver (Luo, Warren et al. 2009).

Moreover, adenoviral overexpression of murine PCSK9 (mPCSK9) led to an increase in total cholesterol and non-HDL cholesterol of about 2-fold and 5-fold, respectively and these effects appeared to be dependent on LDLR because the same adenoviral infection in mice lacking LDLR did not show any effects. The increase in LDL cholesterol was associated to a reduction of LDLR protein but not of mRNA, that remained unchanged (Maxwell and Breslow 2004).

On the contrary, knocking out mPCSK9 increased hepatic LDLR expression and the clearance of circulating lipoproteins, resulting in a reduction of plasma cholesterol levels (Rashid, Curtis et al. 2005).

Analysis of conditional mice lacking PCSK9 in the liver confirmed that the liver is the major contributor to circulating PCSK9 levels and that the absence of PCSK9 in the liver contributes to two third of the hypocholesterolemic profile that was described in PCSK9 complete knockouts (Zaid, Roubtsova et al. 2008). The absence of both PCSK9 and LDLR did not modify cholesterol profile compared to single PCSK9 knockout, suggesting that PCSK9 exerts its effects on cholesterol almost exclusively through its action on LDLR (Zaid, Roubtsova et al. 2008).

It is undeniable that PCSK9 regulates LDLR, but it is also possible that LDLR regulates PCSK9 levels. It was recently demonstrated that the only way to remove PCSK9 from the circulation is via LDLR. In fact, turnover studies using hPCSK9 at physiological levels showed that the clearance of PCSK9 in WT mice was rapid, with a half-life of about 5 minutes, it was faster in hLDLR transgenic mice (about 3 minutes) and very slow in LDLR deficient mice (about 50 minutes) (Tavori, Fan et al. 2013).

Although the interaction with LDLR is well known, some studies have raised the possibility that PCSK9 may target other members of the LDLR gene family. In particular, Poirier and his colleagues demonstrated that PCSK9 and its naturally occurring variant D374Y enhance the degradation of other two members of the family of LDLR, VLDLR and apoER2; moreover, the degrading capacity of PCSK9 and the affinity for those receptors do not seem to be related to the presence of LDLR (Poirier, Mayer et al. 2008). The same group very recently showed that PCSK9 is able to induce the degradation of LRP1 in different cell lines and that, even if LRP1 does not seem to be essential for the regulation of LDLR by PCSK9, it might compete with LDLR for the binding to PCSK9 (Canuel, Sun et al. 2013).

PCSK9 undergoes cleavage after the R218 residue with the formation of a 55 kDa truncated form. The proteolytic cleavage of PCSK9 is mediated in hepatocytes by two convertases, called furin and PC5/6A, as demonstrated using hepatic conditional knockout mice (Benjannet, Rhainds et al. 2006; Essalmani, Susan-Resiga et al. 2011). Cleaved PCSK9 circulates in human and mouse plasma contributing to 15–40% and 30-50% of total circulating PCSK9, respectively (Benjannet, Rhainds et al. 2006; Essalmani, Susan-Resiga et al. 2011). It was recently demonstrated that, although less efficiently than the mature form, the furin-cleaved PCSK9 is able to regulate LDLR and consequently cholesterol levels (Lipari, Li et al.).

2.3 Regulation

The mRNA analysis of livers from mice overexpressing or lacking SREBP, revealed a regulation for PCSK9 by SREBP and suggested a possible role for PCSK9 in lipid metabolism (Horton, Shah et al. 2003). Statins have been extensively demonstrated to positively regulate LDLR expression in the liver, thus increasing LDL cholesterol uptake and reducing its plasma concentrations (Corsini, Bellosta et al. 1999). Similarly, statins induce PCSK9 expression both in mice and human hepatoma cell lines, with a mechanism involving SREBP2 (Jeong, Lee et al. 2008; Dong, Wu et al. 2010). Several studies demonstrated that statins increase plasma PCSK9 levels by 14 to 47%, depending on the type and dose of statin used, as well as the duration of treatment (Awan, Seidah et al.; Careskey, Davis et al. 2008; Welder, Zineh et al. 2010). As a consequence, the absence of PCSK9 enhances the hypolipidemic effects of statin administration (Rashid, Curtis et al. 2005), thus suggesting that inhibiting PCSK9 can potentially synergistically work with statins in reducing LDL cholesterol.

PCSK9 expression and plasma levels are regulated by hormonal and nutritional status. Circulating PCSK9 has a marked diurnal rhythm that parallels that of cholesterol biosynthesis, with a nadir between 3 and 9 pm and a peak at 4.30 am. Fasting causes a drop in PCSK9 concentration from 20 to 35% after 18 hours; a 66% reduction is achieved after 66 hours (Persson, Cao et al. 2010). Moreover, gender influences its concentration during adolescence, with an increase in PCSK9 in males and a decrease in females, according to variations in LDL cholesterol during puberty, but the nature of the hormones involved in this regulation is not known yet (Baass, Dubuc et al. 2009).

2.4 Partner proteins

Several proteins seem to be involved in the interaction with PCSK9. Extracellular Annexin A2 is a membrane-associated receptor which binds the C-terminal domain of PCSK9, preventing PCSK9 from binding to the LDLR, thus inhibiting its degradation (Mayer, Poirier et al. 2008). The removal of Annexin A2 in mice reduces not only the levels of LDLR in the liver, but also in adrenals and large intestine, tissues known to be rich sources of Annexin A2 and resistant to PCSK9, suggesting that Annexin A2 might be partially responsible for the poor effect of PCSK9 in those tissues (Seidah, Poirier et al. 2012).

Resistin, an adipokine mainly secreted by adipocytes and macrophages in obese patients, has been recently shown to reduce LDLR protein expression in hepatocytes, thus increasing LDL cholesterol and atherosclerosis in obese subjects. The degradation of LDLR induced by resistin is primarily, but not exclusively, related to the upregulation of PCSK9 protein levels (Melone, Wilsie et al. 2012).

Some studies investigated on a possible effect of PCSK9 on apoB production, since apoB is a structural component of LDL, taken up from the blood stream through LDLR. Sun and colleagues showed that PCSK9 directly interacts with apoB and that the interaction is responsible for an increase in apoB production through inhibition of its intracellular degradation (Sun, Samarghandi et al. 2012).

PCSK9 has been very recently shown to interact with both amyloid precursor protein (APP) and its close family member amyloid precursor protein-like protein 2 (APLP2); the interaction is mediated by its C-terminal domain and is pH dependent. Moreover, APLP2 seems to be involved in the delivery of PCSK9 to the lysosomes, thus influencing PCSK9 function (DeVay, Shelton et al. 2013).

Other researchers investigated on the possible connection between Sec24A, involved in the transport of proteins from the ER to the Golgi, and PCSK9. In particular, Sec24A deficient mice were found to be hypocholesterolemic due to decreased secretion of PCSK9 and thus increased expression of LDLR in the liver. On the basis of this observation, it has been hypothesized a possible association of PCSK9 with a putative transmembrane receptor capable of linking it to a Sec23/Sec24A complex in the cytosol. The receptor might be incorporated into COPII-coated vesicles and reach the Golgi in order to be secreted (Chen, Wang et al. 2013).

2.5 Gain of function mutations (GOF)

Human PCSK9 is a highly polymorphic gene; in fact, more than 150 coding mutations were identified and some of them were classified as GOF or LOF (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/index.php?select_db=PCSK9).

PCSK9 mutations are less common compared to LDLR mutations in patients with ADH. In 2003, Abifadel and colleagues identified GOF mutations in PCSK9 cosegregating with ADH in French families in whom alterations in LDLR or apoB had been previously excluded (Abifadel, Varret et al. 2003). Other mutations in PCSK9 were reported in a kindred from Utah, Norway and United Kingdom and were responsible for cholesterol levels around 500 mg/dl.

The most severe GOF mutation identified consists in the substitution of Asp374 by Tyr (D374Y). This variant was found to have a 6- to 30-fold higher affinity for LDLR compared to normal PCSK9, due to the formation of an additional hydrogen bond between PCSK9 and the EGF-A domain of the LDLR. Individuals carrying this mutation have an increased risk of coronary artery disease (CAD) that exceed the risk related to mutations in the LDLR gene (Bottomley, Cirillo et al. 2009). A mouse line expressing D374Y-PCSK9 was generated by Herbert and colleagues, who demonstrated that the D374Y variant develops atherosclerosis due to both an impairment of LDL clearance and an increase in apoB-containing lipoprotein secretion (Herbert, Patel et al. 2010).

Another mutation responsible for ADH, occurring at the catalytic site, S127R, was first hypothesized to exert its effect intracellularly, by binding to the LDLR and favoring its degradation (Cameron, Holla et al. 2008; McNutt, Kwon et al. 2009). However, recent evidences showed that this mutation works through increasing the binding capacity to LDLR (McNutt, Kwon et al. 2009). On the contrary, the F216L variant has been shown to cause a reduction in furin-mediated cleavage of PCSK9 (Benjannet, Rhainds et al. 2006; Essalmani, Susan-Resiga et al. 2011). The E670G-PCSK9 variant was

found to be positively correlated to LDL cholesterol levels in some studies, but not all (Cariou, Le May et al. 2011).

2.6 Loss of function mutations (LOF)

In 2005, an association between two LOF mutations in PCSK9 and low LDL cholesterol levels was established (Cohen, Pertsemlidis et al. 2005). The two mutations, C679X and Y142X, occurred in 2-2.6% of African Americans and were associated with a 40% reduction in LDL cholesterol and an 88% reduction of cardiovascular risk during a 15-years follow-up (Cohen, Pertsemlidis et al. 2005).

Another LOF mutation, R46L, has been found in about 3.2 % of white subjects. Individuals carriers of the mutation exhibited a reduction in LDL cholesterol of 15%, accompanied by a 47% reduction in coronary heart disease risk (Cohen, Boerwinkle et al. 2006).

Another substitution at the PCSK9 cleavage site (Q152H) has been recently described. This mutation prevents autocatalytic processing, thus affecting PCSK9 secretion, and reducing LDL cholesterol by 48% (Mayne, Dewpura et al.).

Moreover, two young women with total PCSK9 deficiency have been identified. The subjects present extremely low LDL cholesterol levels (14 mg/dl and 16 mg/dl) but no adverse clinical events have been reported (Zhao, Tuakli-Wosornu et al. 2006).

It has been reported by Cariou a LOF double mutant R104C/V114A that acts a dominant negative (Cariou, Ouguerram et al. 2009). The mutation has been associated with an impaired autocatalytic processing of pro-PCSK9 and a reduction of PCSK9 secretion. Consequently PCSK9 was not detected in the

circulation and LDL cholesterol was nearly absent (7mg/dl), due to an increased catabolic rate.

2.7 Therapeutic approaches

The identification of healthy individuals with homozygous LOF mutations in PCSK9 and no detectable levels of it, has suggested the idea that pharmacological intervention to inhibit PCSK9 can be safe and can help to reduce cardiovascular risk, especially in light of the fact that most of the patients with hypercholesterolemia do not reach the goal of LDL reduction with the currently available lipid lowering agents.

Several monoclonal antibodies directed toward PCSK9 have been developed by different pharmaceutical companies and have been currently tested in clinical trials. Phase I and II trials of AMG145 were published and demonstrated its safety and efficacy. In a phase I single-dose study in healthy volunteers and hypercholesterolemic subjects on a stable statin therapy, subcutaneous or intravenous infusion of AMG145 reduced free PCSK9 levels and LDL cholesterol by up to 60% in a dose-dependent way; the reduction was not dependent on the type or dose of statin administered. In the same study, AMG145 was shown to reduce apoB and lipoprotein (a) (Lp(a)) levels (Dias, Shaywitz et al. 2012). In a phase II randomized, placebo-controlled study, conducted in hypercholesterolemic patients who were receiving statins with or without ezetimibe, the administration of different doses of AMG145 every 2 or 4 weeks reduced LDL cholesterol by 42 to 66%, without any increase in adverse events compared to placebo (Giugliano, Desai et al. 2012).

Another antibody developed by Sanofi/Regeneron, SAR236553/REGN727, showed, in a phase I study in healthy subjects, a significant reduction in LDL cholesterol, ranging from 33 to 46% and from 28

to 65% after subcutaneous and intravenous administration, respectively (Stein, Mellis et al. 2012). In a 12-week phase II study in patients with LDL cholesterol over 100 mg/dl and already on treatment with atorvastatin, administration of the highest dose of SAR236553/REGN727 (150 mg) every 2 weeks reduced LDL cholesterol up to 70% (Stein, Mellis et al. 2012). Recent phase III trial with SAR236553/REGN727 reported its efficacy in monotherapy in reducing LDL cholesterol after 24 weeks in patients with primary hypercholesterolemia and moderated risk of cardiovascular diseases (CVD) (REGENERON Oct 16 2013).

Besides monoclonal antibodies, other approaches directed toward PCSK9 include the use of recombinant adnectins, antisense RNA interference or small molecule inhibitors. It has been recently concluded a randomized, single-blind, placebo-controlled, phase I trial, with ALN-PCS, a small RNA interference inhibiting PCSK9 synthesis. The study showed a reduction in PCSK9 levels up to 70% and a consequent decrease in LDL cholesterol by 40% compared to placebo, with no increased adverse events (Fitzgerald, Frank-Kamenetsky et al. 2013).

2. Familial hypercholesterolemia (FH)

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by severely elevated serum cholesterol levels, xantomas and increased risk of coronary heart disease (CHD) (Hopkins, Toth et al. 2011).

The incidence of homozygous FH (HoFH) is approximately one in one million people and the levels of LDL cholesterol in affected individuals range from 700 to 1000 mg/dl (Goldstein and Brown 1973). If left untreated, they

develop premature atherosclerosis and do not survive the second decade of their life (Kwiterovich 2008). Heterozygous FH (HeFH) is more common and affects around 1 every 300-500 individuals; it is less severe, with LDL cholesterol levels around 300 mg/dl and an onset of CHD at 40 to 50 years of age in non-treated patients (Alonso, Mata et al. 2008). There are some populations, such as Christian Lebanese, French Canadians, Ashkenazi Jews, Dutch Afrikaners and Asian Indians, in which the incidence is much higher (1 in 50 to 100 individuals) due to an increased prevalence of LDLR mutations in the founder populations (Hobbs, Brown et al. 1987; Zeegers, van Poppel et al. 2004).

In more than 85% of affected individuals, mutations in LDLR are responsible for FH and over 1600 mutations have been identified (Varret, Abifadel et al. 2008). The LDLR is located on chromosome 19 and consists of 18 exons encoding for a transmembrane protein of 860 amino acids (Sudhof, Goldstein et al. 1985). Cholesterol homeostasis in normal subjects is tightly dependent on LDLR expression and hepatic surface LDLR is strictly regulated on the basis of cholesterol requirements (Marais 2004). Crystal structure analyses of LDLR were performed to better understand which binding sites are involved in LDL interaction and how mutations can affect the binding and internalization of LDL. The extracellular domain of LDLR (Figure 3) consists of a ligand-binding domain, located at the N-terminus of the protein, an epidermal growth factor (EGF) precursor domain consisting of two cysteine reach EGF-like domain (EGF-A and EGF-B) separated from a third EGF repeat (EGF-C) by a six-bladed β-propeller domain and an O-linked sugar-rich domain (Yamamoto, Davis et al. 1984; Rudenko, Henry et al. 2002) . When the LDLR is present on the cell surface, the extracellular domain assumes an extended conformation and exposes the ligand-binding domain to LDL. After LDL binding, the

receptor-ligand complex is internalized and reaches the endosomes, where the LDLR folds back onto itself, bringing the β-propeller region closer to the ligand-binding domain and allowing the release of LDL in the endosome and the recycle of the LDLR to the cell surface (Rudenko, Henry et al. 2002).

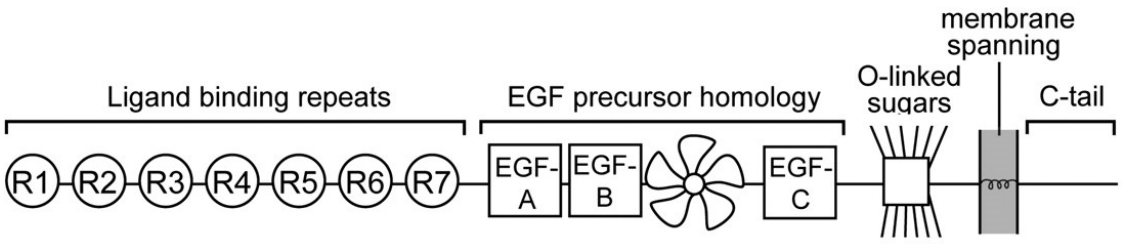


Figure 3. Structure of the extracellular domain of LDLR. *from Zhang D.W. et al. JBC, 2007, 282(25): 18602-18612.*

Mutations in LDLR result in a reduction of the capacity of LDLR to clear LDL cholesterol from the circulation. On the basis of LDLR functionality, patients are classified in receptor negative, with 2% of LDLR activity due to defective synthesis or intracellular transport, or receptor defective, with a 2 to 25% of LDLR activity, due to a reduced binding capacity to LDL (Hobbs, Russell et al. 1990). In general, there is a direct correlation between LDL cholesterol levels and residual activity of LDLR (Rader, Cohen et al. 2003).

Disease	Defective gene	Prevalence	Plasma LDL-C level	Metabolic defect
Autosomal dominant				
FH	*LDLR*			↓LDL clearance (1°) ↑LDL production (2°)
Heterozygous FH		1 in 500	+++	
Homozygous FH		1 in 1×10^6	+++++	
FDB	*APO8*			↓LDL clearance
Heterozygous FDB		1:1,000	++	
Homozygous FDB		1 in 4×10^6	+++	
FH3	*PCSK9*			
Heterozygous FH3		<1 in 2500	+++	Unkown
Autosomal recessive				
Recessive autosomal Hypercholesterolemia (ARH)	*ARH*	<1 in 5×10^6	++++	↓LDL clearance
Sitosterolemia	*ABCG5* or *ABCG8*	<1 in 5×10^6	- to +++++	↓Cholesterol excretion (1°) ↓LDL clearance (2°)

Table 1. Major monogenic diseases that cause hypercholesterolemia *from Rader, DJ et al., JCI, 2003, 111(12) 1795-1803*

Other forms of hypercholesterolemia have been described (Table 1). Hypercholesterolemia due to apoB mutations is referred to as Familial Defective apoB100 (FDB) (Innerarity, Mahley et al. 1990). The most common mutation of apoB responsible for FDB is Arg3500Gln, occurring in 1 in 1000 individuals in Northern Europe but rare in other populations (Varret, Abifadel et al. 2008). FDB is characterized by elevated LDL cholesterol levels but normal levels of triglycerides, tendon xanthomas, and premature onset of atherosclerosis. Homozygous FDB (HoFDB) is less severe than HoFH. In fact, HoFDB subjects have plasma LDL cholesterol levels more similar to those in HeFH rather than HoFH and the progression of atherosclerosis is slower, since the diagnosis is usually done over 40 years of age (Myant 1993).

A third form of autosomal dominant hypercholesterolemia is attributable to mutations in PCSK9, which in turn increases the degradation of LDLR and the levels of LDL cholesterol in the circulation (Horton, Cohen et al.

2009). This is a rare cause of FH, compared to LDLR and apoB mutations and explains less than 5% of FH cases (Varret, Abifadel et al. 2008).

Another form of hypercholesterolemia, called autosomal recessive hypercholesterolemia (ARH), has been attributed to mutations in the LDLR accessory protein 1 (LDLRAP1), involved in the association of LDLR with clathrin-coated pits (Garcia, Wilund et al. 2001). Rare forms of hypercholesterolemia include also sitosterolemia due to ABCG5 or ABCG8 deficiency and hypercholesterolemia caused by deficiency of cholesterol 7-alpha hydroxylase (CYP7A1) (Soutar and Naoumova 2007).

Patients with FH should be treated as soon as the disease is diagnosed. The goal, according to the National Lipid Association (NLA) recommendations, is to achieve a reduction of LDL cholesterol of about 50% with high-doses statins or levels of LDL cholesterol lower than 100 mg/dl in those patients who are at high risk for CVD (Hopkins, Toth et al. 2011). Treatment of FH patients first requires a modification of lifestyle including following a healthy diet, exercise, weight control, blood pressure control, smoking cessation (Hopkins, Toth et al. 2011). The use of statins is considered the first line therapy for reducing CVD risk in FH patients. Moderate and high dose statins have been demonstrated to be safe and to reduce mortality in people at high risk of CVD disease (McKenney, Davidson et al. 2006; Davidson and Robinson 2007).

No placebo-controlled CVD endpoint trials are available specifically for FH subjects, since the absence of treatment prescription in FH patients for management of hypercholesterolemia is considered non ethical. However, several outcomes trials in populations of severely hypercholesterolemic people have been performed. Two placebo-controlled statin trials in populations with severely elevated LDL cholesterol, but not specifically FH,

found that moderate doses of simvastatin or pravastatin reduced the risk of nonfatal coronary events and CVD death (1994; Shepherd, Cobbe et al. 1995). In particular, the Scandinavian Simvastatin Survival Study (4S), performed in a secondary prevention population, found that, after a five-years follow-up, simvastatin administration caused a 30% reduction in both stroke and total mortality (1994). Also atorvastatin 80 mg was found to be safe and efficacious in a long-term secondary prevention study (Davidson and Robinson 2007).

Although some HeFH are responsive to therapy with current hypocholesterolemic agents, HoFH are difficult to treat because on one hand they need to reach much lower LDL cholesterol levels and on the other hand the majority of them totally lack LDLR and treatment with high-dose statins is often ineffective (McGowan 2013).

Other non-statin treatments are commercially available and have been found to reduce CVD events in severely hypercholesterolemic populations. Ezetimibe, an inhibitor of cholesterol absorption, in association with statins, was found to reduce LDL cholesterol levels by 43% to 70% in HeFH and 21% in HoFH (an additional 15% reduction compared to statins alone), depending on the dose of statin prescribed (Hamilton-Craig, Kostner et al.; Gagne, Gaudet et al. 2002; Kastelein, Akdim et al. 2008). However, the additional CVD risk reduction of ezetimibe in combination with statins has not been definitely determined (Robinson and Davidson 2006).

Two large placebo-controlled trials demonstrated that monotherapy with bile acid sequestrants, colestipol and cholestyramine, reduced cholesterol levels of about 13 to 20% and thus cardiovascular events in a primary prevention population (Dorr, Gundersen et al. 1978; 1984). Only one trial is available for combination therapy of statins plus fibrate, Action to

Control Cardiovascular Risk in Diabetes (ACCORD), and did not show any additional reduction in CVD risk in patients with type 2 diabetes, except for those with high triglycerides or low HDL (Ginsberg, Elam et al. 2010).

Although niacin in combination with statins has been found to be safe and effective in reducing cholesterol levels in patients with hypercholesterolemia, no trials have been concluded evaluating the benefits of combination therapy with high-dose statins in FH patients (Hopkins, Toth et al. 2011).

PCSK9 has recently emerged has a possible therapeutic target for the management of hypercholesterolemia, also in FH patients. AMG145, a monoclonal antibody against PCSK9, showed up to 55% reduction in LDL cholesterol in a randomized, double blind, placebo-controlled phase II trial in HeFH patients (Raal, Scott et al. 2012). Another trial with PCSK9 monoclonal antibody, REGN727, showed a 29 to 68% reduction in LDL cholesterol in HeFH patients, compared to placebo (Stein, Gipe et al. 2012). Mipomersen, an antisense oligonucleotide directed against apoB, and the MTP inhibitor lomitapide, have been recently approved by the FDA as an additional therapy to lipid lowering agents and diet modifications in HoFH (Raal, Santos et al. 2010). In particular, lomitapide received the approval only for use in patients with HoFH in a program named JUXTAPID Risk Evaluation and Mitigation Strategy, administered by Aegerion Pharmaceutical.

Besides diet modifications and lipid lowering drug therapy, LDL apheresis (LDL-A) is another highly efficacious option for management of LDL cholesterol in severe FH patients who do not achieve sufficient reduction of LDL cholesterol (Thompson, Barbir et al. 2010). However, the use of this technique is limited by high costs (from $45000 to $100000 (Moriarty 2006)),

difficult access (only 40 centers in the territory of US (Ito, McGowan et al.)) and time and frequency of the treatment (3 hours every 1 or 2 weeks (Ito, McGowan et al.)).

2.1 LDL apheresis (LDL-A)

LDL-A has been approved by the FDA in patients refractory to other therapies and who follow one of the ensuing criteria: LDL cholesterol over 500 mg/dl and HoFH, LDL cholesterol over 300 mg/dl and HeFH without coronary artery disease (CAD) or LDL cholesterol over 200 mg/dl and HeFH with CAD (Thompsen and Thompson 2006). Also the NLA recommended the use of LDL-A in patients who responded to the criteria in Table 2.

LDL apheresis is an FDA-approved medical therapy for patients who are not at LDL cholesterol treatment goal or who have ongoing symptomatic disease.

