Université de Montréal

PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9 IN HUMAN DISEASE

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RÉSUMÉ

Les maladies cardiovasculaires (MCV) demeurent au tournant de ce siècle la principale cause de mortalité dans le monde. Parmi les facteurs de risque, l'hypercholestérolémie et l'obésité abdominale sont directement liées au développement précoce de l'athérosclérose. L'hypercholestérolémie familiale, communément associée à une déficience des récepteurs des lipoprotéines de basse densité (LDLR), est connue comme cause de maladie précoce d'athérosclérose et de calcification aortique chez l'humain. La subtilisine convertase proprotéine/kexine du type 9 (PCSK9), membre de la famille des proprotéines convertases, est trouvée indirectement associée aux MCV par son implication dans la dégradation du LDLR. Chez l'humain, des mutations du gène *PCSK9* conduisent soit à une hypercholestérolémie familiale, soit à une hypocholestérolémie, selon que la mutation entraîne un gain ou une perte de fonction, respectivement. Il demeure incertain si les individus porteurs de mutations causant un gain de fonction de la PCSK9 développeront une calcification aortique ou si des mutations entraînant une perte de fonction provoqueront une obésité abdominale. Dans cette étude, nous avons examiné : **1)** l'effet d'une surexpression de PCSK9 dans le foie de souris sur la calcification aortique ; **2)** les conséquences d'une déficience en PCSK9 (*Pcsk9* KO), mimant une inhibition pharmacologique, sur le tissu graisseux.

Nous avons utilisé un modèle de souris transgénique (*Tg*) surexprimant le cDNA de PCSK9 de souris dans les hépatocytes de souris et démontrons par tomographie calculée qu'une calcification survient de façon moins étendue chez les souris PCSK9 *Tg* que chez les souris déficientes en LDLR. Alors que le PCSK9 *Tg* et la déficience en LDLR causaient tous deux une hypercholestérolémie familiale, les niveaux seuls de cholestérol circulant ne parvenaient pas à prédire le degré de calcification aortique. Dans une seconde étude, nous utilisions des souris génétiquement manipulées dépourvues de PSCK9 et démontrons que l'accumulation de graisses viscérales (adipogenèse) apparaît régulée par la PCSK9 circulante. Ainsi, en l'absence de PCSK9, l'adipogenèse viscérale augmente vraisemblablement par régulation post-traductionnelle des récepteurs à lipoprotéines de très basse densité (VLDLR) dans le tissu adipeux.

Ces deux modèles mettent en évidence un équilibre dynamique de la PCSK9 dans des voies métaboliques différentes, réalisant un élément clé dans la santé cardiovasculaire. Par conséquent, les essais d'investigations et d'altérations biologiques de la PCSK9 devraient être pris en compte dans un modèle animal valide utilisant une méthode sensible et en portant une attention prudente aux effets secondaires de toute intervention.

Mots clés:

Subtilisine convertase proprotéine/kexin type 9, récepteur des lipoprotéines de faible densité, hypercholestérolémie familiale, calcification aortique, métabolisme des graisses.

ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death in the 21st century. Among risk factors, hypercholesterolemia and abdominal obesity are directly linked to premature development of atherosclerosis. Familial hypercholesterolemia, commonly due to low-density lipoprotein receptor (LDLR) deficiency, is known to cause premature atherosclerosis and aortic calcification in humans. Proprotein convertase subtilisin/kexin 9 (PCSK9), a member of the proprotein convertase family, is indirectly associated with CVD through enhanced LDLR degradation. Mutations in the human *PCSK9* gene lead to either familial hypercholesterolemia or hypocholesterolemia, depending on whether the mutation causes a gain or a loss of function, respectively. It is uncertain if individuals carrying mutations causing a gain-of-function of PCSK9 will develop aortic calcification or whether loss-of-function mutations will lead to abdominal obesity. In this thesis, we investigated: **1)** the effect of PCSK9 overexpression on aortic calcification; **2)** the consequences of PSCK9 deficiency, mimicking pharmacological inhibition of PCSK9 on fat tissue.

We employed a transgenic (*Tg)* mouse model overexpressing mouse PCSK9 and illustrated by micro-computerized tomography that calcification occurs to a lesser extent in PCSK9 *Tg* mice than in *LDLR*-deficient mice. While both PCSK9 *Tg* and *LDLR* deficiency caused familial hypercholesterolemia, circulating cholesterol levels alone could not dictate the degree of aortic calcification. In another study, we used genetically modified mice lacking PCSK9 and demonstrated that visceral fat accumulation (adipogenesis) is regulated by circulating PCSK9. Thus in the absence of PCSK9, visceral adipogenesis increases likely *via* post-translational regulation of very-low-density lipoproteins receptors (VLDLR) in the adipose tissue.

In conclusion, these two studies highlight the dynamic balance of PCSK9 in different metabolic pathways, making it a key element in cardiovascular health. Consequently, attempts to survey and/or alter PCSK9 biology should be performed in a valid animal model using sensitive methods and with careful attention to side effects of any given intervention.

Key words:

Proprotein convertase subtilisin/kexin 9, low-density lipoprotein receptor, familial hypercholesterolemia, aortic calcification, fat metabolism.

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LIST OF ABBREVIATIONS

ABCA1	*ATP-binding cassette, sub-family A1*
AoCS	*aortic calcium score*
apoER2	*apolipoprotein E receptor 2*
BMI	*body mass index*
CETP	*cholesteryl ester transfer protein*
CVD	*cardiovascular disease*
DEXA	*dual-energy X-ray absorptiometry*
EGF	*epidermal growth factor-like repeat*
ER	*endoplasmic reticulum*
FDB	*familial defective apolipoprotein B*
FFA	*free fatty acid*
FH	*familial hypercholesterolemia*
HDL	*high-density lipoprotein*
HDL-C	*high density lipoprotein cholesterol*
HMG-CoA	*hydroxymethylglutaryl coenzyme A reductase*
HSPG	*heparin sulfate proteoglycans*
IDL	*intermediate-density lipoprotein*
KO	*knockout*
LCAT	*lecithin:cholesterol acyl transferase*
LDL	*low-density lipoprotein*
LDL-C	*low-density lipoprotein cholesterol*
ldlr $^{-/-}$	*ldlr gene knockout in mice*
LDLR	*low-density lipoprotein receptor*
LNA	*locked nucleic acid*
LPL	*lipoprotein lipase*
LRP	*LDL-receptor-related protein*
mAb	*monoclonal antibody*
micro-CT	*micro computed tomography*
mRNA	*messenger RNA*
PC	*proprotein convertase*
PCNA	*proliferating cell nuclear antigen*
PCR	*polymerase chain reaction*
PCSK	*PC subtilisin/kexin*
Pcsk9 $^{-/-}$	*Pcsk9 gene knockout in mice*
PCSK9	*proprotein convertase subtilisin/kexin type 9*
PLTP	*phospholipid transfer protein*
QPCR	*quantitative polymerase chain reaction*
RAP	*receptor-associated protein*
RCT	*reverse cholesterol transport*
RGD	*arginine-glycine-aspartic acid*
S1P	*site 1 protease*
siRNA	*small interfering RNA*
SKI-1	*subtilisin kexin isozyme-1*
SNP	*single nucleotide polymorphism*
SR-BI	*scavenger receptor BI*
SREBP	*sterol regulatory element binding protein*
TC	*total circulating cholesterol*

LIST OF ABBREVIATIONS (cnt'd)

Tg	*transgenic*
TG	*triglycerides*
TGN	*trans-Golgi network*
UWW	*underwater weighing*
VLDL	*very-low-density lipoprotein*
VLDLR	*VLDL receptor*
WC	*waist-circumference*
WD	*Western diet*
WHO	*World Health Organization*
WHR	*waist-hip ratio*
WT	*wild type*

I dedicate this thesis to my wife, kids, parents and siblings for their solid support and caring, without whom, none of this would have been possible.

I extend this dedication also to my academic family, mentors and colleagues from whom the source of my energy, continuity and search for knowledge has been acquired.

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CHAPTER 1

INTRODUCTION

LITERATURE REVIEW

CHAPTER 1
LITERATURE REVIEW

1. Pandemic of cardiovascular disease

Cardiovascular disease (CVD) is the number one killer disease in developed countries (*Murray et al 1997, Yusuf et al 2001*). According to the World Health Organization (WHO) report this has been also the case in developing countries during last year (WHO 2011). The rapid urbanization of the developing world has led to the pandemic of CVD with significant impact on individuals, societies and global well-being (*Ruff et al 2010*). It is estimated that around 80% of CVD deaths will strike low- to middle-income countries with limited medical resources. In less than 20 years close to 24 millions of individuals will die from CVD worldwide. The largest percentage will occur in the Middle East and the largest absolute number of deaths alone will take place in the Indian subcontinent and the Far East (WHO 2011).

This is in accord with epidemiological studies provided insight into the shifting etiology of CVD from the drastic infection-related rheumatic heart and syphilitic aortitis (*Nakazone et al 2010*) to an insidious and slowly progressing etiology related to physical inactivity and caloric excess. This shift has led to the blowup of secondary dyslipidemia that gradually emerged from affluent societies to less wealthier ones. The emphasis today is to tackle modifiable risk factors such as hypercholesterolemia and improve treatment strategies to lessen this burden (*Ruff et al 2010*).

In this chapter I will discuss hypercholesterolemia and abdominal obesity and the challenges facing disease detection and cure. Chapter 2 will be an application of an imaging method extensively used in human studies that has been applied to two hypercholesterolemic animal models to evaluate arterial calcification. Chapter 3 will explore the effect of a hypothetical pharmacological approach to treat hypercholesterolemia and the potential abdominal obesity as a side effect. Finally I will conclude and summarize the overall progress in Chapter 4.

1.1. Risk factors

Diabetes mellitus, abdominal obesity and hypercholesterolemia are among the top modifiable risk factors of CVD along with hypertension and smoking (*Yusuf et al 2004*). Individuals with features of the first three risk factors are diagnosed with the metabolic syndrome and therefore at a higher risk of atherosclerosis and vascular calcification

(*Ellison et al 2005*) **(Table I)**. Other important behavioral risk factors of CVD are Western diet and inactivity.

Table I: Risk factors for cardiovascular disease †	
Risk factor	Population-attributable risk (%)
Diabetes mellitus *	**12.3**
No exercise	12.5
No fruit and vegetable intake	12.9
No alcohol intake	13.9
Hypertension	23.4
Psychosocial factors ‡	28.8
Abdominal obesity *	**33.7**
Current smoking	36.4
Hypercholesterolemia* (High Apo B:Apo A1 ratio)	**54.4**

†From the INTER-HEART study. * Risk factors for atherosclerosis and arterial calcification. ‡Depression, perceived stress at home or work (general stress), low locus of control, and major life events. (Abbreviations: Apo, apolipoprotein; MI, myocardial infarction). Taken from *Ruff et al. Nat. Rev. Cardiol; 2010.*

Behavioral risk factors are responsible for about 80% of CVD and are usually modifiable, but genetic risk factors including gender and age, are not (*Ruff et al 2010*). Adherence to treatment guidelines to modify risk factors is a current priority. Although not always easy to treat, failing to succeed in reducing modifiable risk factors will aggravate chronic illness to a new unanticipated dimension. Along the same lines, advancement in biomedical research leading to our better understanding of complex disease should translate into higher and earlier detection rate that will most certainly enhanced treatment modalities and engender new pharmacological approaches.

1.1.1. Hypercholesterolemia

Hypercholesterolemia is caused by excessive circulating cholesterol levels above the 95th percentile of a given population (*Bae et al 1993*). Hypercholesterolemia is a well known risk factor to CVD (*Goldstein et al 1987*). In the INTER-HEART study (an international heart study designed to assess the importance of risk factors on CVD worldwide) it had the highest attributable risk (54%) for development of myocardial infarction (*Ruff et al 2010*) **(Table I)**. In order to understand how excess cholesterol may leads to medical complications we have to study how cholesterol is carried in the blood

and the essential role of different players and mediators like the essential role of the low-density lipoprotein receptor (LDLR) in disease progression and treatment.

1.1.1.1. Lipid metabolism and the role of LDLR

Lipoprotein particles including chylomicrons, very-low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) carry cholesterol in the blood. Lipoprotein particles also transfer and accept cholesterol, triglycerides (TG) and proteins from other lipoprotein particles and tissues. These lipoprotein particles are separated and classified according to their densities and diameters that reflect their load of cholesterol, TG and apolipoproteins **(Figure 1)** (*Grundy et al 1990*). A single lipoprotein particle is usually composed of apolipoproteins, phospholipids, TG, free cholesterol and cholesteryl esters.

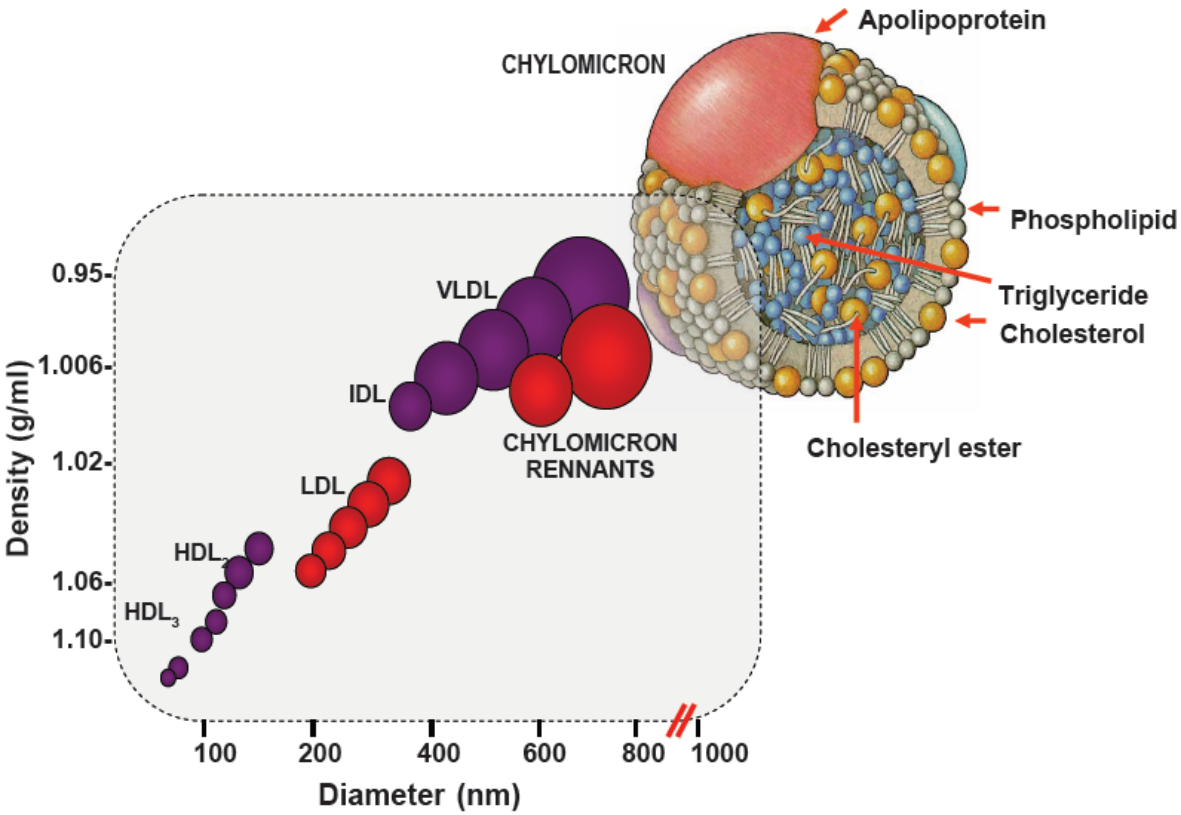

Figure 1. Lipoprotein classes and subclasses. A lipoprotein particle is composed of apolipoprotein, phospholipids, triglycerides, cholesterol and cholesteryl esters. Taken from *Grundy et al. Cholesterol and atherosclerosis; 1990.*

Lipoproteins are subjected to plasma and tissue enzyme actions and are removed from the circulation through receptor–mediated uptake by three pathways (*Brown et al 1983, Von Eckardstein et al 2001*) **(Figure 2)**:

1. The exogenous pathway, which dictates the fate of dietary lipids.
2. The endogenous pathway, which dictates the fate of lipid synthesized in liver.
3. The reverse cholesterol transport pathway, which dictates the fate of peripheral tissue lipids and the excess return back to the liver.

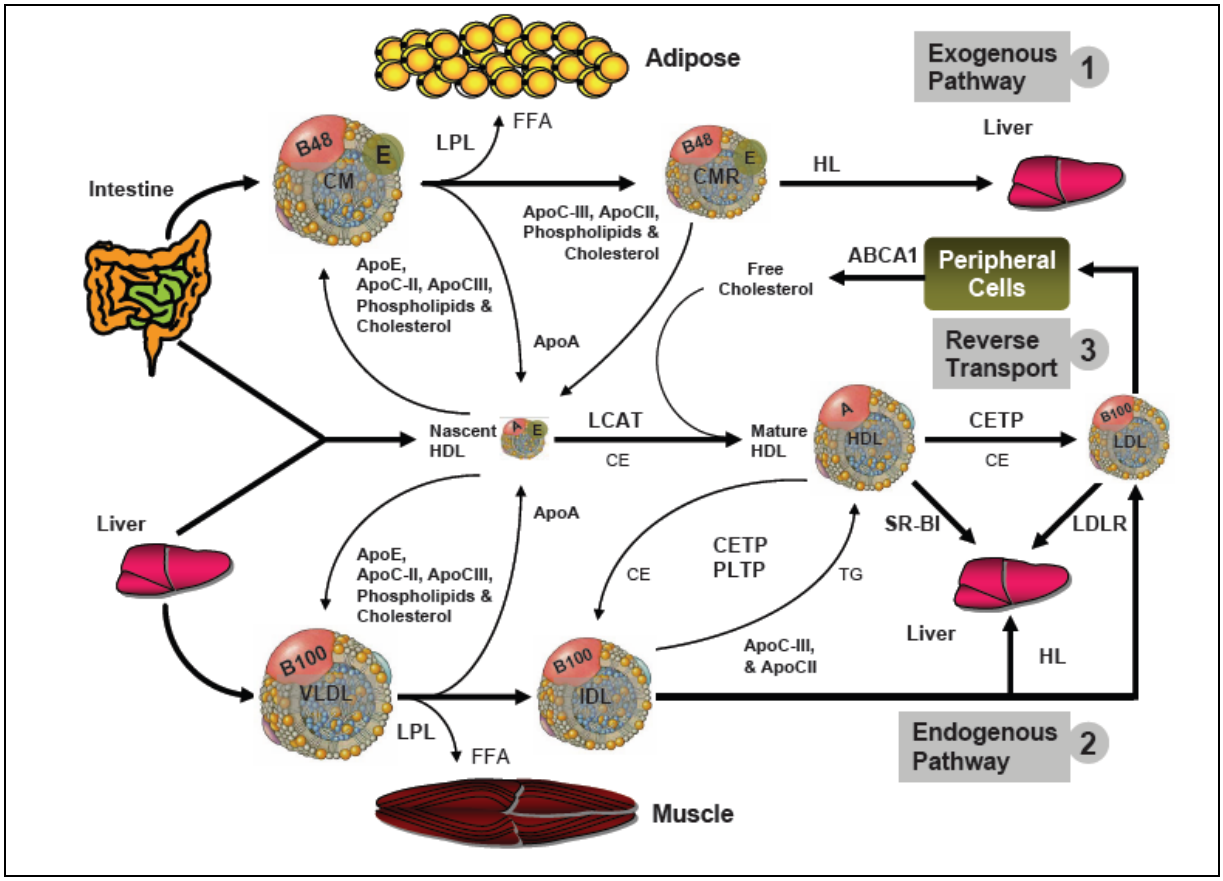

Figure 2. Lipid metabolism; exogenous, endogenous and reverse cholesterol transport pathways are depicted. VLDL, CM and HDL initial and mediate the exogenous, endogenous and reverse cholesterol transport processes respectively in the human body. (Abbreviation – ABCA1: ATP-binding cassette 1, CETP: Cholesteryl ester transfer protein, CM: chylomicron, FFA: free fatty acid, HDL: high-density lipoprotein, IDL: intermediate-density lipoprotein, LCAT: lecithin:cholesterol acyl transferase, LDL: low density lipoprotein, LPL: lipoprotein lipase, PLTP: phospholipid transfer protein, VLDL: very low density lipoprotein). Taken from *Marcil M et al*. Hypoalphalipoprotéinémie familiale. Aspects cliniques, génétiques, cellulaires et moléculaires. Doctoral Thesis, Université de Montréal, 1999; p. 17.

The exogenous pathway. The intestinal epithelium assembles dietary TG and cholesterol, along with apolipoproteins (apo) B-48 and apoA and phospholipids into chylomicron (CM) particles. This facilitates its travel inside the aqueous plasma to deliver lipid to different tissue after having a meal. Following their secretion into the plasma, an exchange of components takes place between chylomicrons and HDL, wherein plasma the cholesterol, apoE, apoC-II, apoC-III and phospholipids are transferred from HDL to chylomicrons and apoA is transferred from chylomicrons to HDL. ApoC-II in the chylomicrons activates the lipoprotein lipases (LPL) found on the epithelium of the muscle and adipose tissue (*Saxena et al 1991*) and associated with heparan sulfate proteoglycans (HSPG). When a VLDL particle docks on a VLDL receptor (VLDLR), the TG in the core are hydrolyzed by LPL. Newly liberated free fatty acids (FFA) then enter either the muscle cells (metabolized for energy via β-oxidation) or the adipocytes synthesized back to TG for storage (*Jensen et al 2003*). As a result, the chylomicrons shrink in size and apoC-II, apoC-III, phospholipids and free cholesterol are transferred back to HDL particles. Chylomicron remnants containing apoB-48, apoE and cholesteryl esters are internalized and catabolized after binding the hepatic apoE receptor (*Brown et al 1983, Gregory et al 2001*).

The endogenous pathway. *De novo* synthesized lipids by the liver are incorporated in nascent VLDL particles. These VLDL, contain TG, cholesterol, apoB-100 and phospholipids assembled and secreted by the hepatocytes into the plasma when the body is in a fasting state. In the plasma, apoC-II, apoC-III and apoE are transferred to VLDL from HDL. Apo C-II activates LPL to hydrolyze TG from VLDL and yield a smaller and denser intermediate-density lipoprotein (IDL) particle. Some IDLs particles are removed by the liver *via* an apoE receptor (*Brown et al 1983*), while the remaining particles transfer CE to HDL in exchange for TG. Conversion of IDL to LDL is complete after the cholesteryl esters are transferred from HDL to IDL, while TG and apolipoproteins (except apo B-100) are removed from IDL. LDL delivers cholesterol to hepatocytes and peripheral cells (e.g. adrenal gland) *via* LDL receptor (LDLR) uptake. Scavenger cells (e.g. macrophages) in blood vessels may pick up oxidized LDL particles, in case of hypercholesterolemia, leading to atherosclerotic plaque formation (*Brown et al 1983, Gregory et al 2001*).

Reverse cholesterol transport. HDL particles mediate the return of cholesterol from peripheral cells to the liver to protect the vessels form the harmful effect of excess lipids. Nascent HDL particles containing apoA1, apoE are assembled and secreted by the liver and intestine. Free cholesterol from other lipoproteins or peripheral cells is transferred to HDL and esterified by LCAT. The cholesteryl esters can be transferred to other lipoproteins such as IDL and LDL to be derived back to the liver or transported directly by HDL particles to the liver through scavenger receptor class B type I (SR-BI) (*Von Eckardstein et al 2001*).

The LDLR is mainly a hepatic cell surface receptor (*Brown et al 1983*) that recognizes the apoB-100 ligand, but also apoE carried on VLDL remnant, IDL and some classes of HDL (*Innerarity et al 1990, Kane et al 2001*). Shortly after the LDLR discovery in 1979, Brown and Goldstein won the Nobel Prize for what was considered the key element in the cholesterol endogenous pathway (*Brown et al 1983*).The receptor-mediated endocytosis of the LDL particles by LDLR occurs in most, but not all, cells in a clathrin coated vesicles assisted by the adapter proteins ARH (*Garcia et al 2001*). Then when internalized the low pH in the vesicles facilitate the dissociation of the LDLR from its ligand. LDLR then recycles back to the surface to be associated with more LDL particle and eventually cholesterol levels fall in the circulation (*Goldstein et al 1985*). The liver then utilizes the cholesterol and TG to synthesize VLDL particles which are then exported once again to the circulation and/or excreted in the intestine as bile acids.

The LDLR is a 839-amino acids transmembrane glycoprotein that is encoded on chromosome 19 (*Brown et al 1987*) **(Figure 3)**. Its N-terminal ligand-binding domain is 292-amino acids long that compose a cysteine-rich sequence. Its C-terminal cytoplasmic domain composes 50-amino acids long and serves to direct the receptor to the coated pits. In addition the extracellular portion of the receptor contains a 22-amino acid membrane-spanning region, a 58-amino acid region of 18 O-linked carbohydrate chains and a 400-amino acid region that is homologous to the precursor for epidermal growth factor (EGF) (*Brown et al 1987*).

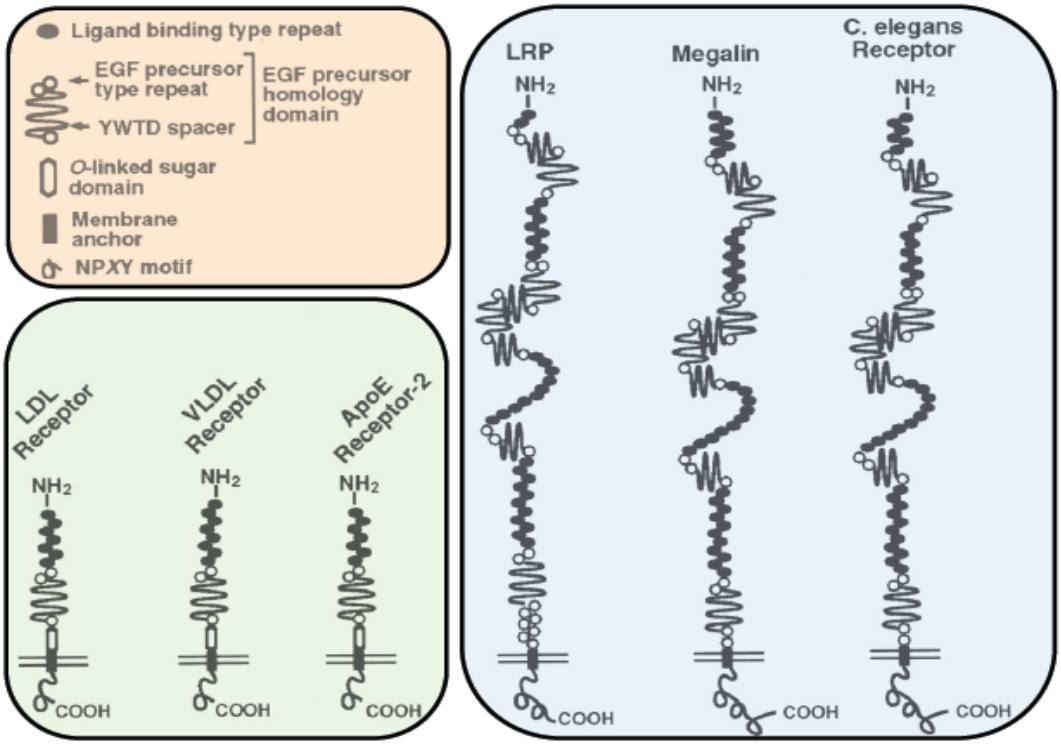

Figure 3. The LDL receptor superfamily. The structural organization of some members of the LDL receptor gene family is depicted. The closest member to the LDLR is the VLDLR followed by the ApoER2. Taken from *Schneider et al*. *J Am Soc Nephrol*; 1999.

The EGF precursor homology domains consist of EGF repeats and YWTD spacer regions whish are involved in the pH-dependent release of the ligands from LDLR. The LDLR belongs to the LDLR superfamily that shares structural similarity with members of the LDL receptor gene family (Figure 3). Sequence alignment programs revealed that the closest members to the LDLR in structure were VLDLR (59% identity) and apolipoprotein E receptor 2 (ApoER2) (46% identity) followed by LRP, Megalin and *C-elegans* receptor (*Poirier et al 2009*).

There are over one thousand mutations identified in the *LDLR* gene to date (*Guardamagna et al 2009*) and they are classified into the 4 following groups (*Goldstein et al 1987, Hobbs et al 1990*):

1. Null mutations or LDLR defective synthesis (e.g. >15kb deletion *in LDLR* genes, a common French-Canadian mutation).

2. LDLR defective transport from the endoplasmic reticulum (ER) to the trans-Golgi network (TGN) (or gain-of-function mutation in PCSK9 gene which targets intra-cellular LDLR).
3. LDLR defective binding (or loss of function mutation in *APOB* gene encoding the apoB-100 ligand).
4. LDLR defective internalization where receptors do not cluster in the coated pits, thereby minimizing LDL internalization (e.g. mutations in autosomal recessive hypercholesterolemia *"ARH"* gene encoding the adaptor protein ARH).

1.1.1.2. The etiology of hypercholesterolemia

Monogenic hypercholesterolemia (or familial hypercholesterolemia - Fredrickson classification IIa) is the most common cause of markedly elevated serum cholesterol concentrations. Familial hypercholesterolemia (FH) is diagnosed by plasma low-density lipoprotein cholesterol (LDL-C) elevations 2-fold (heterozygote) or 3- to 5-fold (homozygote) above the average of a given population, age- and gender–matched, while serum TG concentrations remains within normal range (*Rader et al 2003*). Mixed hypercholesterolemia (familial combined dyslipidemia, Fredrickson classification IIb) has both LDL-C and TG elevations. This condition is caused by a susceptible genotype aggravated by one or more risk factors, including atherogenic diet (saturated fat, trans-fat, and cholesterol) (*Hu et al 1997*), obesity, and lack of exercise. Monogenic hypercholesterolemia is associated with xanthelasma and cutaneous xanthomas that can be sometimes extensive and disfiguring in homozygous FH. More importantly, affected FH patients are at increased risk for premature coronary artery disease and death at a younger age. Homozygous FH can experience their first cardiac event in childhood and only few survive to reach adulthood (*Awan et al 2008*).

The carrier frequency of mutations leading to FH in the general population is 1:500 (*Brown et al 1983*); however, this frequency increases in the French-Canadian descent who may suffer the "founder effect" of the first French settlers. This is due to many years of geographical isolation and a small genetic pool. The carrier frequency in some of the rural sites in Quebec can reach 1:80 (*Scriver 2001*). This phenomenon can be observed not only in the French-Canadian population but in similar culturally isolated environments in South-Africa and in some regions of Italy, Scandinavia and Lebanon (*Khachadurian 1964*).

From a practical point of view, human mutations in FH are of two major types depending on LDLR residual activity: < 2% (receptor-negative, poor response to statin) or between 2-25% (receptor-defective, good response to statin) (*Rader et al 2003*). Thus levels of residual LDLR activity correlates negatively with plasma levels of LDL-C. Etiologically speaking hypercholesterolemia can be a problem of cholesterol clearance (primary) or a problem of over production (secondary):

A. Primary or genetic etiologies are collectively called FH; classically a monogenic disorder caused by mutations in *LDLR* gene leading to either an absence or low activity of the LDLR. Since LDLR seminal discovery by *Brown et al*, three additional gene mutations have been discovered as primary cause of FH **(Figure 4)**: *APOB* gene (FDB, familial defective apolipoprotein B) (*Humphries et al 2006*), *ARH* gene (encoding the adaptor protein, also known as autosomal recessive hypercholesterolemia) (*Garcia et al 2001*) and the recently described gain-of-function mutations in *PCSK9* gene. PCSK9 will be discussed in greater details in Section 2.

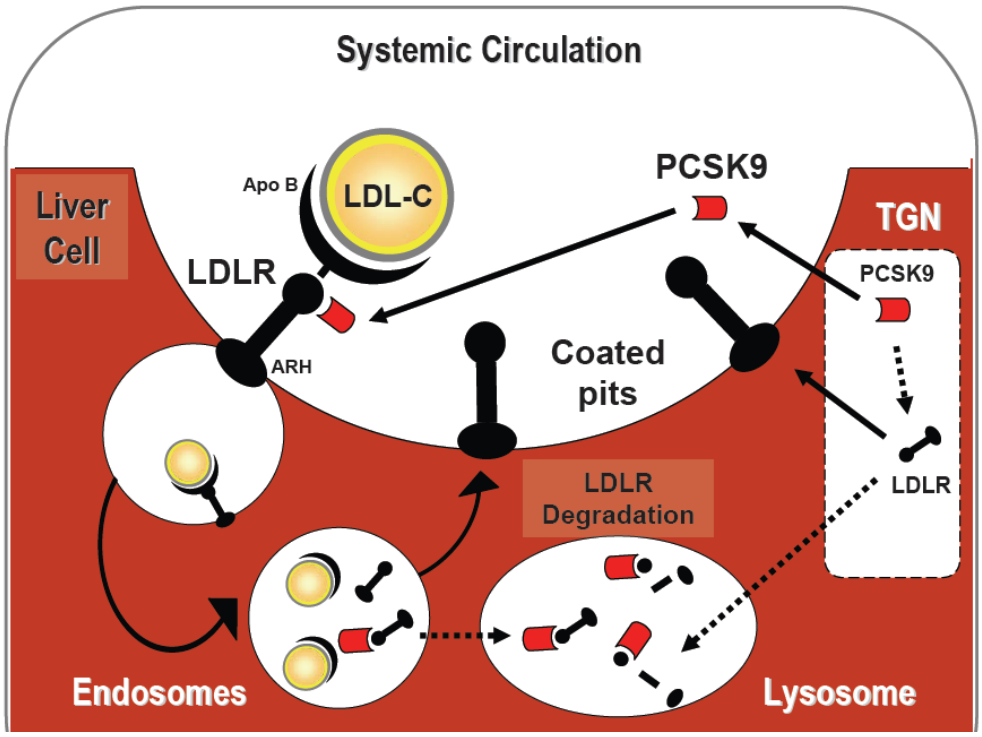

Figure 4. LDLR recycling and associated genetic defect. Familial Hypercholesterolemia (FH) is caused by mutations in *LDLR* gene leading to either an absence or low activity of the LDLR, mutations in *APOB* gene (Familial defective apolipoprotein B), mutations *ARH* gene (Autosomal Recessive Hypercholesterolemia) or gain-of-function mutations in *PCSK9* gene (encoding the LDLR regulatory protein).

B. Secondary or acquired etiologies of hypercholesterolemia may include metabolic disorders like liver disease (bile duct obstruction), renal failure (nephrotic syndrome) (*Awan et al 2009*) and hypothyroidism (*Abrams et al 1981*).

1.1.1.3. Treatment of hypercholesterolemia

Before the statin era, cholestyramine (bile acid binder) and niacin (vitamin B3 analog) (*Goldstein et al 1987, Grundy et al 1981*) were among the commonly used drugs to lower cholesterol, but were limited in usage due to their intolerability. Statins on the other hand represent a class of medication that inhibits the hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase (*Goldstein et al 1987*), a rate limiting enzyme in endogenous cholesterol synthesis. As a consequence statins, by reducing the endogenous levels of cholesterol synthesis, increase the uptake of circulating LDL particles, with significantly lower side effects leading to higher patient compliance. The mechanism of statins' action is mediated by the upregulation of the levels of cell surface LDLR, which is found ubiquitously in all cells, but predominantly in hepatocytes (*Goldstein et al 1987*). Upregulation of the LDLR at the surface of hepatocyte increases LDL particles uptake in the liver and effectively lowers plasma cholesterol in blood (*Goldstein et al 1987*). The *LDLR* gene is under the influence of sterol regulatory element binding protein 2 (SREBP-2) which binds specific sterol regulatory elements in the promoter of the *LDLR* gene, thus up regulating the expression of LDLR in response to low sterol (*Horton et al 2002*). The transcription factor SREBP-2, in response to statin, also upregulates a molecule termed PCSK9 to counter regulate the LDLR effect (*Dubuc et al 2004*). PCSK9 binds to the LDLR *via* its catalytic domain, which interacts with the epidermal growth factor (EGF) A domain on the extracellular region of the LDLR (*Zhang et al 2007*) **(Figure 3)**. The EGF precursor domain modulates LDL particle binding to the LDLR and mediates the acid-dependent dissociation of the ligand from the LDLR. Although the EGF-like repeats on LDLR are homologous to other LDLR family members (*Christensen et al 1999*) **(Figure 3)**, there was no *in vivo* evidence (until our study) that PCSK9 binds other members of the LDL receptor gene family like the closely related VLDLR and the apoER2, although this was reported in cell lines (*Poirier et al 2008*).

Statins not only increase LDLR and decrease cholesterol levels, but also have been associated with a 20-25% reduction in CVD in over 14 randomized clinical trials (*Baigent et al 2005*). The additional protective role on CVD beyond cholesterol lowering can be

explained by the pleiotropic effects of statins (*Davignon et al 2004*). These pleiotropic properties influence various aspects of cell functions, inflammation, coagulation and vasomotor activity (*De Lorenzo et al 2006*). However, in patients lacking LDLR or having significantly lower LDLR activity, the statin class of medications is not efficient to lower cholesterol on their own, and ezetimibe (*Rader et al 2003*), a class of specific inhibitors of intestinal cholesterol absorption, are usually added. If all pharmacological attempts to lower cholesterol fail, a drastic invasive technique, LDL apheresis (Rader *et al* 2003), is then required. LDL apheresis, a type of dialysis to filtrate lipoproteins, has allowed homozygous FH patients to live beyond their life expectancy; however they are not free of disease as they experience arterial calcification as a late complication of the original disease (*Awan et al 2008*).

In summary lipoproteins and lipoprotein disorders play an important role in the onset and progression of CVD. Available treatments to date, including statins and LDL apheresis, are not sufficient to eliminate cardiovascular risk and the search for newer and safer cholesterol-lowering agents continues. Therefore, classes of drugs, such as the PCSK9 inhibitors are viable candidates, as will be discussed in section 2.

1.1.2. Abdominal obesity

A state of excess visceral fat in the abdomen is termed abdominal obesity. The WHO terms abdomen obesity a worldwide epidemic. Public health leaders marked obesity and inactivity as the second leading cause of preventable deaths in the West (*Mokdad et al 2005*). In the INTER-HEART study (an international heart study designed to assess the importance of risk factors on CVD worldwide) abdominal obesity carried a 33.7% attributable-risk of developing myocardial infarction **(Table I)**. The WHO was also alarmed by the rise of childhood obesity (*Koletzko et al 2002*). Excess calories in children fuel the formation of new adipocytes and lead to hyperplastic obesity. This differs from adulthood caloric-excess that leads to hypertrophic obesity. Individuals with hyperplastic obesity are more likely to encounter difficulties in losing weight, since diet alone decreases the size but not the number of adipocytes. Childhood overweight and obesity is associated with higher CVD risk (*Teixeira et al 2001*) and the persistence of obesity is linked to the development of multiple risk factors (*Srinivasan et al 1996*), which in addition to developing CVD may lead to diabetes and cancer (*Haslam et al 2005*).

One billion adults and one tenth of the world's children are now classified as overweight or obese and will experience diminished life expectancy (*Haslam et al 2005*). For instance obesity has been shown to decrease life expectancy by 7 years at the age of 40 (*Peeters et al 2003*).

1.1.2.1. Fat metabolism and the role of VLDLR

Similar to the LDLR in hepatocytes, the very-low-density lipoprotein receptor (VLDLR) plays a major role in adipocyte fat metabolism. VLDLR is highly expressed in adipose tissue (*Takahashi et al 2004, Tacken et al 2001*). In rabbits, the mRNA expression of VLDLR was significant in muscles and adipose tissues, but not detectable in the liver. This indicates a key role of VLDLR in the fatty acid metabolism of peripheral tissues (*Oka et al 1994*). Different from the LDLR, the VLDLR is not regulated by cellular sterols and does not bind efficiently LDL particles. However estradiol, thyroid hormone and active vitamin D modulate VLDLR expression (*Tacken et al 2001*). Feeding status *per se* did not influence mRNA levels of VLDLR, but atherogenic diets upregulate the expression of VLDLR in different animal models (*Tiebel et al 1999*).

Initially the involvement of VLDLR in lipoprotein metabolism could not be easily illustrated using a VLDLR knockout mouse. Apart from a 15-20% adipose mass reduction, lipid parameters were not significantly altered (*Frkman et al 1995*). It was only later, when VLDLR and LDLR double knockout mice became available, that the contribution to fatty acid delivery from TG-rich lipoprotein particles in peripheral tissues was elucidated (*Tacken et al 2000*). VLDLR-deficient mice were protected against diet-induced obesity and insulin resistance (*Goudriaan et al 2000*). VLDLR binds apoE-enriched chylomicrons and VLDLs, IDLs, as well as LPL that hydrolyzes TG into FFA **(Figure 5)**. These interactions are facilitated by HSPG that binds LPL as well (*Takahashi et al 2004, Tacken et al 2001*), whereas binding to ApoC-I, inhibits FFA hydrolysis (*Jong et al 1996*). Once the binding is facilitated by VLDLR **(Figure 5)**, LPL starts to hydrolyze TG from TG-rich particles and FFAs diffuse into the cell were they become esterified or reform back to TG and contribute to the lipid core.

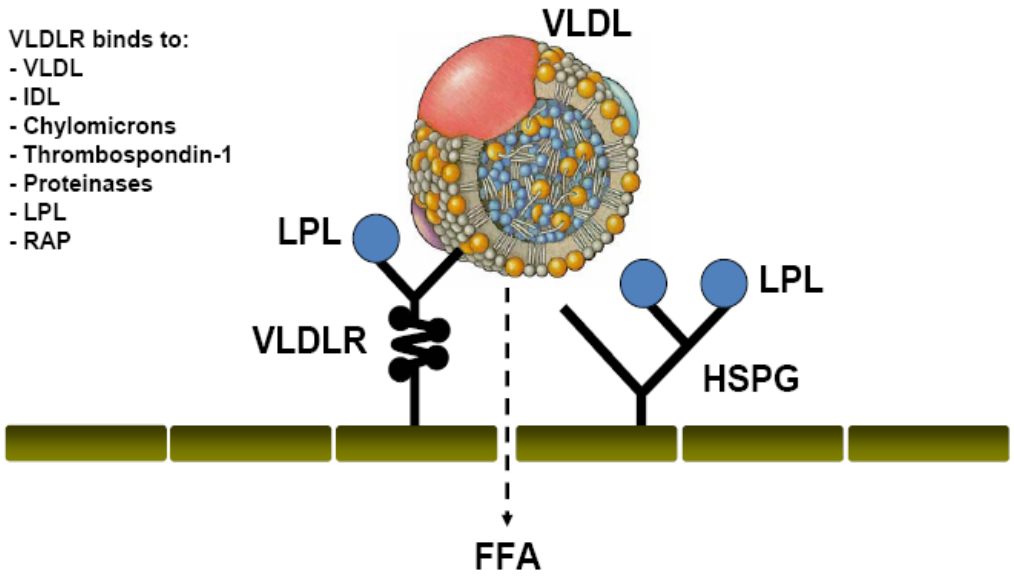

Figure 5. The VLDLR role in fat metabolism. The VLDLR and HSPG facilitate LPL proximity to VLDL leading to TG hydrolysis and FFA entry to the cell. Taken and modified from *Tacken et al*. *Curr Opin Lipidol*; 2001.