In patients who, after 6 months, do not have an adequate response to maximum tolerated drug therapy, LDL apheresisis is indicated according to these guidelines:

1. Functional homozygous FH patients with LDL cholesterol ≥ 300 mg/dL (or non-HDL cholesterol ≥ 330 mg/dL).
2. Functional heterozygous FH patients with LDL cholesterol ≥ 300 mg/dL (or non-HDL cholesterol ≥ 330 mg/dL) and 0-1 risk factors.
3. Functional heterozygous FH patients with LDL cholesterol ≥ 200 mg/dL (or non-HDL cholesterol ≥ 230 mg/dL) and high risk characteristics such as ≥ 2 risk factors or high lipoprotein (a) ≥ 50 mg/dL using an isoform insensitive assay.
4. Functional heterozygotes with LDL cholesterol ≥ 160 mg/dL (or non-HDL cholesterol ≥ 190 mg/dL) and very high risk characteristics (established CHD, other cardiovascular disease, or diabetes).

Table 2. National lipid association (NLA) recommendations for identifying candidates for LDL-A. adapted *from Ito et al., Journal of clinical lipidology, 2011; 5(3 suppl 1):S38-S45.*

LDL-A is a procedure that selectively removes apoB–containing lipoproteins from plasma, thereby lowering LDL cholesterol by 70% to 80%. Moreover, it is the only treatment that significantly reduces Lp(a) by 50 to 80% (McGowan 2013). Depending on the LDL-A method, also HDL can be partially removed from plasma at an extent that vary between 12 and 20% (Hovland, Hardersen et al. 2009).

Five different techniques can be clinically used to carry out LDL-A: adsorption mediated by dextran sulfate cellulose beads columns (Liposorber), Heparin Extracorporeal LDL Precipitation (HELP), Polyacrylate Full Blood Adsorption (PFBA), Immunoadsorption and Filtration plasmapheresis. Among these procedures, the HELP and the Liposorber system are those currently approved by the FDA for FH treatment. A more detailed description of the Liposorber system can be found in Methods.

Besides cholesterol lowering, LDL-A can also affect other markers involved in inflammation and consequently in the progression of atherosclerosis. Some literature data suggest that plasma separator columns stimulate the formation of complement factors, such as C3a, C5a which bind to anaphylatoxin receptors and exert highly potent pro-inflammatory effects. The same components were found to be adsorbed on the LDL-A columns, demonstrating that LDL-A might induce the activation of complement, but at the same time removes the generated pro-inflammatory factors (Hovland, Hardersen et al. 2012). Also C-reactive protein (CRP), a pro-inflammatory marker involved in the pathogenesis of atherosclerosis, is reduced after LDL-A, both in patients with peripheral artery disease and with HeFH (Kojima, Shida et al. 2003; Hershcovici, Schechner et al. 2004). Although with some dissimilarities probably due to the different properties of adsorption to the

columns, LDL-A methods lower the plasma levels of several pro-inflammatory cytokines, such as MCP-1, TNF-alfa, IL-1α, IL-1β and IL6 (Stefanutti, Vivenzio et al. 2011; Stefanutti, Vivenzio et al. 2011). Moreover, all the LDL-A techniques reduce fibrinogen, the precursor of fibrin, whose levels contribute to the assessment of CVD risk (Hovland, Hardersen et al. 2010).

Although invasive, LDL-A is considered to be a safe procedure, but side effects can occur. In a group of 64 patients who underwent LDL-A treatments using Liposorber columns, the main reported side effects were hypotension, nausea and vomiting, abdominal pain, lightheadness, headache, flushing, fainting and anemia (Gordon, Kelsey et al. 1998). They were mainly due to the activation of the intrinsic pathway of coagulation, which in turn activated bradykin, causing vasorelaxation. Some of the symptoms indicated above, such as lightheadness, hypotension, nausea and vomiting, were associated to the concomitant administration of ACE inhibitors, responsible for the activation of the bradykinin system; thus it has been recommended to suspend the administration of ACE inhibitors 24 to 48 hours before the procedure or to switch the ACE inhibitors to angiotensin type 2 antagonists (McGowan 2013).

4 Atherosclerosis

Atherosclerosis is a multifactorial disease in which, especially in FH, LDL plays a strong role. Atherosclerosis has long been considered a disease characterized only by the progressive retention of apoB-containing lipoproteins in susceptible areas of large and medium size arteries (Mayerl, Lukasser et al. 2006). Nowadays this definition has been flanked by the concept of inflammation, involving both innate and adaptive immunity, which modulate the development and progression of lesions. Atherosclerotic plaques are thus characterized by the accumulation of lipids in the arterial wall together with the infiltration of macrophages and other immune cells (Ross 1999; Hansson and Libby 2006).

Key early steps in lesion formation are the activation of endothelium by pro-inflammatory lipoproteins accumulated into the arterial wall and the recruitment of monocytes from the circulation (Figure 4) (Glass and Witztum 2001).

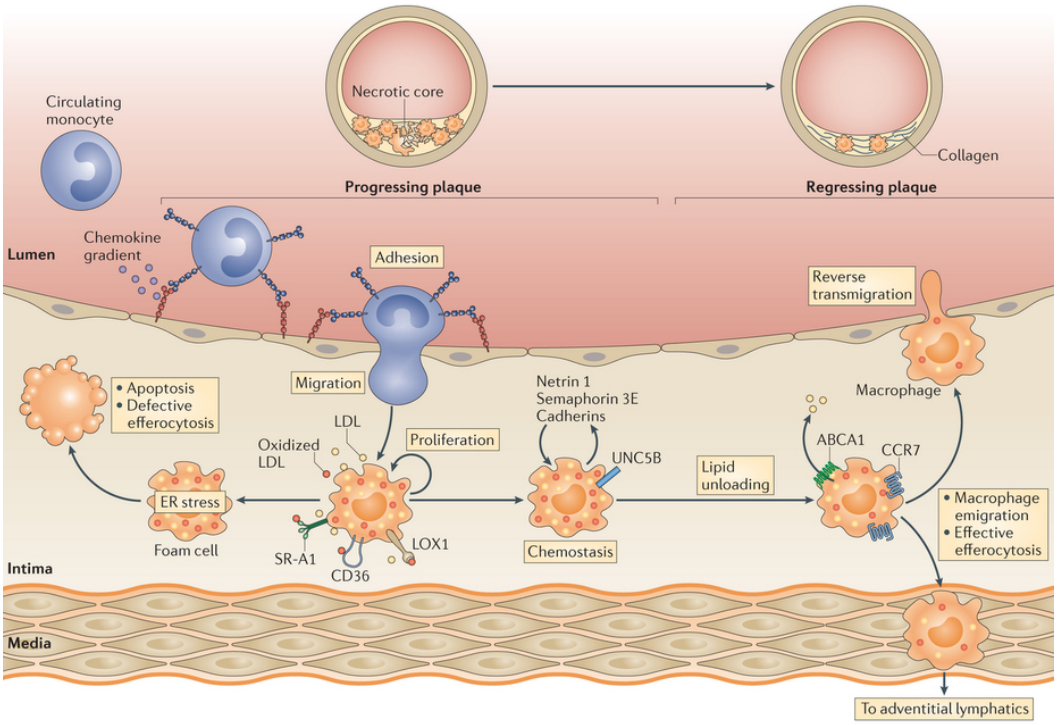


Figure 4. Mechanisms regulating monocytes recruitment and accumulation in plaques *from Moore K.J., Nature review immunology, 2013; 13:709-721.*

4.1 Monocyte subsets in atherosclerosis

Monocytes originate from bone marrow-derived progenitor cells (Volkman 1970). In addition to those produced in bone marrow, it was recently shown that spleens can also provide an extramedullary source of undifferentiated monocytes that are readily mobilized to inflammatory sites and accumulate in the atheroma (Robbins, Chudnovskiy et al. 2012). In mice, monocytes are distinguished from other blood cells by the expression C-C chemokine receptor type 2 (CCR2), CX3C chemokine receptor 1 (CX3CR1) and Ly6C surface antigen (Geissmann, Jung et al. 2003). Circulating murine monocytes consist of two major subsets: $Ly6C^{high}$ and $Ly6C^{low}$, which correspond to human $CD14^{hi}CD16^{+}$ and $CD14^{low}CD16^{+}$. Several literature data

suggest that Ly6C^{high} monocytes are associated to acute inflammation and enter more readily atherosclerotic plaque in the early stages of atherosclerosis, while Ly6C^{low} are mainly related to resolution of inflammation. Moreover, Ly6C^{high} monocytes are believed to convert to Ly6C^{low}, but the conversion is compromised in the context of atherosclerosis (Sunderkotter, Nikolic et al. 2004). In humans, CD14^{hi}CD16^{+} usually represent about 85% of total monocytes in healthy subjects, while the remaining are CD14^{low}CD16^{+} but the role of each subset in atherosclerosis is not completely defined (Passlick, Flieger et al. 1989). However, recent studies suggested a possible role for the different subsets on CVD risk prediction in obese and people with CHD (Hristov, Leyendecker et al. 2010).

Hypercholesterolemia has been associated to an increase in the number of circulating monocytes. In fact, when apoE$^{-/-}$ mice were fed a high fat diet, not only the circulating monocytes were increased by 50% compared to wild type (WT) animals consuming the same diet, but monocytes number was also 4-fold higher compared to apoE$^{-/-}$ fed a chow diet. In particular, monocytosis resulted in a 14-fold increase of the Ly6C^{high} subpopulation (Swirski, Libby et al. 2007). Some researchers suggested that cholesterol engulfment by hematopoietic stem and progenitor cells stimulates their proliferation through an upregulation of interleukin-3 (IL-3) and granulocyte macrophage-colony stimulating factor (GM-CSF) receptor; moreover the expression of factors that promote efflux is able to revert this proliferative effect and decrease the risk of atherosclerosis (Yvan-Charvet, Pagler et al.).

Several adhesion molecules are involved in the firm adhesion of monocytes and their transmigration across the endothelium. The processes of tethering and rolling mainly depend on the interaction of chemokines. In

particular, monocytes roll on endothelial cells through the interaction of P-selectin glycoprotein ligand 1 (PSGL1), present on monocyte surface, with endothelial selectins, such as E-selectin and P-selectin (Mestas and Ley 2008). The firm adhesion of monocytes to the endothelium is mediated by the binding of vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) to very late antigen 4 (VLA4) and lymphocyte function-associated antigen 1 (LFA1), respectively; the differences in expression of LFA1 between $Ly6C^{high}$ and $Ly6C^{low}$ monocytes may explain the ability of $Ly6C^{low}$ monocytes to adhere but not enter the arterial wall (Woollard and Geissmann 2010). Several chemokines, secreted by endothelial cells, macrophages and SMCs provide the chemotactic stimulus for monocyte entry. In particular, studies on $apoE^{-/-}$ mice showed that the removal of CCR2, CX_3CR1 and CCR5 and consequently of the interaction with their respective ligands, is able to almost completely abolish $Ly6C^{high}$ and $Ly6C^{low}$ monocytosis and atherosclerosis (Combadiere, Potteaux et al. 2008). CCR2 and CX3CR1 are not only involved in the transmigration of monocytes, but it seems they are directly able to influence the number of monocytes in the plaque. In fact, CCR2 drives extravasation of $Ly6C^{high}$ cells from the bone marrow (Serbina and Pamer 2006) and CX3CR1 promotes monocyte survival through the inhibition of apoptotic signals (Landsman, Bar-On et al. 2009).

4.2 Macrophage subsets in atherosclerosis

Once in the intima, driven by macrophage-colony stimulating factor (M-CSF) and other differentiation factors, monocytes differentiate to macrophages or dendritic cells and interact with atherogenic lipoproteins (Johnson and Newby 2009). Two different subsets of macrophages have been identified both in mice and humans. M1 macrophages, or classically activated

macrophages, originate from Ly6C^{high} monocytes after differentiation by type I cytokines or lipopolysaccharide (LPS) (Liu and Yang 2013). LPS is a microbial product that binds to Toll-like receptor 4 (TLR4) and activates downstream pathways that in turn activate Nuclear factor-kappa B (NF-kB), activator protein 1 (AP1), cAMP response element-binding protein (CREB) and signal transducer and activator of transcription 1 (STAT1), thus inducing both pro-inflammatory and anti-inflammatory mediators expression (Akira 2001). M1 macrophages secrete pro-inflammatory cytokines, such as IL-1β, IL-6, IL-12, IL-23, CCL5, CXCL10 and Tumor Necrosis Factor-α (TNF-α), and express inducible NO synthase (iNOS) and cyclooxygenase 2 (COX2), whose products aggravate oxidative stress in the plaque (Liu and Yang 2013). M2 macrophages are generally characterized by reduced secretion of IL-12 and they are further subdivided into three groups. M2a, or alternatively activated macrophages, are thought to differentiate from Ly6C^{low} monocytes in the presence of IL-4, IL-13 or IL-21; M2b are generally activated by immune complexes or by agonists of TLRs or IL-1 receptor type 1 (IL1R1) and M2c, induced by IL-10, Transforming Growth Factor-β (TGF-β) and glucocorticoids. M2 macrophages produce large amounts of IL-10, TGF-β1 and the anti-inflammatory marker arginase I, which favors wound healing through generation of polyamines and proline (Gordon and Taylor 2005). M2 macrophages and in particular M2a are also characterized by high levels of non-opsonic receptors, such as the mannose receptor (Gordon 2003). Gene activation of M1 and M2 different phenotypes is driven by NF-kB and Peroxisome Proliferator-Activated Receptor γ (PPARγ) pathways, respectively (Adamson and Leitinger 2011). Histologic analysis of human atherosclerotic plaques revealed M1 macrophages to be more prone to accumulate lipids and localized in areas of the plaque distincted from those where M2 usually accumulate. Moreover,

the distribution of phenotypes varies during the different stages of atherosclerotic plaque development (Stoger, Gijbels et al. 2012). In fact, both M1 and M2 numbers increase during atherogenesis and both are equally present in the fibrous cap. When lesion progresses, M1 subtype mainly populates rupture-prone shoulder regions, while M2 is predominant in the adventitia. In vitro and animal studies led to distinguish M1 macrophages as promoting inflammation and M2 as resolving inflammatory response (Moore, Sheedy et al. 2013). Another study compared human carotid and femoral lesion composition and found that M1 macrophage markers were mostly localized in carotid arteries, whereas M2 markers were increased in femoral arteries, suggesting that the accumulation of M1 macrophages might be related to symptomatic lesions (Shaikh, Brittenden et al. 2012).

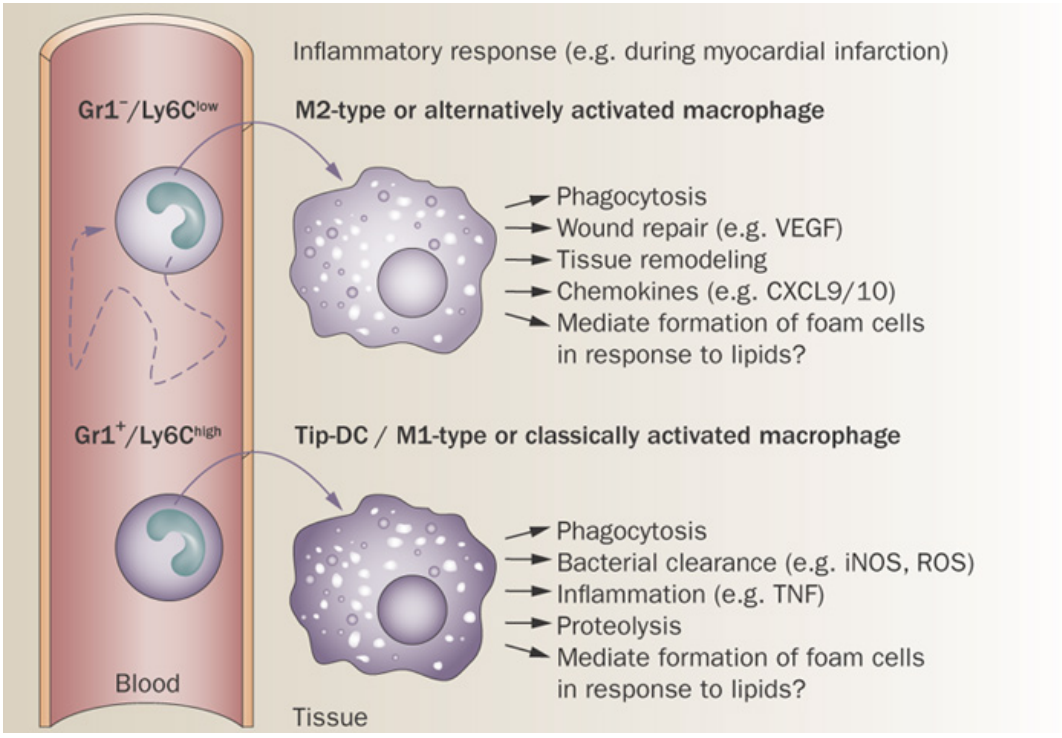


Figure 5. Macrophage subsets in atherosclerosis. *from Woollard et al,. Nat Rev Cardiology, 2010; 7: 77-86*

A third macrophage phenotype was recently discovered, Mox, characterized by an increased expression of Nuclear factor erythroid 2-related factor2 (NRF2) and induced by oxidized phospholipids present in oxidized LDL (Kadl, Meher et al. 2010). The relative abundance of macrophage subtypes was estimated to be 40% M1, 30% Mox and 20% M2 in both human and $LDLR^{-/-}$ mice atherosclerotic plaques (Kadl, Meher et al. 2010).

The number of macrophages in the plaque depends on different processes: on one hand, recruitment in the lesion and proliferation, on the other hand emigration and cell death (Moore, Sheedy et al. 2013).

Macrophages proliferate within the intima and amplify the inflammatory processes by releasing several growth factors, cytokines and metalloproteinases (MMPs) (Libby, Okamoto et al. 2010). Among the secreted pro-inflammatory cytokines, TNF-α has been defined the "master regulator", because of its fundamental role in controlling the production of the whole inflammatory cascade (Parameswaran and Patial 2010). It is rapidly released after traumas, infections, or exposure to LPS and, together with interferon γ (IFNγ), activates both macrophages and endothelial cells via NF-kB, inducing the secretion of cytokines and adhesion molecules, that amplify the inflammatory response in the plaque (Wesemann and Benveniste 2003). Besides inflammation, it regulates multiple functions including macrophage proliferation, differentiation and apoptosis. The secretion of TNF-α and nitric oxide by macrophages can also induce apoptosis of SMCs through the activation of Fas apoptotic pathway, thus contributing to the vulnerability of the plaque (Boyle, Weissberg et al. 2003). Another contributor of fibrous cap thinning is represented by the family of MMPs, proteolytic enzymes responsible for the degradation of the extracellular matrix (Libby, Okamoto et

al. 2010). Enhanced MMP expression has been detected in the atherosclerotic plaque and activation of MMPs appear to facilitate atherosclerosis, plaque destabilization and platelet aggregation (Galis, Sukhova et al. 1994). Several MMPs, such as MMP1 and MMP10, are upregulated in macrophages polarized to the M1 phenotype through activation of NF-kB and also colocalize with M1 macrophages in atherosclerotic plaques (Huang, Sala-Newby et al.). MMP9 levels were found to be higher in patients with unstable plaques compared to those with stable plaques; moreover analysis by gelatin zymography showed a higher expression of MMP2 and MMP9 in people with acute coronary syndromes compared to subjects with stable angina, suggesting a role for them as possible biomarkers of CAD (Alvarez, Ruiz et al. 2004).

Foam cells formation begins at a very early stage of atherogenesis as a consequence of lipoprotein uptake. Macrophages express several receptors involved in the internalization of apoB-containing lipoproteins, including LDLR, LDLR-related protein (LRP), and scavenger receptors. LDLR mediates the uptake of native LDL and is thought to exert a peripheral role in plaque progression because of its cholesterol-dependent downregulation (Fogelman, Haberland et al. 1981). However, it has been demonstrated that also native LDL can be taken up by activated macrophages and mediate foam cells formation in vitro (Kruth, Huang et al. 2002). Moreover, Linton and his colleagues demonstrated that the lack of macrophage LDLR in mice reduces the formation of foam cells, highlighting a possible athero-protective role of macrophage LDLR (Linton, Babaev et al. 1999). LRP is another member of the superfamily of LDLR that modulates atherogenesis through multiple pathways in addition to its effect as a lipoprotein internalizing receptor (Overton, Yancey et al. 2007) (Yancey, Blakemore et al. 2010; Yancey, Ding et al. 2011).

In fact, deletion of LRP1 in macrophages has been associated to an increased expression of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and MMPs, such as MMP9, and impairment of the pAkt survival pathway and efferocytosis (Overton, Yancey et al. 2007; Yancey, Blakemore et al. 2010). Scavenger receptors were originally characterized by their capacity of internalization of modified LDL. In particular type A1 scavenger receptor (SRA1) and CD36 seem to be the major determinants of foam cell formation. In fact, the combined deletion of the two receptors greatly affects the formation of foam cells; moreover, the extent of inflammation and macrophage apoptosis is greatly reduced, suggesting a more complex role of the two receptors in the development of the plaque (Manning-Tobin, Moore et al. 2009). Other members of the scavenger receptors family, such as type B1 scavenger receptor (SRB1), lectin-like oxidized LDLR 1 (LOX-1), macrophage receptor with collagenous structure (MARCO), were found to be involved in the lipoprotein uptake and thus contribute to foam cell formation. Once internalized by the cells, the cholesterol esters (CE) in lipoproteins are hydrolyzed into the lysosomes to free cholesterol (FC) and fatty acids. Since excess of FC can be dangerous for cell survival, FC is then targeted to the ER where is esterified to CE by the enzyme acyl-CoA cholesterol acyltransferase 1 (ACAT1) cells (Moore, Sheedy et al. 2013). A failure in FC esterification can lead on one hand to the accumulation of FC in the ER membrane and impairment of esterification through ACAT1; on the other hand FC can activate inflammatory signaling pathways involving TLRs and NF-kB (Figure 4) (Moore and Tabas 2011).

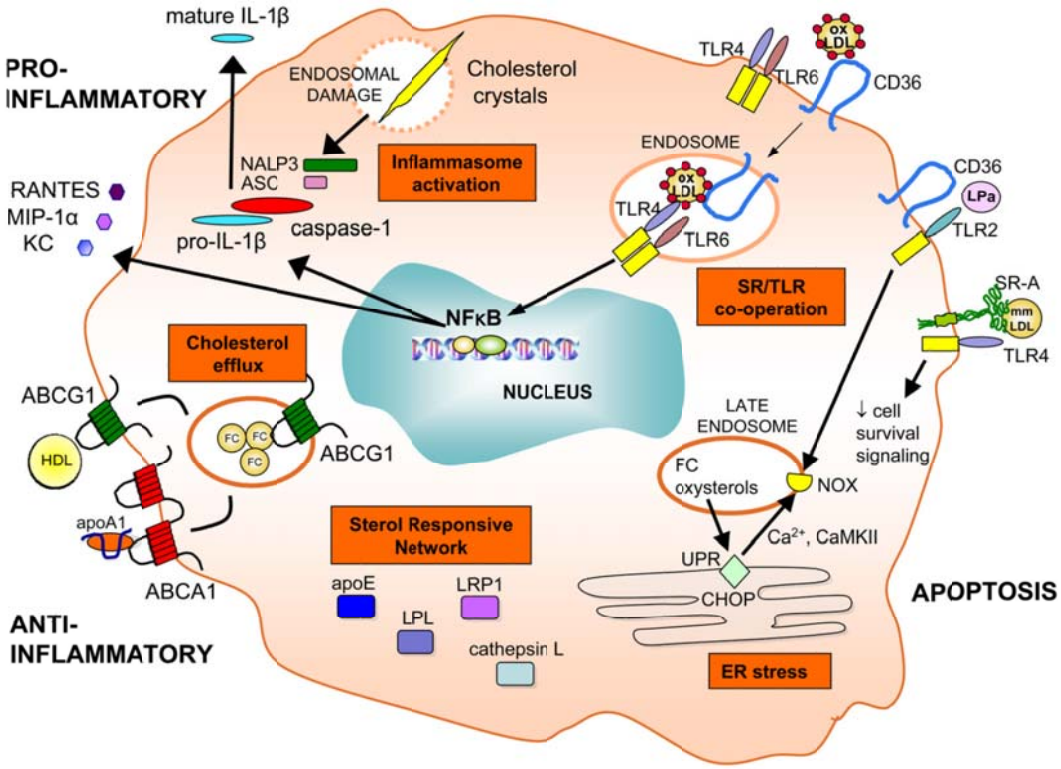


Figure 6. Signaling pathways activated in lesional macrophages. *from Moore et al., Cell, 2012 145(3): 341-355*

Moreover, the accumulation of cholesterol within the cells induces the expression of transcription factors, such as LXRα and LXRβ, which in turn increase the expression of transmembrane proteins involved in the efflux of cholesterol (Leitinger and Schulman 2013). In fact, cholesterol efflux is one of the major processes that determine plaque regression and is mediated by transporters belonging to the family of the ATP binding cassette transporters, or by passive diffusion of cholesterol to cholesterol poor HDL (Rothblat and Phillips 2010). ABCA1 and ABCG1 deliver cholesterol to apoA-I and HDL, respectively (Figure 4); the double deletion of ABCA1 and ABCG1 impairs cholesterol efflux and thus increases of atherosclerosis (Tall, Yvan-Charvet et al. 2008). On the contrary, the absence of only one of the two transporters

does not cause atherosclerosis, mainly due to the activation of compensatory mechanisms linked to the induction of the other counterpart (Tarling, Bojanic et al. 2010).