1.1.2.2. The etiology of obesity

A simplified mechanism of obesity is energy disequilibrium leading to excessive calories stored in adipose tissue when energy expenditure is reduced (*Rosenbaum et al 1997*). However, the exact mechanism of obesity is not fully understood and is likely multifaceted (*Bouchard et al 1991*) **(Figure 6)**. Evidence to support the fact that obesity is under genetic influences comes from identical twins studies (*Bouchard et al 1990*). Depending on the method used, genetic variation is estimated to account for up to 50% of the weight variability (*Bluchard et al 1988*). The total transmission effect to obesity represents 35% when estimated by body mass index (BMI), but only 5% are linked to a genetic defect (*Bouchard et al 1991*). Hence, in contrast to overall weight, BMI is more sensitive to lifestyle and environmental conditions. Genetics can explain up to 25% of the individual differences in percent body fat and mass (*Bouchard et al 1991*). Therefore genetic predisposition alone does not necessarily translate into obesity. Although the transmission of a way of life and some genetic factors influence how the body regulates appetite and energy expenditure (*Grundy et al 1998*), the total transmission effect alone could not contribute significantly to the endemic rise of obesity **(Figure 6)**. Therefore classification of obesity by genetic means may require more studies (*Pi-Sunyer 2000*).

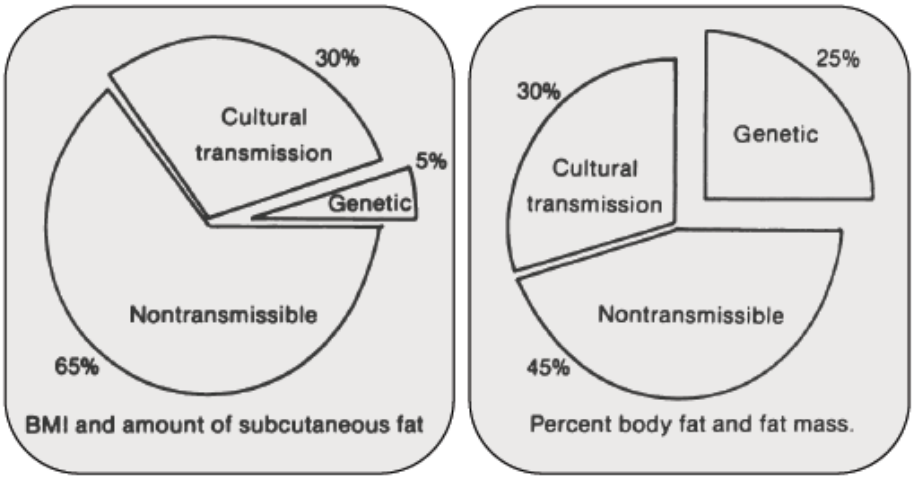

Figure 6. Etiology of obesity: genetic vs. non-genetic factors. Total transmissible variance and its genetic component for body mass index (BMI), subcutaneous fat (skin-fold thickness) and for total body fat from underwater weighing. Taken from *Bouchard et al. Am J Clin Nutr; 1991.*

The only fully studied gene that causes direct obesity is the gene leading to congenital leptin deficiency (*Rosenbaum et al 1997*). Mouse model simulating the human disease is designated *ob/ob* mice and becomes morbidly obese and insulin resistant on a regular diet (*Ingalla et al 1950*). Conversely polygenic mutation in over 15 genes has been linked to Bardet-Biedl syndrome that is characterized by obesity, mental retardation, and hypogonadism (*Green et al 1989, Farooqi et al 2005*). Interestingly, single-nucleotide polymorphism (SNP) in the gene termed proprotein convertase 1 gene (*PCSK1*) leads to hypoinsulinemia and elevated proinsulin and pro-opiomelanocortin (POMC). These SNPs were associated with severe childhood obesity (*Heni et al 2010, Farooqi et al 2004*).

On the other hand, environmental factors such as eating habits and physical inactivity play a major role in weight gain in westernized communities. The type and quantity of refined carbohydrates and saturated fat consumed is a direct cause of the alarming incidence of obesity (*Hu FB 2010*). Obesity usually gives rise to many chronic diseases such as hypertension, diabetes, dyslipidemia and CVD (*Haslam et al 2005*). Obesity can also be a side effect of chronic diseases such as in Cushing's syndrome and hypothyroidism, but the cause of obesity is sometimes hard to distinguish (*Longe et*

al 2006). For instance, depression in patients may be a cause of obesity, or commonly depression may lead to overweight and obesity **(Table II)**. Antipsychotic and antidepressant medications *per se* may increase weight likely by activating the SREBP transcription factors as seen in human primary hepatocytes, with subsequent up-regulation of downstream genes involved in cholesterol and fatty acid biosynthesis (*Raeder et al 2006*). A list of medications that may lead to weight gain and obesity are indicated in **Table II**. Therefore, whenever designing newer drugs that influence lipid metabolism, weight gain must be examined.

Table II: Medications that may lead to weight gain and obesity

- **Antipsychotics:** especially olanzepine
- **Antidepressants:** tricyclics, selective serotonin-reuptake inhibitors, monoamine oxidase inhibitors, mirtazepine, lithium
- **Anticonvulsants:** phenytoin, sodium valproate
- **Antihistamines:** many antihistamines, though weight gain is greater with older agents.
- **Antimigraine:** pizotifen, used as a prophylactic migraine treatment
- **Corticosteroids**
- **Oral contraceptive** and progestagenic compounds
- **β-blockers**
- **Oral hypoglycaemic agents:** glitazones (peripheral rather than visceral gain), sulphonylureas
- **Insulin**

Taken from *Haslam et al. Lancet; 2005.*

1.1.2.3. Measurement of obesity

The absolute measurement of body fat is difficult and no single method is readily available for routine clinical use (*Pi-Sunyer 2000*). Chemical analysis of fat content in animals are commonly performed while in humans were only performed in cadavers during the 60's (*Widdowson 1965, Sheng et al 1979*) and the outcome from these studies have shown a wide range of body fat content among different individuals. The underwater weighing (UWW) method or hydrodensitometry is based on the assumption that the body is divided into two compartments: adipose tissue and lean body mass (muscle and bone); as fat has a lower density under water therefore the body weighs less. Apart from being tedious and labor intensive, this method does not take into

account the high density of bones and muscles in athletes, which leads to body fat percentage underestimation, while the body fat of an osteoporotic patient may be overestimated (*Haslam et al 2005*). In contrast dual-energy X-ray absorptiometry (DEXA) is based on a three compartment measurement: bones, muscles and fat tissues. This technique is based on the assumption that bone content is directly proportional to the amount of X-ray absorbed by the bone being studied. In a comparative study, DEXA and UWW provided complementary information with DEXA values being lower. Thus, we used absolute measurement of body fat in mice for accurate results (Chapter 3). Nevertheless, DEXA is becoming the new gold standard method for human studies (*Kennedy et al 2009*).

Relative fat measurement assessing total body weight and body mass index (BMI) is also used in human studies. Obesity has conventionally been determined as a 20% increment in weight relative to subjects having the lowest death rate, matched for age and gender (*Laquatra et al 2000, Longe et al 2006*). This has been replaced completely by BMI, which is the ratio of weight (kg) to squared height (m^2). WHO now adopts and accepts a BMI of 25 kg/m^2 or more as overweight and obese when the BMI reaches 30 kg/m^2 or more (WHO 2000). This is relevant as dyslipidemia gradually develop when BMI increases above 21 kg/m^2, and when BMI reaches 30 kg/m^2, small and dense LDL particle increases the risk of coronary heart disease by 3.6-fold (*Willett et al 1995*). In addition, associated low levels of HDL cholesterol and high concentrations of TG further and independently aggravate CVD risk (*Wannamethee et al 1998*).

More importantly the pattern of fat distribution will lower the threshold at which CVD of obesity starts. Obese patients with metabolic syndrome have an apple-like abdominal obesity and are at earlier risk for diabetes, than pear-shaped fat distributed around hips and thighs (*Lebovitz et al 2003*). The former is associated with cytokines release into the circulation, such as the tumor necrosis factor, leading to a proinflammatory state, insulin resistance and CVD (*Lebovitz et al 2003*). From the 52 countries participating in the INTER-HEART study (*Yusuf et al 2004*) the predictive ability of the waist/hip ratio (WHR) is superior compared to waist circumference (WC) or BMI, although WC measurements are more practical. In contrast, in another study BMI and WC were found to perform better over WHR for fatness assessments (*Neovius et al 2005*). A waist circumference of greater than 102 cm in men and 88 cm in women is an established risk factor for insulin

resistance, diabetes mellitus and CVD (*Pi-Sunyer 2000*). However the most accurate way to measure abdominal obesity is by magnetic resonance imaging (MRI) or computed tomography (CT) scanning, although both are expensive for routine clinical use (*Pi-Sunyer 2000*).

In summary, the surge in obesity rate continues to threaten our communities and economies. Health problem related to obesity does not depend on the absolute amount of fat but rather the distribution. Quantitatively and qualitatively ways to measure fat is required to prevent chronic disease and/or detect side effect of treatment modalities. The development of safe PCSK9 inhibitors must not lead to abdominal obesity as a side effect, as this would defeat their purpose, since abdominal obesity may lead to an increased incidence of atherosclerosis and CVD.

1.1.3. Atherosclerosis

The true incidence of atherosclerosis is impossible to compute, because it remains asymptomatic for years before a person starts to complain. Therefore the frequency of clinical manifestations of atherosclerosis is used instead. The process of atherosclerosis begins during childhood with the development of fatty streaks (*McGill et al 2000*). These lesions can be found in the aorta shortly after birth and appear in increasing numbers between 8 and 18 years (*Guardamagna et al 2009*). But serious lesions begin to develop in high risk groups when an individual reaches the fourth and fifth decades of life (*Goldstein et al 1987*).

However, we must make a distinction between arteriosclerosis and atherosclerosis. Arteriosclerosis occurs in smooth muscle cell layer of the tunica media, while the atherosclerosis occurs in sub-endothelial layer of the tunica intima (*Porter et al 2004*). Arteriosclerosis refers to vessel hardening or loss of elasticity and occurs diffusely in medium size arteries. It does not cause narrowing of the lumen. Arteriosclerosis occurs as a consequence of aging, diabetes, metabolic bone diseases, renal failure, hypertension and osteoporosis **(Table III)**. Atherosclerosis on the other hand refers to vessel thickening due to plaque build up. It occurs as patches at sites of low shear stress and causes narrowing of the lumen. Atherosclerosis occurs as a consequence of hypercholesterolemia, diabetes, hyperhomocysteinemia and excessive smoking **Table III** illustrates the main differences between arteriosclerosis and atherosclerosis.

Table III. Arteriosclerosis *vs.* Atherosclerosis	
Arteriosclerosis	**Atherosclerosis**
Vessel hardening (loss of elasticity)	Vessel thickening (plaque build up)
Diffuse (medium size arteries)	Patchy (low shear stress sites)
Does not cause narrowing of the lumen	Cause narrowing of the lumen
Age, diabetics, metabolic bone disease, renal failure, hypertension and osteoporosis.	Age, hypercholesterolemia, hyperhomocysteinemia and smoking.

Summarized and taken from *wikipedia. 2011*

1.1.3.1. The pathogenesis of atherosclerosis

Atherosclerosis is characterized by foam cells and plaque formation that potentially may erupt and cause thrombosis. This may lead to blood obstruction in vital organs, like the heart or the brain. Nevertheless, atherosclerotic plaques may also regress in response to statins and become less susceptible to eruption, but the associated calcific lesions may not regress (*Chan KL et al 2010*). The oxidized LDL particles that find their way into the sub-endothelium space as a result of endothelium dysfunction are engulfed by macrophages. Foam cell are then formed and subsequently die to give fatty streaks and plaques. The plaque formation process stimulates the cells of vascular bed to differentiate and produce substances that accumulate in the sub-endothelium. Notably, calcium deposition and calcified connective tissue start to form. The inner layer of the arterial wall thickens, the artery diameter shrinks and the blood flow starts to be compromised.

1.1.3.2. The progression of arterial calcification

Aortic calcification is complex and the exact mechanism is unknown, but damage to the endothelium may be the initial hit. Damage can be due to high cholesterol, blood glucose, blood pressure or cigarette smoking (*Demer et al 2008*). The role of triglycerides in aortic calcification is unclear (*Awan et al 2008*). High levels of triglycerides are often associated with diabetes, obesity, and low levels of high-density lipoprotein cholesterol, features of the metabolic syndrome and another cause of calcific atherosclerosis (*Ellison et al 2005*). The latter are all modifiable, while heredity, sex and age are not. Some people experience calcification in their 50's or 60's, others have rapid

progressive atherosclerosis and/or calcification during their 20's, e.g., when they have a genetic predisposition as in FH (*Alrasadi et al 2009*).

From over 30 prospective clinical studies, arterial calcification was associated with most conventional cardiovascular risk factors and was deemed an independent risk factor for CVD (*Rennenberg et al 2009*). Arterial calcification is a cause of morbidity and mortality at any site: coronary arteries, peripheral arteries or aortic wall (*Budoff et al 2007, Blacher et al 2001, Awan et al 2008*). Plaque instability is a feature of calcified plaque, inducing mechanical failure leading to plaque rupture (*Hoshino et al 2009*). Aortic rigidity also leads to systolic hypertension, myocardial enlargement, myocardial infarction, heart failure, aortic aneurysm, ischemic limbs and death (*Shao et al 2010, Awan et al 2008, London et al 2002, Budoff et al 2007*). Calcification occurs most commonly in renal failure (*Moe et al 2004*), followed by uncontrolled diabetes, aggressive smoking and hypercholesterolemic disorders (*Shao et al 2010*). However, more extensive lethal calcification occurs in rare metabolic mutations as in homozygous deficiency of the enzyme nucleotide pyrophosphatase 1, a potent calcification inhibitor leading to infantile CVD (*Rutsch et al 2008*). Patients with *fibrodysplasia ossificans progressiva*, caused by a mutation in the BMP type-I receptors, are characterized by ectopic bone formation in muscles and soft-tissues that are mediated by cells of vascular origin (*Hegyi et al 2003*). FH patients develop premature severe calcific aortopathy and valvulopathy, which underscores the role of hyperlipidemia and/or the LDLR-deficiency. Correction of the hyperlipidemia does not alter the progression of calcification (*Awan et al 2008*). Furthermore a gene-dose effect of aortic calcification was observed in FH independent of cholesterol levels (*Alrasadi et al 2009*). Similarly loss of other genes like the *NOTCH* gene may lead to aortic valve calcification in susceptible individuals by favoring osteoblast differentiation (*O'Brien 2006*).

For over one hundred years, pathologists observed extra skeletal bone formation in a vascular bed (*Bunting et al 1906, Virchow et al 1863*). Later when the field of lipids expanded, calcified plaques were thought to occur passively in degenerative tissues. In the last few years, numerous mechanisms have been implicated in arterial calcification (*Budoff et al 2007*). Atheromatous calcification is currently considered a highly regulated process involving cellular components and regulatory factors (*Wallin et al 2001, O'Brien 2006*). Whether atherosclerosis activates the calcification machinery, or calcification

leads to the hardening of the vessels is still an enigma. Likely both occur in parallel, but one mechanism might be the initiating event **(Table IV)**. In the case of kidney disease, mineral imbalance is an important contributor since the calcium, phosphate and calcification inhibitors and stimulators are dysregulated once kidney function becomes abnormal (*Touyz et al 2009*).

Arterial calcification is divided into two main histological categories: Mönckeberg and atheromatous calcification **(Table IV)**:

Mönckeberg type of calcification is a cellular independent and dependent process. Amorphous mineral is believed to be deposited first, leading to the formation of amorphous calcified matrix that may be fueled by losing inhibitory calcification factors or acquiring pro-calcification factors (*Sage et al 2010*). Mineral imbalance is caused by excess calcium, phosphate and parathyroid hormone elevation. This induces a cellular differentiation process leading to chondro-osseous formation and eventual bone formation in the arterial wall (*Cecilia et al 2004*).

Atheromatous type of calcification is classically related to hypercholesterolemia (oxidized LDL), excess glucose, high homocysteine (*Van Campenhout et al 2009*) or oxidative stress (smoking) (*Marangon et al 1998, Ker-Zabel et al 2003*). Endothelial dysfunction arises from any of the previously mentioned insults. Scavenger cells infiltrate the site of insult and plaque is formed in a patchy manner, leading to inflammation that drives local cellular differentiation to an osteoblast-like phenotype, which later may form bone in the arterial wall.

Table IV: Associated calcification mechanisms*	
Arteriosclerosis	**Atherosclerosis**
Mönckeberg calciphylaxis	Atheromatous calcification
Smooth muscle layer (Tunica media)	Sub-endothelium layer (Tunica intima)
Diffuse (circumferential around elastic laminae)	Patchy (within atheromatous plaques in Type I collagen)
Cellular independent and dependent process	Cellular dependent process
Amorphous matrix formation followed by chondro-osseous formation	Osteochondrogenesis followed by mineralization
Mineral imbalance related mechanism: • Calcium, phosphate and pyrophosphate imbalance. • Losing inhibitory factors. • Acquiring promoting factors. • Cellular differentiation.	Metabolic excess related mechanism: • Endothelial dysfunction. • Oxidative stress and inflammation. • Macrophage infiltration. • Plaque formation. • Cellular differentiation.

* Taken from *Sage et al. Nature review; 2010.*

Thus, in both types of calcification, differentiation of bone-like cells ultimately appears to occur, producing a calcified matrix. Although the initiating events may differ, ultimately the calcifications many mechanisms may overlap. Furthermore, both types of calcification can simultaneously occur in the same individual and/or the same vascular bed (*Sage et al 2010*).

Arterial calcification can also be re-classified in four categories based on their underlying mechanisms: **1)** imbalance in mineral metabolism (*Cecilia et al 2004, Dellegrottaglie et al 2006*), **2)** loss of calcification inhibitory factors (*Weissen-Plenz et al 2008*), **3)** gain of calcification activator factors (*Touyz et al 2008*) **(Table V)**, and **4)** chondro-osteogenic cell differentiation (*Shao et al 2006*).

Table V: Factors that affects vascular calcification*	
Activators	**Inhibitors**
• Inorganic phosphates • TGFb1 • 25-hydroxycholesterol • cAMP • MAP kinase • Acetylated LDL • Homocysteine • Glucose • Endothelin-1 • Elastin degradation products • Pit-1 • Leptin • Reactive oxygen species • BMP2-Msx2-Wnt • Wnt–β-catenin–lrP-5 • Vitamin D • Collagen type I • Alkaline phosphatase	• Pyrophosphate • Statins • N-3 fatty acids • Tropoelastin • Bisphosphonates • Matrix Gla Protein • Osteopontin • Osteoprotegrin • NPP-1 via PPi • Fetuin-A (Ahsg) • Smad6 • Klotho • Carbonic anhydrase-2 • Calcimimetics • BMP-7 • CaR • Vitamin K • Estrogen

* Taken from *Touyz et al. Cardiovasc Res; 2009.*

The pathophysiology of arterial calcification in FH is likely to be different from the vascular calcification observed in patients with renal disease treated with hemodialysis, where the imbalance in calcium and phosphate homeostasis, parathyroid hormone and vitamin D may contribute to the differentiation of local vascular cells to an osteoblast-like phenotype (*Johnson et al 2006*). Imbalance in mineral metabolism was not found in aortic calcification of FH patients, as addressed in our recent publication (*Awan et al 2010*).

Cells in the arterial walls of patients with either type of vascular calcification share many features with osteoblasts, including expression of bone morphogenic protein 2 (BMP2), core binding factor α1 (Cbfa1) and osterix, which have been isolated from calcified arterial wall lesions **(Figure 7)**. This osteoblast-like phenotype of cells in the vessel wall is further supported by the prevalence of aortic valve calcification seen in individuals harboring a mutation in the *NOTCH* gene leading to the activation of the Runx2/Cbfa1 pathway (*O'Brien 2006*), one of two known pathways involved in osteoblast formation, with the other being the Wnt/Lrp5/β-catenin pathway (*O'Brien 2006, Shao et al 2010*).