FC accumulation in the cells results in ER stress and activation of apoptosis, a process of programmed cell death that occurs in all stages of atherosclerotic (Tabas 2010). Apoptosis is difficult to detect in early lesions, because apoptotic cells are rapidly and efficiently taken up by the surrounding macrophages in the plaque, a process known as efferocytosis (Hegyi, Skepper et al. 1996). An efficient efferocytosis prevents plasma membrane damage and release of toxic intracellular materials, activates in efferocytes both anti-inflammatory responses, mediated by IL-10 and TGF-β, and cell survival pathways, through Akt and NF-kB (Moore and Tabas 2011). Efferocytosis is mediated by different receptors, such as tyrosine kinase MER (MERTK) and LDLR related protein 1 (LRP1). Some studies have shown that the MERTK receptor plays a role in efferocytosis of lesional apoptotic macrophages (Thorp, Cui et al. 2008). However, other studies demonstrated that also macrophage LRP is involved in efferocytosis of apoptotic cells both in cultured macrophages and atherosclerotic lesions (Yancey, Blakemore et al. 2010). In advanced lesions, the mechanisms by which apoptotic macrophages are cleared up are compromised and this impairment contributes to plaque necrosis, thus increasing the lipid core and the vulnerability of plaques (Tabas 2010). The mechanisms responsible for defective efferocytosis are not completely known and some hypotheses have been proposed. However, exaggerated apoptosis does not seem to be the major cause, because its induction mediated by genetic manipulation in early lesions is counteracted by an efficient process of efferocytosis (Tabas 2010).

Emigration of monocyte-derived cells from inflammatory sites is generally required for resolution of inflammation, but egression from the plaque fails as atherosclerosis progresses. Emigration from the plaque has always been thought to be a characteristic of early lesions, progressively reduced once the plaque turns to a more advanced state (Gerrity and Naito 1980). However, more recent studies have focused their attention toward possible strategies to restore the capacity of monocytes to leave the plaque in advanced lesions. Different animal models have been considered for regression studies and in all of them a reduction in the macrophage number in the plaque was achieved (Rong, Li et al. 2001; Feig, Parathath et al. 2011). Multiple explanations has been provided to clarify the mechanisms involved in the reduction of macrophage content; some of them involve the upregulation of the retention factors semaphorin3E and netrin1 and of the motility factor CCR7 and the downregulation of adhesion molecules, mainly belonging to the cadherin family (Moore, Sheedy et al. 2013). Another mechanism that can explain monocyte regression might be associated to a phenotypic change of macrophages in the plaque, from the most inflammatory M1 phenotype, to the least inflammatory M2 phenotype (Feig, Parathath et al. 2011).

Taken together, all of these observations highlight the fundamental role of monocytes and macrophages in the pathogenesis of atherosclerosis thus suggesting that targeting the processes that trigger migration, inflammation, efferocytosis and egression might be a good strategy for reducing atherosclerosis progression and cardiovascular risk.

Part II

Introduction

PCSK9 was identified in 2003 as the third cause of autosomal dominant hypercholesterolemia, a genetic disorder characterized by high levels of LDL cholesterol, development of xanthomas and early CAD (Abifadel, Varret et al. 2003). While GOF mutations in PCSK9 are responsible for increased cholesterol levels, LOF mutations have the opposite effect and are associated with lower LDL cholesterol levels and a significant reduction of cardiovascular risk (Abifadel, Varret et al. 2003; Cohen, Pertsemlidis et al. 2005). Therefore, inhibition of PCSK9 action has been recently developed as a therapeutic approach for the management of hypercholesterolemia.

PCSK9 is mainly synthesized by the liver as a precursor of 75 kDa protein (pro-PCSK9) that undergoes auto-catalytic processing in the ER to form the mature structure. PCSK9 is then secreted into the circulation, where it binds to and degrades hepatic LDLR, thus increasing LDL cholesterol and promoting atherosclerosis (Zhang, Lagace et al. 2007; Zaid, Roubtsova et al. 2008). Circulating PCSK9 is present in different molecular forms: the mature 62 kDa form, the furin-cleaved 55 kDa fragment and higher molecular weight forms, mainly dimers and trimers. We and others demonstrated that around 40% of PCSK9 is associated with LDL in plasma (Kosenko, Golder et al. 2013; Tavori, Fan et al. 2013).

LDL-A is a FDA approved procedure to remove LDL cholesterol from plasma in patients with FH in whom the treatment with lipid lowering agents is not sufficient to achieve the desired goal in terms of LDL cholesterol reduction. LDL-A removes apoB-containing lipoproteins from plasma, thereby lowering LDL by more than 70%. Since hPCSK9 binds to LDL, the first aim of our study was to determine whether LDL-A could be able to affect plasma PCSK9 levels (Project I).

Moreover, although LDLR is the main target of PCSK9, some literature data suggested a possible role for PCSK9 in the degradation of other members of the

LDLR family, such as apoER2, VLDLR and LRP1 (Poirier, Mayer et al. 2008; Canuel, Sun et al. 2013).

Macrophage LDLR and LRP1 are known to exert different roles in the development of atherosclerotic plaques. In particular, our group has demonstrated that macrophage LDLR has a pro-atherogenic effect by showing, in bone marrow transplantation (BMT) studies, that the absence of LDLR reduces the accumulation of macrophages into the plaque and thus atherosclerosis progression (Linton, Babaev et al. 1999). On the contrary, the deletion of LRP1 in hyperlipidemic, atherosclerosis-prone mice increased both lesion size and inflammation, contributing to the development of the atheroma.

Since little is known about the role of PCSK9 in macrophages the second aim of our study was to determine the effect of hPCSK9 on LDLR and LRP1 levels and its contribution to the development of the atherosclerotic plaque in hyperlipidemic mice (Project II).

Methods

Patients

Six subjects with severe FH (according to Simone Broome criteria) (1991) and history of CAD undergoing LDL-A every 2 weeks at Vanderbilt University Medical Center (VUMC) were studied. Control subjects were not taking lipid-lowering agents. The study protocol was approved by VUMC's institutional review board (IRB 101615). All participants gave informed consent.

Collection of pre- and post-LDL apheresis plasma samples

Plasma from pre- and post-LDL-A treatments was used to measure lipids and proteins. In the LDL-A procedure (Figure 7), the patient is subjected to blood withdrawal via a venous access and the blood enters a machine that separate plasma. The remaining blood, containing red blood cells, leukocytes and platelets, and the plasma (pre-apheresis) exit from the separator through the bottom outlet and the side outlet, respectively. The plasma is pumped into one of two LDL absorption columns, where the apoB-containing lipoproteins are selectively absorbed by the dextran-sulfate cellulose beads. The LDL-depleted plasma (post-apheresis) exits the bottom outlet of the column and is recombined with the previously separated blood cells and returned to the patient through a second venous access. Once one column is full, flow is automatically directed toward a second column, allowing the first one to regenerate and facilitating a continuous apheresis. The column is washed and then eluted into a waste bag using lactated Ringer's solution (0.6% Sodium chloride, 0.31% sodium lactate, 0.03% potassium chloride and 0.02% calcium chloride) and 5% NaCl (column eluate).

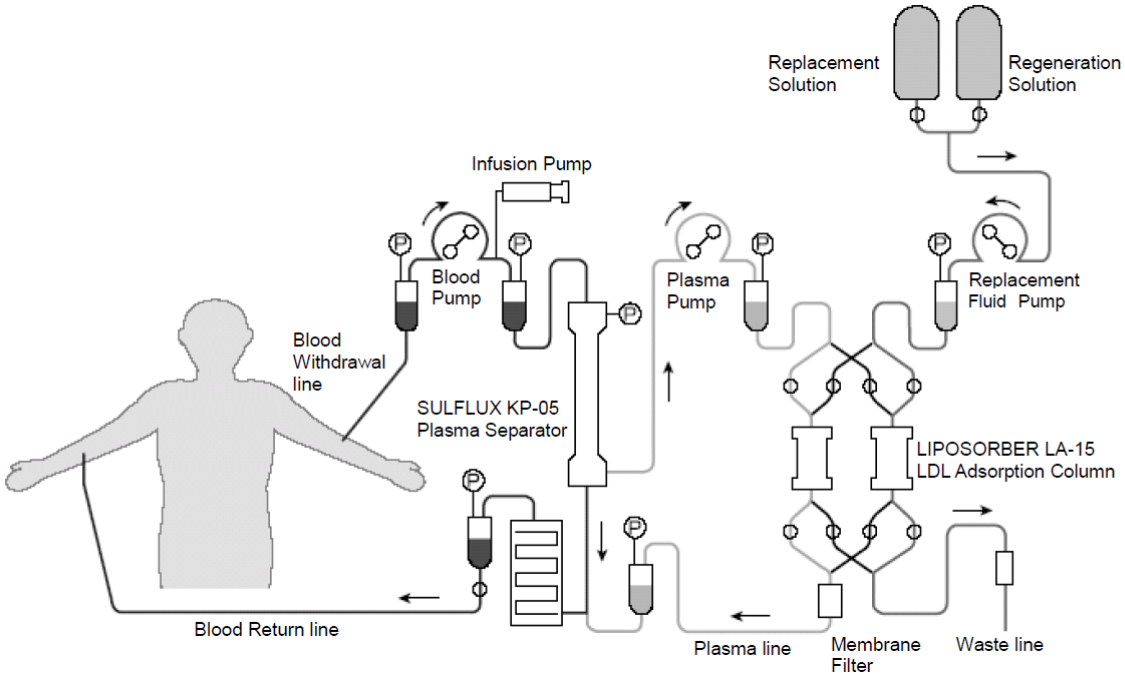


Figure 7. The Liposorber system.

Experimental dextran sulfate column

Using commercial-grade dextran-sulfate cellulose beads (from an unused Liposorber® column), we gravity-packed 1 mL of beads into a column for 30 minutes and we washed with 5 volumes of 0.9% NaCl. Six mL of plasma were loaded on the column at 0.2 ml/minute; 6 ml of eluate were collected. Sequentially, using 2 volumes of Ringer's solution, 2 volumes of 5% NaCl and 2 volumes of Ringer's solution (total volume of 6 mL) were used to elute LDL and proteins from the column.

Plasma PCSK9 levels

The levels of human PCSK9 in plasma of FH patients pre- and post-LDL-A, column eluate, ultracentrifuge fractions and purified GST-tag protein were

determined by ELISA kits, as recommended by the manufacturer (MBL-International). A more detailed description regarding hPCSK9 measurement can be found in the paragraph about serum analysis.

Generation of pcDNA-PCSK9-GST construct

DNA vector pGEX-2T (GE Healthcare) was used as a template to PCR the GST encoding DNA fragment with a stop codon TGA at the end. The following primers were used: 5'gacaattctgcagattcccctatactagg3' and 5'gaccaaggcgcaacttaggtcgtgtcaccg3'. The plasmid pcDNA-PCSK9 was digested by EcoRV and both the PCR product and the digested pcDNA-PCSK9 DNA were ligated using In-FusionTM 2.0 CF Dry-Down PCR cloning Kit. The DNA construct pcDNA-PCSK9-GST was confirmed by DNA sequencing.

Recombinant PCSK9 with Glutathione S-transferase (GST) affinity tag

HEK293T cells were transfected with pcDNA-PCSK9-GST, using a Mammalian Transfection System from Promega, as previously described (Fan, Yancey et al. 2008) and the full-length PCSK9 protein with a C-terminal GST tag was purified from the culture medium using a glutathione-agarose column and protein concentrations were then determined by Lowry assay.

Plasma lipoprotein separation

Lipoproteins were isolated from plasma of FH patients pre- and post-LDL-A by natural gradient media (Optiprep) using a TLN100 rotor and OptiSeal tubes at 90,000 rpm for 2.5 hours.

Mice

WT, apoE $^{-/-}$ and LDLR$^{-/-}$ mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed at the Vanderbilt University Medical Center. All animal experiments were carried out in compliance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Generation of hPCSK9 transgenic Mice

The generation of hPCSK9 transgenic (hPCSK9 tg) mice was done, as previously described by Fan and colleagues (Fan, Yancey et al. 2008). Briefly, the pc3.1 DNA-hPCSK9 DNA construct was digested by BglII and PsiI and the yielded fragment of 3800-bp was used for transgenic injection. The injection was performed by Vanderbilt Transgenic mouse/Embryonic Stem Cell Shared Resource. Transgenic expression was confirmed by Southern Blot for DNA insertion and PCR. Transgenic mice for hPCSK9 were then backcrossed to apoE $^{-/-}$or LDLR $^{-/-}$ mice to generate hPCSK9 tg/apoE$^{-/-}$or hPCSK9 tg/LDLR$^{-/-}$, respectively.

Atherosclerosis analyses

For experiments comparing atherosclerosis in hPCSK9 tg/apoE$^{-/-}$, apoE$^{-/-}$, hPCSK9 tg/LDLR$^{-/-}$ and LDLR$^{-/-}$, 8 to 10 week old female mice were fed a Western-type diet for 8 weeks and then the extent of atherosclerosis was examined.

Bone Marrow Transplantation (BMT)

As summarized in Figure 8, recipient apoE$^{-/-}$ and LDLR$^{-/-}$ mice were lethally irradiated (9 Gy) using a cesium γ source and transplanted with 5

million bone marrow cells from hPCSK9 tg/apoE$^{-/-}$ or apoE$^{-/-}$ and hPCSK9 tg/LDLR$^{-/-}$ or LDLR$^{-/-}$ mice, respectively, through injection in the retro-orbital venous plexus, as previously described (Linton, Atkinson et al. 1995; Fazio, Babaev et al. 1997). After 4 weeks, all mice were placed on a Western-type diet for 8 weeks and then the extent of atherosclerosis was examined.

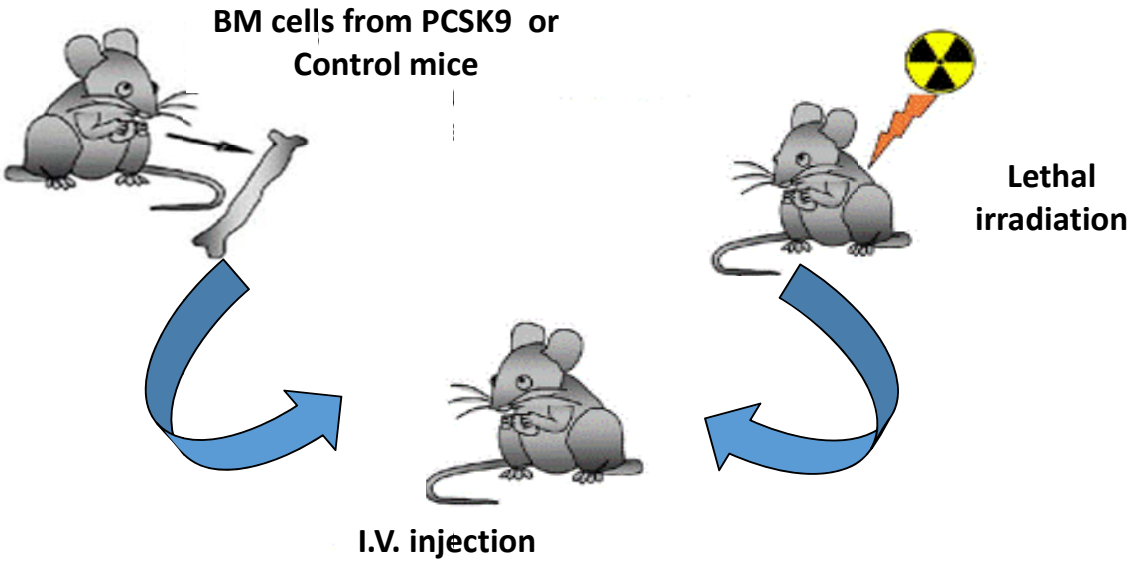


Figure 8. Summary of BMT procedure

Serum Analysis

Total serum cholesterol (TC) and triglyceride (TG) levels were determined by enzymatic colorimetric assay. Apo-b levels were measured by ELISA, as recommended by the manufacturer. Mouse lipoprotein profile was determined by fast protein liquid chromatography (FPLC). hPCSK9 and mPCSK9 levels were determined by ELISA (MBL international), with a measured 5% cross reactivity between mPCSK9 and hPCSK9 within the kit's linear range. Briefly, serum samples were diluted with the appropriate buffer and 100 µl of solution were transferred into each well and incubated for 1 h at room temperature, shaking. After washing 5 times, 100 µl of HRP conjugated

detection antibody were added for 1 h at room temperature. At the end of incubation, each well was washed 5 times and 100 µl of substrate reagent were transferred for 15 minutes in the dark. Then, 100 µl of stop solution were added and the absorbance was measured at a dual wavelengths of 450/540 nm using a spectrophotometric micro plate reader. Results were expressed as ng/ml.

Aorta lesion analysis

Frozen sections of 10 µm thickness were taken in the region of the proximal aorta starting from the end of the aortic sinus and for 300 µm distally, as previously described (Linton, Atkinson et al. 1995). Sections were stained with Oil Red-O and counterstained with hematoxylin. Quantitative analysis of lipid-stained lesions was performed on 15 alternate cryosections. Oil red-O-stained cross-sections of the proximal aorta were analyzed using the KS300 imaging system (Kontron Elektronik GmbH), as described previously (Linton, Atkinson et al. 1995).

Immunocytochemistry

For lesion staining of hPCSK9 and MOMA-2, 5 µm cryosections of the proximal aortas were sent to the laboratory of Rinat-Pfizer Inc (South San Francisco, CA). To analyze lesion content of the pro-inflammatory monocyte marker Ly6C, serial 5-µm cryosections of the proximal aortas were fixed in acetone for 20 minutes, washed with PBS and blocked with background buster solution (Innovex Biosciences) for 1 h at 37°C. Then the sections were incubated overnight at 4°C using rat anti-mouse Ly6C biotin (1:50; BD Pharmingen #557359). After washing with PBS, the sections were treated with Streptavadin-AlexaFluor 488 (Invitrogen #S11223) at a dilution of 1:150 for 1 h at 37°C. Then the sections were washed in PBS and mounted with

Vectashield containing DAPI (Vector Labs) to stain the nuclei. Ly6C^{high} positive and negative cells in the lesion were then counted in 4 sections per mouse using Image J software and the results were expressed as percentage of positive cells.

Flow Cytometry Analyses of Blood and Spleen Ly-6C^{high} Monocytes

hPCSK9 tg/apoE$^{-/-}$ (n=3), apoE$^{-/-}$ (n=3), hPCSK9 tg/LDLR$^{-/-}$ (n=6), LDLR$^{-/-}$ (n=7) mice were fed a Western-type diet for 8 weeks. Mice were then sacrificed and spleens were collected and weighted. Spleens were homogenized by disruption in sterile PBS pH 7.4 through a 70µM mesh screen. Splenocytes were then centrifuged to pellet the cells, counted and subsequently suspended at a concentration of 10 million cells/ml in PBS/2%FBS pH 7.4. One hundred microliters of splenocytes (1 million cells) per test mouse were added to 5ml FACS tubes containing 2µl Fc Block (Pharmingen Catalog #553142) and mixed gently by pipetting. Then 1µl each of rat anti-mouse CD90.2, B220, GR1 (Pharmingen #553003, #553087 and 553126, respectively) and NK cells (Caltag RM7901), all with the FITC fluorochrome tag was added to each sample, as well as rat anti-mouse CD11b-PE (Pharmingen #557397) and rat anti-mouse Ly6C conjugated with biotin (Pharmingen #557359). Cells were then incubated for 20 minutes at room temperature in the dark before being diluted to 4ml in PBS/2%FBS and pelleted by centrifugation. Cells were then suspended in 100µl PBS/2%FBS before the addition of streptavidin-linked AlexaFluor 647 (Invitrogen #S32357) for other 20 minutes at room temperature in the dark. Cells were washed and the pellets were suspended in 1X Permaflow (Invirion # 55001) and incubated overnight at 4°C to fix the cells and lyse red blood cells. After three to four washes with PBS/2%FBS, cells were suspended in PBS for analysis in flow

cytometry. Cells were analyzed and gated for monocytes, defined as CD90.2, B220, GR1-FITC-negative, CD11b-PE-positive cells. These cells were then gated for high levels of Ly6C biotin-streptavidin-AlexaFluor 647.

Collection of Macrophages from the peritoneal cavity

Macrophages were collected in PBS three days after peritoneal injection of 5% thioglycollate in all mice. The collected cell suspension was centrifuged at 1200 rpm for 10 minutes at 4°C and suspended in PBS for cell counting. Cells were used for Western blot and flow cytometry analysis of LDLR and LRP1 levels.

Immunoprecipitation

For immunoprecipitation (IP) of hPCSK9, livers from hPCSK9 tg mice were collected and homogenized in IP buffer. Samples were centrifuged at 10000 rpm for 10 minutes at 4°C and the supernatants were used for the analysis. Protein concentration was measured by Lowry and 500μg of protein were used for IP. Lysates were precleared by incubating with protein G beads (New England Biolabs) for 1 hour at 4°. Then 5 μg of rat anti-hPCSK9 (R&D) or IgG were added to the precleared lysates and incubated overnight. The antibody/PCSK9 complex was pulled down using protein G beads, washed, and eluted with IP buffer. Samples were incubated at 70°C for 5 minutes and loaded onto a 4-12% Bis-Tris gel (Life Technologies).

Western Blotting

Cells were lysed in RIPA buffer containing a cocktail of protease inhibitors and protein concentration was measured by Lowry (Lowry, Rosebrough et al. 1951). Samples were loaded onto NuPage 4-12% Bis-Tris precast gels (In Vitrogen) for electrophoresis and the size separated proteins

were transferred onto nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with blocked with 3% skim milk in 10 mM PBS pH 7.4, 0.05% Tween 20 for 1 h at 37°C before adding the primary antibodies against LDLR (1:1000, Abcam), LRP1 (1:5000, Novus biological), GAPDH (1:3000, Novus biological) and β-actin (1:2000, Abcam) overnight at 4°C. Membranes were washed and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. Signal was detected using a mixture of luminol, p-coumaric acid and hydrogen peroxide in 100mM Tris pH 8.5. Intensity quantification of the bands was obtained using Image J software and normalized to β-actin or GAPDH.

Flow Cytometry analysis of LDLR and LRP1 levels

Single-cell suspensions of MPM were blocked for 15 minutes at RT in a 1:50 dilution of Fc receptor block (BD Biosciences) in FACS buffer (1X HBSS, 1% BSA, 4.1 mM sodium bicarbonate, and 3 mM sodium azide). To stain LRP, we fluorescently labeled 5A6 LRP clone (gift from Dr. Dudley Strickland, University of Maryland) with APEX Alexa Fluor-488 antibody labeling kit (Invitrogen), according to manufacturer protocol. The fluorescently labeled LDLR and LRP1 antibodies were diluted 1:100 in FACS buffer and incubated with cells for 45 minutes at 4°C. Mouse IgG2b-Alexa Fluor 488 or RatIgG2b-PE were used as isotype control (BD Biosciences). Labeled cells were analyzed on a MACSquant seven-color flow cytometer (Miltenyi Biotech) and data analyzed with FlowJo software (Tree Star).

Statistical Analyses

All of the statistical analyses were carried out using GraphPad Prism Software. Differences between two groups of values were compared by Student's paired t-test. Analysis of variance (ANOVA) and Bonferroni's post-

hoc test were used to compare the mean values among more than two groups. $p<0.05$ was considered to be significant. Results are presented as means $\pm$ SD or as percentage $\pm$ CV.

Project I
“Does LDL-A affect plasma PCSK9 levels?”

PCSK9 has been found to bind LDL in both murine and human plasma and the percentage of PCSK9 bound to LDL in human plasma is about 30 to 40% (Fan, Yancey et al. 2008; Kosenko, Golder et al. 2013; Tavori, Fan et al. 2013). LDL-A is a procedure that removes huge amounts of LDL from the circulation (McGowan 2013). Therefore, we expected PCSK9 to be removed together with LDL during LDL-A. Our study was performed in 6 patients with FH undergoing LDL-A with dextrose sulfate cellulose columns at the VUMC. We collected samples at three consecutive cycles of treatment. In Table 3, we summarized some characteristics of the recruited patients, such as age, height, weight, body mass index (BMI), race and a list of the medications taken by each subject.