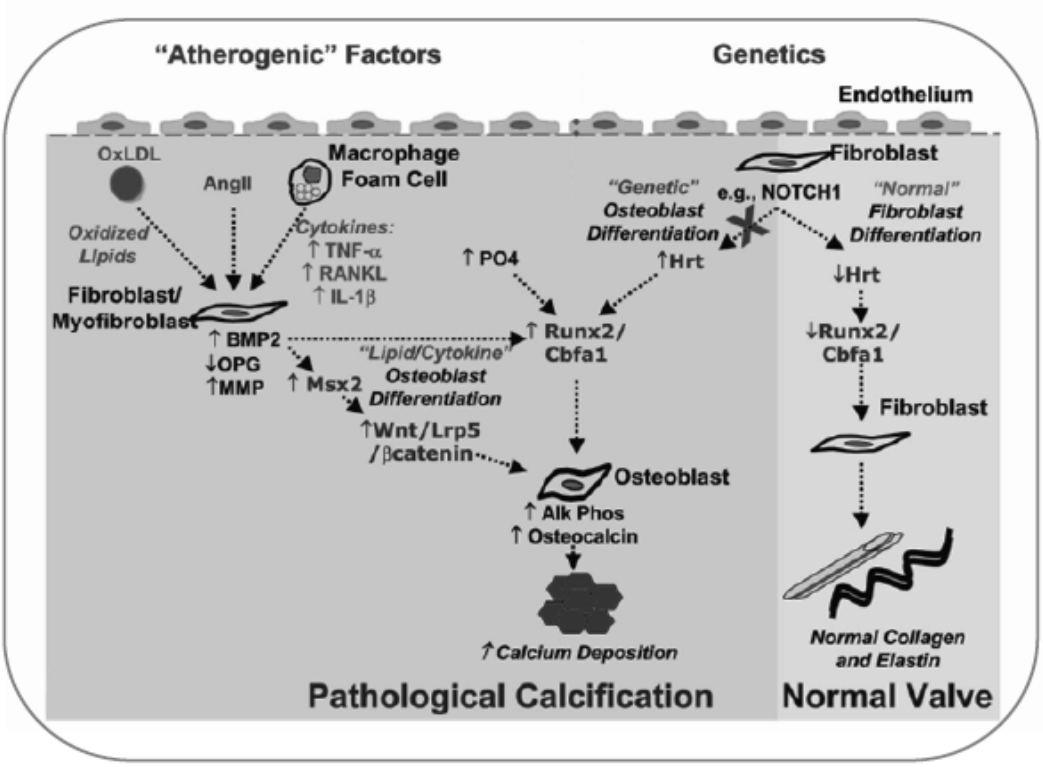

Figure 7. Potential interplay of lipids and inflammation with genetics in the pathogenesis of calcification. Oxidized lipids, may induce osteoblastic differentiation of fibroblasts by upregulating expression of BMP2, which activates the Wnt/Lrp5/B-catenin pathway through upregulation of the transcription factor, Msx2. In addition, multiple cytokines, including TNF-, IL-1, and RANKL, may also promote calcification by activation of this pathway. Hypercholesterolemia is also associated with activation of the Runx2/Cbfa1 pathway in aortic valve disease. Increased phosphate (PO4) levels, as are seen in chronic kidney disease, might promote valve calcification through upregulation of Runx2/Cbfa1. Genetic factors may interact to further promote osteoblastic differentiation. NOTCH1 is shown as one example of how a genetic abnormality might contribute to both valvular morphological abnormalities and valvular calcification. Taken from *O'Brien. Arterioscler Thromb Vasc Biol; 2006.*

Similarly, other mutations associated with FH may affect these pathways **(Figure 7)**. One possible mechanism is that similar signaling dysregulation in one pathway may be involved with *LDLR*$^{-/-}$ cells causing vascular calcification, as observed in *Ldlr*$^{-/-}$*, APOB*$^{100/100}$ mice. Controversy about the origin of these osteoblast-like cells, *i.e.* whether they represent phenotypic changes in vascular smooth muscle cells, pericytes or differentiated mesenchymal stem cells is unresolved. Nevertheless, the pathophysiology of arterial calcifications in FH may well be caused by an interplayed between atherogenic factors and underlined genetic predisposition.

Furthermore, it would be of interest to test the remaining mechanisms of arterial calcification in an animal model. Before embarking on approaches to examine mechanisms, a sensitive examination method to measure calcification in soft tissue must be established in the same animal model that resembles human disease. This will validate any claims of cure or attenuation of arterial calcification.

1.1.3.3. Measurement of arterial calcification

For many years, physicians have made the diagnosis of atherosclerotic calcification during physical examination by simple means of palpation of hard arteries, auscultation by stethoscope for carotid bruits and more recently and accurately by Doppler examination. Vascular calcification in humans can be also detected on a standard x-ray or quantified more precisely by CT scan (*Iijima et al 2011; Alrasadi et al 2009*). Computed tomography or CT scan enabled physicians to measure the degree of calcified atherosclerotic plaques in the aorta and coronary arteries **(APPENDIX I)**. This step is critical in patients at risk of aortic calcification undergoing major heart or aortic surgery to prevent intra-operative complications (*Awan et al 2008*).

Although conventional histology allows quantification of calcification, this occasionally leads to destruction of sample integrity and reduces *in vivo* structural preservation. In the proceeding chapter **(CHAPTER 2)**, we applied micro-CT, a miniaturized version of CT, to detect and quantify aortic calcification in mice with a resolution of 5-20 μm. The micro-CT used (SkyScan) was initially developed to evaluate bone density in human teeth and skeletal parts of rodents. Therefore the apparatus imposed technical challenges to adopt a soft tissue mouse aorta to fit a small slot designed for holding small hard objects.

In summary, atherosclerosis and arterial calcification are complex processes. They require good novel tools to elucidate the cause of progression and evaluate potential therapeutic approaches. Similar to LDLR mutations, PCSK9 gain-of-function mutations carry at risk of atherosclerosis and arterial calcification. The understanding of PCSK9 biology is advancing each day and will benefit from innovative research tools.

2. The Proprotein Convertase Family

Proprotein convertases (PCs) are calcium-dependent serine endoproteases that play a key role in the post-translational processing of precursors of bioactive peptides. Many precursors of neuropeptides, hormones, enzymes, receptors or growth factors are synthesized as inactive precursors (prohormones) and are activated by proteolytic cleavage; this catalytic function is carried out by specific PCs (*Seidah et al 1999, Fugère et al 2002*).

Mammalian PCs belong to a family of 9 subtilisin/kexin proteases (*Seidah et al 2008*). The genes coding for PCs are designated by the letters *PCSK* and they are numbered from 1 to 9. *PCSK*s products are critically involved in various physiological processes depending on their protease activities resulting in activation/inactivation events, some of which could lead to cardiovascular homeostasis (*Seidah et al 2007, Tall 2006*) **(Figure 8A)**.

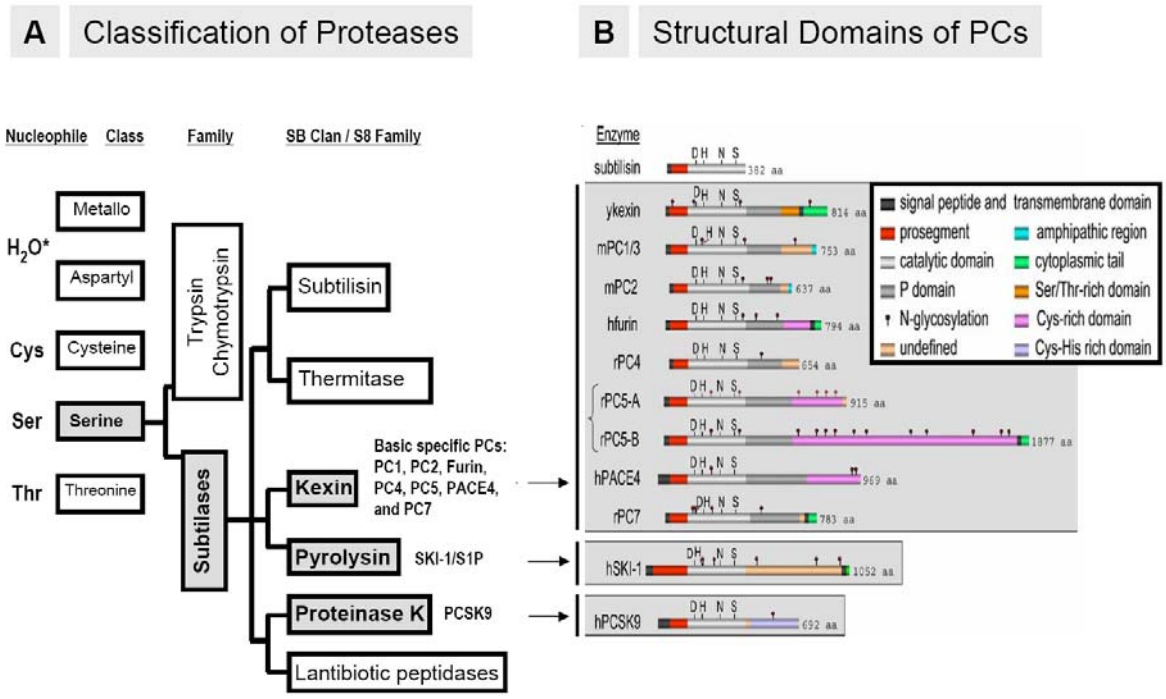

Figure 8. The Proprotein Convertases Family. (A) Classification of proteases of the genome with emphasis on the PCs. **(B)** Schematic representation of the structure of bacterial subtilisin, yeast kexin and the mammalian PCs. The different domains and active site residues are shown. Taken and modified from *Seidah et al. 2006 and 2007.*

All PCSK products have an N-terminal signal peptide, a pro-segment domain, a catalytic domain and a P domain (*Seidah et al 2008*) **(Figure 8B)**. The catalytic domains are highly conserved between many species. The 80-90 amino acids length pro-segment first is cleaved by an auto-catalytic mechanism in the endoplasmic reticulum (ER). The pro-segment acts both as an intramolecular chaperone to promote proper folding and as a competitive inhibitor of PCs function; this seems to prevent PCs from being accidentally activated during synthesis. The second round of cleavage occurs after a second set of basic amino acid residues in the pro-segment releasing it from the catalytic domain leading to activation of the PCs (*Bergeron et al 2000, Seidah et al 2008*).

2.1. Mammalian proprotein convertases in lipid metabolism

The first seven members of the mammalian PCs are similar in that they cleave protein precursors at basic amino acids. Out of these seven PCs, the fifth member, proprotein convertase subtilisin/kexin 5 (PCSK5), has relevance to lipid metabolism. Variation in the coding sequence of *PCSK5* gene affects the HDL cholesterol levels as shown in a recent study (*Iatan et al 2009*). HDL cholesterol is inversely correlated to cardiovascular disease. This is achieved by the direct inactivation of endothelial lipase by PCSK5. PCSK5 also indirectly cleave and activate angiopoetin-like protein 3, a natural inhibitor of endothelial lipase and, consequently, affects atherosclerotic CVD risk (*Iatan et al 2009*) **(Figure 8B)**.

The eighth member, PCSK8 also known as subtilisin kexin isozyme-1 (SKI-1) or site 1 protease (S1P), is known to cleave membrane-bound transcription factors, including sterol regulatory element binding proteins (SREBP-1 and -2), in the luminal domain of the Golgi apparatus, which when further cleaved by S2P, results in the release of their DNA-binding domain in absence of sterols. After cleavage, the cytosolic N-terminal domain becomes water soluble and translocates to the nucleus. This activated SREBP then binds to specific sterol regulatory element DNA sequences, thus upregulating the synthesis of enzymes involved in sterol/lipid biosynthesis. Sterols inhibit the cleavage of additional SREBP through a negative feedback loop, and thus synthesis of additional sterols is turned down (*Horton et al 2002*). Therefore, SREBPs are named master regulators of lipid homeostasis (*Eberlé et al 2004*) **(Figure 9)**.

The ninth member, PCSK9 formerly named NARC1 (Neural Apoptosis Regulated Convertase-1) (*Seidah et al 2003*) is involved in the post-translational regulation of the protein levels of the LDLR, affecting circulating cholesterol.

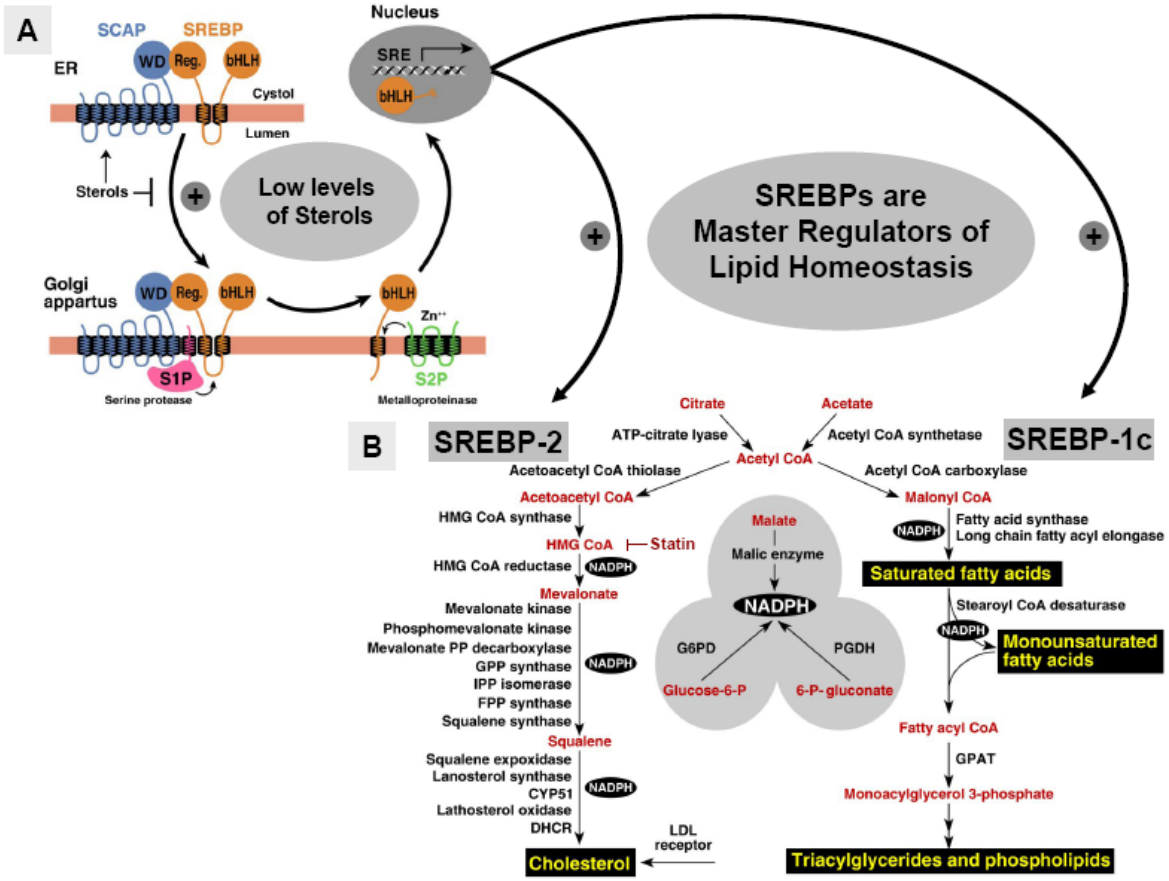

Figure 9. SREBPs are master regulators of lipid homeostasis. Sterol regulatory element binding protein (SREBP) are activators of the complete program of cholesterol and fatty acid synthesis in the liver. Taken from *Horton et al. J Clin In vest; 2002.*

2.1.1. PCSK9 domains and crystal structure

The human 25-kb *PCSK9* gene lies on the short arm of chromosome 1, and comprises 12 exons and 11 introns (*Seidah et al 2007, Davignon et al 2010*). After its identification in 2003, the crystal structure of PCSK9 was revealed in 2007 (*Cunningham et al 2007, Piper et al 2007*) **(Figure 10B)**. The PCSK9 692 amino acid product has an N-terminal signal peptide, a prosegment domain, a catalytic domain, a hinge region (of unknown function) and a C-terminal and Cys-His rich domain. Unlike the first seven

basic PCs it has no P domain (*Seidah et al 2003, Naureckiene et al 2003*). Sequence analysis of the catalytic domain indicates that three conserved residues; Asp_{186}, His_{226}, and Ser_{386} comprise the active catalytic center **(Figure 10A)**. The PCSK9 S1 pocket that recognizes the cleavage P1 site of its prosegment is shallower compared to other subtilases. This is due to the replacement of one glycine residue on the side of the S1 pocket by an Alanine raising the pocket floor (*Piper et al 2007*). In all domains, gain or a loss of function mutations have been reported (*Davignon et al 2010*) and summarized in **Figure 11**. Even variations in the PCSK9 hinge region (e.g. R434W) have mysteriously resulted in a loss of function, with a lower level of circulating cholesterol (*Dubuc et al 2010*) **(Figure 10)**. Interestingly, these highly conserved amino acids differ when compared to other subtilases.

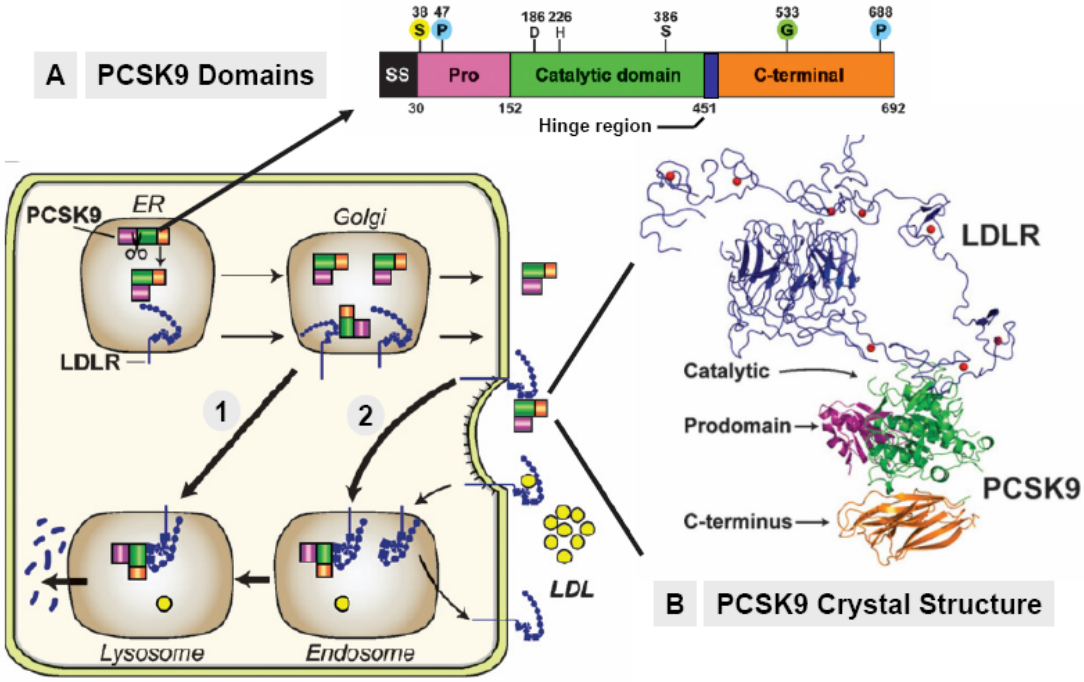

Figure 10. PCSK9 domains, biology and crystal structure. (A) PCSK9 is a protease that is synthesized as an enzyme precursor. Following synthesis, PCSK9 undergoes autocatalytic cleavage, which is required for secretion from the cell. Although cleaved, the pro-segment and catalytic domains of PCSK9 remain together. **(B)** When LDL cholesterol binds to the LDL receptor, they are both internalized into endosomes and upon binding to PCSK9; the LDL receptor is redirected for lysosomal degradation and prevented from recycling to the cell surface. 1 and 2 represent PCSK9 endogenous and exogenous pathways. Taken from *Horton et al. J Lipid Res, Vol. 50, S172-S177, April 2009.*

2.1.2. PCSK9 site of synthesis and distribution

PCSK9 is synthesized as a precursor, like all members of the PCs family, that undergoes autocatalytic cleavage of its N-terminal pro-segment in the endoplasmic reticulum, a step required for its exit from this compartment and its efficient secretion. Different from other secreted PCs, PCSK9 remains associated with its pro-segment **(Figure 10B)**. PCSK9 is expressed in many tissues, but mainly in liver, small intestine, and kidney (*Seidah et al 2003, Zaid et al 2008*). Depending on the technique used for detection, levels are also high in the pancreas (*Seidah et al 2003, Mbikay et al 2010*). For this reason some investigators have already started to examine the role of PCSK9 in many organs at different pathological conditions. Recent data indicate an important role of PCSK9 in liver regeneration (*Zaid et al 2008*). Another important role for PCSK9 is the prevention of lipotoxicity in pancreatic cell by limiting the LDLR action to internalize excessive lipids inside β-cells. In theory the loss of this protection may lead to lipotoxicity induced diabetes when a PCSK9 inhibiter is given (*Cnop et al 2002, Roehrich et al 2003, Ishikawa et al 2008, Mbikay et al 2010*). In light of all of these strong biological involvements, we investigated the role of PCSK9 in vascular calcification, fat distribution and deposition in mice **(CHAPTER 2)**.

2.1.3. PCSK9 natural substrate and activity

Unlike other proprotein convertases, PCSK9 has no known substrate other than itself (*Zaid et al 2008*). PCSK9 binds to the EGF-like domain A of the LDLR (*Cunningham 2007*) and is thought to target the LDLR to the endosomal/lysosomal pathway (*Maxwell et al 2004, Benjannet et al 2004*). The low pH in these compartments facilitates PCSK9 binding to the LDLR (*Fisher et al 2007*). There are two proposed sites where PCSK9 can come in close proximity to the LDLR: in the TGN (intracellular route) (*Nassoury et al 2007, Poirier et al 2009*) and on the cell surface of hepatocytes (extracellular route) (*Lagace et al 2006*) **(Figure 10)**. Interestingly other LDLR family members like VLDLR and apoER2 also share homology in the EGF-like domain **(Figure 1)**. Because human PCSK9 targets *ex vivo* human VLDLR (*Poirier et al 2008*) and binds *in vitro* mouse VLDLR (*Shan et al 2008*), PCSK9 has the potential to target human VLDLR *in vivo*. In theory that would affect tissues in which VLDLR is abundant, i.e.; muscle and adipose. Muscles usually burns fat while adipose tend to store energy as fat droplets for later use by the body. Therefore it would of interest to examine the VLDLR as a potential *in vivo* target of PCSK9 in adipose tissue **(CHAPTER 3)**.

2.1.4. PCSK9 and lipid metabolism

PCSK9 favors the post-translational degradation of the LDLR by an unclear mechanism. By modulating the level of LDLR it acts as a regulatory checkpoint. Induction of PCSK9 levels by the statins will decrease LDL-C statin-lowering potential in human. That is to say that statins would have had a higher capacity to decrease LDL-C if it was not for the concomitant rise in PCSK9 level following statin administration. Moreover it was shown that PCSK9 transcription could be suppressed by fasting and induced by insulin, likely by activating liver X receptor and SREBP-1c (*Costet et al 2005*).

Family studies of coronary heart disease patients led to the mapping of *PCSK9* gene to familial hypercholesterolemia (*Abifadel et al 2003*). This was supported by gain-of-function mutations exhibiting high serum levels of LDL-C, while loss-of-function mutations are known to lower serum levels of LDL-C and protect from coronary heart disease (*Cohen et al 2005*). Likewise *Pcsk9* in KO mice exhibit higher LDLR expression in the liver leading to the decrease in serum cholesterol. In contrast, the overexpression of *Pcsk9* reduces LDLR levels, resulting in increased serum cholesterol levels (*Maxwell et al 2004*).

Until now, one compound heterozygote loss of function mutation and one homozygote mutation were reported in humans (*Zhao 2006 et al, Hooper et al 2007*). One of the individuals harboring these mutations, a 32-year-old fertile apparently healthy female, had been described with undetectable circulating PCSK9 resulting in a strong reduction of LDL-C by 86% (*Zhao et al 2006*). Lowering cholesterol earlier in life is believed to produce greater impact on preventing CVD. This has been shown in a study of natural *PCSK9* loss-of-function mutations associated with 28 percent reduction in mean LDL-C and 88 percent reduction in the risk for cardiac events (*Cohen et al 2006*). A list of mutations discovered in PCSK9 is presented in **Figure 11** (*Davignon et al 2010*).

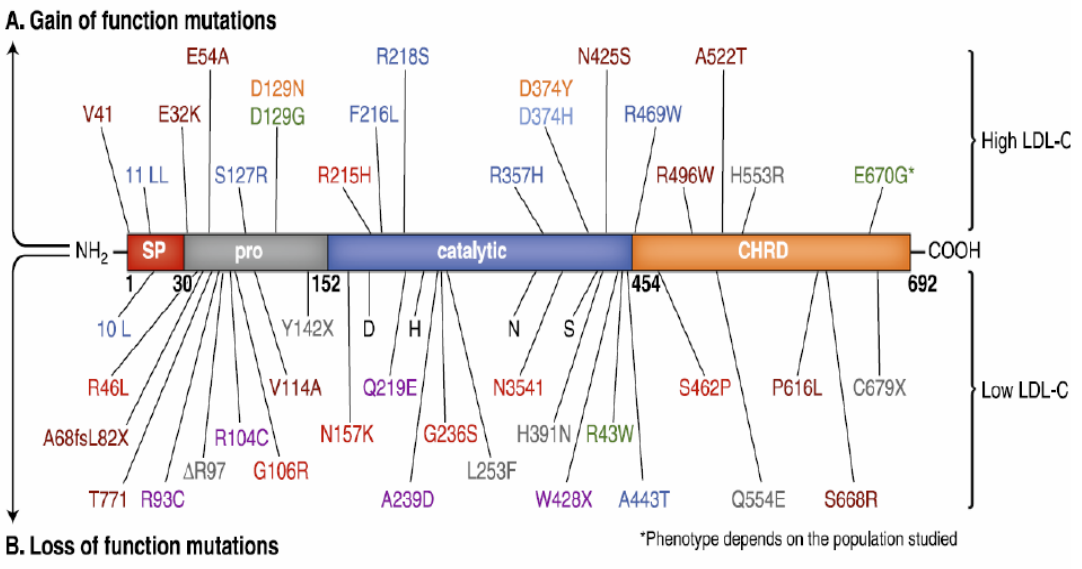

Figure 11. The PCSK9 protein and mutations influencing plasma LDL-C. The PCSK9 primary structure and domains – SP: signal peptide, pro: pro-segment domain, catalytic: catalytic domain and CHRD: Cysteine-Histidine rich domain. Key residues, aspartic acid (D), histidine (H), asparagine (N) and serine (S) are depicted. PCSK9 becomes active after auto-catalytic cleavage of the pro-segment. The position and the amino acid substitutions of the mutations that are associated with high LDL-C levels are shown above the protein, those that are associated with a reduced plasma LDL-C are shown below. Taken from *Davignon et al. Curr Atheroscler Rep; 2010.*

Therefore, an inhibitor of PCSK9 is thought to be beneficial. To further investigate the effect of PCSK9 inhibition, scientists have generated total and conditional knockout of the *Pcsk9* gene (*Pcsk9* $^{-/-}$) in mice on a C57BL/6 background, as previously described (*Zaid et al 2008*). These models have allowed us to advance our understanding of PCSK9 role in different organs and different metabolic conditions. *In vivo* studies have shown that conditional knockout in livers, extreme metabolic conditions like partial hepatectomy results in delayed liver regeneration and formation of liver lesions (*Zaid et al 2008*). Fortunately, when a high-cholesterol diet was supplemented after partial hepatectomy, the lesions where absent, which indicates an indirect role of PCSK9 in hepatic regeneration capacity. Furthermore *in vivo* studies had shown that PCSK9 deficiency was associated with a 2-fold decrease in postprandial triglyceride levels, suggesting enhanced triglyceride clearance (*Le May et al 2009*) and a role of PCSK9 in TG metabolism.

2.1.5. PCSK9 as a potential therapeutic target

As previously mentioned, PCSK9 favors the post-translational degradation of the LDLR, therefore decreasing its capacity to lower LDL-C. PCSK9 therefore acts as regulatory checkpoint for the LDLR. This makes PCSK9 a promising therapeutic target (*Lilly et al 2007, Seidah et al 2007, 2009, Lambert et al 2006, Lopez 2008, Hedrick et al 2009*) and several companies have developed various approaches to inhibit PCSK9: **1)** poly- and monoclonal antibodies targeted against PCSK9 (*Chan JC et al 2010*), **2)** antisense small interfering RNA (siRNA) (*Frank-Kamenetsky et al 2008*) or locked nucleic acid (LNA) (*Gupta et al 2010*), **3)** small-molecule inhibitors. Phase one clinical trials based on siRNA targeting PCSK9 have unfortunately suffered from adverse clinical off target effects. In contrast, the development of conventional small-molecule therapeutics to inhibit PCSK9 continues to present challenges (*Hedrick et al 2009*) **(Figure 12)**. The method that will move to clinically application will depend on long-term safety, cost and ease of administration (*Davignon et al 2010*). For instance, Chan *et al* reports remarkable cholesterol lowering in monkeys with the use of a single injection of monoclonal antibody (mAb) against PCSK9. This effect on plasma cholesterol levels occurs after the infusion of humanized mAbs that bind to PCSK9 and inhibits binding to LDLR. A single injection reduces the LDL-C levels by 80 % for more than a week. In the same study, they show a synergistic effect of mAb and statins on the induction of LDLR levels. Thus, this suggests that inhibiting PCSK9 in combination with statin would lead to more lowering effects on hypercholesterolemia (Chan JC 2010). Furthermore on the same line, phase one trial in human have already started by many pharmaceutical companies like Regeneron© .Some safety data from the use of their antibody have been released in an abstract in a resent international conference (*Swergold et al 2010*). In this report they claim an LDL-C reduction in health human volunteers reaching 60% and lasting for 30 days with no significant elevation in liver transaminases. Additionally, locked nucleic acid antisense oligonucleotides (LNA ASO) that targets both human and mouse PCSK9 have similarly shown great efficiency in reducing PCSK9 (*Gupta et al 2010*) **(Figure 12)**.

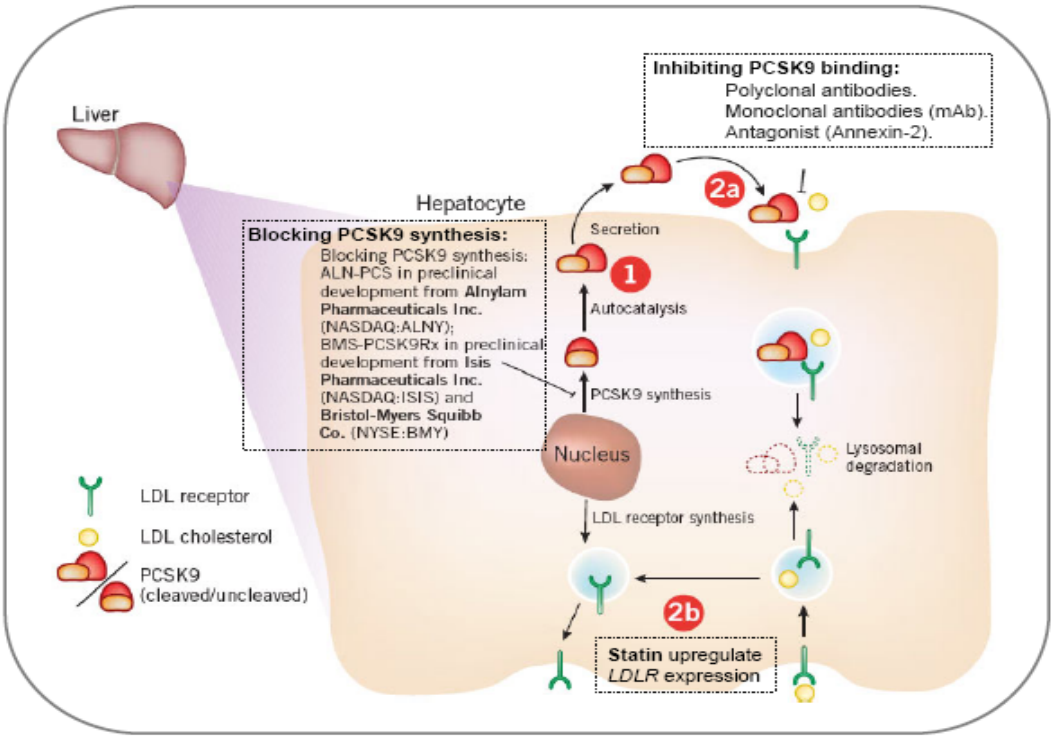

Figure 12. Novel pharmacology approaches to treat hypercholesterolemia. (1) PCSK9 undergoes autocatalytic cleavage. Although cleaved, the two domains of PCSK9 remain together. When LDL cholesterol binds to the LDL receptor, they are both internalized into the cell. **(2a)** If the cholesterol-bound LDLR is also bound to PCSK9, the LDL receptor is redirected for lysosomal degradation and prevented from recycling to the cell surface. **(2b)** If an internalized LDL receptor is not bound to PCSK9, the receptor is recycled to the cell surface, where it continues to remove LDL-C from circulation. A number of companies are developing compounds that stifle PCSK9 activity, either by blocking synthesis of the protein or by inhibiting PCSK9 binding to the LDLR. Taken from *Lou et al. Sci BX; 2009.*

Although protein and small peptide injection are not particularly attractive for lifelong treatment, such treatments would likely be accepted by patients suffering side effects from current anti-lipidemic agents or high risk patients striving to achieve lower LDL-C levels. For example, heterozygous familial hypercholesterolemia patients, for whom initial LDL-C levels starts around three times that of the general population, are unable to achieve even a 50 percent reduction with available oral agents and often require may LDL apheresis, a form of dialysis to eliminate the LDL-C from the blood (*Rader et al 2003*).

Despite its apparent safety, there are concerns about the idea of inhibiting PCSK9, since we know little about its entire biological function. Therefore, before we start to assess their clinical implications or embark on more clinical trials, careful experiments must be conducted to evaluate the consequences of inhibiting PCSK9. Animal modeling is a proven strategy to screen new drug modality. Of course, unanticipated and disqualifying toxic side effects (e.g; Ab-PCSK9 immune complexes) can only be ruled out in large-scale, multicenter trials. Therefore, much remains to be done to bring any of these PCSK9 inhibitors to the bedside (*Steinberg et al 2009*) **(Figure 12)**.

3. Hypothesis and objectives

3.1. First hypothesis and objectives

Although severe atherosclerosis should be the primary target for most interventions to prevent mortality from CAD, secondary targets in surviving patient should shift to arterial calcification to lessen the morbidity of this disease as our population grows older and develop extensive arterial calcifications. These secondary interventions will help to lessen the burden of expensive procedures and lengthy hospitalizations. In previous studies, we have shown that FH patients carrying null mutation for the LDLR are at higher risk of premature aortic calcifications **(APPENDIX I)** and the degree of atherosclerotic calcification was not correlated with cholesterol levels, neither initially nor on treatment (*Awan et al 2008*). Calcification rather depends on the degree of LDLR deficiency (homozygote vs. heterozygote) (*Alrasadi et al 2009*). To date no information is available on other gene mutations that lead to FH and whether they are similarly affected. Therefore, it was interesting to document the occurrence and degree of aortic calcification associated with transgenic mice overexpressing PCSK9 (mimicking LDLR-deficiency), which reflects “gain-of-function mutations” in human. Thus, **we hypothesized that the degree of the LDLR deficiency (whether by knocking the *Ldlr* gene or by overexpression *Pcsk9* gene to over degrade the LDLR) will have a major impact on the degree of aortic calcification and will be independent of diet.** To perform this in a methodological way we first replicated the findings obtained in human investigations (images and histology) **(APPENDIX I)** in our LDLR knockout mice and measure aortic calcifications by this sensitive method. We then will apply the same methodology to transgenic mice overexpressing PCSK9 as compared to wild-type and *Ldlr* knockout mice in an age- and diet-dependent manner.

3.2. Second hypothesis and objectives

In our laboratory, we recently noticed that lacking PCSK9 mice tend to accumulate visceral fat **(APPENDIX II)**. It is possible to imagine that PCSK9 inhibition could lead to fat tissue dysfunction and insulin resistance in mice and humans. *In vivo* studies had shown that PCSK9 deficiency was associated with a 2-fold decrease in postprandial triglyceride levels, suggesting enhanced triglyceride clearance (*Le May et al 2009*). No report to date has systematically addressed the mechanism by which PCSK9 influences fat tissue metabolism. Thus, **we hypothesized that VLDLR, a closely related family member of the LDLR abundant in adipose tissue, could be a target of PCSK9**. Accordingly, it was shown that PCSK9 enhances VLDLR degradation *ex vivo* (*Poirier et al 2008*). We proposed to study, the functional aspect of adipose tissue in mice lacking *Pcsk9* gene with (single knockout) or without *Ldlr* (double knockout) gene to establish a direct *in vivo* role for PCSK9. By immunohistochemistry, we will show that VLDLR levels were affected in the adipose tissue and that this will cause a direct impact on fat metabolism. Additional, we investigated *in vivo* and *ex vivo* the metabolism of FFA uptake and TG synthesis in the adipose tissue of PCSK9 KO mice.

CHAPTER 2

PCSK9 AND AORTIC WALL CALCIFICATION

Article 1

CHAPTER 2:

PCSK9 AND AORTIC WALL CALCIFICATION

4. Foreword: Article 1

From our previous work, we noticed that FH patients present with premature aortic calcification **(APPENDIX I)**. A gene-dosage effect of the LDLR has been correlated with aortic calcification. Despite cholesterol lowering modalities, the progression of the disease seem to be independent from levels of circulating cholesterol. In addition, our evaluation of calcium homeostasis and bone density could not explain this finding. Therefore in the following study we examine this phenomenon in a laboratory animal model to assess the degree of LDLR-deficiency in relation to the extent of calcium deposition at sites of vascular injury in the presence or absence of a Western diet. We applied an animal model for aortic calcification using genetically modified mice. Therefore we studied *Ldlr*$^{-/-}$ and PCSK9 *Tg* mice using a sensitive imaging technique. Extensive calcifications were observed in old *Ldlr*$^{-/-}$ chow-fed mice. In WT mice fed a Western diet, similar cholesterol levels to *Ldlr*$^{-/-}$ chow-fed mice were achieved, however no significant calcification was observed. The magnitude of cholesterol level and calcification degree in PCSK9 *Tg* mice was between WT and *Ldlr*$^{-/-}$ mice but more toward WT, suggesting that LDLR availability may be required for preventing extensive calcification. This article also reports a novel use of micro-CT to quantify calcification in mice.

Animal dissections, sample treatments, image analyses, compiling data, statistical analysis, as well as manuscript writing and editing, were conducted by Zuhier Awan (first author) and Maxime Denis (second author).

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4.1. The LDLR Deficient and PCSK9 Gain-of-function Mouse as Models for Aortic Calcification and Quantification by Micro-Computed Tomography

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4.1.1. Abstract

Objective: Patients with Familial hypercholesterolemia (FH) due to LDLR mutations suffer premature aortic calcification, an effect that is age- and gene dosage-dependent and unaltered to cholesterol reduction later in life. To better understand this process, we examined murine models. **Methods**: We compared chow fed *Ldlr*$^{-/-}$ mice to controls at 6, 12 and 18 months and on a Western diet (WD) at 6 months. Additionally, we compared controls to *Ldlr*$^{-/-}$ mice and transgenic (*Tg*) mice overexpressing PCSK9 protein (PCSK9 *Tg*), which promotes LDLR degradation. Aortas were perfused-fixed, embedded in paraffin, and sections were stained with alizarin red. We used micro-computerized tomography (micro-CT) to quantify vascular calcification. **Results:** *Ldlr*$^{-/-}$ mice develop calcification in the ascending, transverse aorta and neck vessels with a distribution similar to that of human. Histology confirmed that the calcification were predominantly sub-intimal. Calcification was most prominent in 18-month-old *Ldlr*$^{-/-}$ mice fed a chow diet and in 6-month-old *Ldlr*$^{-/-}$ mice fed a WD. Interestingly; PCSK9 *Tg* mice fed a WD develop aortic calcification. **Conclusions:** The two mouse models develop aortic calcification in an age- and diet-dependent manner. Further analysis of these mouse models of aortic calcification with the micro-CT imaging may provide a better understanding of the links between FH and arterial calcification.

Word count: 5351

Word count of Abstract: 199

Figures and Tables: 4 Figures, 2 Tables and 1 Supplementary video

Key Words: Familial Hypercholesterolemia, LDLR, PCSK9, Aortic Calcification and Micro-Computed Tomography

4.1.2. Introduction

Patients with homozygous familial hypercholesterolemia (FH) due to mutations of the low-density lipoprotein receptor (*LDLR*) gene have severe coronary artery disease (CAD) manifesting in childhood or young teens and later developing into severe aortic stenosis. The advent of extracorporeal LDL removal techniques in the 1980s coupled with pharmacological therapy has allowed homozygous FH patients to reach adulthood. The development of calcific aortic stenosis in homozygous FH is a condition well known to clinicians. Several case series in the surgical literature have described extensive calcification of the ascending aorta in homozygous FH, a condition often referred to as “porcelain aorta” (1). This creates a surgical challenge during aortic valve replacement, requiring the simultaneous replacement of the ascending aorta using a Cabrol or Bental procedure (1). By using computerized tomography (CT) scan of the thoraco-abdominal aorta, we have previously shown that homozygous FH patients have extensive, premature calcification of the entire aorta, with an aortic calcium score (AoCS) reaching 5,000 Agatston units by the second decade of life (2). In heterozygous FH, age-dependent calcification also occurs, but 20 years later (3). In contrast, in subjects without mutations at the LDLR locus, such vascular calcification is not encountered until the 7th decade of life.

The mechanism of arterial calcification in FH is not well understood. Clinical observations in homozygous FH suggest that the calcification process continues unchecked. Despite the reduction of total and LDL-cholesterol (LDL-C) levels by conventional therapy and the reduction in the progression of CAD, severe calcific aortic stenosis develops and often warrants valve replacement. Aortic wall calcification also has untoward effects on cardiovascular health, including higher systolic blood pressure, aneurysm and rupture of the aorta (4). Furthermore, calcified lesions in the aorta have the potential to: 1) impinge on coronary arteries causing myocardial ischemia (5); 2) contribute to ischemic stroke and peripheral vascular disease as a result of dislodged lesions (6, 7, 8); and 3) complicate angiography during catheterization and surgical procedures (1). In addition, calcification within the aorta can create a technical artifact when attempting to determine both carotid stenosis by Doppler sonography (9, 10) and bone mineral densitometry by DEXA scan (11, 12).

To better understand the role of the *Ldlr* gene and LDLR protein in the process of FH arterial and valvular calcification, we sought to characterize mouse models that potentially recapitulate this phenotype. Since mice and humans share many important physiological characteristics, we believed mouse models would provide a suitable counterpart to study the pathogenesis of human arterial calcification, malformation, and the mineralization of the aortic valve. Herein, we demonstrate that inactivation of the *Ldlr* gene or degradation of the LDLR protein *in vivo* results in premature aortic calcification as seen in FH. Degradation of the LDLR protein was achieved by over-expressing the *Pcsk9* gene that encodes the PCSK9 protein, the ninth member of the proprotein convertase family.

Lakoski et al reported that a 100 ng/ml increment in plasma PCSK9 level was associated with a 4.5 mg/dL increase in plasma LDL-C concentration in women and 3.2 mg/dL increase in LDL-C in men. (13). PCSK9 exerts this effect on cholesterol metabolism through post-translational downregulation of the LDLR (14). PCSK9 binds to the epidermal growth factor motif of the LDLR and the complex is endocytosed and targeted for intracellular degradation (14, 15). We have previously postulated that the LDLR may play a key role in vascular calcification based on observational studies in patients with homozygous and heterozygous FH due to LDLR defects (2). Therefore, we wanted to use an independent mouse model to examine the role of the LDLR on aortic calcification. In addition, as the high expression transgenic mice resemble gain-of-function mutations in *PCSK9* and causes autosomal dominant hypercholesterolemia (16), we also presently tested the hypothesis that if LDLR-deficiency was necessary in initiating aortic calcification; therefore calcification should then be observed in PCSK9 *Tg* mice on either a chow (regular diet) or high-fat Western Diet (WD).

4.1.3. Material and methods

Animal protocol

Our maintained colony of *Ldlr*$^{-/-}$ mice (C57BL/6J back-ground; stock 002529) (*n*=30) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). PCSK9 *Tg* mice overexpressed mouse PCSK9 under the control of ApoE promoter (17) and were since backcrossed to C57BL/6J for 10 generations. Suitable age- and diet-matched C57BL/6J wild-type (WT) mice (*n*=45) were used as controls for both genotypes. Mice were fed either a normal chow diet (2018 Teklad Global) or a WD diet when indicated (Harlan Teklad # TD 88137) from weaning until sacrifice. Mice were kept in a pathogen free facility under 12 hour light/dark cycles. Mice were fasted for 3 hours prior to being sacrificed and 86 adult mice were used in this study. All procedures were approved by the IRCM animal care committee.