	Age	Sex	Height (cm)	Weight (Kg)	BMI	Race	List of Medication
Patient #1	17	Male	183	63.5	19.0	Caucasian	Zetia, Metoprolol succinate, Aspirin, Crestor, Niaspan, Cozaar
Patient #2	61	Female	170	90.2	31.2	Caucasian	Aspirin , Plavix, Atenolol, Furosemide , Advair Diskus, Cetirizine, Fluticasone, Potassium chloride, Prevacid, Cyanocobalamin, Alamast Ophthalmic Soln, Medtronic insulin pump, INSPRA, Gabapentin, Ketoconazole, Diclofenac sodium, Amlodipine, Lunesta, Humalog, Fish Oil, Astepro, Docusate sodium.
Patient #3	62	Female	145	69.4	33.0	Caucasian	Ambien, Sertraline, Hydrocodone-acetaminophen, Buspirone, Amlodipine, Livalo.
Patient #4	21	Female	160	99.3	38.8	Caucasian	Lopressor, Niaspan, WelChol, Zetia, Amlodipine, Aspirin, Claritin, Crestor, Mirena, Senna, Warfarin Furosemide, Spironolactone, Oxycodone-acetaminophen.
Patient #5	56	Female	157	42.1	17.1	Caucasian	Premarin patch, Aspirin, Atorvastatin, Hyoscyamine sulfate, Lactobacillus acidoph & bulgar, Lactulose, Levofloxacin, Levothyroxine, Lisinopril, Metoprolol tartrate, Nitroglycerin, Nystatin, Omeprazole, Sennosides, Simethicone, Warfarin, Zolpidem, Plavix, Protonix, Donnatal TID, Zetia, Crestor, Carvedilol.
Patient #6	57	Male	168	114.8	40.7	Caucasian	Endocet, Patanol, Fluticasone, Flovent HFA, Vimovo, Miralax, Diazepam, Nexium, Metoprolol tartrate, Crestor, Simethicone, Aspirin, Soma, Nitroglycerin.

Table 3. Characteristics of the patients with FH recruited for LDL-A.

As shown in Table 4, before LDL-A treatment, patients presented very high cholesterol levels, primarily due to exaggerated LDL levels. LDL-A drastically removed LDL cholesterol, lowering its levels by 77±4%. The procedure also reduced TG, HDL and Lp(a) by 49±13%, 18±2% and 75±15%, respectively.

	TC (mg/dL)		TG (mg/dL)		HDL (mg/dL)		LDL (mg/dL)		Lp(a) (mg/dL)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Patient #1	342±35	117±7	121±65	47±13	38±3	30±2	279±27	78±5	N/A	N/A
Patient #2	315±7	96±3	217±31	110±17	45±2	35±0	226±12	39±3	2.9±1.8	0.1±0.1
Patient #3	308±43	116±5	229±96	102±14	55±14	45±12	207±38	50±49	5.3±1.2	0.2±0.1
Patient #4	265±21	80±8	83±12	53±21	36±2	29±2	213±16	40±5	2.9±0.3	0.1±0.1
Patient #5	337±27	127±13	132±24	92±14	54±4	42±5	257±29	64±17	17±4	6±4
Patient #6	244±0	65±8	177±102	91±46	36±6	28±4	173±14	19±2	6.1±4.4	0.1±0.1
% reduction		65±4		49±13		18±2		77±4		75±15
p Value (pairwise)		0.0000015		0.004		0.03715		0.0000082		0.0096

Table 4. Plasma lipids and protein levels of 6 patients with FH, pre- and post-LDL-A. Values are expressed as mean±SD or percentage±coefficient of variation (n=3 treatment cycles). *p* values were calculated using pairwise analysis with Prism 4 software. TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; Lp(a), lipoprotein(a).

On the contrary, no changes in plasma levels of other control proteins, such as albumin, creatinine, and CK-MB were found after LDL-A (Table 5).

	Albumin (g/dL)		Creatinine (mg/dL)		CK-MB (ng/ml)		PCSK9 (ng/ml)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Patient #1	4.1±0.3	3.8±0.2	0.61±0.05	0.66±0.03	1.04±0.38	1.08±0.19	496±38	251±21
Patient #2	4.0±0.1	3.6±0.1	0.87±0.1	0.8±0.1	1.5±0.1	1.3±0.1	394±42	171±10
Patient #3	3.9±0.1	3.9±0.1	0.74±0.2	0.55±0.01	0.83±0.2	0.7±0.2	362±18	184±17
Patient #4	3.6±0.0	3.4±0.1	0.93±0.04	0.83±0.02	1.14±0.2	1.14±0.1	522±54	224±19
Patient #5	N/A	N/A	N/A	N/A	N/A	N/A	452±35	230±13
Patient #6	3.9±0.2	3.7±0.3	1.1±0	1.0±0	0.9±0.2	1.1±0.1	395±17	186±13
% reduction		6±3		8±7		8±12		52±5
p Value (pairwise)		0.08		0.19		0.80		0.000034

Table 5. Plasma protein levels of 6 patients with FH, pre- and post- LDL-A. Values are expressed as mean±SD or percentage±coefficient of variation (n=3 treatment cycles). *p* values were calculated using pairwise analysis with Prism 4 software. CK-MB, creatine kinase MB; PCSK9, pro-protein convertase subtilisin/kexin type 9.

As hypothesized, LDL-A significantly reduced PCSK9 levels by 52±5% (Table 5 and Figure 9A). Figure 9B shows a representative Western Blot of PCSK9 levels in plasma pre- and post- LDL-A; both the mature 62 kDa form and the 55 kDa furin-cleaved form were reduced. Interestingly, the reduction in total PCSK9 was much higher than the one predicted on the basis of LDL reduction alone. In fact, taking into account that around 80% of LDL is removed and that about 40% of PCSK9 binds LDL, the estimated reduction should be approximately 32%.

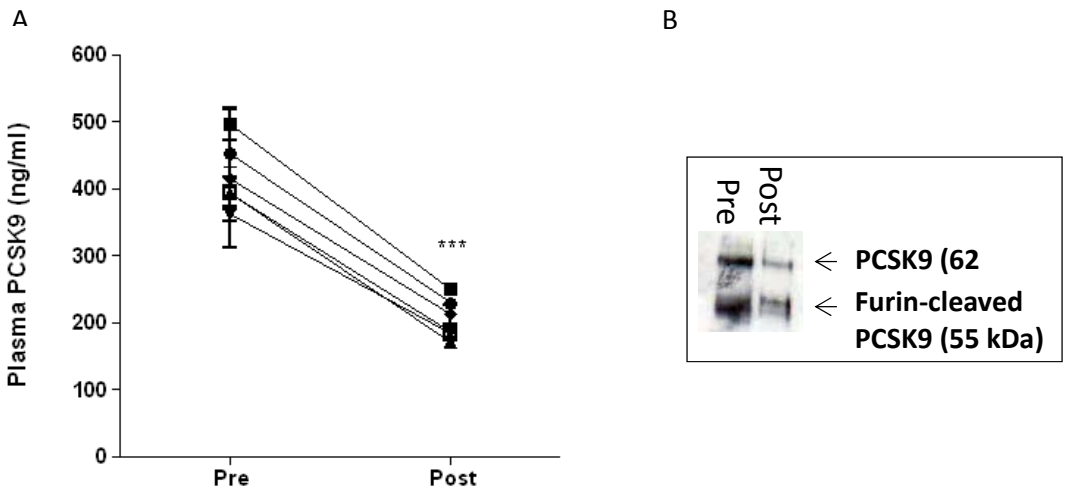


Figure 9. (A) Plasma PCSK9 levels in 6 patients with FH pre- and post- LDL-A. (B) Representative Western Blot of PCSK9 from pre- and post-LDL-A samples ***P<0.001.

We also measured PCSK9 variations during 3 consecutive treatments, each one carried out every 2 weeks. PCSK9 drastically dropped down at the end of each cycle, and two weeks after, the levels returned to those of pre-LDL-A in all 6 patients (Figure 10).

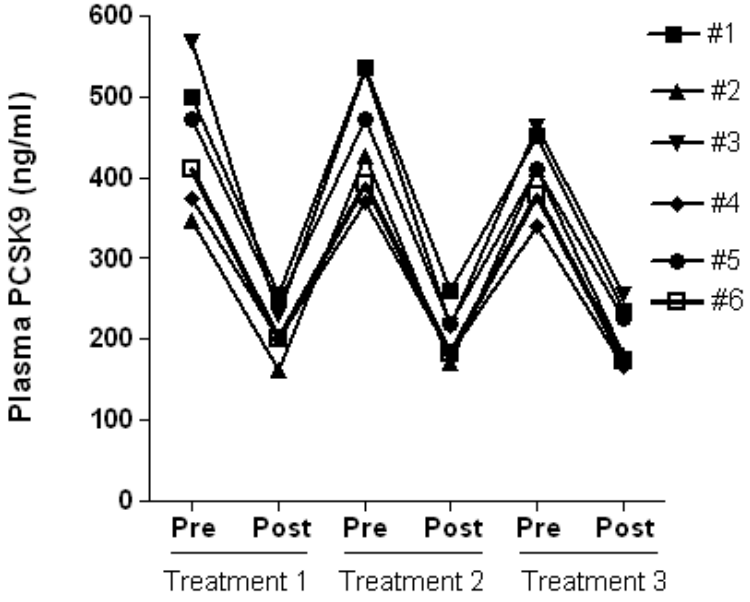


Figure 10. Plasma PCSK9 levels pre- and post- LDL-A in 6 patients with FH during 3 consecutive treatments.

As shown in Figure 11, the decrease in PCSK9 observed after LDL-A was significantly correlated with the LDL reduction achieved after the treatment ($p=0.0322$; $r^2=0.26$).

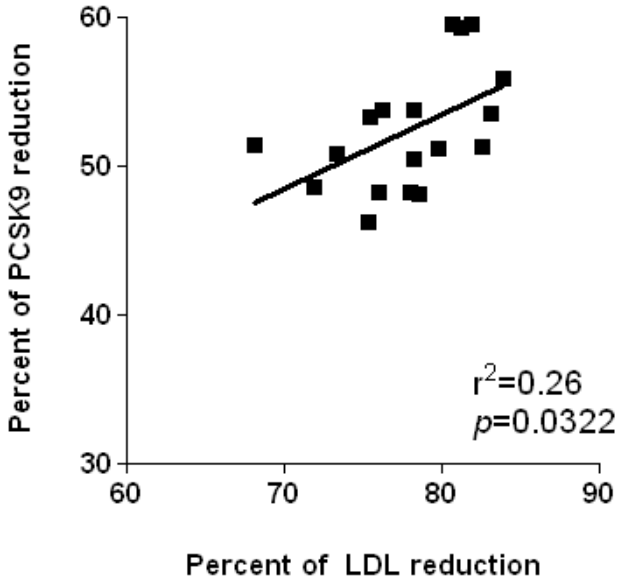


Figure 11. Correlation between LDL cholesterol and PCSK9 reduction after LDL-A in 6 patients with FH. Linear regression analysis was performed with Graph Pad PRISM 4.

Since LDL-A did not only reduce LDL cholesterol, but also TG and HDL, we performed a linear regression analysis to investigate whether PCSK9 reduction was associated with a change in other lipid parameters. No correlation was found between PCSK9 reduction and either TG or HDL variations after LDL-A (Figures 12 and 13).

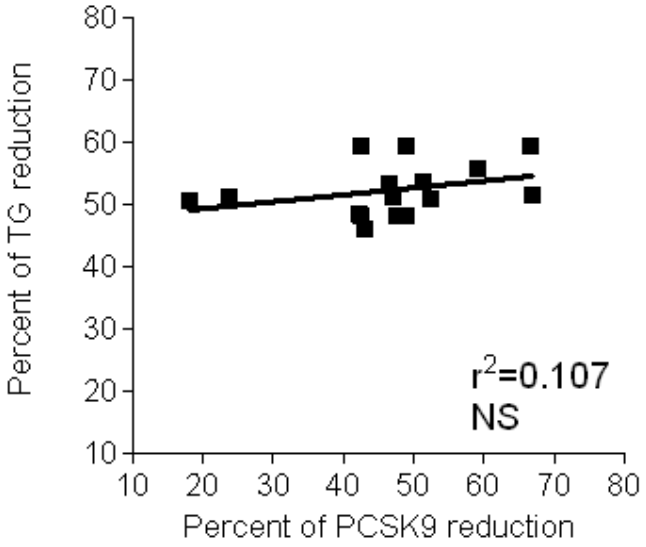


Figure 12. Correlation between TG and PCSK9 reduction after LDL-A in 6 patients with FH. Linear regression analysis was performed with Graph Pad PRISM 4.

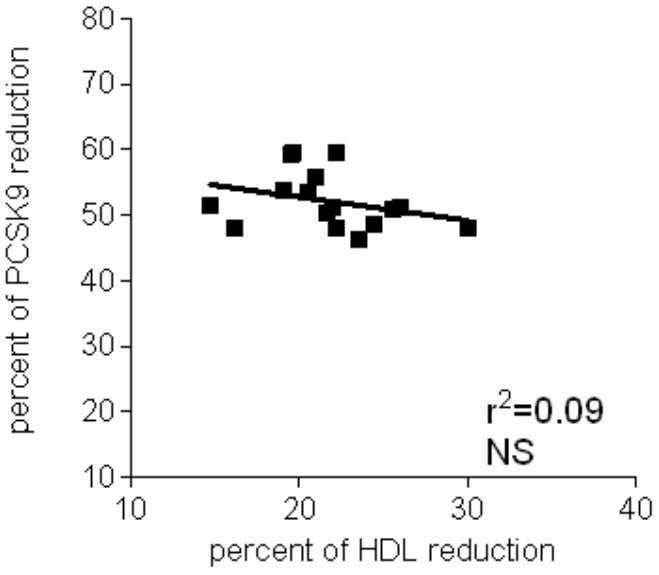


Figure 13. Correlation between HDL and PCSK9 reduction after LDL-A in 6 patients with FH. Linear regression analysis was performed with Graph Pad PRISM 4.

We previously showed that PCSK9 is present in both LDL and apoB-free fractions (Tavori, Fan et al. 2013). Since the decrease in PCSK9 after LDL-A was greater than expected, we decided to measure the degree of PCSK9 reduction in the different plasma fractions separated by ultracentrifugation using natural gradient media. Figure 14 shows a representative picture of the different fractions separated by ultracentrifugation from the plasma of one of the patients before and after LDL-A and of the eluate from the LDL-A column. The purity of each fraction is shown in figure 15.

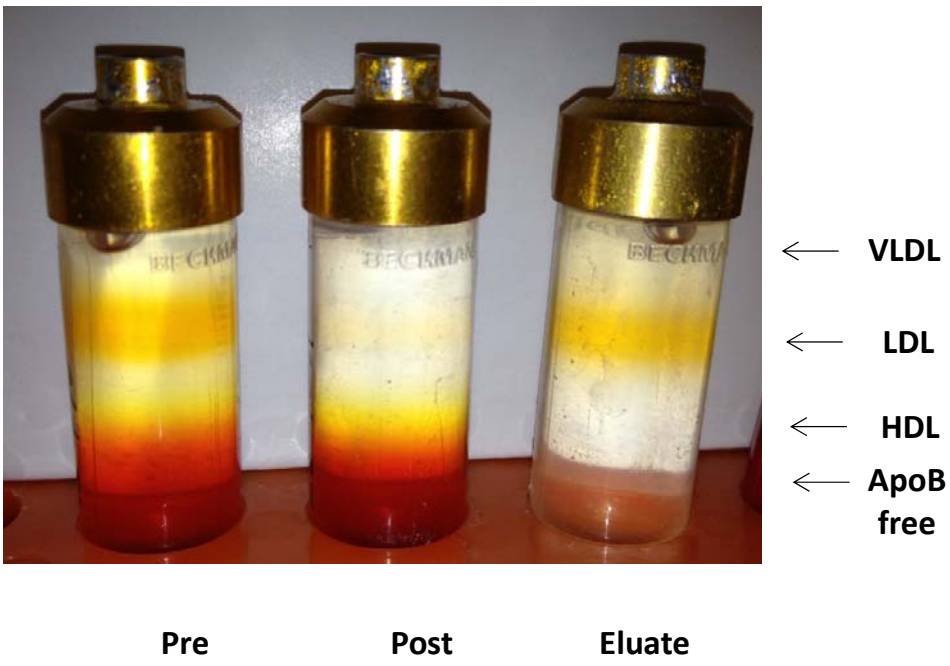


Figure 14. Representative picture of the fractions separated by ultracentrifugation from the plasma of one of the patients with FH, pre- and post- LDL-A and from the column eluate.

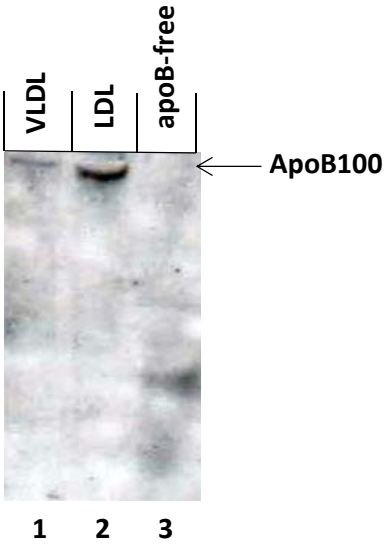


Figure 15. Western Blot of apoB in pre-LDL-A plasma fractions, isolated by ultracentrifugation using natural gradient media. Lane 1: VLDL, lane 2: LDL, lane 3: apoB-free fraction.

We found that the percentages of PCSK9 removal from LDL-bound and apoB-free fractions were 81±11% and 48±14%, respectively (Figure 16). Thus the combined reduction completely explains the high removal of PCSK9 from the circulation. Moreover, in the column eluate we recovered 82±11% of the total PCSK9 removed from the circulation.

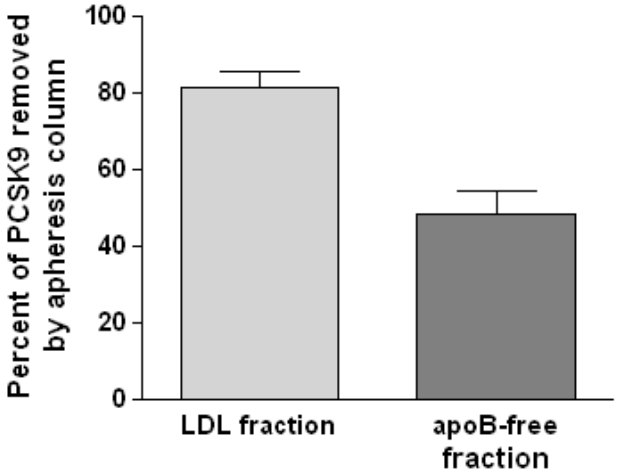


Figure 16. Percentage of PCSK9 removal from LDL and apoB-free fractions of plasma after LDL-A (n=6).

Our group and others have previously demonstrated that PCSK9 binds to LDL, but not to VLDL in serum from WT mice subjected to ultracentrifugation (Fan, Yancey et al. 2008) (Sun, Samarghandi et al. 2012; Tavori, Fan et al. 2013). Similar results were obtained in human plasma samples from healthy subjects (Kosenko, Golder et al. 2013) (Tavori, Fan et al. 2013). Western blot analysis of PCSK9 in plasma from FH patients showed that no PCSK9 binds the VLDL fraction (Figure 17, lanes 1, 2). Moreover, only the mature 62 kDa form of PCSK9 associates to LDL (Figure 17, lanes 3,4), whereas the apoB-free fraction contains higher molecular weight forms, probably dimers or trimers, and the 55 kDa furin-cleaved form (Figure 17, lanes 5,6).

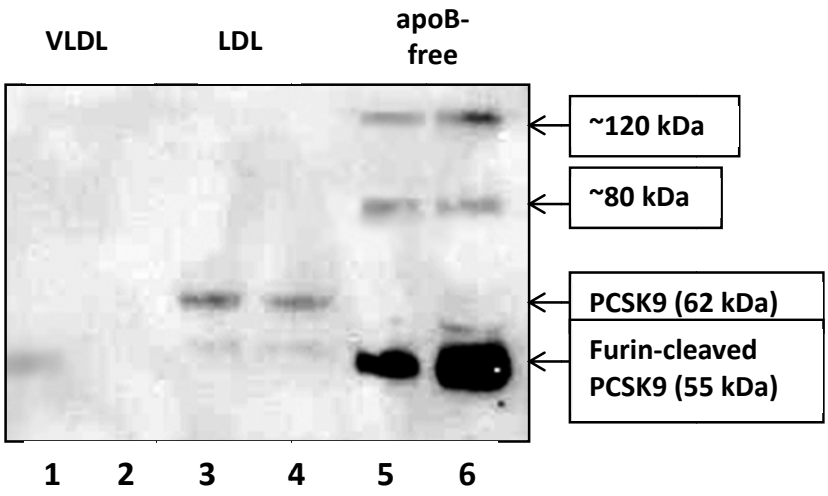


Figure 17. PCSK9 Western blot of plasma fractions isolated by ultracentrifugation from 2 FH patients. Lanes 1, 2: VLDL; lanes 3, 4: LDL; lanes 5, 6: apoB-free (2-fold concentration for apoB-free fractions to better visualize the higher molecular weight bands).

To investigate on PCSK9 removal from each fraction, we loaded plasma from LDL-A patients on a scaled-down dextrose sulfate cellulose beads column. As shown in Figures 18 and 19A, the result was a 61±4% reduction of cholesterol, principally LDL cholesterol, and a 51±7% removal of PCSK9. When we loaded the LDL purified fraction on the column we found that 68±16% of

cholesterol and 61±5% of PCSK9 were removed. The removal of cholesterol from the apoB-free fraction was much lower, around 17±9%, mainly HDL; also PCSK9 was removed in lower percentages, 38±6%.

We also produced and purify a GST-tag PCSK9 in transfected HEK293T cells and we loaded purified PCSK9 dissolved in a saline solution (pH=7.4) on the dextran sulfate column. Figure 19A shows that GST-tag PCSK9 was not removed from the solution and Figure 19B confirmed the purity of the GST-tagged PCSK9 produced.

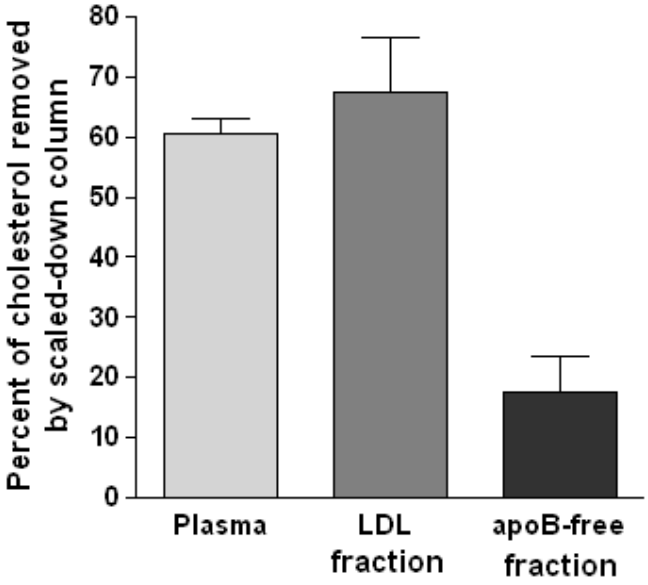


Figure 18. Percentage of total cholesterol removal from plasma, LDL and apoB-free fractions obtained using a scaled-down, dextran sulfate cellulose beads column (n=3).

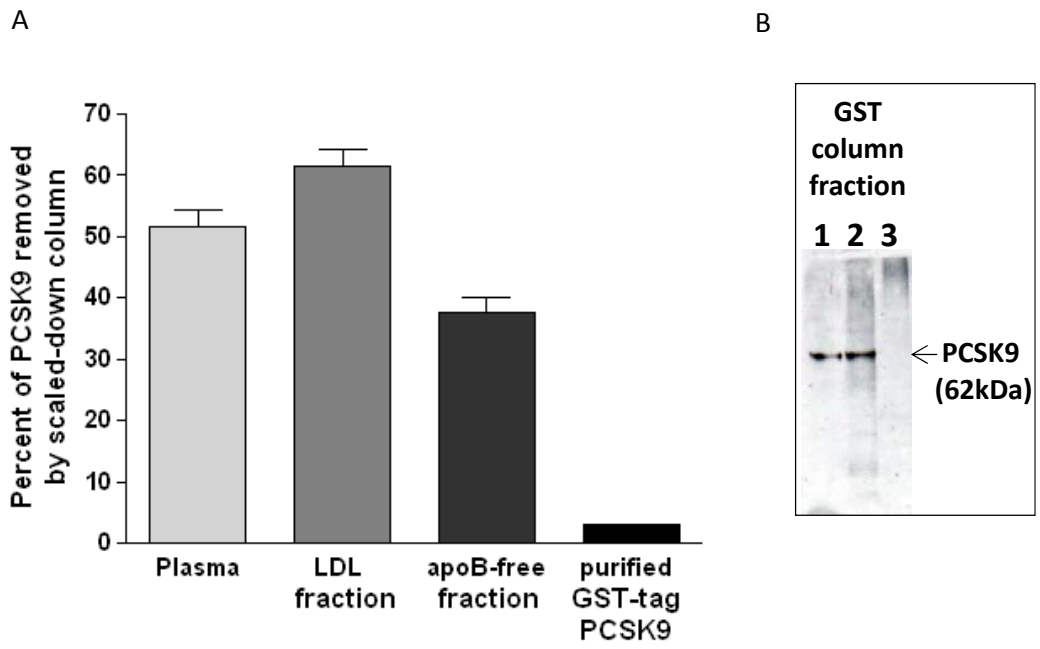


Figure 19. (A) Percentage of PCSK9 removal from plasma, LDL and apoB-free fractions and purified GST-tagged PCSK9 obtained using a scaled-down dextran sulfate cellulose beads column (n=3). (B) PCSK9 Western blot of GST-tagged fractions after elution from a glutathione agarose column of media collected from HEK293T cells transfected with pcDNA-PCSK9-GST.