Aorta isolation and fixation

Aortic isolation was carried out as previously published (18), with some modifications. In brief, mice were anesthetized with isoflurane at a rate of 2% in 1 L of oxygen. Blood was removed via intracardiac puncture through an intact diaphragm and the circulatory system was flushed with 5-6 mL of phosphate buffered saline (PBS) through the left ventricle at a pump rate of 0.4 mL per minute. To keep the aortic wall from collapsing, the aorta was perfused and fixed in situ with 4% phosphate-buffered paraformaldehyde (PBP). The aorta was extracted in bulk by opening the chest wall and gently lifting the heart and excising posterior to the thoracic aortic and abdominal aorta until the renal artery. The apex of the extracted aorta was excised and the aortic tissue was fixed ex vivo in 4% PBP at 4°C for 24-48 hours. After fixation, microscopic fine dissection was performed and each intact aorta was embedded in a single paraffin block.

Tissue sampling and processing

As mentioned previously, blood was collected from mice at the induction of anesthesia by puncture of the left ventricle, and then transferred immediately to heparin anti-coagulated tubes on ice. Whole blood was used to measure glucose via a commercial glucometer (Bayer Diabetes Care, USA). Plasma was separated by centrifugation (20 minutes at 850*g* and 4°C) and kept at –20°C until assayed. Total cholesterol and triglyceride levels were measured by Infinity Total Cholesterol (Thermo Fisher Scientific Inc, MA, USA) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan) kits, respectively, and according to the manufacturer's recommendations.

Calcification and lesion analysis

After dissection, the entire aorta was exposed to digital imaging capture. Aortas were then embedded in paraffin blocks for calcium measurement. The calcium content of the entire aorta and aortic valve was determined by digital X-ray quantification on a micro computed tomography (micro-CT) scanner (Skyscan-1072, TomoNT version 3N.5, Belgium) adopting the following parameters: 40 kV voltage; 248 A current; 50 X zoom; 5.63 m pixel size; no filter applied; 0.9 rotation step, 2.2 second between frames at a 10 micron slice thickness. The calcium score was determined using dedicated software (CTAn v.1.8.1.2). Aortic calcification score (AoCS) was expressed as the ratio of calcified volume (mm3) over aorta volume (mm3) multiplied by 100 and expressed in arbitrary units (A.U.). While being rotated on a vertical axis, a three-dimensional (3D) reconstruction image and video were produced using commercial software (ANT 3D creator v.2.4 and NRecon v.1.4.4) to further analyze the aorta and aortic valve (Supplemental video image online). The blocks were then sectioned and stained with Alizarin red S solution (25 mg/l alizarin red S in 1% potassium hydroxide) to confirm calcium deposition in regions corresponding to calcified segments on micro-CT (Figure 1).

4.1.4. Results

Micro-computed tomography characterizes aortic calcification

Vascular calcification in humans can be detected on a standard x-ray or quantified more precisely by CT scan (19, 3). In the present study, we applied micro-CT, a miniaturized version, to detect and quantify aortic calcification in mice with a resolution of 5-20 μm. Although conventional histology allows quantification of calcification, this occasionally leads to destruction of sample integrity and reduces *in vivo* structural preservation. To test the superiority of micro-CT scanning, murine aortas were imbedded in paraffin blocks to retain anatomical position while calcium content was determined. We found that micro-CT was rapid and allowed a 3D-virtual visualization of the aorta for better lesion quantification and characterization. Thus, *Ldlr*$^{-/-}$ mice develop calcification in the ascending, lower part of the transverse aorta and neck vessels with a distribution strikingly similar to that observed in humans. The aorta was subsequently sectioned and stained with alizarin red to confirm calcification (Figure 1A). Representative X-ray scans and 3D-reconstructions are shown in Figure 1B and 1C. The AoCS was determined from histological and 2D and 3D micro-CT analysis. Consistent with previous reports, there was a good agreement of calcium volume estimates between histological and micro-CT analyses (data not shown).

Age-dependent aortic calcification in *Ldlr*$^{-/-}$ mice on chow diet

Characteristics of the mice are shown in Table 1. As shown in previous studies (20), body mass remained relatively stable in *Ldlr*$^{-/-}$ mice (34, 38 and 39 g) compared to that of control mice that increased with time (36, 44 and 50 g at ages 6, 12 and 18 months, respectively). Body mass differences between *Ldlr*$^{-/-}$ and control mice reached statistical significance only at 18-months (*P*-value < 0.05). There were no significant differences between liver sizes (% of body weight) or glucose levels between groups except at 12 months where *Ldlr*$^{-/-}$ mice are significantly higher (Table 1). Total cholesterol levels were increased with age in both *Ldlr*$^{-/-}$ and WT mice. As expected, circulating plasma cholesterol and triglyceride (TG) levels were 3-fold higher in *Ldlr*$^{-/-}$ mice relative to WT mice (*P*-values ≤ 0.001) consistent with previous reports (21). Fed a chow diet, *Ldlr*$^{-/-}$

mice developed spontaneous calcification that increased with age, with a statistically 9-fold increase of the AoCS between 12 and 18 moths (*P*-values = 0.02) (Table 1, Figure 2D), recapitulating the age effect seen in human FH (3). WT mice did not develop significant calcification at any age. Analysis of 3D images of aortas from 18-month-old *Ldlr*$^{-/-}$ mice identified the areas of low shear stress as being the most calcified (Figure 1). Alizarin red staining of paraffin sections at the level of the aortic root and carotid artery confirmed the presence of calcium (Figure 2). As shown in Figure 2, vascular calcification was predominantly observed in the sub-endothelial, sub-intimal space and was associated with atherosclerotic plaques. This is similar to our previous report in human FH subjects that underwent aortic valve replacement and aortic surgery (2). These data suggest that LDLR deficiency and/or elevated total cholesterol and/or TG levels initiate aortic calcification, and that the severity of calcification increases with age.

Accelerated aortic calcification in LDLR-deficient mice fed a WD

Ldlr$^{-/-}$ mice fed a WD for 6 months maintained a lower body mass (37 versus 49 g for controls; *P*-value ≤ 0.001) and lower liver size (% of body weight) (5.8 versus 10.3 g for controls, *P*-value ≤ 0.001). However, glucose levels were not affected (Table 2). As expected, circulating cholesterol and TG levels were the highest in *Ldlr*$^{-/-}$ mice maintained on a WD, with average levels of 2248±862 mg/dL and 509±118 mg/dL, respectively (Table 2). Circulating cholesterol levels were also significantly increased in WT mice fed a WD as compared to age-matched WT mice fed a normal chow diet (509±118 mg/dL versus 101±9 mg/dl; *P*-value ≤ 0.01). By micro-CT quantification, *Ldlr*$^{-/-}$ mice of 6 months of age were found to develop extensive aortic calcification when fed a WD (Figure 3A and 3B). The degree and pattern of calcification were similar to that of 18-month-old *Ldlr*$^{-/-}$ mice fed a chow diet, with an AoCS 300-fold higher (Figure 3C) than for age-matched controls (3.01±2.0 versus 0.01±0.0 A.U.; *P*-value <0.001). This indicates that a WD accelerates arterial calcification.

Elevated cholesterol alone was not sufficient to produce severe aortic calcification

WT mice fed a WD for 12 months had a lower AoCS than *Ldlr*$^{-/-}$ mice of the same age maintained on a normal chow diet (0.75±1.1 versus 0.02±0.0 A.U.) (Table 1 and 2), even though they exhibited higher total cholesterol levels (440±151 mg/dL versus 316±65 mg/dL for age-matched *Ldlr*$^{-/-}$ mice fed a chow diet). This suggests that elevated cholesterol levels alone do not result in severe aortic calcification. Taken together, these data suggest that absence of the LDLR is the major contributor to aortic calcification, and that the process is accelerated in the presence of a WD (Figure 4A).

Aortic calcification in *Pcsk9* high expression transgenic mice

Using the calcification rate of *Ldlr*$^{-/-}$ mice as an indicator, we chose to study two time points in PCSK9 *Tg* mice where calcification should be present if LDLR-deficiency does impact the process of calcification: 18 months on a chow diet and 12 months on a WD. Unlike *Ldlr*$^{-/-}$ mice, 18-month-old PCSK9 *Tg* mice exhibited a body mass and liver size similar to that of WT mice (Table 1). Glucose levels were also similar. Although lower than in *Ldlr*$^{-/-}$ mice, total cholesterol and TG levels found in PCSK9 *Tg* mice were higher than controls. Of the two mice that were assisted by micro-CT, only one developed calcification making the difference non-significant in compare to WT, and therefore future analysis was conducted in PCSK9 *Tg* mice at 12 months of age fed a WD (Table 2 and Figure 3D-F). When fed a WD for 12 months, PCSK9 *Tg* mice had equivalent total body and liver weight to that of control mice (Table 2). Their blood sugar levels were also similar. Cholesterol levels of PCSK9 *Tg* mice were significantly higher than that of age-matched controls (1043±236 versus 440±151 mg/dL), and TG levels were also significantly increased relative to controls (Table 2). However, cholesterol levels of PCSK9 *Tg* mice (1043±236 mg/dL) were still lower than those obtained in 6-month-old *Ldlr*$^{-/-}$ mice fed a WD (2248±862 mg/dl). Similar to *Ldlr*$^{-/-}$ mice, 12-month-old PCSK9 *Tg* mice fed a WD had aortic calcification, with AoCS increased significantly in compare to age-matched controls on a WD (1.69±1.2 versus 0.02±0.00 A.U.; PCSK9 *Tg* versus controls, *P*-value <0.01) and exhibited an intermediate calcification level with an AoCS 85-fold higher than in controls (Figure 3D, 3E and 3F).

4.1.5. Discussion

Arterial calcification in human is associated with considerable mortality and morbidity. The pathophysiology of arterial calcification is poorly understood and has been the subject of recent reviews (22, 23). Once thought to be a passive process related to age, arterial calcifications are now believed to be due to a complex interplay between vascular injury, inflammation, and osteogenesis (24, 25). Therefore arterial calcification can be classified into four categories based on machinery: 1) imbalance in mineral metabolism (26, 27), 2) loss of calcification inhibitory factors (28), 3) gain of calcification activator factors (29), and 4) chondro-osteogenic cell differentiation (30). Imbalance in mineral metabolism occurs most commonly in renal failure patients (31). Renal calcification resembles the most extensive calcification in a chronic disease followed by diabetes, heavy smoking and hypercholesterolemia (25). Rare deficiencies in the nucleotide pyrophosphates 1 enzyme, a potent inhibitor of calcification leads to lethal infantile calcification and cardiovascular diseases in homozygous patients (32). Similarly, loss of both calcification inhibitors osteopontin and matrix Gla protein leads to spontaneous and extensive arterial calcification in a double knockout mouse model (33). Additionally, patients with fibrodysplasia ossificans progressiva caused by a mutation in the BMP type I receptor are characterized by earlier onset of ectopic bone formation in muscles and soft-tissues that are mediated by cells of vascular origin (34). In FH patients, neither the onset nor the site of calcification fits the phenotype of genetic inhibitors or activators of calcification. Furthermore the pathophysiology of arterial calcifications in FH is likely to be different from that of renal disease treated with hemodialysis, and whose balances in phosphate and calcium homeostasis, parathyroid hormone and vitamin D are significantly altered, as addressed in our recent publication (11). Therefore, it is of interest to examine alternative mechanisms in animal models.

Using mice either lacking totally (*Ldlr*$^{-/-}$) or exhibiting lower LDLR protein levels, (PCSK9 *Tg*) we provided evidence for the existence of a correlation between LDLR levels and vascular calcification. Relative to controls, *Ldlr*$^{-/-}$ mice on any diet exhibited significantly elevated cholesterol, triglyceride levels, and accelerated aortic calcification as quantified by micro-CT. Conversely, only PCSK9 *Tg* mice fed WD, exhibited significant aortic calcification although at a lesser extent than *Ldlr*$^{-/-}$ mice fed WD for a shorter period (Table 2). In contrast, calcification was undetectable in WT mice on chow and there was insignificant calcification when hypercholesterolemia was induced by WD

(Figure 4A). Thus, LDLR-deficiency increases aortic calcification independently of cholesterol levels, as observed in human studies (2). Since calcification is significantly milder in PCSK9 Tg mice than in *Ldlr*$^{-/-}$ mice. We propose that the decreased severity of calcification in PCSK9 Tg is due to both the less severe hyperlipidemia and to the residual LDLR proteins, not affected by PCSK9 (17). This is consistent with previous observations made between men carrying a null-mutation of the LDLR gene (the French Canadian deletion >15 Kb) that includes the promoter region, exon 1 and part of intron 1) and low LDLR activity due to missense mutations (3). Therefore, PCSK9 *Tg* mice may represent the intermediate levels of calcification observed in heterozygote FH patients. Nevertheless, because 12 month-old WT mice start to develop mild calcification on WD, hypercholesterolemia must have been the initiating factor whereas the absence of LDLR significantly enhances the progression of calcification.

We have observed previously that arterial calcifications in surgical specimens of homozygous FH patients undergoing aortic valve and ascending aorta replacements are predominantly sub-intimal (2), unlike renal failure patients (35, 36, 37). The mouse model presented herein reproduces the same histological changes observed in humans (Figure 1 and Figure 2). Arterial calcification within the intimal layer (as compared to medial calcification associated with renal failure) is a common feature of atherosclerosis in animal models and in human disease. Various cell types therefore may participate in this process. Macrophages, mast cells, and vascular smooth muscle cells (SMC) found in plaques are postulated to be the primary cells involved in atherosclerotic intimal calcification, with a smaller contribution from SMC (38). Alternatively, calcification may be promoted by mesenchymal stem cells which arise from various cellular elements in the vascular wall and which can mimic the repertoire of bone marrow stem cells in their capacity to differentiate into chondrocytic and osteoblastic cells (39). Bone-marrow derived osteoblast-like cells have also been suggested to participate in the development of vascular calcification (40).

The process of cell-induced calcification involves the activation of fibroblasts and myofibroblasts through a variety of stimuli including oxidized LDL, angiotensin II, and cytokines (TNFα, RANKL, and IL-1β) secreted by macrophages. In turn, these events trigger the bone morphogenic protein 2 activation of MX-2 and the Wnt/Lrp5/beta catenin pathway, as well as the Runx2 mediated activation of osteoblast function. The *Ldlr*$^{-/-}$ mouse model fed high-fat diet has been used previously to study aortic valve

calcification (41). In this model, the authors concluded that TNFα induced the Msx2-Wnt pathway to promote vascular calcification. Another model of aortic stenosis uses old (17-25 months) *Ldlr*$^{-/-}$ *ApoB*$^{100/100}$ hypercholesterolemic mice; here aortic stenosis and calcifications were demonstrated by echocardiography, hemodynamic measurements and histology (42). However, aortic calcification in this case may be critically dependent on multiple metabolic abnormalities (elevated oxidized lipoproteins, diabetes, obesity, inflammation), and not necessarily on the absence of the LDLR protein. Neither of these previous studies addressed the potential role of the LDLR in progression of aortic wall calcification. In the present model, the mice were neither diabetic nor obese (Table 1, 2). Thus, the pathogenesis of hypercholesterolemia-induced aortic calcifications in the obese, hyperlipidemic, diabetic mice does not apply here.

4.1.6. Clinical perspective

Studying vascular calcification mechanisms may lead also to a better understanding of the development of calcific aortic stenosis, a common disease in older individuals for which, presently, the only option is valve replacement (surgical or transcutaneous). Lowering of plasma cholesterol with statins has been shown to inhibit hypercholesterolemia-induced calcification of the aortic valve in animal models via the LRP5/β-catenin pathway (43). However, clinical trials testing the ability of statins to reduce the progression of calcific aortic stenosis have not been met with success (44). In our experience in patients with homozygous and heterozygous FH due to mutations in the *LDLR*, despite normalization of plasma cholesterol levels, vascular calcification proceeds in an age- and gene-dosage fashion, irrespective of cholesterol levels (2,3). Therefore, it remains to be determined whether long-term statin use and/or lifelong LDLR reduction is associated with an altered prevalence of aortic stenosis. With the use of a representative animal model of FH, such as *Ldlr*$^{-/-}$ or PCSK9 *Tg* mice, the ability of combination therapy using anti-inflammatory agents, hormonal modulation, calcium channel blockers, and/or chelating agents to attenuate arterial calcification can be explored further.

In conclusion, the results of the current study suggest that aortic calcification is associated with age and LDLR function, and is accelerated by a WD. We provide a mouse model of extensive vascular calcification that replicates the disease observed in

humans. Furthermore, we provide a precise and quantifiable method of measuring aortic calcification in mice using micro-CT. The implications of these findings are two-fold. First, we replicate human premature aortic calcification in two animal models resembling absolute LDLR deficiency and low LDLR availability. Second, similar to humans, we developed a novel technique to reliably assess the site and extent of aortic calcification digitally in these animals. This should now facilitate studies on both the pathophysiology of arterial calcification and the efficacy of different treatment modalities.

4.1.7. Acknowledgment

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4.1.8. References:

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4.1.9. Tables:

Table 1. Attributes of WT, *Ldlr* $^{-/-}$ and *Tg (Pcsk9)* mice on regular (chow) diet

Age (months)	6		12		18		
Number (total)	*n*=10	*n*=10	*n*=10	*n*=6	*n*=10	*n*=9	*n*=2
Gene	*WT*	*Ldlr* $^{-/-}$	*WT*	*Ldlr* $^{-/-}$	*WT*	*Ldlr* $^{-/-}$	*Tg*
Number	*n*=10	*n*=10	*n*=10	*n*=6	*n*=10	*n*=6	*n*=2
Body weight (g)	36±5	34±2	44±8	38±10	50±5	39±12*	36±2*
Liver (% of weight)	3.9±0.5	4.1±0.3	4.5±0.7	5.0±1.2	5.4±1.2	5.4±1.4	4.6±1.0
Cholesterol (mM)	101±9	375±38***	151±22	316±65***	199±43	561±247***	213±16
Triglycerides (mM)	40±10	122±47***	30±9	126±17***	20±7	125±77***	50±3***
Number	*n*=3	*n*=5	*n*=4	*n*=6	*n*=4	*n*=5	*n*=2
AoCS (A.U.)	0.00±0.00	0.00±0.00	0.00±0.00	0.75±1.10	0.00±0.00	6.73±4.86*	0.01±0.01

Table 2. Attributes of WT, *Ldlr* $^{-/-}$ and *Tg (Pcsk9)* mice on western diet

Age (months)	6		12	
Number (total)	*n*=10	*n*=8	*n*=5	*n*=9
Gene	*WT*	*Ldlr* $^{-/-}$	*WT*	*Tg*
Number	*n*=10	*n*=8	*n*=5	*n*=6
Body weight (g)	49±3	37±8***	57±7	56±16
Liver (% of weight)	10.3±1.1	5.8±1.0***	8.7±1.7	7.5±1.0
Cholesterol (mM)	509±118	2248±862***	440±151	1043±236 ***
Triglycerides (mM)	34±10.3	574±285***	35±19	119±39**
Number	*n*=9	*n*=6	*n*=5	*n*=9
AoCS (A.U.)	0.01±0.04	3.01±2.05**	0.02±0.04	1.69±1.2*

4.1.10. Figure legends:

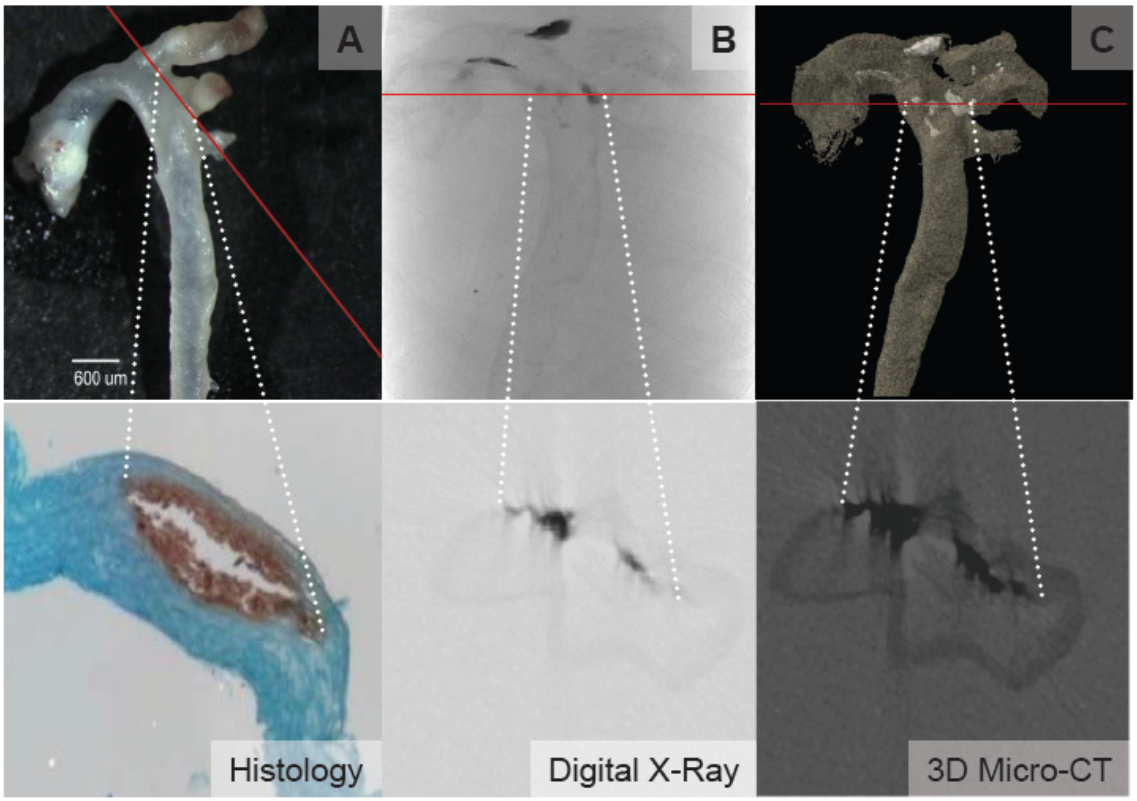

Figure 1: Measurement of aortic calcification in mice by micro-computed tomography. **A:** Digital image of an atherosclerotic aorta after fine dissection (top panel) and paraffin section symbolized by the red lane and stained with alizarin red to confirm calcification (bottom panel). **B:** Corresponding digital X-ray images from top panel in (*A*) and a cross-section symbolized by the red line. **C:** Corresponding 3D reconstructed images.