Literature data show that HoFH patients have higher PCSK9 levels compared to HeFH whose levels are in turn higher than normal subjects (Raal, Panz et al. 2013). We decided to measure PCSK9 levels in patients with extremely low cholesterol levels due to inherited diseases and we compared them to normal controls. As shown in figure 20, the levels of PCSK9 were 29% lower in low cholesterol patients compared to normal controls.

Moreover, we measured the levels of LDL-bound PCSK9 and PCSK9 associated to the apoB-free fraction in normal subjects and we compared them with those of LDL-A patients before undergoing the procedure. FH patients have much higher LDL-bound levels of PCSK9 compared to control (262$\pm$54 ng/ml in FH vs 89$\pm$9 ng/ml in controls, Figure 21A). Also the ratio LDL-bound/apoB-free PCSK9 is significantly higher in FH (40$\pm$7 % in FH vs 32$\pm$3 % in controls, Figure 21B).

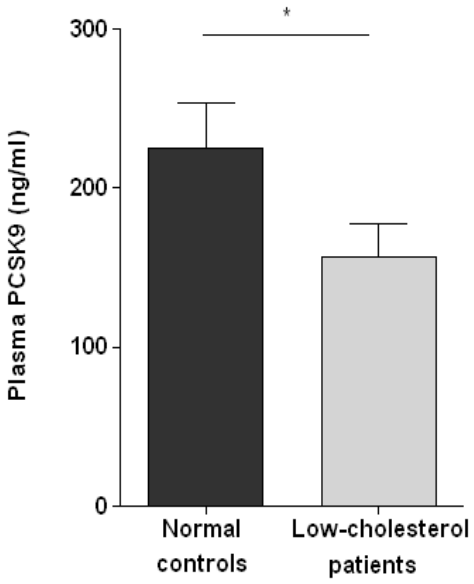


Figure 20. Plasma PCSK9 levels in normal controls (n=8) and low cholesterol patients (n=4).

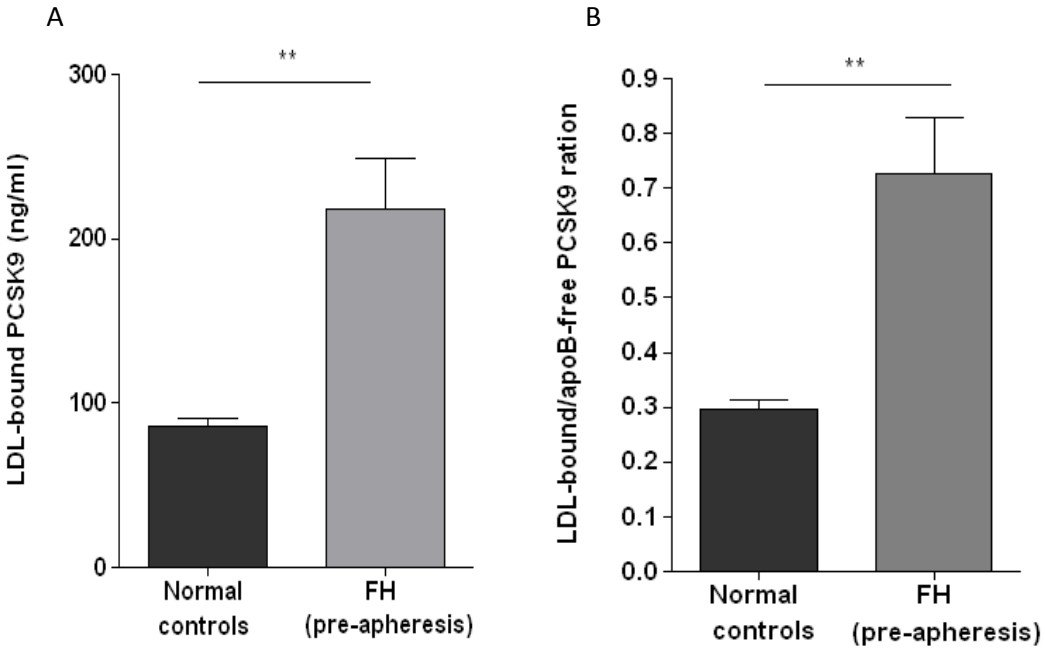


Figure 21. (A) Plasma PCSK9 levels in LDL fractions from normal controls (n=4) and FH patients before LDL-A (n=6). (B) LDL-bound PCSK9 and PCSK9 apoB-free ratio in normal controls (n=4) and FH patients before LDL-A (n=6).

Project II
“Does PCSK9 affect atherosclerotic plaque?”

To test the effect of hPCSK9 expression on atherosclerosis we developed transgenic mice expressing hPCSK9 in multiple tissues (Tavori, Fan et al. 2013). We first demonstrated that both mPCSK9 and hPCSk9 are expressed at the mRNA level and secreted in the culture medium by murine peritoneal macrophages (MPM) (Figures 22A and 22B).

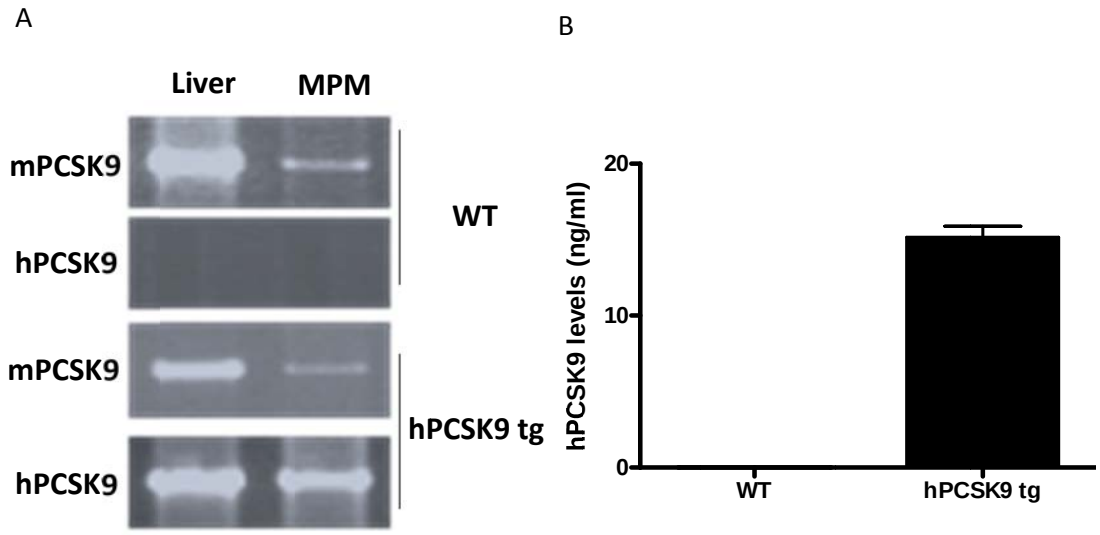


Figure 22. (A) mRNA levels of mPCSK9 and hPCSK9 in livers and murine peritoneal macrophages (MPM) from WT and hPCSk9 tg mice, measured by PCR (B) hPCSK9 secretion in medium by MPM, measured by ELISA.

As previously demonstrated in both hepatocytes and small intestine (Tavori, Fan et al. 2013), hPCSK9 reduced the levels of LDLR by 66% in transduced J774A.1 cells, a murine macrophage cell line; the D374Y-PCSK9 mutant further reduced LDLR levels by 79% (Figure 23A). Moreover, Western blot analysis showed that hPCSK9 reduced the levels of LDLR both in inflammatory (thioglycollate elicited) or resident MPM and that the effects on LDLR levels were much more evident in resident macrophages compared to the elicited ones (Figure 23B). Flow cytometry analysis displayed that the

surface levels of LDLR were strongly affected by hPCSK9; the quantified reduction is 52% (Figure 24A and 24B).

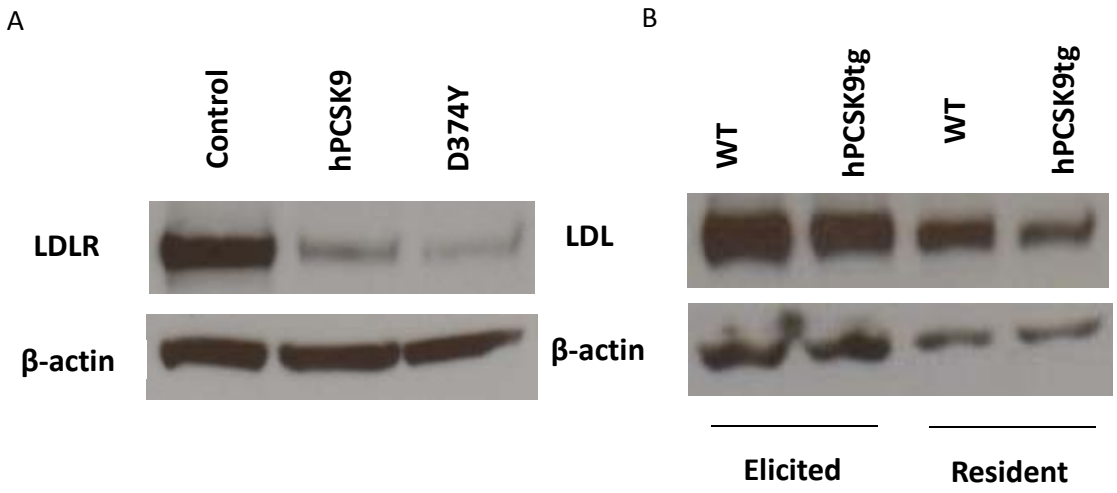


Figure 23. Western blot analysis of LDLR levels in (A) J774.1 cells transduced with hPCSK9 or the GOF mutant D374Y-PCSK9 or (B) elicited and resident MPM from WT and hPCSK9 tg mice. β-actin was chosen as housekeeping protein.

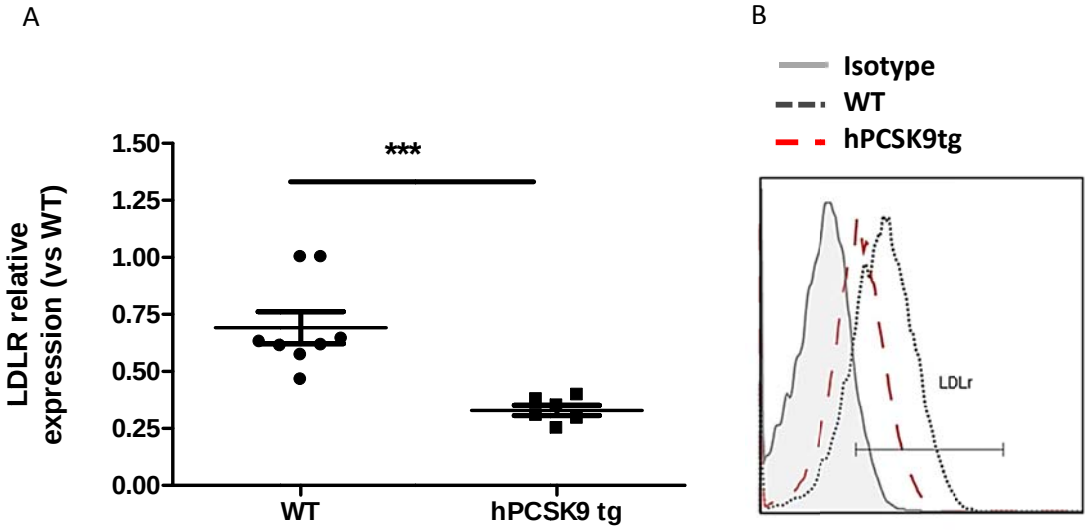


Figure 24. (A) Flow cytometry analysis of surface LDLR levels in elicited MPM from WT and hPCSK9 tg mice (B) Representative diagram of surface LDLR expression analyzed by flow cytometry. ***p<0.001.

On the contrary, culturing macrophages in medium supplemented with either FBS or LPDS completely abolished the degrading capacity of hPCSK9 on LDLR (Figure 25A and 25B).

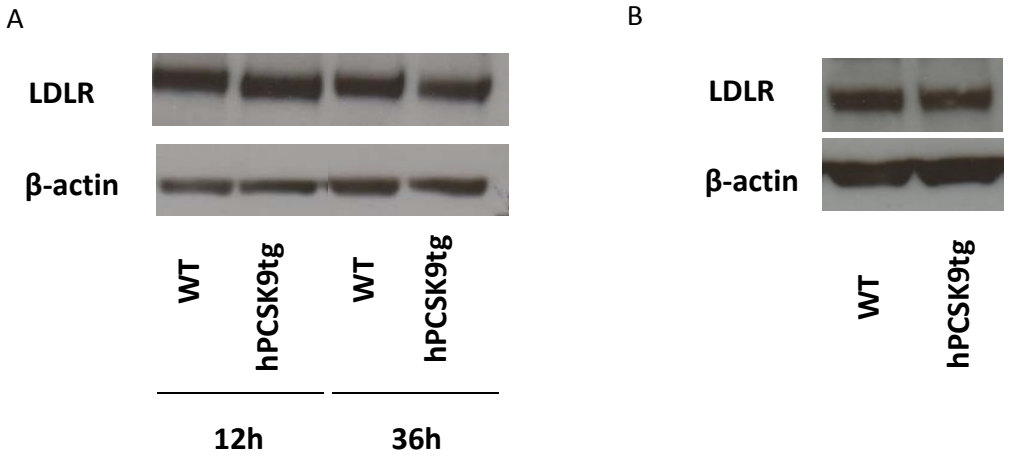


Figure 25. Western blot analysis of LDLR levels in MPM from WT and hPCSK9 tg mice. Cells were collected and cultured for either 12 or 36 hours in medium supplemented with FBS (A) or LPDS (B). β-actin was chosen as housekeeping protein.

Co-immunoprecipitation experiments with livers from hPCSK9 tg mice confirmed a direct interaction of hPCSK9 with LDLR (Figure 26). Moreover, we found that hPCSK9 was able to interact with LRP1, another member of the LDLR gene family, involved in the development of atherosclerosis through its effects on macrophage inflammatory responses and promotion of cell survival (Overton, Yancey et al. 2007; Yancey, Blakemore et al. 2010) (Figure 25).

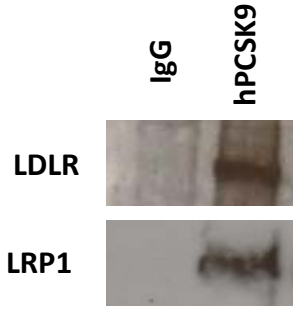


Figure 26. Immunoprecipitation of hPCSK9 in liver samples from WT and hPCSK9 tg mice and western blot analysis of LDLR and LRP1. IgG were used as negative control.

Recently published literature data demonstrated that PCSK9 reduced LRP1 expression in transduced HEK293T cells (Canuel, Sun et al. 2013). We wanted to see whether hPCSK9 was able to affect LRP1 levels. We transduced J774.1 cells with hPCSK9 or the GOF mutant D374Y; moreover, we measured LRP total expression in MPM. As shown in Figure 26A, we found that the interaction of hPCSK9 with LRP1 did not affect total levels of LRP1, neither in J774.1, nor in MPM, as analyzed by Western blot. Interestingly, flow cytometry analysis in MPM revealed a slight although statistically significant increase in surface levels of the receptor in hPCSK9 tg MPM (Figure 27).

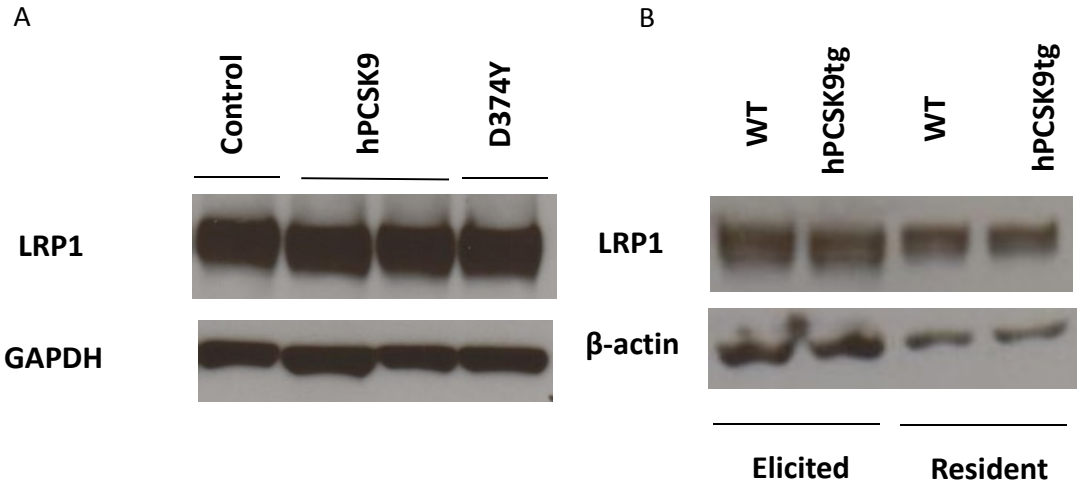


Figure 26. Western blot analysis of LRP1 levels in (A) J774.1 cells transduced with hPCSK9 and the GOF mutant D374Y or (B) elicited or resident MPM from WT and hPCSK9 tg mice. β-actin or GAPDH were chosen as housekeeping proteins.

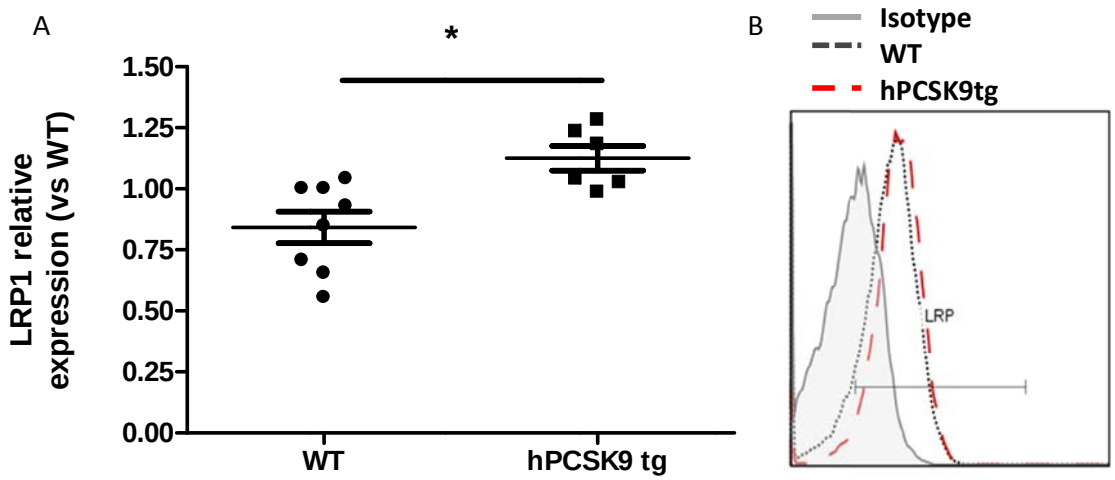


Figure 27. (A) Flow cytometry analysis of surface LRP1 levels in elicited MPM from WT and hPCSK9 tg mice (B) Representative diagram of surface LRP1 expression analyzed by flow cytometry. *p<0.05.

To study the effects of hPCSK9 in the atheroma, hPCSK9 tg mice on WT background were fed a high fat diet for 8 weeks. After 8 weeks, we measured the expression levels of hPCSK9 and mPCSK9 in serum (Figure 28 and 29). As shown in figure 29, serum levels of endogenous PCSK9 were increased of about 5-fold in transgenic mice compared to WT controls (274.75±155.19 vs 45.77±28.29 ng/ml).

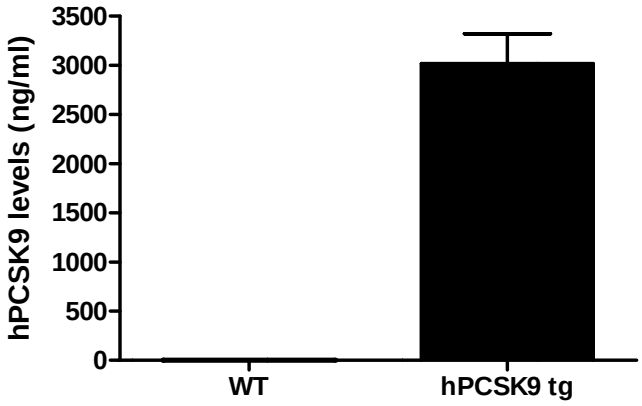


Figure 28. Serum hPCSK9 levels in hPCSK9 tg mice fed a high fat diet for 8 weeks.

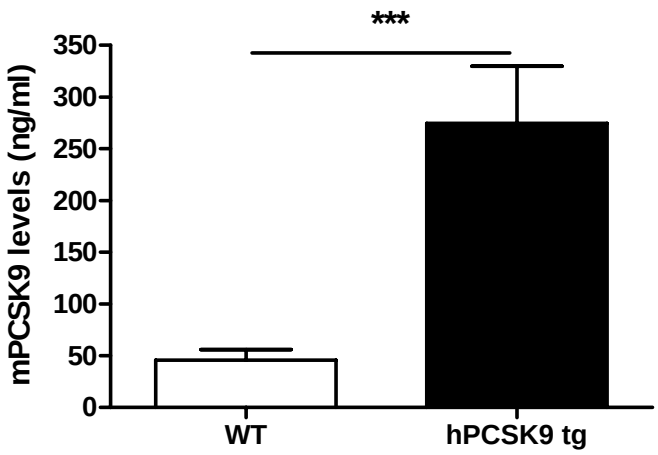


Figure 29. Serum mPCSK9 levels in hPCSK9 tg mice fed a high fat diet for 8 weeks, taken into account 5% cross-reactivity with hPCSK9. *** p<0.001.

We also measured the levels of different lipid parameters in serum. Figure 30 showed that expression of hPCSK9 caused a 2-fold increase in total cholesterol (325.3±64.0 vs 157.8±43.9 mg/dl), compared to controls. As shown by the FPLC profile in figure 31, this was mainly due to increased LDL cholesterol levels.

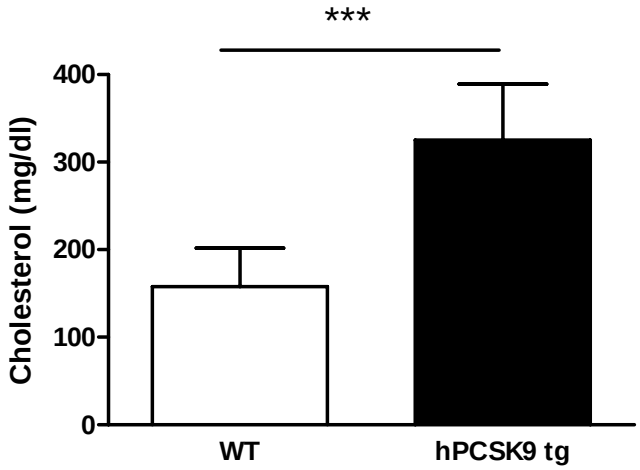


Figure 30. Total serum cholesterol levels in hPCSK9 tg mice fed a high fat diet for 8 weeks. Results are expressed as mg/dl. *** p<0.001.

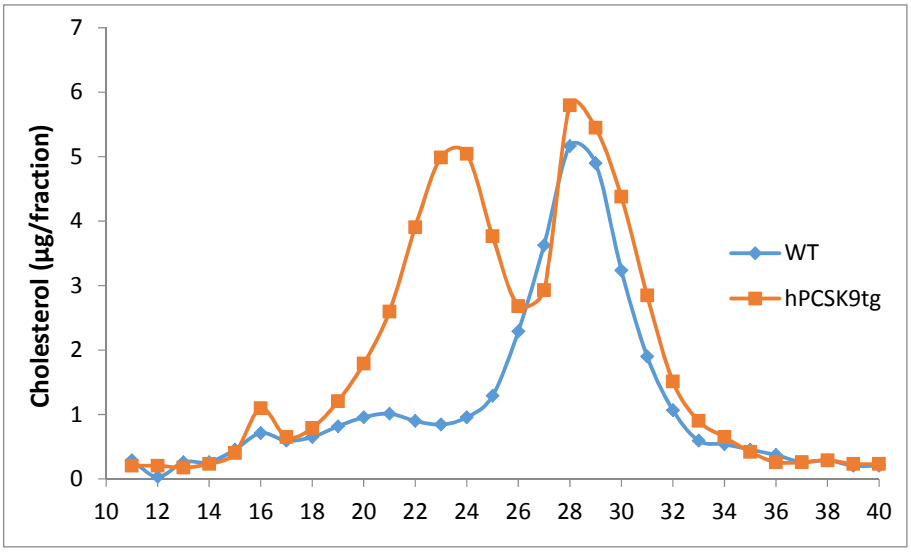


Figure 31. Serum fast protein liquid chromatography lipoprotein profiles in hPCSK9 tg mice fed a high fat diet for 8 weeks. Results are expressed as µg per fraction. Each profile represents pooled serum from 3 mice. Fractions: VLDL, 12 to 17; LDL, 18 to 27; HDL, 28 to 34; and lipoprotein-deficient serum, 35 to 40.

On the contrary, TG levels were not significantly affected by hPCSK9 compared to WT (69.3±12.3 vs 65.7±4.0 mg/dl) (Figure 32).

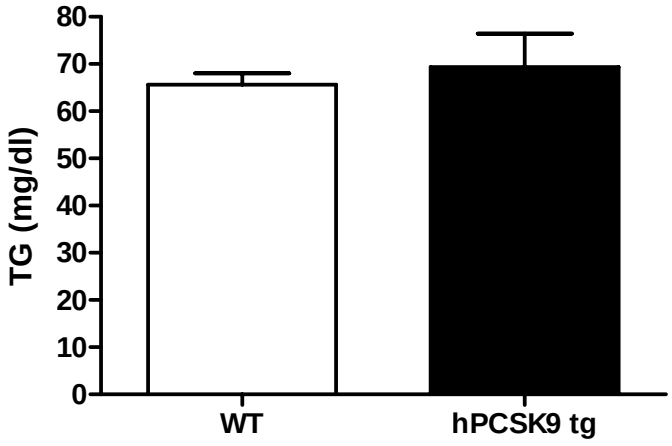


Figure 32. Triglyceride levels in hPCSK9 tg mice fed a high fat diet for 8 weeks. Results are expressed as mg/dl.

As shown in figure 33, since apoB is the major component of LDL, also apoB levels were significantly increased in hPCSK9 tg mice compared to WT (99.8±6.0 vs 62.5±14.8 mg/l, respectively)

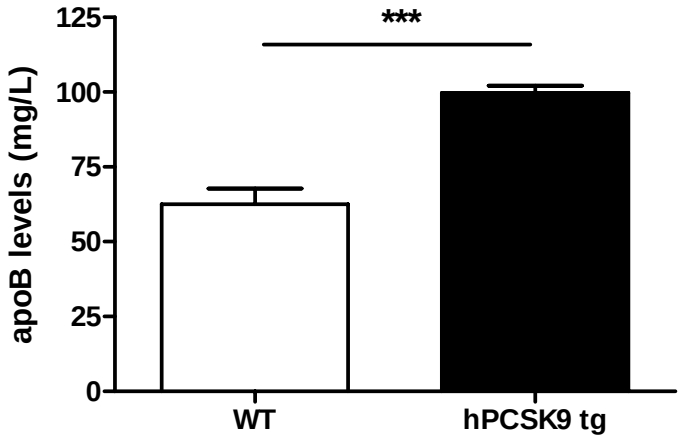
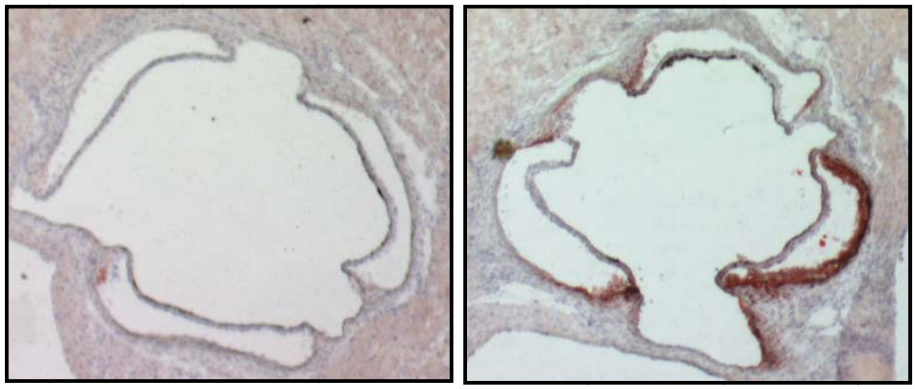


Figure 33. apoB levels in hPCSK9 tg mice fed a high fat diet for 8 weeks. Results are expressed as mg/L. ***p<0.001.

The increase in cholesterol levels observed in transgenic mice were enough to cause the development of atherosclerotic lesions, compared to WT mice that do not develop lesions even on high fat diet, as shown by Oil-Red-O staining of proximal aortas and quantitation of proximal lesion area (Figure 33A and 33B). Moreover, we stained the same sections of proximal aortas for hPCSK9. As shown in Figure 34, hPCSK9 was detected in atherosclerotic plaques of hPCSK9 transgenic mice, demonstrating that hPCSK9 is both secreted and internalized by macrophages through the LDLR. No signal was detected in WT mice, demonstrating the almost absence of cross reactivity of the antibody with mPCSK9.

A



WT hPCSK9tg

B

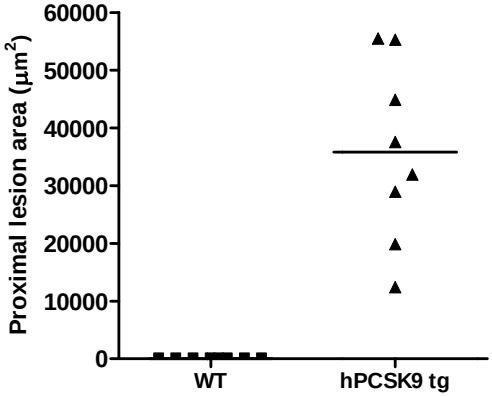


Figure 33. (A) Representative images showing Oil-red-O stained cross-sectional area of proximal aortas of WT and hPCSK9 tg mice fed a high fat diet for 8 weeks. (B) Quantitation of proximal lesion area. Results are expressed in μm^2.

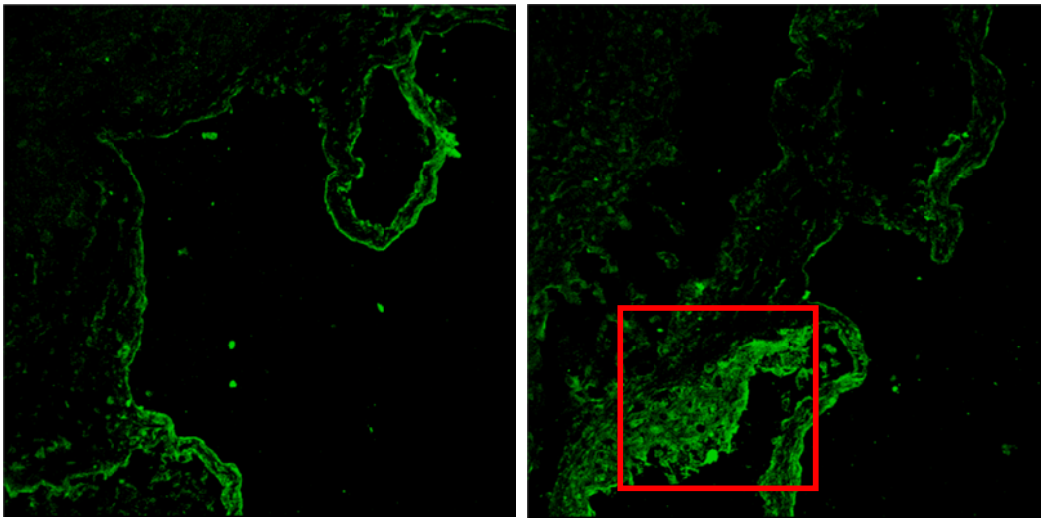


Figure 34. Representative images showing hPCSK9 in proximal aortas of hPCSK9 tg mice. The areas positive for hPCSK9 are squared in red.

To study the effect of hPCSK9 in a more pro-atherogenic scenario, transgenic mice expressing hPCSK9 were backcrossed with apoE$^{-/-}$ mice, a commonly used mouse model for atherosclerosis studies, to generate hPCSK9 tg/apoE$^{-/-}$ mice. To induce atherosclerosis, we fed the animals on high fat diet and after 8 weeks we measured serum hPCSK9 and endogenous PCSK9 levels (Figures 35 and 36). As shown in figure 36 and previously observed in WT mice, the presence of the transgene increased mPCSK9 levels of about 4-fold (986.4±274.4 vs 214.2±40.3 ng/ml), confirming that the clearance of PCSK9 is LDLR independent.

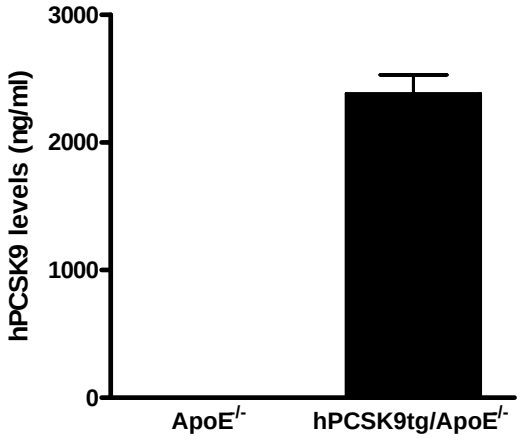


Figure 35. Serum hPCSK9 levels in hPCSK9 tg/apoE$^{-/-}$ mice fed a high fat diet for 8 weeks.

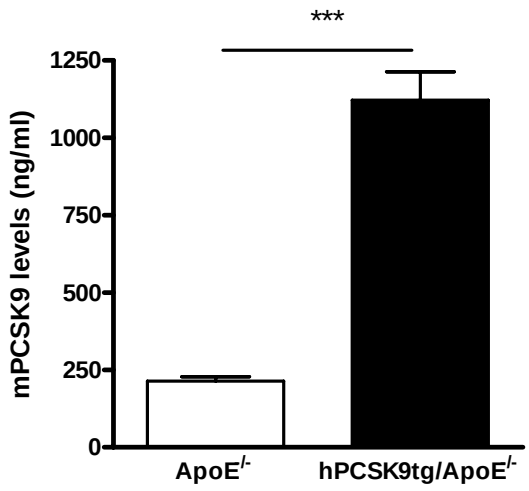


Figure 36. Serum mPCSK9 levels in hPCSK9 tg/apoE$^{-/-}$ and apoE$^{-/-}$ mice fed a high fat diet for 8 weeks. ***p<0.001.

Surprisingly, no significant changes were found in total cholesterol levels in mice transgenic for hPCSK9 compared to apoE$^{-/-}$ controls (1066.4±188.1 vs 964.4 ±161.5 mg/dl) and also the cholesterol FPLC profiles were almost similar (Figures 37 and 38)

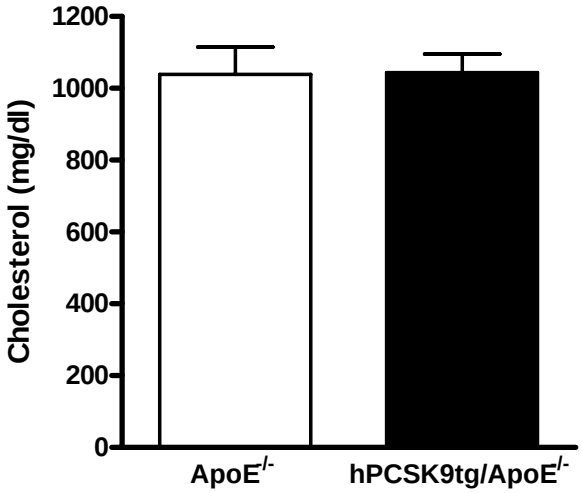


Figure 37. Total serum cholesterol levels in hPCSK9 tg/apoE$^{-/-}$ or apoE$^{-/-}$ mice fed a high fat diet for 8 weeks. Results are expressed as mg/dl.

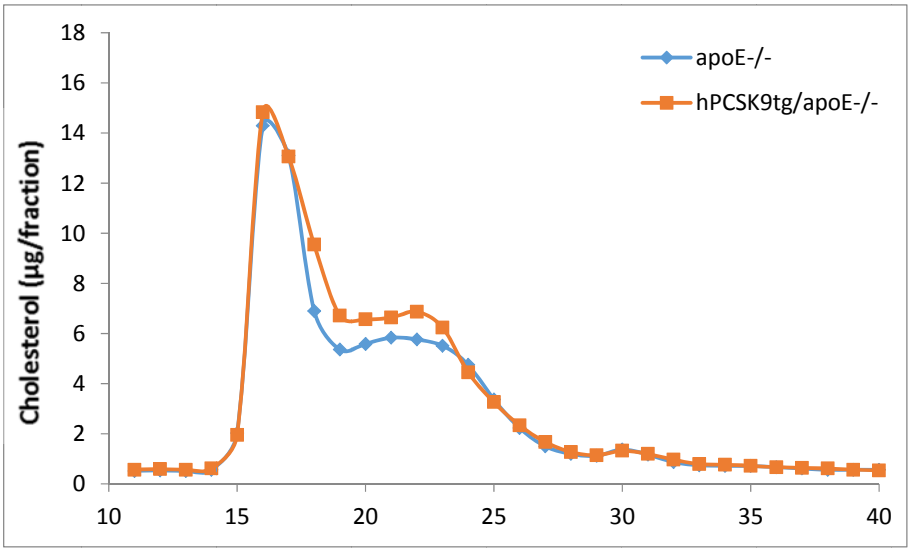


Figure 38. Serum fast protein liquid chromatography lipoprotein profiles in hPCSK9 tg/apoE$^{-/-}$ and apoE$^{-/-}$ mice fed a high fat diet for 8 weeks. Results are expressed as µg per fraction. Each profile represents pooled serum from 3 mice. Fractions: VLDL, 12 to 17; LDL, 18 to 27; HDL, 28 to 34; and lipoprotein-deficient serum, 35 to 40.

Also TG levels were not significantly affected by the presence of the hPCSK9 transgene (106.5±22.7 vs 90.8±22.7 mg/dl of controls apoE$^{-/-}$), as shown in Figure 39.

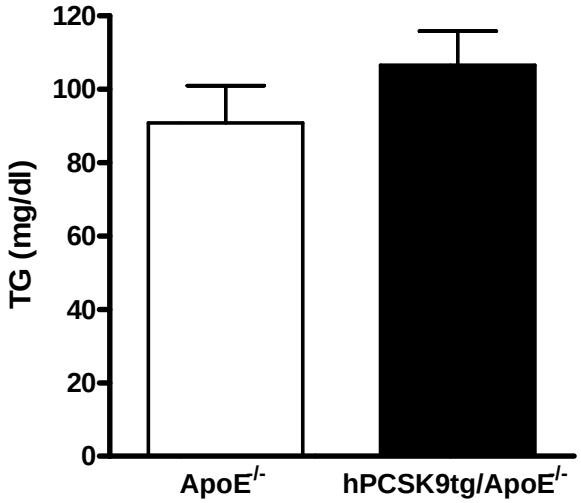


Figure 39. Triglyceride levels in hPCSK9 tg/apoE$^{-/-}$ and apoE$^{-/-}$ mice fed a high fat diet for 8 weeks. Results are expressed as mg/dl.

Sections of proximal aortas were stained for hPCSK9, showing a huge accumulation in the plaque. Moreover, the areas positive for the macrophage MOMA2 staining were also found to be strongly positive for hPCSK9, to demonstrate that hPCSK9 was not only produced but also internalized by macrophages in the atherosclerotic plaque (Figure 40).

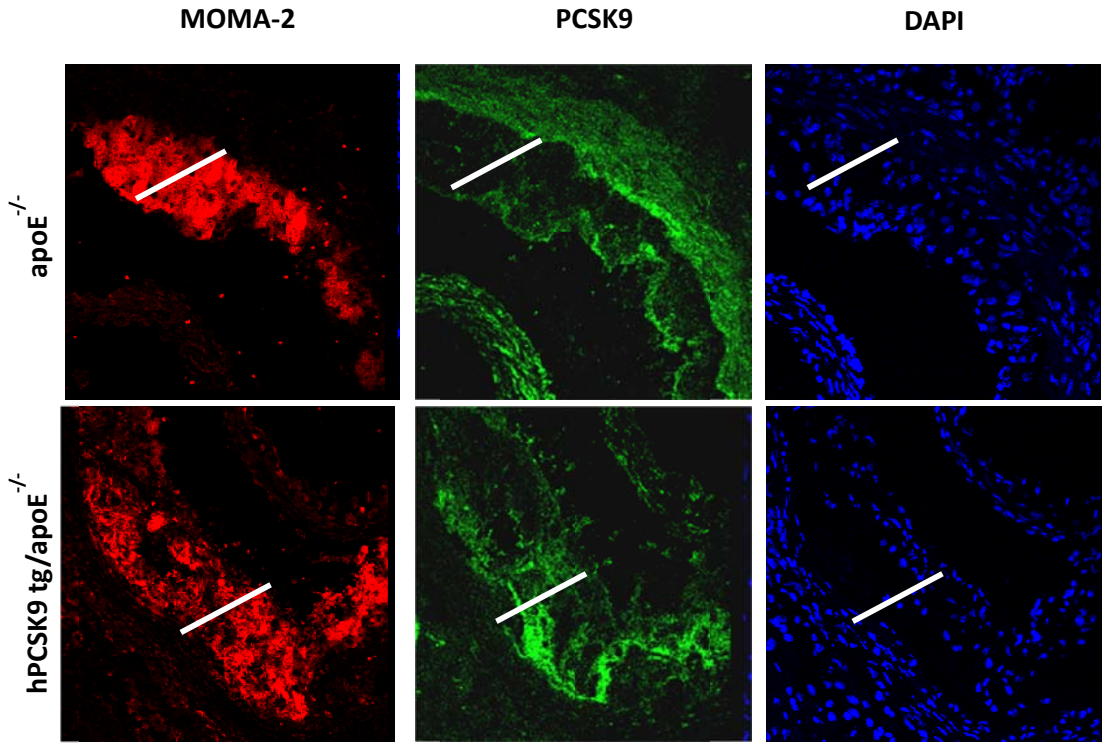
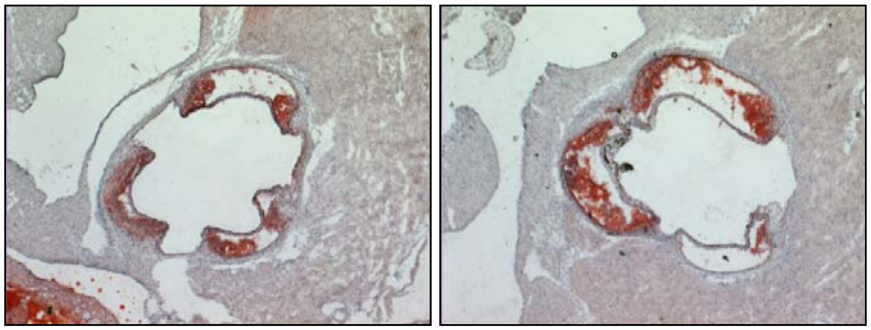


Figure 40. Representative images showing hPCSK9 staining of lesions of proximal aortas in apoE$^{-/-}$ and hPCSK9 tg/ apoE$^{-/-}$ mice fed a high fat diet for 8 weeks. In red: MOMA2 staining; green: hPCSK9 staining; blue: DAPI

Even in the absence of changes in cholesterol levels, hPCSK9 tg mice were characterized by a statistically significant increase of lipids in proximal lesion area compared to apoE$^{-/-}$, as shown by Oil-Red-O staining (Figures 41A and 41B).

A



apoE$^{-/-}$ hPCSK9 tg/apoE$^{-/-}$

B

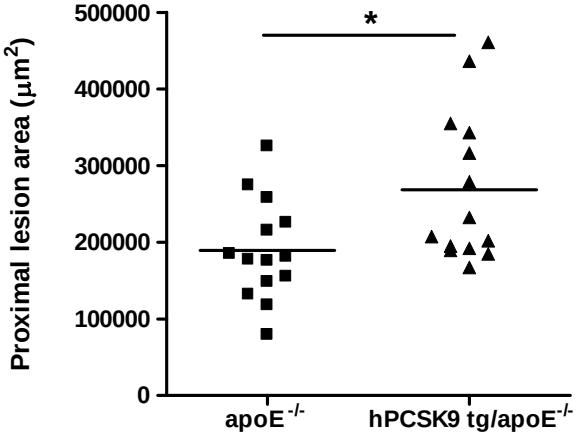
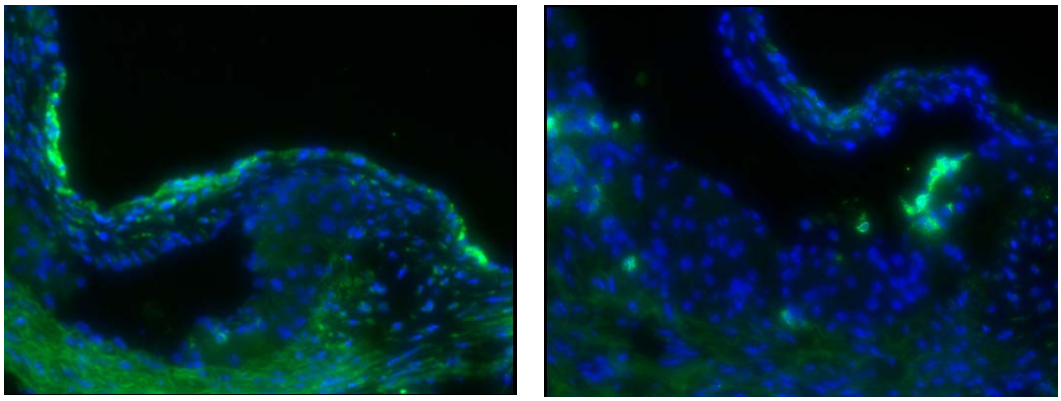


Figure 41. (A) Representative images showing Oil-red-O stained cross-sectional area of proximal aortas of apoE$^{-/-}$ and hPCSK9 tg/apoE$^{-/-}$ mice fed a high fat diet for 8 weeks. (B) Quantitation of proximal lesion area. Results are expressed in µm^2. *p<0.05.

Even more interestingly, lesion composition analysis showed significantly higher levels of Ly6C^{high} positive cells in PCSK9 lesions compared to apoE$^{-/-}$ (6.7±0.2% vs. 5.7±0.4%, respectively, p<0.05) (Figures 42A and 42B).

A



apoE $^{-/-}$ hPCSK9 tg/apoE $^{-/-}$

B

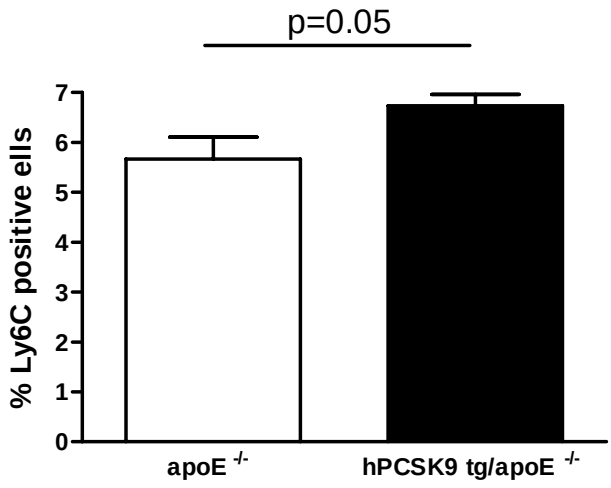


Figure 42. (A) Representative images showing Ly6C positive cells in lesions of apoE$^{-/-}$ and hPCSK9 tg/ apoE$^{-/-}$ mice fed a high fat diet for 8 weeks. (B) Quantitation of the percentage of Ly6C positive cells compared to total number of cells in the lesion.

Moreover, the analysis of spleen lysates revealed an increase in inflammatory Ly6C ^{high} cells in the spleens of our hPCSK9 tg mice compared to the control apoE $^{-/-}$mice (Figure 43), suggesting that PCSK9 might exerts pro-inflammatory effects thus favoring the development of atherosclerosis.

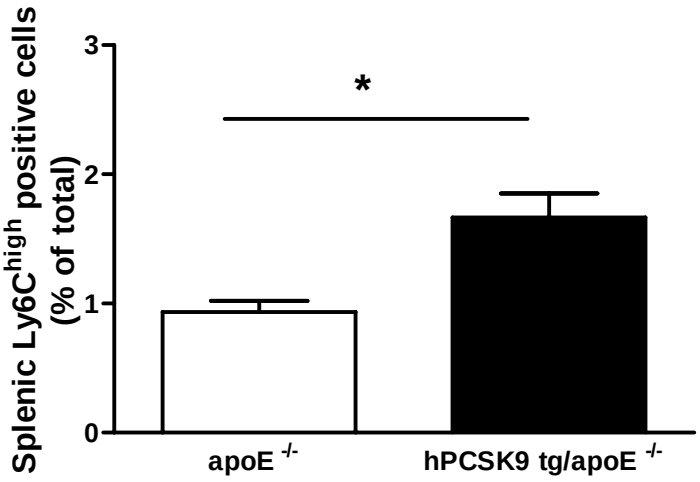


Figure 43. Ly6C^{high} positive in spleens of apoE$^{-/-}$ and hPCSK9 tg/apoE$^{-/-}$ mice fed a high fat diet for 8 weeks. Analysis of Ly6C^{high} positive cells was performed by Flow cytometry.* $p<0.05$

To study whether the effect on inflammation observed in hPCSK9 transgenic mice was dependent on the presence of its target LDLR, mice expressing hPCSK9 transgene were backcrossed with $LDLR^{-/-}$ mice to generate hPCSK9 tg/$LDLR^{-/-}$ mice. We previously demonstrated that LDLR represents the main route of elimination of PCSK9 (Tavori, Fan et al. 2013). In fact, hPCSK9 levels were extremely high in transgenic mice on $LDLR^{-/-}$ compared to the levels in WT (5832.4±1709.5 vs 3016.6±1008.76 ng/ml, respectively) (Figure 44), demonstrating the reduced clearance of PCSK9 in the absence of LDLR. As previously shown in animals on chow diet (Tavori, Fan et al. 2013), after 8 weeks of high fat diet, serum levels of mPCSK9 were extremely increased in $LDLR^{-/-}$ mice compared to WT (2587.5±197.8 vs 45.8±28.3 ng/ml), but did not significantly differ from hPCSK9 tg/$LDLR^{-/-}$ mice (2587.5±197.8 vs 2312.9±593.8 ng/ml) (Figure 45).