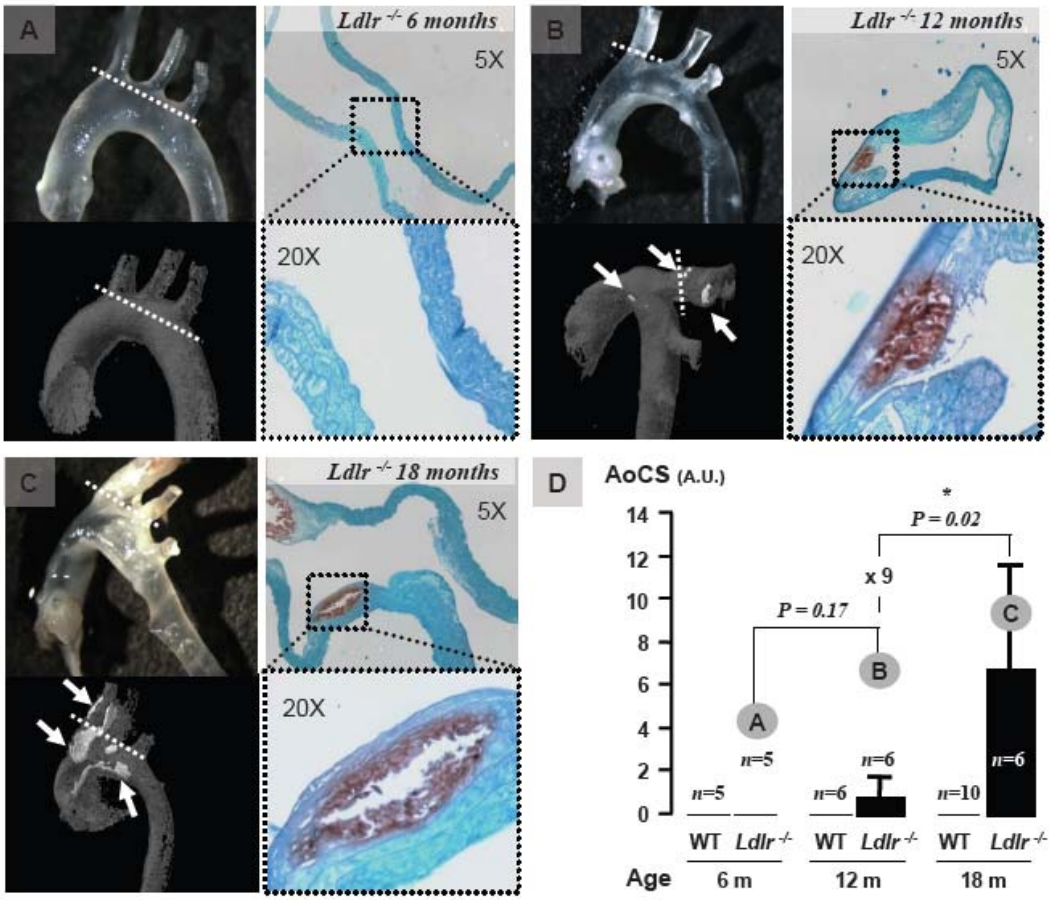

Figure 2: Age-dependent aortic calcification in *Ldlr*$^{-/-}$ mice. Aortic calcification in *Ldlr*$^{-/-}$ mice fed a regular chow diet at 6 (**A**), 12 (**B**) and 18 (**C**) months of age. For each age group, representative images of aortas after fine dissection (top left panel), 3D reconstructions of calcification (bottom left panel), and alizarin red stained sections of calcified lesions (top and bottom right panels) are shown. Arrows indicate site of calcification. (**D**) Age-dependence of the AoCS in *Ldlr*$^{-/-}$ versus age-matched WT mice.

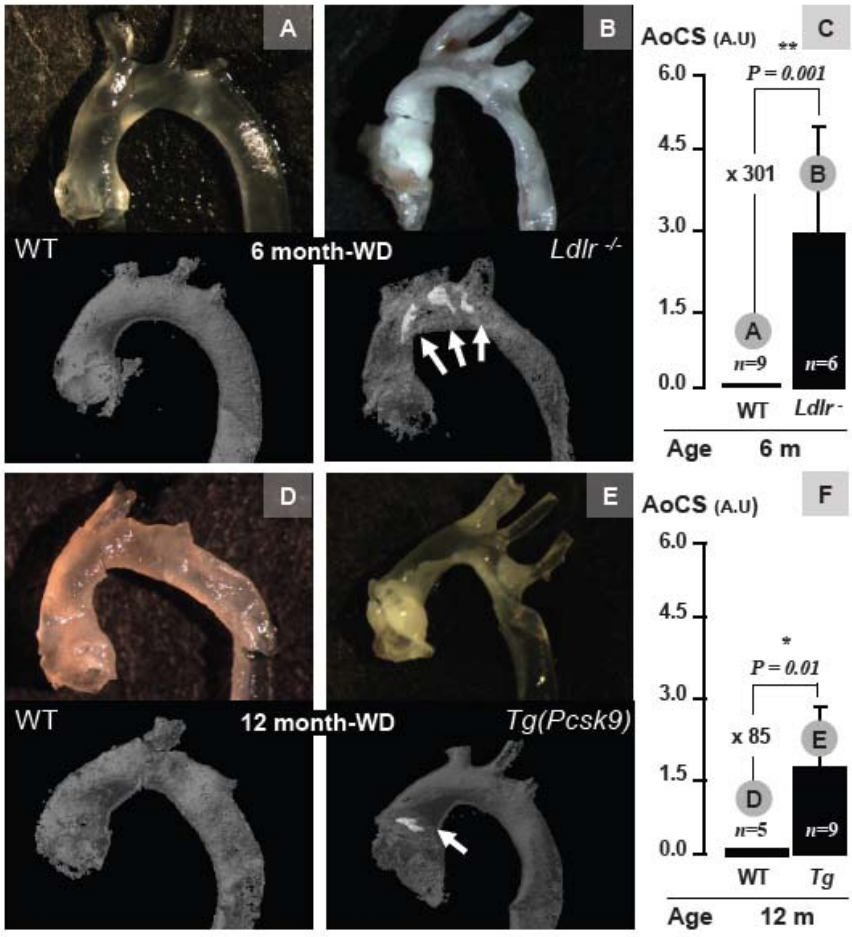

Figure 3: Aortic calcification in *Ldlr*$^{-/-}$ and PCSK9 *Tg* mice on a WD. Aortic calcification in WT (**A**) and PCSK9 *Tg* mice (**B**) mice at 12 months of age on a WD. A representative image of an aorta after fine dissection is presented (top panel), and a corresponding 3D reconstructed image (bottom panel). Arrows indicate site of calcification. **(C)** AoCS histogram of WT mice compared to PCSK9 *Tg* mice on WD at 12 months of age. Aortic calcification in WT (**D**) and PCSK9 *Tg* mice (**E**) at 12 months of age on a WD for comparison purpose, again presented as a digital image and a corresponding 3D micro-CT reconstructed image showing the extensive calcification **(F)** AoCS histogram of WT mice compared to *Ldlr*$^{-/-}$ mice on WD at 6 months of age.

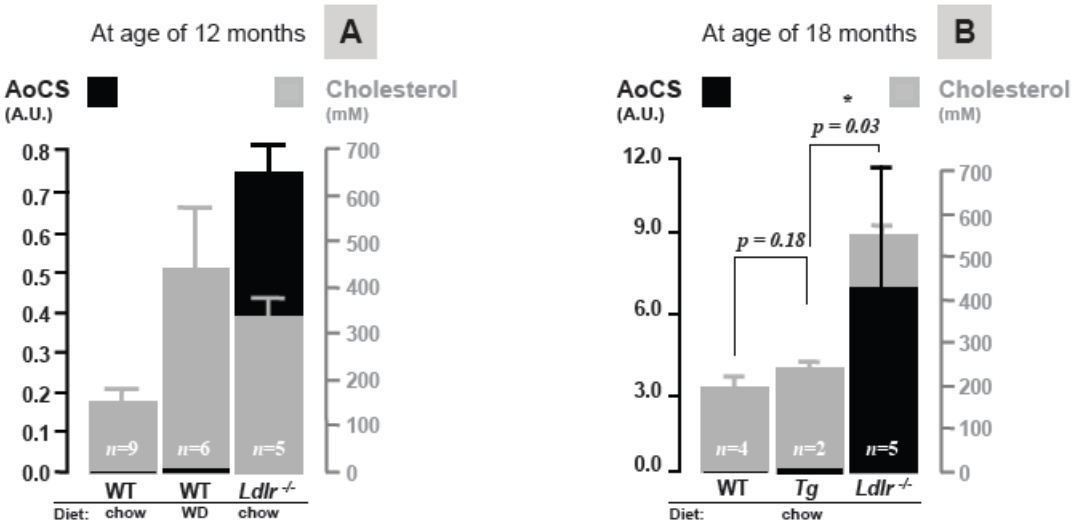

Figure 4: The degree of AoCS in relation to circulating cholesterol. (A) AoCS overlying cholesterol histogram comparing WT on WD to age-matched *Ldlr*$^{-/-}$ mice on normal chow diet at ages 6 and 12 month. **(B)** AoCS overlying cholesterol histogram comparing WT, PCSK9 *Tg* and *Ldlr*$^{-/-}$ mice at ages 18 months on a chow diet.

CHAPTER 3

PCSK9 AND FAT METABOLISM

Article 2

CHAPTER 3:

PCSK9 AND FAT METABOLISM

5. Foreword: Article 2

In the following chapter, we will focus on the effect of PCSK9 deficiency on fat metabolism. In this article, we compared adipose tissue of WT mice versus *Pcsk9*$^{-/-}$ mice in the presence or absence of LDLR. By dissecting visceral adipose we confirmed that at 6 months of age, *Pcsk9*$^{-/-}$ mice accumulate 80% more visceral adipose tissue than WT mice in an LDLR-independent manner **(APPENDIX II)**. *In vivo* and *ex vivo* functional analyses of adipose tissue demonstrated an increased fatty acid uptake and a higher triglyceride synthesis in *Pcsk9*$^{-/-}$ males and females. In addition, immunohistochemistry data illustrated clearly an increase in VLDLR surface availability in adipose tissue. Since VLDLR is a close member of LDLR family, absence of PCSK9-mediated degradation of VLDLR is likely the mechanism of VLDLR accumulation in *Pcsk9*$^{-/-}$ mice. Finally PCSK9 liver specific knock-out and knock-in mice were used to illustrate that the sole source of circulatory PCSK9 is of hepatic origin. These sets of experiments are an attempt to predict the impact of drugs targeting PCSK9 as a method of lowering cholesterol and the safety profile surrounding this method.

All experiments, animal sampling, data compiling and analyzing as well as parts of the manuscript preparation was conducted by Anna Roubtsova (first author) and Zuhier Awan (second author), with the exception of the *in vivo* and *ex vivo*.

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5.1. Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue

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5.1.1. Abstract

Objective- Proprotein convertase subtilisin/kexin 9 (PCSK9) promotes the degradation of the low-density lipoprotein receptor (LDLR), and its gene is the third locus implicated in familial hypercholesterolemia. Herein, we investigated the role of PCSK9 in adipose tissue metabolism.

Methods and Results- At 6 months of age, *Pcsk9*$^{-/-}$ mice accumulated ~80% more visceral adipose tissue than wild type mice. This was associated with adipocyte hypertrophy and increased *in vivo* fatty acid uptake and *ex vivo* triglyceride synthesis. Moreover, adipocyte hypertrophy was also observed in *Pcsk9*$^{-/-}$ *Ldlr*$^{-/-}$ mice, indicating that the LDLR is not implicated. Rather, we show here by immunohistochemistry that *Pcsk9*$^{-/-}$ males and females exhibit 4- and ~40-fold higher cell surface levels of very- low-density lipoprotein receptor (VLDLR) in perigonadal depots, respectively. Expression of PCSK9 in the liver of *Pcsk9*$^{-/-}$ females re-established both circulating PCSK9 and normal VLDLR levels. In contrast, specific inactivation of PCSK9 in the liver of wild type females led to ~50-fold higher levels of perigonadal VLDLR.

Conclusions- *In vivo*, endogenous PCSK9 regulates VLDLR protein levels in adipose tissue. This regulation is achieved by circulating PCSK9 that entirely originates from the liver. PCSK9 is thus pivotal in fat metabolism: it maintains high circulating cholesterol levels via hepatic LDLR degradation, but also limits visceral adipogenesis likely via adipose VLDLR regulation.

Key words: PCSK9, VLDL receptor, adipose tissue metabolism, fatty acid uptake, proprotein convertase

5.1.2. Introduction

PCSK9 is the ninth member of the family of proprotein convertases (PCs; encoded by the genes *PCSK1* to *PCSK9*) that share identity with subtilisin and kexin[1]. The first 8 members of the family cleave protein precursors of hormones, growth factors, receptors and transmembrane transcription factors that transit through or reside in the secretory pathway[2]. In contrast, PCSK9 has no known substrates but itself. It undergoes an autocatalytic cleavage of its N-terminal prosegment[1, 3], which remains trapped in the catalytic pocket[4].

PCSK9 shortens the half-life of the LDLR[5], a process independent of its catalytic activity[6]. Gain-of-function mutations in the *PCSK9* gene lead to autosomal dominant hypercholesterolemia[7], as do mutations in the *LDLR* and *APOB* genes. Other *PCSK9* mutations or polymorphisms responsible for loss of function result in hypocholesterolemia[8].

PCSK9 is highly expressed in the liver[1], where it binds the LDLR and promotes its internalization and degradation in endosomal/lysosomal compartments[5]. Thus, mice lacking PCSK9 (*Pcsk9*$^{-/-}$) exhibit a 2 to 3-fold increase of the LDLR protein in liver homogenates, and a substantial accumulation of the receptor at the hepatocyte cell surface[9, 10]. This leads to hypocholesterolemia (-40%), with a ~5-fold drop in LDL-cholesterol levels. In humans, where 70% of cholesterol is associated with LDLs, versus only 30% in mice, the hypocholesterolemia due to PCSK9-deficiency is even more dramatic. Two women lacking functional PCSK9 exhibited LDL-cholesterol levels of ~0.4 mM (15 mg/dL)[11, 12]. PCSK9 is now considered as a promising target to treat hypercholesterolemia and prevent coronary heart disease.

Human PCSK9 is abundant in plasma[13] and analysis of mice that lack PCSK9 specifically in liver revealed that circulating PCSK9 originates primarily from this tissue[10]. Whether physiological levels of circulating PCSK9 affect the LDLR protein levels in peripheral tissues, independent of its local expression, is yet to be demonstrated.

Another issue is the ability of PCSK9 to target other receptors. The major binding site of PCSK9 to the LDLR involves its catalytic domain, which interacts with EGF-A, one of three EGF-like repeats present in the extracellular domain of the LDLR[14]. In the LDLR family, VLDLR and apoER2 that are the two closest receptors to LDLR also contain an

EGF-A repeat that shares ~60% identity with the LDLR one. *Ex vivo*, the levels of both receptors were shown to be efficiently reduced upon PCSK9 overexpression[15, 16], suggesting that they are *in vivo* targets of PCSK9. While apoER2 is mainly present in brain, VLDLR is highly expressed in heart, skeletal muscle and adipose tissue, where it lays at the surface of endothelial cells (for reviews, see[17, 18]). Different from the LDLR, the VLDLR is not regulated by cellular sterols and does not bind LDLs efficiently. It rather contributes to the delivery of fatty acids that derive from triglyceride-rich lipoproteins to peripheral tissues. It binds apoE-enriched chylomicrons and VLDLs, intermediate-density lipoproteins (IDLs), as well as lipoprotein lipase that hydrolyses triglycerides into free fatty acids. These interactions are facilitated by heparan sulfate proteoglycans that bind lipoproteins and lipoprotein lipase. The latter, like the VLDLR, is abundant in adipose tissue, heart and muscle. Finally, free fatty acid internalization is facilitated by several proteins, including the translocase FAT/CD36[19].

Although PCSK9 is a key regulator of circulating LDL-cholesterol levels, the question of its implication in the regulation of adipogenesis was raised by our seminal observation that mice lacking PCSK9 (*Pcsk9*$^{-/-}$) accumulated abdominal fat. Furthermore, it has been recently reported that postprandial triglyceride levels were 2-fold lower in *Pcsk9*$^{-/-}$ mice, suggesting enhanced clearance[20].

We therefore compared the adipose tissue of WT versus *Pcsk9*$^{-/-}$ mice. *Pcsk9*$^{-/-}$ mice indeed accumulated more visceral fat in an LDLR-independent manner. *In vivo* and *ex-vivo* functional analyses demonstrated an increased fatty acid uptake and a higher triglyceride synthesis in the adipose tissue of *Pcsk9*$^{-/-}$ males and females. VLDLR protein levels were significantly increased in the visceral adipose tissue of *Pcsk9*$^{-/-}$ mice, consistent with the hypothesis that circulating PCSK9 promotes the degradation of the VLDLR in this tissue.

5.1.3. Methods

Animals and treatments

All procedures were approved by the Clinical Research Institute of Montreal animal care committee. *Pcsk9*$^{-/-}$ mice lacking the *Pcsk9* proximal promoter and exon 1 were described[10], and were since backcrossed 10 times to C57BL/6J mice. *Ldlr*$^{-/-}$ (C57BL/6J background; stock 002529) and *Vldlr*$^{-/-}$ (stock 002207) mice were from The Jackson Laboratory. Mice were housed in a specific pathogen-free facility under 12-hour light/12-hour dark cycles, and fed a normal chow diet (Agribrands, St-Hubert, QC).

Supplemental Methods available online at http://atvb.ahajournals.org

5.1.4. Results

The absence of PCSK9 leads to fat accumulation in perigonadal depots

In 10 week-old mice fed a chow diet, perigonadal and perirenal masses were increased with significant or close to significant *P* values ($P \leq 0.075$) in *Pcsk9*$^{-/-}$ males and *Pcsk9*$^{-/-}$ females (Figure 1). In 26 week (6 month)-old mice, the increase of perigonadal mass reached 77% and 60% in *Pcsk9*$^{-/-}$ males and females, respectively, while the increase of perirenal mass reached 108% and 76%. No significant variations were observed in the mass of inguinal, pectoral or brown fat pads (Supplemental Figure I), indicating that fat accumulation mainly occurred in visceral adipose tissue. However, *Pcsk9*$^{-/-}$ mice were not prone to obesity. Their body weights only slightly increased, by 4 to 8%, even when mice were fed a Western diet for 25 weeks after weaning (Supplemental Figure II).

To determine whether fat accumulation was due to adipocyte hypertrophy or hyperplasia, paraffin sections of WT and *Pcsk9*$^{-/-}$ perigonadal adipose tissue were analyzed (Figure 2). The average number of adipocytes per mm^2 did not differ between WT males and females, but was strongly reduced in both *Pcsk9*$^{-/-}$ males (-46%) and females (-53%). With an estimated average adipocyte sectional area of ~4080 μm^2

versus 2060 μm^2, the *Pcsk9*$^{-/-}$ adipocytes are estimated to be ~2.8-fold larger than WT adipocytes.

To elucidate whether hypertrophy is due to the loss of PCSK9 in perigonadal fat, we evaluated its expression level by quantitative RT-PCR. PCSK9 mRNA was ~160-fold less abundant than in the liver (Supplemental Figure), the major expression site of PCSK9, thus raising the question of the potential role of circulating PCSK9.

Circulating triglycerides levels are slightly higher in mice lacking PCSK9

Circulating cholesterol was 32% and 40% lower in *Pcsk9*$^{-/-}$ males and females, respectively, as previously reported for male mice[9, 10]. Conversely, plasma triglycerides were slightly increased in *Pcsk9*$^{-/-}$ males (+35%; not significant) and females (+46%; P = 0.03). In agreement, FPLC analyses showed triglyceride enrichment in VLDL fractions in both males and females (Supplemental Figure IV).

Dietary lipid uptake is increased in *Pcsk9*$^{-/-}$ adipose tissue

To better define the molecular mechanisms leading to fat accumulation, WT and *Pcsk9*$^{-/-}$ mice received a ^{3}H-oleate fat load by gavage after overnight fasting. Plasma triglycerides were measured over 3 hours (Supplemental Figure V). Their clearance was not significantly affected in *Pcsk9*$^{-/-}$ mice, although a trend for faster clearance was observed in *Pcsk9*$^{-/-}$ females. After 3 hours, mice were sacrificed to evaluate the combined ^{3}H-triacylglycerol and ^{3}H-diacylglycerol content of perigonadal and inguinal pads, muscle and liver (Figure 3A). ^{3}H-oleate uptake into tissue represents the overall process of lipoprotein hydrolysis (primarily chylomicrons) coupled to fatty acid uptake and esterification into triglycerides. In males, ^{3}H-oleate incorporation increased in all *Pcsk9*$^{-/-}$ tissues tested, especially in perigonadal and inguinal pads. In *Pcsk9*$^{-/-}$ females, ^{3}H-oleate incorporation increased in perigonadal and inguinal pads, although to a lesser extent than in *Pcsk9*$^{-/-}$ males, while the incorporation in muscle was the same in WT and *Pcsk9*$^{-/-}$ females. Thus, the loss of PCSK9 favors a higher uptake of fatty acids by the adipose tissue, in both genders. In contrast to *Pcsk9*$^{-/-}$ males that exhibited 78% increased incorporation in the liver, *Pcsk9*$^{-/-}$ females showed a 41% lower incorporation.