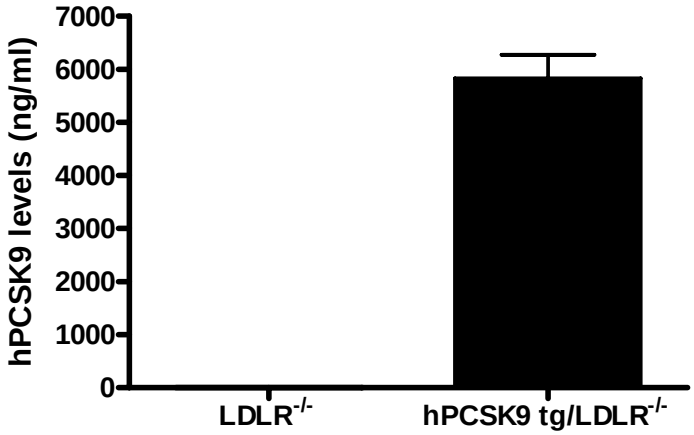


Figure 44. Serum hPCSK9 levels in hPCSK9 tg/ $LDLR^{-/-}$ mice fed a high fat diet for 8 weeks.

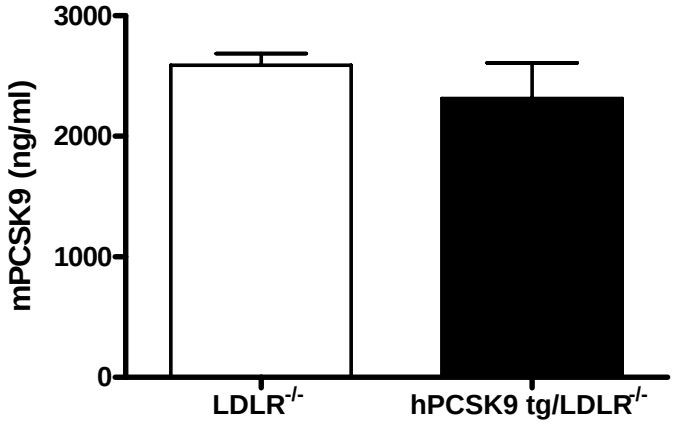


Figure 45. Serum mPCSK9 levels in hPCSK9 tg/LDLR$^{-/-}$ and LDLR$^{-/-}$ mice fed a high fat diet for 8 weeks.

A more moderate but still significant increase in total cholesterol levels was observed in mice transgenic for hPCSK9 compared to LDLR$^{-/-}$ controls (1294.1±91.3 vs 1052.0±232.8 mg/dl, $p<0.05$) (Figure 46).

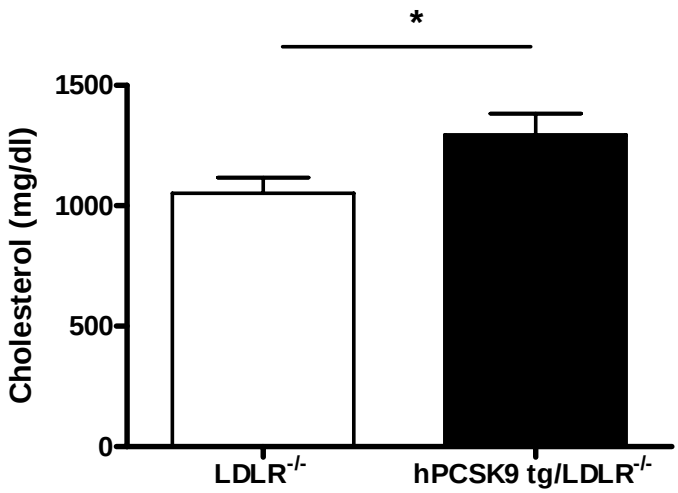


Figure 46. Total serum cholesterol levels in hPCSK9 tg/LDLR$^{-/-}$ and LDLR$^{-/-}$ mice fed a high fat diet for 8 weeks. Results are expressed as mg/dl. * $p<0.05$.

As shown in figure 47, also TG levels were significantly increased (210.3±28.9 vs 275.1±89.3 mg/dl).

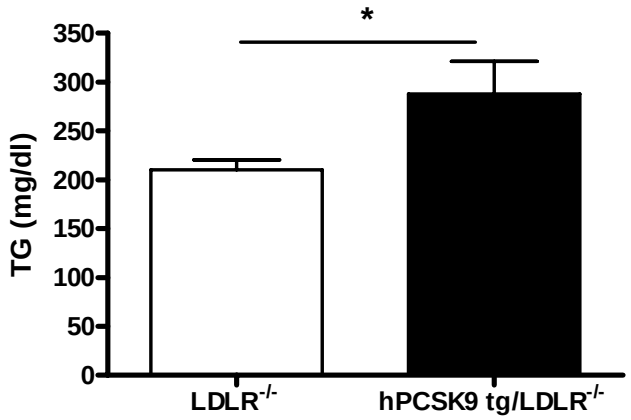


Figure 47. Triglyceride levels in hPCSK9 tg/LDLR$^{-/-}$ and LDLR$^{-/-}$ mice fed a high fat diet for 8 weeks. Results are expressed as mg/dl. * p<0.05.

As expected, the increase in cholesterol and TG levels determined an increase in the serum concentration of apoB (Figure 48).

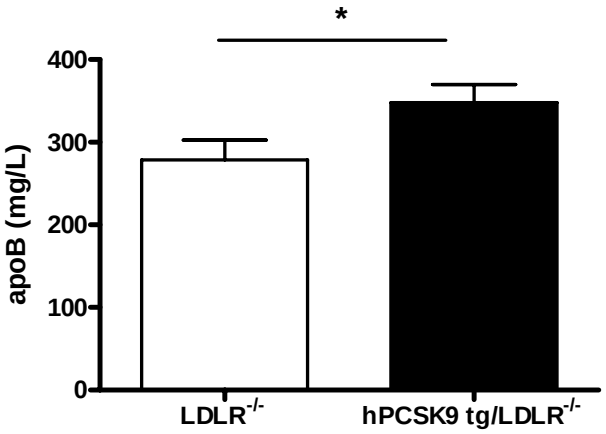
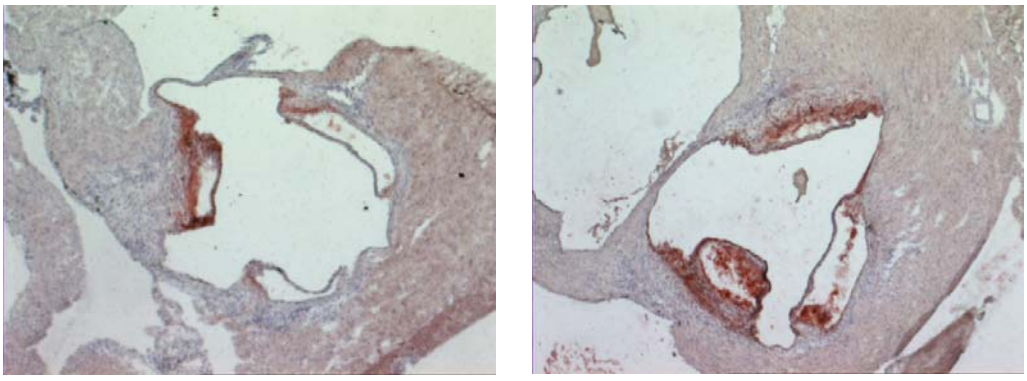


Figure 48. apoB levels in hPCSK9 tg/LDLR$^{-/-}$ and LDLR$^{-/-}$ mice fed a high fat diet for 8 weeks. Results are expressed as mg/L. *p<0.05.

The analysis of sections of proximal aorta revealed a significantly increased lesion area in transgenic mice compared to controls (Figures 49A and 49B). Surprisingly, as shown in figure 50, the analysis of hPCSK9 in the plaque revealed that even if detectable, hPCSK9 was less abundant, nevertheless the concentration of both hPCSK9 and mPCSK9 in the blood were much higher compared to hPCSK9 tg on WT background.

A



LDLR $^{-/-}$ hPCSK9 tg/LDLR $^{-/-}$

B

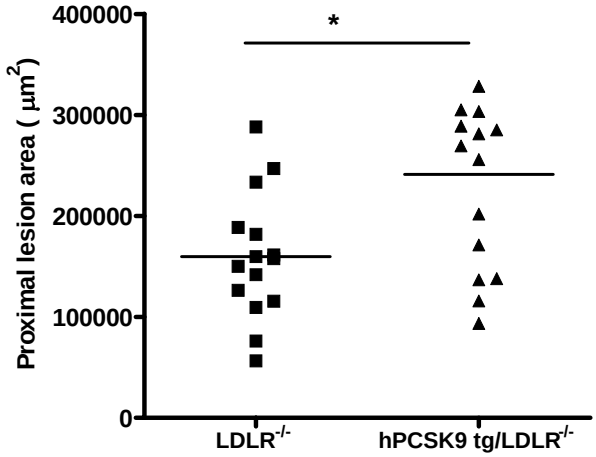


Figure 49. (A) Representative images showing Oil-red-O stained cross-sectional area of proximal aortas of hPCSK9 tg/LDLR$^{-/-}$ and LDLR$^{-/-}$ mice fed a high fat diet for 8 weeks. (B) Quantitation of proximal lesion area. Results are expressed in µm^2. * p<0.05

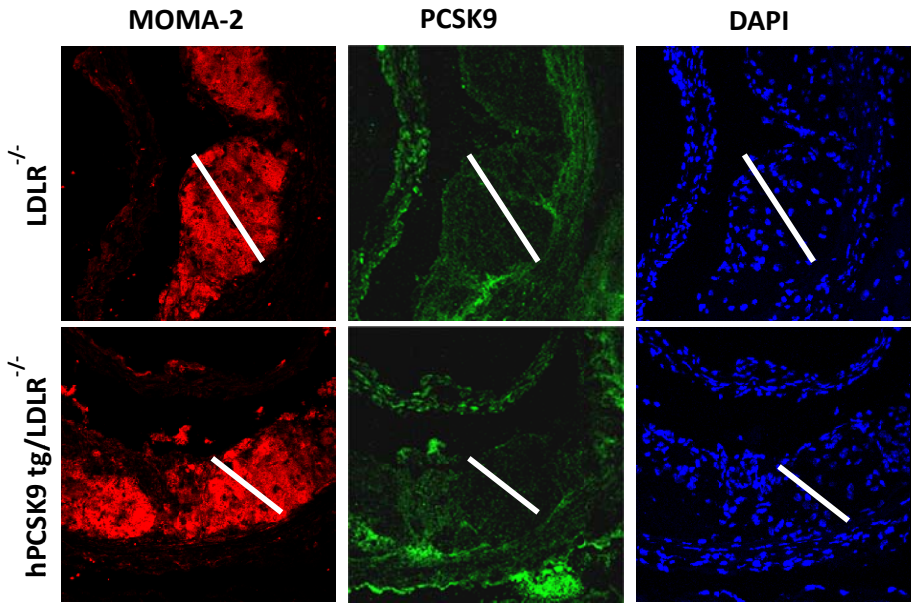


Figure 50. Representative images showing hPCSK9 staining of lesions of proximal aortas in LDLR$^{-/-}$ and hPCSK9 tg/LDLR$^{-/-}$ mice fed a high fat diet for 8 weeks. In red: MOMA2 staining; green: hPCSK9 staining; blue: DAPI

Contrary to the previous analysis in apoE$^{-/-}$ atherosclerotic lesion, the analysis of lesion composition did not show any differences between the two groups in terms of Ly6C^{high} positive cells (5.9±0.9% in LDL$^{-/-}$ vs. 5.9±0.8% in hPCSK9 tg/LDLR$^{-/-}$) (Figure 51).

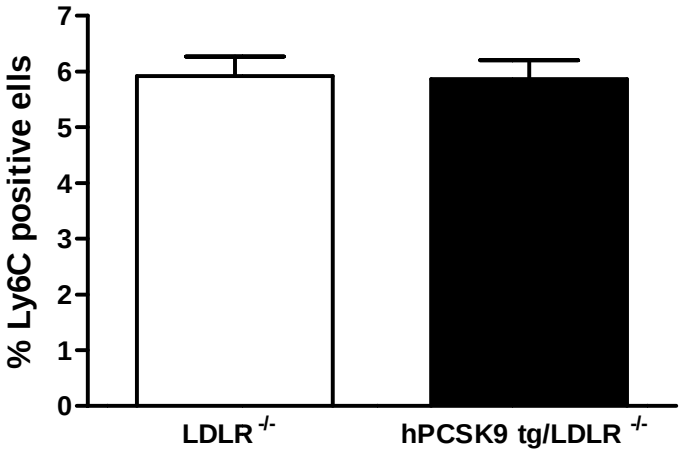


Figure 51. Quantitation of the percentage of Ly6C positive cells compared to total number of cells in the lesions of LDLR$^{-/-}$ ot hPCSK9 tg/LDLR$^{-/-}$ fed a high fat diet for 8 weeks.

Moreover, hPCSK9 did not affect the percentage of splenic Ly6C^{high} positive cells, because even if there was a trend toward an increase, this was not statistically significant (Figure 52).

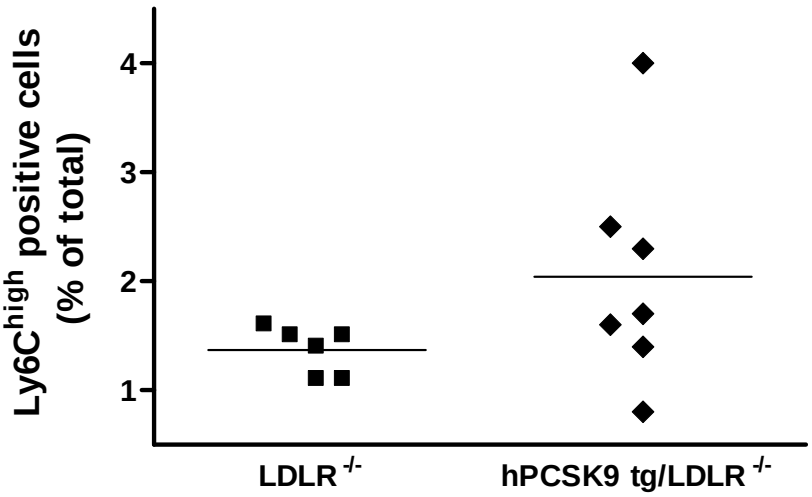


Figure 52. Ly6C^{high} positive in spleens of LDLR$^{-/-}$ and hPCSK9 tg/LDLR$^{-/-}$ mice fed a high fat diet for 8 weeks. Analysis of Ly6C^{high} positive cells was performed by Flow cytometry.

To study the effect of macrophage PCSK9 in the atheroma, bone marrow cells from PCSK9/apoE$^{-/-}$ (PCSK9) or apoE$^{-/-}$ (Control) mice were transplanted into apoE$^{-/-}$ recipients. After 8 weeks on high fat diet, we measured the levels of hPCSK9 in the circulation. hPCSK9 was detected in serum from PCSK9 mice, further demonstrating that macrophages secrete PCSK9 (65.4±27.5 ng/ml) (Figure 53).

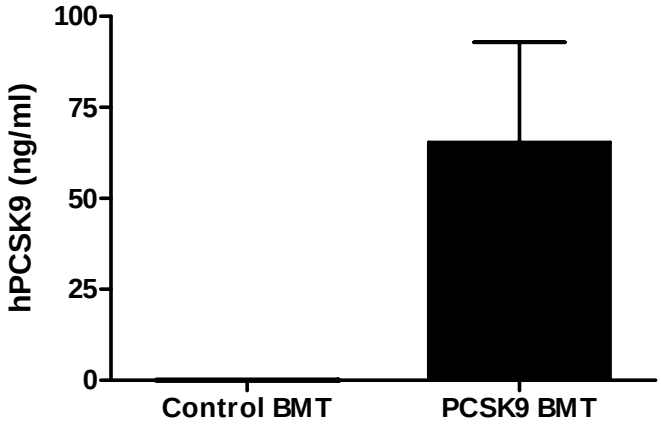


Figure 53. Serum hPCSK9 levels measured apoE$^{-/-}$ mice transplanted with bone marrow from apoE$^{-/-}$ (Control BMT) or hPCSK9 tg/ apoE$^{-/-}$ (PCSK9 BMT).

Moreover, macrophage hPCSK9 was found in the atherosclerotic plaques of PCSK9 BMT mice and in MOMA2-positive areas (macrophage-rich areas), confirming both production and internalization of PCSK9 by macrophages in the atherosclerotic plaque (Figure 54).

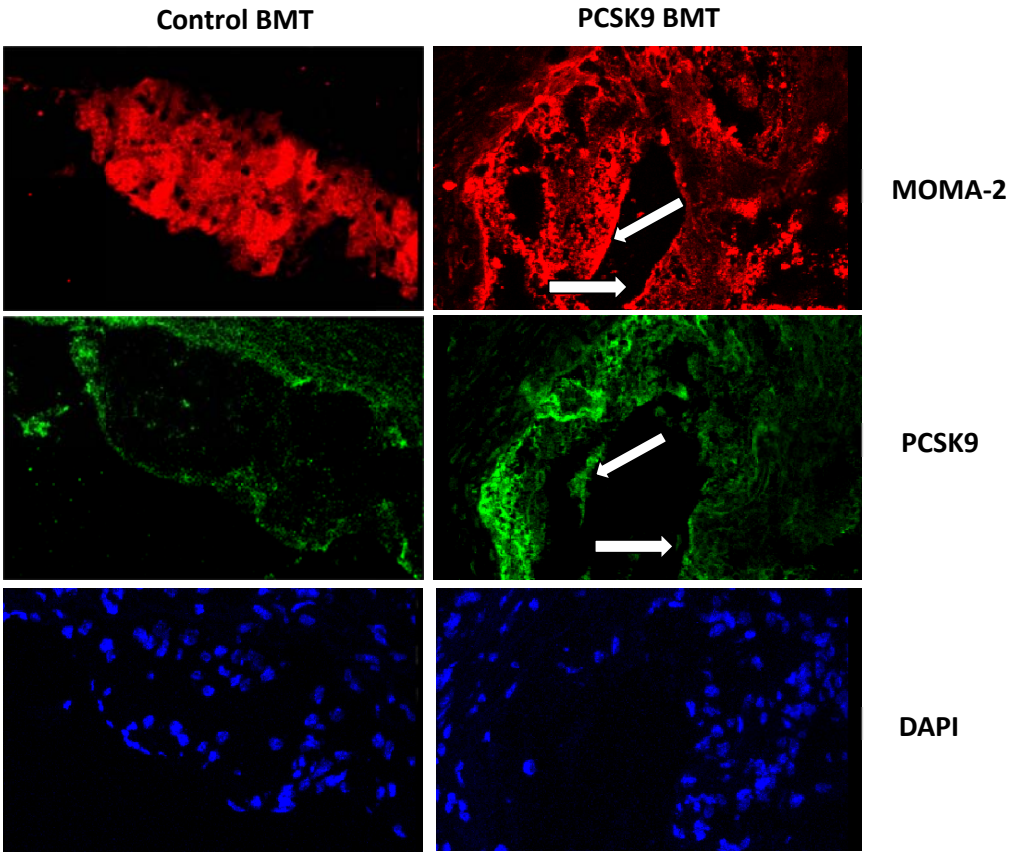


Figure 54. Representative images showing hPCSK9 staining of lesions of proximal aortas in apoE$^{-/-}$ mice transplanted with bone marrow from apoE$^{-/-}$ (Control BMT) or hPCSK9 tg/ apoE$^{-/-}$ (PCSK9 BMT). In red: MOMA2 staining; green: hPCSK9 staining; blue: DAPI

No differences were found between the two groups in terms of cholesterol levels (861±206 mg/dl in Control vs 881±137 mg/dl in PCSK9) or proximal lesion area (286402±24800 μm^2 in Controls vs 274615±47130 μm^2 in PCSK9) (Figure 55 and 56).

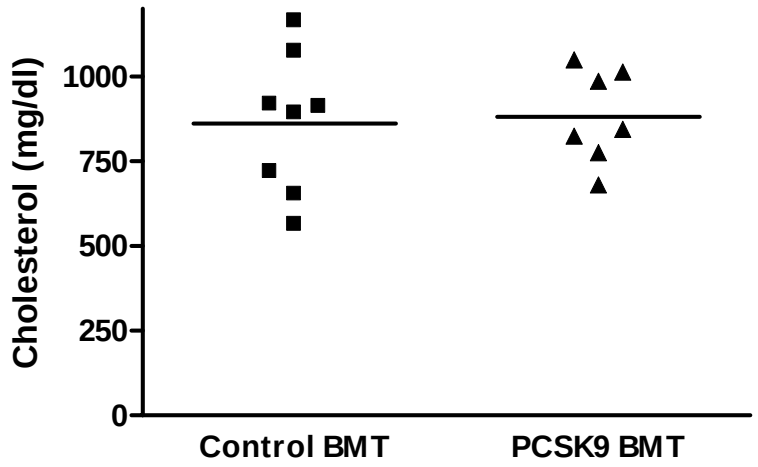


Figure 55. Total serum cholesterol levels in apoE$^{-/-}$ mice transplanted with bone marrow from apoE$^{-/-}$ (Control BMT) or hPCSK9 tg/ apoE$^{-/-}$ (PCSK9 BMT). Results are expressed as mg/dl.

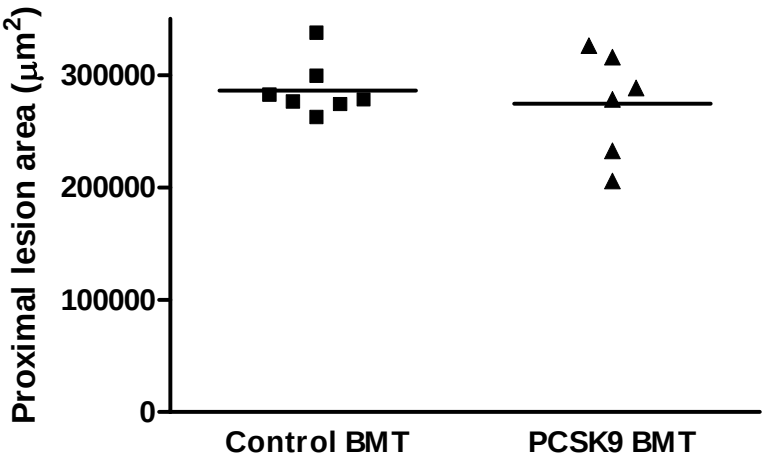


Figure 56. Quantitation of proximal lesion area in Oil-red-O stained proximal aortas of apoE$^{-/-}$ mice transplanted with bone marrow from apoE$^{-/-}$ (Control BMT) or hPCSK9 tg/ apoE$^{-/-}$ (PCSK9 BMT). Quantitation of proximal lesion area. Results are expressed in μm^2.

As previously observed in transgenic mice on apoE$^{-/-}$ background fed on high fat diet, lesion composition analysis showed significantly higher levels of Ly6C^{high} positive cells in PCSK9 lesions compared to Control (7.4±1.5% vs. 5.6±1.1%, respectively, p<0.05) (Figure 57).

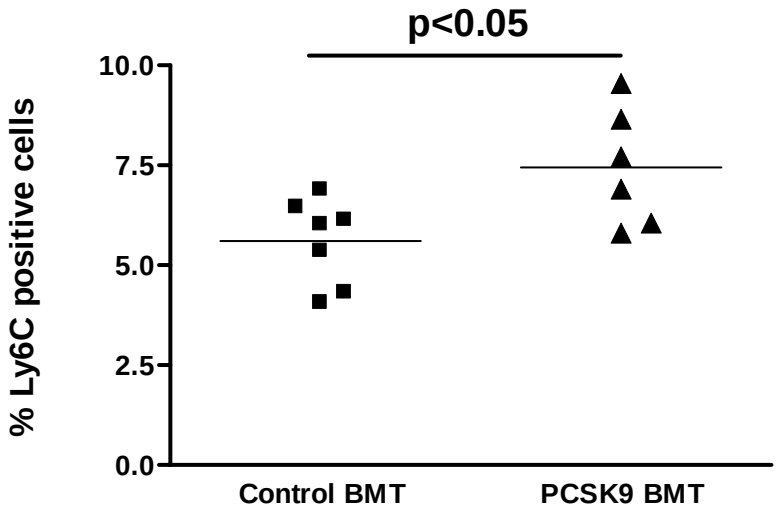


Figure 57. Quantitation of the percentage of Ly6C positive cells compared to total number of cells in the lesions of apoE$^{-/-}$ mice transplanted with bone marrow from apoE$^{-/-}$ (Control BMT) or hPCSK9 tg/ apoE$^{-/-}$ (PCSK9 BMT).

To investigate whether the inflammatory effect of macrophage hPCSK9 was dependent on the binding and degradation of LDLR, we transplanted bone marrow cells from hPCSK9 tg/LDLR $^{-/-}$ and LDLR $^{-/-}$ mice into LDLR $^{-/-}$ recipients. The levels of hPCSK9 in the circulation were ten times higher in LDLR $^{-/-}$ recipients (829$\pm$232 ng/ml) compared to apoE$^{-/-}$ recipients and this was due to the accumulation of PCSK9 in the blood over time, since its target was absent (Figure 58).

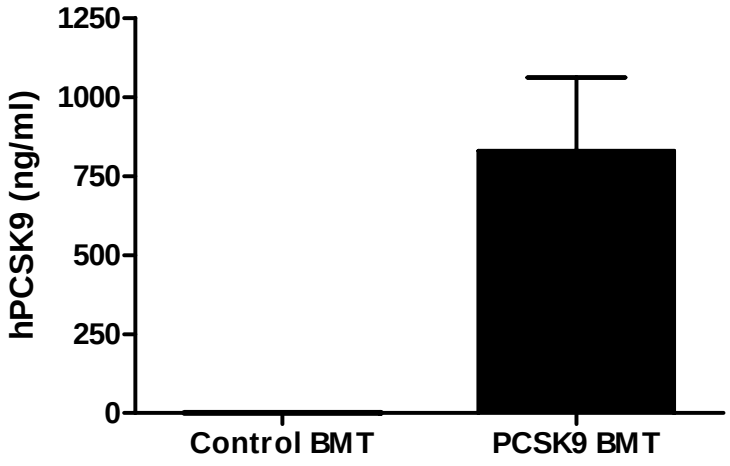


Figure 58. Serum hPCSK9 levels measured LDLR$^{-/-}$ mice transplanted with bone marrow from LDLR$^{-/-}$ (Control BMT) or hPCSK9 tg/LDLR$^{-/-}$ (PCSK9 BMT).

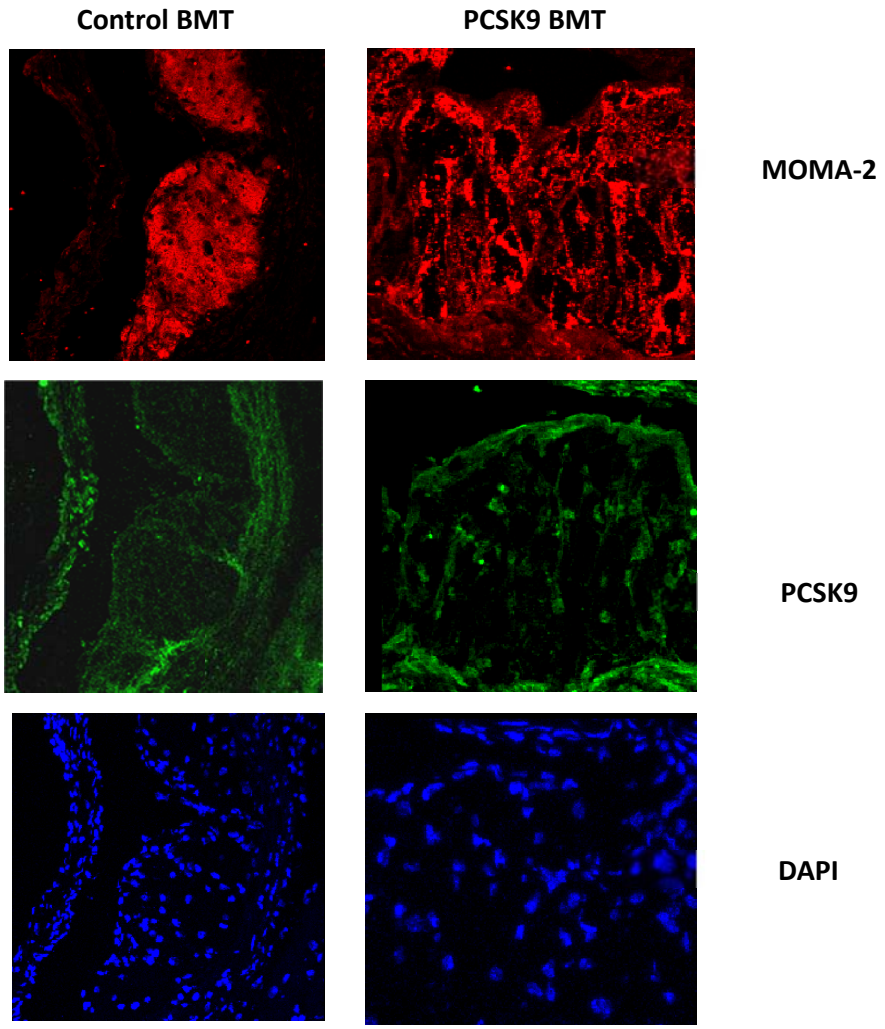


Figure 54. Representative images showing hPCSK9 staining of lesions of proximal aortas in LDLR$^{-/-}$ mice transplanted with bone marrow from LDLR$^{-/-}$ (Control BMT) or hPCSK9 tg/LDLR$^{-/-}$ (PCSK9 BMT). In red: MOMA2 staining; green: hPCSK9 staining; blue: DAPI

As in apoE recipients, no changes were found between the two groups in cholesterol levels (996±26 mg/dl in Controls vs 966±31 mg/dl in PCSK9) or lesion size (110700± 20440 μm^2 in Controls vs 104400±15950 μm^2 in PCSK9) (Figure 60 and 61).

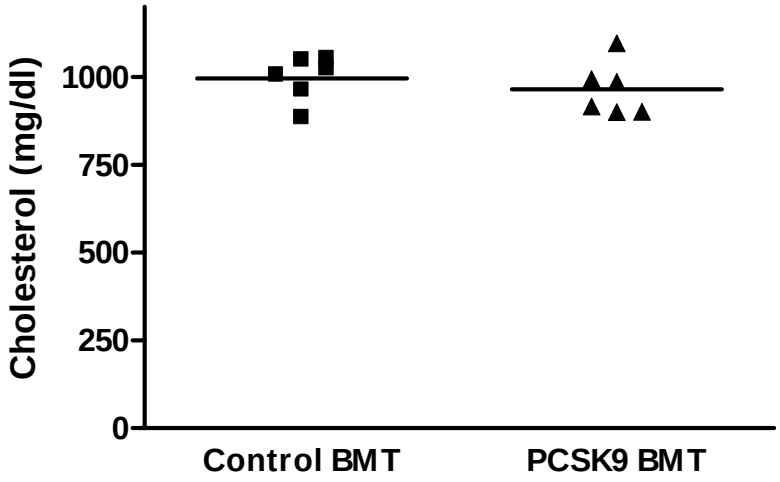


Figure 60. Total serum cholesterol levels in $LDLR^{-/-}$ mice transplanted with bone marrow from $LDLR^{-/-}$ (Control BMT) or hPCSK9 tg/$LDLR^{-/-}$ (PCSK9 BMT). Results are expressed as mg/dl.

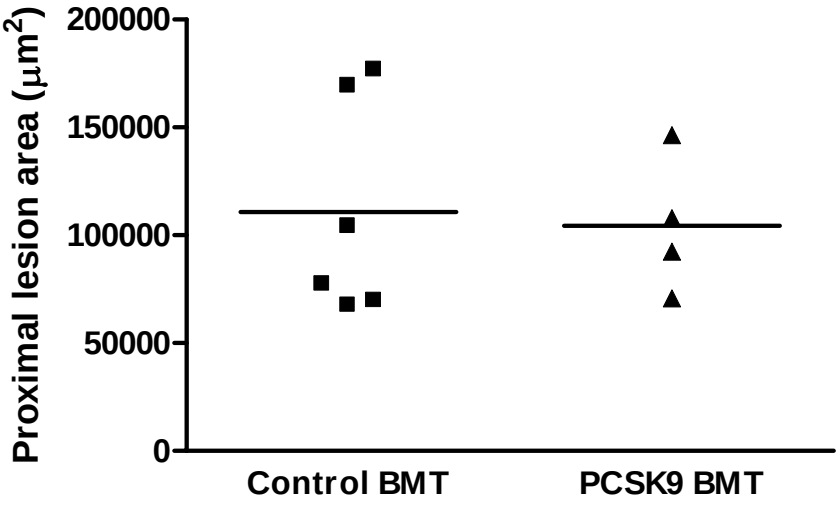


Figure 61. Quantitation of proximal lesion area in Oil-red-O stained proximal aortas of $LDLR^{-/-}$ mice transplanted with bone marrow from $LDLR^{-/-}$ (Control BMT) or hPCSK9 tg/ $apoE^{-/-}$ (PCSK9 BMT). Quantitation of proximal lesion area. Results are expressed in μm^2.

Moreover, the analysis of Ly6C ^{high} positive cells in the plaque revealed no differences in the percentage of positive cells in Controls and PCSK9 (5.5±0.2% vs. 5.7±0.2%, respectively) (Figure 62), suggesting a possible role for LDLR in mediating an inflammatory effect of macrophage PCSK9.

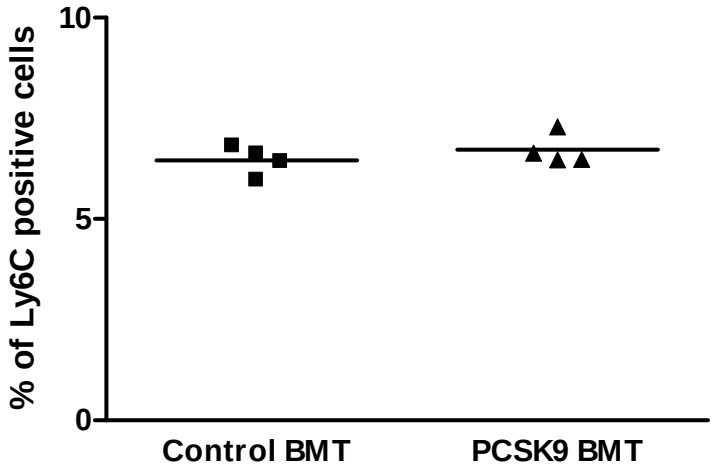


Figure 62. Quantitation of the percentage of Ly6C positive cells compared to total number of cells in the lesions of apoE$^{-/-}$ mice transplanted with bone marrow from apoE$^{-/-}$ (Control BMT) or hPCSK9 tg/ apoE$^{-/-}$ (PCSK9 BMT).

Discussion

Since we previously demonstrated that one third of PCSK9 is associated with LDL in human plasma (Tavori, Fan et al. 2013) and LDL-A is a FDA approved procedure for LDL removal, we wanted to test whether also plasma PCSK9 levels were affected by LDL-A. We therefore collected samples from FH patients undergoing LDL-A and we measured PCSK9 before and after the treatment. We found that LDL-A reduced PCSK9 levels and the reduction strongly correlated with the drop in LDL cholesterol, but not TG and HDL. Moreover, the analysis of different lipoprotein fractions separated by ultracentrifugation revealed that PCSK9 was removed from both the LDL and apoB-free fraction.

FH is a genetic disease caused in the majority of cases by mutations in LDLR and less frequently by defects in apoB or PCSK9 (Soutar and Naoumova 2007). Despite the genetic causes, all FH patients are characterized by elevated LDL cholesterol levels. Therefore, when drug medications are not enough to reach the goal of LDL reduction, LDL-A can be considered a safe procedure to remove apoB containing lipoproteins, thus reducing cardiovascular risk (McGowan 2013).

Our aim was to investigate on the effects of LDL-A on plasma levels of PCSK9. We found that LDL-A reduces PCSK9 levels by 52%; moreover, 82% of the removed PCSK9 is present in the eluate that comes out of column. Such a great reduction of PCSK9 levels is similar to that obtained after administration of low-dose antiPCSK9 antibody that causes a reduction in LDL cholesterol of about 25%. This suggests that the effect observed on PCSK9 levels after LDL-A can be partially helpful in delaying the increase in LDL cholesterol levels immediately after the procedure. After 10 to 14 days, LDL cholesterol levels return to the levels of pretreatment; we found that the levels of PCSK9 follow

the same timetable with an extreme reduction after the treatment and a return to baseline two weeks after. Since PCSK9 reduction is strictly correlated to the reduction in LDL (R^2=0.26), we can conclude that the previous literature data, which studied this correlation (Lambert, Ancellin et al. 2008; Lakoski, Lagace et al. 2009), should consider not only the effect of PCSK9 on LDLR levels, but also the physical association between PCSK9 and LDL.

We also showed that both LDL-bound PCSK9 and apoB-free PCSK9 are removed by LDL-A and, according to previous data from transgenic mice expressing hPCSK9 (Fan, Yancey et al. 2008; Tavori, Fan et al. 2013), we found that PCSK9 is present in different forms depending on the fraction of plasma analyzed. Moreover, we confirmed that no PCSK9 binds to VLDL. Previous studies demonstrated that the most active form of PCSK9 is the 62kDa form, while both the dimeric and trimeric forms and the furin-cleaved form are less active (Fan, Yancey et al. 2008). A Western blot analysis of PCSK9 expression in LDL fraction and apoB-free fraction demonstrates that the 62 kDa form is only present on LDL, whereas all the other forms can be found in the apoB-free fraction. This suggests that the binding of mature PCSK9 to LDL might prevent PCSK9 from inactivation by furin or dimerization, thus increasing its potential effects on LDLR. On the basis of these observations, we can conclude that the destiny of PCSK9 and its action on LDLR are both dependent on its traditional ligand. Moreover, considering the binding of PCSK9 to LDL, the administration of monoclonal antibodies against PCSK9 might give rise in the circulation to immune complexes that can be removed through LDLR or accumulate in the atherosclerotic plaque together with LDL. In addition, if the only active form of PCSK9 is the one bound to LDL, the ELISA methods currently available might be replaced with specific methods to quantify only the mature form.

We also studied the interactions that could be responsible for the removal of PCSK9 from the circulation. The columns currently used for LDL-A at the VUMC are made of dextran sulfate cellulose beads and thus negatively charged. According to the calculation made using a Web-based tool (http://isoelectric.ovh.org/)(Sillero and Maldonado 2006), we hypothesized that PCSK9 in plasma is negatively charged. Therefore a direct interaction between the column and PCSK9 is uncertain. We produced and purified a GST-tag PCSK9, which is negatively charged, and we mimic the conditions of LDL-A using an experimental column. The column did not remove GST-tag PCSK9, suggesting that the loss of PCSK9 might be due to interactions with partner proteins. Since in our study we only considered one of the LDL-A methods, the evaluation of the effects of other procedures on PCSK9 levels might help to better understand the possible mechanisms responsible for the reduction of PCSK9 levels we observed.

Moreover, it was recently shown by Raal and colleagues that PCSK9 levels in hoFH subjects are higher than those in heFH patients, which in turns are higher than controls. We measured PCSK9 levels in patients with extremely low levels of LDL and in patients with FH. PCSK9 was reduced by 29% in low LDL patients compared to normal subjects. Moreover, we showed that PCSK9 LDL-bound levels are higher in patients with FH and the ratio PCSK9 LDL-bound to PCSK9 apoB-free is higher too.

Taken together, these results suggest that LDL can directly influence PCSK9 and a modulation of LDL levels might in turn exert a positive effect in terms of PCSK9 reduction. Moreover, the results of a clinical trial with AMG145, a monoclonal PCSK9 antibody, showed a reduction in LDL cholesterol in hoFH. Therefore a therapy with anti-PCSK9 antibodies might be

considered together with LDL-A to reduce the frequency of treatment and improve the beneficial effects on LDL cholesterol in FH patients.

Since PCSK9 was identified as the third gene involved in autosomal dominant hypercholesterolemia and GOF mutations are associated with increased LDL cholesterol and cardiovascular risk (Abifadel, Varret et al. 2003), in the second part of our study, we wanted to determine the contribution of PCSK9 on atherosclerotic plaque development. We found that mice expressing hPCSK9 on WT and $LDLR^{-/-}$ background receiving a high fat diet, showed increased LDL cholesterol and triglyceride levels and displayed an increase in lesion size. On the contrary, hPCSK9 in $apoE^{-/-}$ mice did not affect cholesterol levels but still influenced lesion size. Moreover, unlike in $LDLR^{-/-}$, the effects on lesion size were associated to a change in lesion composition. Since PCSK9 is expressed and secreted by macrophages, to study the effect of macrophage PCSK9 in the atheroma, we transplanted bone marrow cells from hPCSK9tg/$apoE^{-/-}$ or $apoE^{-/-}$ mice into $apoE^{-/-}$ recipients. We observed no differences in cholesterol levels or lesion size, but lesion composition was altered, with increased percentages of $Ly6C^{high}$ positive cells in the plaque of transgenic mice. When we transplanted bone marrow cells from hPCSK9tg/$LDLR^{-/-}$ or $LDLR^{-/-}$ into $LDLR^{-/-}$ recipients, the effects of PCSK9 on the inflammatory $Ly6C^{high}$ cells in the plaque totally disappears, suggesting that PCSK9 might affect inflammation through LDLR dependent pathways.

In our studies we used transgenic mice, in whom the transgene was designed to deliver hPCSK9 in multiple tissues, including macrophages. We clearly demonstrated that both mPCSK9 and hPCSK9 were expressed in MPM from WT and hPCSK9 tg mice and that hPCSK9 was secreted in the culture medium of MPM. No studies in literature have previously reported data about

PCSK9 expression in cells that normally populate the vascular wall, except one (Ferri, Tibolla et al. 2011). Ferri and colleagues reported that SMCs, but not endothelial cells and monocytes/macrophages, expressed PCSK9. They also measured PCSK9 expression in human atherosclerotic plaques describing a co-localization of PCSK9 with SMCs. Moreover, they found that PCSK9 secreted by SMCs exerted paracrine effects on other cells populating the plaque, since it reduced LDLR expression in the murine macrophage cell line J774 cells. We also provide evidences of the presence of hPCSK9 in atherosclerotic plaques of hpCSK9 transgenic mice and we found that the areas in the lesions enriched in macrophages were the ones in which the expression of PCSK9 was higher. The different specificity of the antibodies used to detect PCSK9 both in cell cultures and in the plaque and the differences between the models studied might be responsible for the discrepancy of our results. However, in line with their conclusions, we found that PCSK9 affects LDLR expression in macrophages. In particular both total and surface levels of LDLR were lowered by hPCSK9, as shown by Western Blot and flow cytometry analysis. Moreover, we performed co-immunoprecipitation studies and we found a direct interaction of PCSK9 with LDLR in both livers and MPM of hPCSK9 transgenic mice. We also found that PCSK9 interacts with another member of the LDLR gene family, LRP1. A few literature data investigated on the effects of PCSK9 on other members of the LDLR family. In particular, in one study, Shan et al. provided the first direct evidence that PCSK9 binds to ApoER2 and VLDLR, as well as to LDLR and that this binding can be mediated by EGF-A domain interaction with PCSK9. Moreover, Lagace and colleagues found no differences in LRP expression in mouse embryonic fibroblasts treated with PCSK9 compared to non-treated controls (Lagace, Curtis et al. 2006). On the contrary, a very recently published paper (Canuel, Sun et al. 2013), provides

evidences for a PCSK9 mediated degradation of LRP in different cell lines transduced with PCSK9, including HEK293T cells. We confirmed the effect of PCSK9 on LRP in HEK293T cells (data not shown), but we found no differences in total LRP expression in other cell lines, such as J774 cells transduced with PCSK9 or with the GOF mutant D374Y-PCSK9. This might be function of the cell type. Moreover, they found that PCSK9 was unable to degrade LRP1 in primary hepatocytes. We also observed that the total levels of LRP were not changed in MPM, as measured by Western blot. The analysis of LRP surface levels by flow cytometry confirmed no PCSK9 mediated degradation of LRP. Intriguingly, we found a slight but significant increase in LRP surface levels. It might be that PCSK9 exerts different actions on different receptors. However, further experiments will be necessary to provide a certain explanation.

In the next series of experiments, we wanted to study the effects of PCSK9 on atherosclerotic plaque development. Therefore, we put hPCSK9 transgenic mice on WT, $LDLR^{-/-}$ and $apoE^{-/-}$ background on high fat diet and we analyzed the extent of atherosclerosis after 8 weeks. We found an increase in both cholesterol and triglyceride levels in transgenic mice on WT and $LDLR^{-/-}$ background and a consequent increase in lesion size. Despite the absence of effects on cholesterol in transgenic mice on $apoE^{-/-}$ background, we still observed an increase in lesion size. Only one literature study described the effects of transgenic expression of PCSK9 on the extent of atherosclerosis (Denis, Marcinkiewicz et al. 2012). Denis et al. generated transgenic mice overexpressing mPCSK9 on WT, $LDLR^{-/-}$ and $apoE^{-/-}$ background and they fed the animals a Western diet for 12 weeks. In line with our results, in their PCSK9 overexpressing transgenic mice on $apoE^{-/-}$ background, they found an increase in lesion size without any changes in cholesterol levels. They did not provide a clear explanation for this observation. We think that the increased

lesion size might be attributable to an effect on inflammation, as suggested by changes in lesion composition observed.

Previous studies of Swirski and colleagues (Swirski, Libby et al. 2007) have shown that apoE$^{-/-}$ mice fed a Western diet are characterized by enhanced Ly6C^{high} monocytosis, derived from increased survival and proliferation of monocytes as well as lower conversion of Ly6C^{high} monocytes to Ly6C^{low} monocytes. According to this study, we observed an increase in the number of Ly6C^{high} monocytes in the spleens of apoE$^{-/-}$ animals fed a high fat diet compared to WT (data not shown). Moreover, we found an increase in the percentage of Ly6C high positive cells in the spleens of transgenic mice on apoE-/- background, that might in part explain the increased accumulation of Ly6C^{high} positive cells in the plaque. Despite increase an in cholesterol levels and lesion size, no effects on Ly6C^{high} positive cells in transgenic mice on LDLR$^{-/-}$ background were observed, neither in the lesions, nor in the spleens. Moreover, in transgenic LDLR$^{-/-}$ plaques, hPCSK9 was less abundant compared to WT and apoE $^{-/-}$ plaques. This suggests a possible role for LDLR in mediating PCSK9 effects on inflammation.

To specifically study the effects of macrophage hPCSK9 on atherosclerotic lesion development we used a BMT approach. We injected bone marrow cells from PCSK9 tg/apoE$^{-/-}$ or apoE$^{-/-}$ control mice into apoE$^{-/-}$ recipients. We further confirmed that the macrophages from our transgenic mice secrete hPCSK9, since we found that even if at low levels hPCSK9 was present in serum of PCSK9 BMT mice. Moreover, hPCSK9 was detected in the plaque. Despite no differences were found among the two groups in terms of cholesterol levels or proximal lesion area, as previously observed in atherosclerosis studies on hPCSK9 tg mice, differences in lesion composition

were identified. To investigate whether the inflammatory effect of hPCSK9 was dependent on the binding and degradation of LDLR, we transplanted bone marrow cells from hPCSK9 tg/LDLR$^{-/-}$ and control LDLR$^{-/-}$ mice into LDLR$^{-/-}$ recipients. The levels of hPCSK9 in the circulation were extremely high compared to apoE$^{-/-}$ recipients and this can be explained by the accumulation of plasma PCSK9 over time, since its receptor and target is absent. Moreover, despite the accumulation in the blood stream, the analysis of hPCSK9 in the plaque revealed much lower, nearly undetectable levels of hPCSK9 in the absence of LDLR, suggesting again that the mechanisms of hPCSK9 uptake by macrophages in atheroma is exclusively based on LDLR. As in apoE$^{-/-}$ recipients, no changes were found between the two groups, neither in terms of cholesterol levels, nor in lesion size. Also the analysis of Ly6C^{high} positive cells in the plaque revealed no differences between controls and PCSK9 mice. It was previously demonstrated that macrophage LDLR has a pro-atherogenic effect in the plaque since, in BMT studies, its absence reduces the accumulation of macrophages into the plaque and thus atherosclerosis progression (Linton, Babaev et al. 1999). Therefore, the absence of LDLR, might mitigate the inflammatory effect of PCSK9 on inflammation.

In conclusion, our results showed that hPCSK9 secreted by macrophages accumulates in plasma and reduces macrophage LDLR levels, while the effects on macrophage LRP1 still need to be further investigated. hPCSK9 expression in macrophages directly influencse atherosclerotic plaque composition in the absence of changes in serum cholesterol levels, suggesting a direct effect of PCSK9 in macrophage inflammation and plaque development. The effect on inflammation is dependent on LDLR, since no effects on lesion composition were identified in its absence. Thus, an anti-

inflammatory effect of PCSK9 inhibitors can be envisioned as additional benefit of therapy for hypercholesterolemia.

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