However, because of high variations between mice, *P* values (0.15 and 0.08) did not reach statistical significance (Figure 3A; right panel).

Pcsk9$^{-/-}$ adipose tissue exhibits increased *ex vivo* triglyceride synthesis

To evaluate the intrinsic ability of each tissue to synthesize triglycerides directly from glucose and fatty acids, pieces of perigonadal and inguinal fat, muscle and liver were incubated with ^{14}C-glucose (Figure 3B). Glucose is the precursor of the intracellular triglyceride backbone, glycerol-3-phosphate. In these experiments, the contribution of lipoprotein binding and lipoprotein lipase-mediated lipolysis of dietary triglycerides is bypassed. Triglyceride synthesis was maintained or increased in all *Pcsk9*$^{-/-}$ tissues tested, including male livers in which triglyceride synthesis strongly increased (+118%). In contrast, female *Pcsk9*$^{-/-}$ liver exhibited a significant 53% drop, consistent with the 41% lower ^{3}H-oleate incorporation observed following the fat load. Altogether, these data suggest that PCSK9-deficiency leads to an increased fatty acid uptake that stimulates triglyceride synthesis in perigonadal (+80% in males) and inguinal (+93% in *Pcsk9*$^{-/-}$ females; *P* = 0.1) pads.

Adipocyte hypertrophy in *Pcsk9*$^{-/-}$ mice is LDLR-independent

To examine whether adipocyte hypertrophy was dependent on PCSK9-mediated LDLR regulation, paraffin sections of perigonadal fat of mice lacking the LDLR and having either normal PCSK9 (*Ldlr*$^{-/-}$) or null PCSK9 (*Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$) levels were analyzed (Figure 4). In an *Ldlr*$^{-/-}$ background, the lack of PCSK9 (*Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$) resulted in adipocyte hypertrophy with 39% and 57% fewer adipocytes per mm^2 in males and females, respectively, similar to the 46% and 53% drops seen in *Pcsk9*$^{-/-}$ mice (Figure 2). Note that in *Ldlr*$^{-/-}$ perigonadal depots, neither the size of the adipocytes (Figure 4) nor PCSK9 expression (Supplemental Figure III) were significantly affected.

VLDLR protein levels increase in perigonadal fat in the absence of PCSK9

Because LDLR regulation by PCSK9 did not seem implicated in the adipose tissue phenotype, and as the VLDLR was shown to be targeted by PCSK9 *ex vivo*[15, 16], we examined the possibility of its *in vivo* regulation by PCSK9.

Western blot analyses were unsuccessful with commercially available antibodies and, to our knowledge, adipose VLDLR has never been visualized by Western blot[21, 22]. Paraffin sections of perigonadal depots from 26-week-old mice were therefore analyzed by immunohistochemistry (Figure 5). Image analysis (*see* Methods) revealed that lack of PCSK9 led to 4- and 43-fold increase in VLDLR labeling in males and females, respectively, revealing a strong VLDLR downregulation exerted by PCSK9 in this tissue, especially in females. In the absence of the LDLR, the loss of PCSK9 led to a similar 36-fold ($P < 0.0001$) increase in VLDLR labeling in females, but not in males. Note that VLDLR was not detected in *Vldlr*$^{-/-}$ control sections (Supplemental Figure VI).

VLDLR mRNA levels were assessed in the same tissues by quantitative reverse transcription-polymerase chain reaction (Supplemental Figure VII). Although similar in the adipose tissue of all male mice, VLDLR mRNA levels in *Pcsk9*$^{-/-}$ females doubled, a difference that cannot account for the 43-fold increase in cell surface VLDLR protein levels. In the absence of LDLR, the loss of PCSK9 in females (*Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$) was accompanied by a significant 35% drop in VLDLR mRNA relative to *Ldlr*$^{-/-}$ females, while the surface protein level was increased 36-fold. VLDLR mRNA upregulation is thus not responsible for VLDLR protein accumulation at the adipocyte cell surface. In the liver, where the VLDLR was not detectable by immunohistochemistry, VLDLR mRNA expression was 6 to 27-fold higher in females than in males ($P \leq 0.000005$; Supplemental Figure VII), depending on the genotype, thus raising the possibility that PCSK9 may also affect VLDLR protein levels in female hepatocytes.

Neither lipoprotein lipase which hydrolyzes triglycerides from triglyceride-rich lipoproteins nor CD36 that translocates free fatty acids were affected in perigonadal fat pads at the transcriptional level by the loss of PCSK9 and/or LDLR, except in *Pcsk9*$^{-/-}$ females where CD36 mRNA almost doubled (+80%; Supplemental Figure VIII). However, this 80% increase may be not essential since CD36 mRNA levels were not affected in *Pcsk9*$^{-/-}$ males, in which fat accumulation and increased ^{3}H-oleate uptake

were even more severe than in *Pcsk9*$^{-/-}$ females, and not affected in the *Ldlr*$^{-/-}$ background.

Circulating PCSK9 regulates adipose VLDLR protein

The poor expression of PCSK9 in perigonadal depots led us to examine the possible role of circulating PCSK9 in VLDLR protein regulation. We took advantage of the liver origin of circulating PCSK9. First, we expressed PCSK9 in the liver of *Pcsk9*$^{-/-}$ mice using a transgene that expresses mouse PCSK9 under the control of the apoE promoter [10] (*Pcsk9*$^{-/-}$ *Tg*$^{+/0}$). This led to a 53-fold reduction of the VLDLR labeling observed in *Pcsk9*$^{-/-}$ females (Figure 6; top panels) and to plasma PCSK9 levels that were ~5-fold higher than WT ones (Figure 6; right panel). Second, we compared *Pcsk9*$^{flox/flox}$ (equivalent to WT) mice with littermates carrying a transgene that expresses the Cre recombinase under the albumin promoter (*Pcsk9*$^{flox/flox}$ *Alb-cre*$^{+/0}$), and thus lacking PCSK9 specifically in hepatocytes[10]. The relatively low levels of VLDLR protein observed in *Pcsk9*$^{flox/flox}$ females, which do not differ from WT females by their levels of circulating PCSK9 (233 versus 238 ng/mL), was dramatically increased (53-fold) when PCSK9 was no longer expressed in the liver (Figure 6; lower panels). In the latter mice, no circulating PCSK9 was detected by ELISA confirming the unique liver origin of PCSK9 and high efficiency of *Pcsk9* excision by Cre in hepatocytes[10].

Interestingly, plasma PCSK9 levels were significantly lower (-45%) in *Vldlr*$^{-/-}$ mice (Figure 6, right panel), suggesting that liver PCSK9 is downregulated in the absence of the VLDLR. Altogether, these data show that circulating PCSK9 is responsible for the reduction of VLDLR protein levels in perigonadal depots.

5.1.5. Discussion

The purpose of our study was to gain insight into the role of PCSK9 in adipose tissue metabolism. We show herein that circulating PCSK9 regulates fat accumulation in adipocytes.

Pcsk9$^{-/-}$ mice exhibited larger perigonadal (+70%) and perirenal (+90%) depots than WT mice, and the average volume of a perigonadal adipocyte was estimated to increase by 2.8-fold. Dietary fat hydrolysis and incorporation was increased in perigonadal and inguinal fat (from 1.5 to 2.6-fold) in both *Pcsk9*$^{-/-}$ males and females, even though the inguinal fat mass was moderately increased in 10 week-old mice. In the same tissues, the intracellular triglyceride synthesis was maintained or increased, and no major changes were observed in WT and *Pcsk9*$^{-/-}$ fatty acid catabolism evaluated by the number of disintegrations per minute (dpm) associated with oxidation products, except an 81% increased oxidation in inguinal pads of *Pcsk9*$^{-/-}$ females (*not shown*), likely leading to an underestimation of the ^{3}H-incorporation in this tissue.

Although males and females shared the above phenotypes, a striking difference was observed in their liver metabolism: ^{3}H-oleate incorporation and triglyceride synthesis approximately doubled in the liver of *Pcsk9*$^{-/-}$ males, but were about 2-fold reduced in the liver of *Pcsk9*$^{-/-}$ females (Figure 3; right panels). In addition, livers from 26 week-old *Pcsk9*$^{-/-}$ mice did not exhibit any steatosis (*not shown*). This suggests that, following a gavage, *Pcsk9*$^{-/-}$ males have a higher potential to internalize abundant chylomicron remnants than *Pcsk9*$^{-/-}$ females.

We examined the adipose tissue of *Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$ mice and found that lack of PCSK9 led to adipocyte hypertrophy independently from the LDLR. This is in agreement with low LDLR levels in perigonadal fat, in which LDLR mRNA is ~8-fold lower than in the liver. (Supplemental Figure VIII).

As a candidate target of PCSK9 in fat, the VLDLR presented several key properties. It is ~60% identical to LDLR; it was downregulated *ex vivo* by PCSK9[15, 16]; and it is a major lipoprotein receptor in adipose tissue implicated in triglyceride metabolism[18]. When compared to WT mice, *Pcsk9*$^{-/-}$ males and females had 4- and 43-fold more cell surface VLDLR in perigonadal fat, respectively. In *Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$ females, a similar 36-fold increase was observed. Altogether, these data reveal that PCSK9 exerts, in an

LDLR-independent manner, a strong control on adipocyte VLDLR, especially in females. It is tempting to attribute the increased visceral adipogenesis to VLDLR upregulation. In agreement with this hypothesis, *Vldlr*$^{-/-}$ mice were reported to exhibit 15 to 20% lower body weights, reduced (-40%) perigonadal fat and adipocyte size, and impaired fatty acid uptake[22], whereas *Pcsk9*$^{-/-}$ mice exhibited slightly higher body weights, fat accumulation, larger adipocytes and higher fatty acid uptake (this study). Additional studies in mice lacking both PCSK9 and VLDLR will be required to verify that the latter phenotypes are lost in the absence of the VLDLR.

Finally, the analysis of different mouse models revealed that circulating, and not local, PCSK9 is responsible for adipose VLDLR protein regulation. Expression of PCSK9 in the liver of *Pcsk9*$^{-/-}$ mice normalized the VLDLR labeling, while removal of PCSK9 from the liver of normal mice led to a dramatic increase in adipose VLDLR protein. Thus, this study provides the first evidence for an *in vivo* role of endogenous levels of circulating PCSK9. In WT mice, the above data suggest that the latter is responsible for the degradation of ~75% and ≥ 95% of the perigonadal surface VLDLR in males and females, respectively.

Curiously, although the absence of PCSK9 led to ~10-fold higher levels of VLDLR in female perigonadal pads than in male ones, similar phenotypes in terms of adipocyte size and perigonadal mass were observed, whether endogenous levels of circulating PCSK9 also target extra-hepatic LDLR remains to be determined. Only huge quantities of PCSK9 obtained by injection of purified PCSK9 or adenoviral overexpression were reported to reduce LDLR levels in lung, adipose, kidney and/or ileum[23].

Because plasma PCSK9 exclusively originates from the liver, its expression in this organ could be pivotal for the coordination between peripheral fatty acid uptake and liver lipoprotein uptake, via the regulation of surface levels of VLDLR and LDLR. The function of liver PCSK9 may reside in its ability to slow down the catabolism of lipoproteins in a coordinated manner, and to maintain proper circulating levels of cholesterol and triglycerides. For example, we have previously shown that lesions observed in the regenerating liver of *Pcsk9*$^{-/-}$ mice were efficiently prevented by feeding the mice a high cholesterol diet, indicating that, in mouse, loss of PCSK9 leads to a critical hypocholesterolemia. In agreement with its key regulatory role, PCSK9 is regulated at

the transcriptional level by sterols[24], insulin, glucagon, ethinylestradiol and fasting[25-27], but also at the protein level by hepatic furin[28].

Because human PCSK9 targets *ex vivo* human VLDLR[15], and binds *in vitro* mouse VLDLR[16], PCSK9 may also target VLDLR in humans. Whether an increased visceral fat deposition also occurs in humans lacking functional PCSK9 remains to be elucidated. Perigonadal fat is part of what is called the visceral adipose tissue, which correlates directly with obesity-related metabolic disease and coronary heart disease. However, it was recently reported that, within obese patients with similar levels of visceral adipose tissue, metabolic complications were more prevalent in those exhibiting higher intra-hepatic triglyceride [29]. In a clinical perspective, since *Pcsk9*$^{-/-}$ mice do not develop liver steatosis and are not prone to obesity, the administration of a PCSK9 inhibitor developed for hypercholesterolemia treatment should not result in adverse effects.

5.1.6. Acknowledgment

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5.1.7. References

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5.1.8. Figures and legends

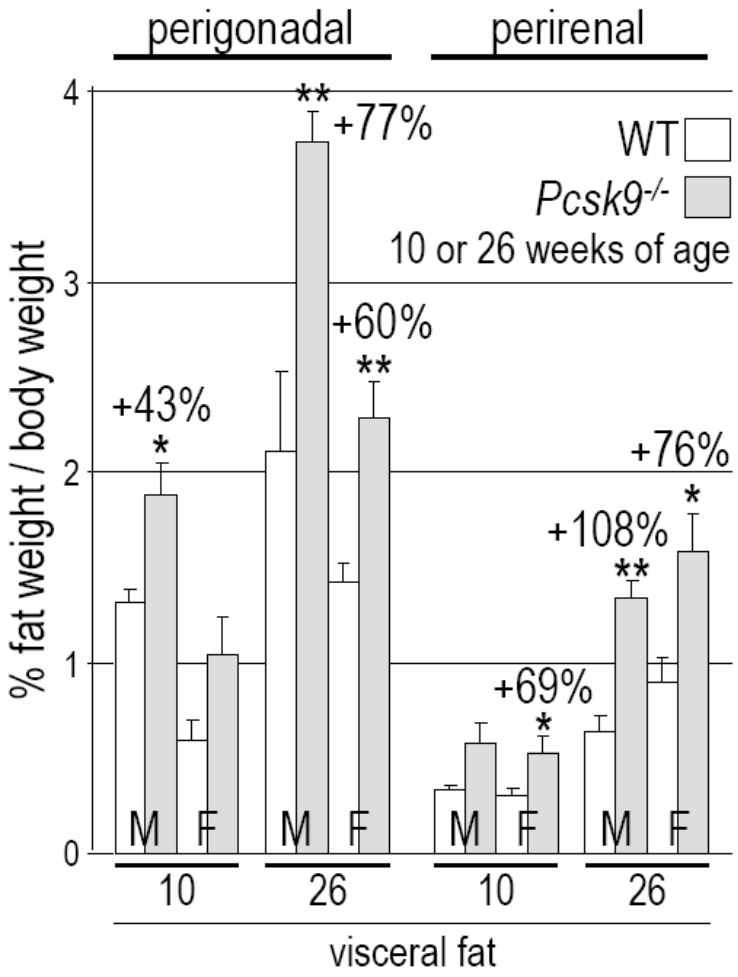

Figure 1. *Pcsk9*$^{-/-}$ mice accumulate fat in visceral adipose tissue. Visceral (perigonadal and perirenal), subcutaneous (pectoral and inguinal) and brown fat was dissected out from 26 and/or 10 week-old males (M) and females (F) and its weight expressed as the percentage of body weight. n = 4 to 15 mice per gender and genotype. Average ± SEM; *, $P \leq 0.05$; **, $P \leq 0.005$.

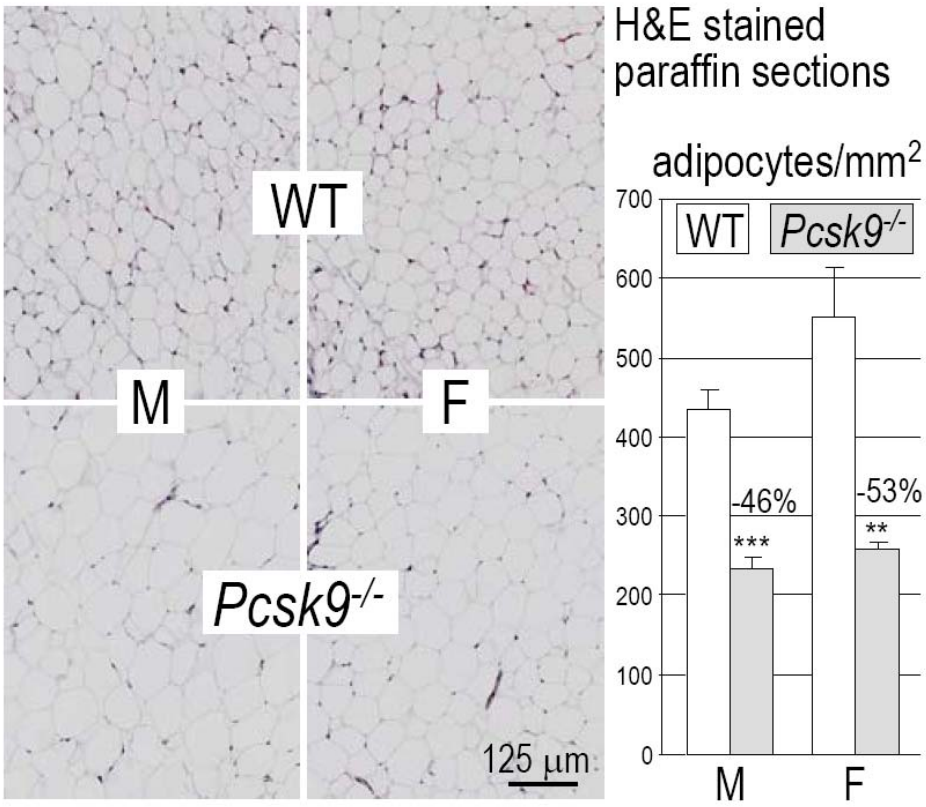

Figure 2. PCSK9 deficiency leads to adipocyte hypertrophy. Paraffin sections of WT and *Pcsk9*$^{-/-}$ perigonadal depots from 26 week-old mice were stained with hematoxylin and eosin (H&E) and the number of adipocytes/mm^2 determined. n = 8 to 11 mice per gender and genotype. Average ± SEM; **, $P \leq 0.005$; ***, $P \leq 0.0005$.

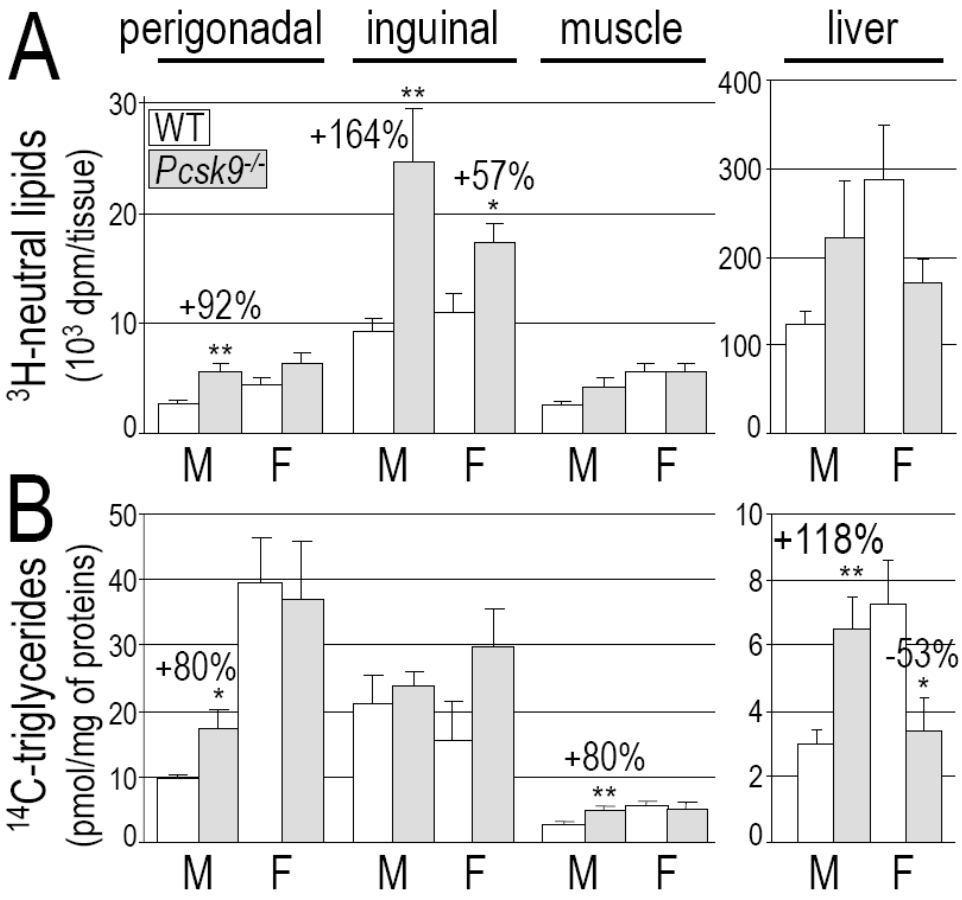

Figure 3. *In vivo* dietary lipid uptake and *ex vivo* triglyceride synthesis are altered in *Pcsk9*$^{-/-}$ mice. A, Mice received a ^{3}H-oleate fat load. After 3 hours, the ^{3}H-oleate uptake into neutral lipids was evaluated in fat depots, muscle and liver and expressed in disintegrations per minute (dpm) /tissue; n = 8 to 10 mice per gender and genotype. B, Indicated tissues were incubated with ^{3}H-glucose for 3 hours and their ^{3}H-triglyceride content was assessed in triplicates; n = 7 to 10 mice per gender and genotype. Average ± SEM; *, $P \leq 0.05$; **, $P \leq 0.005$.

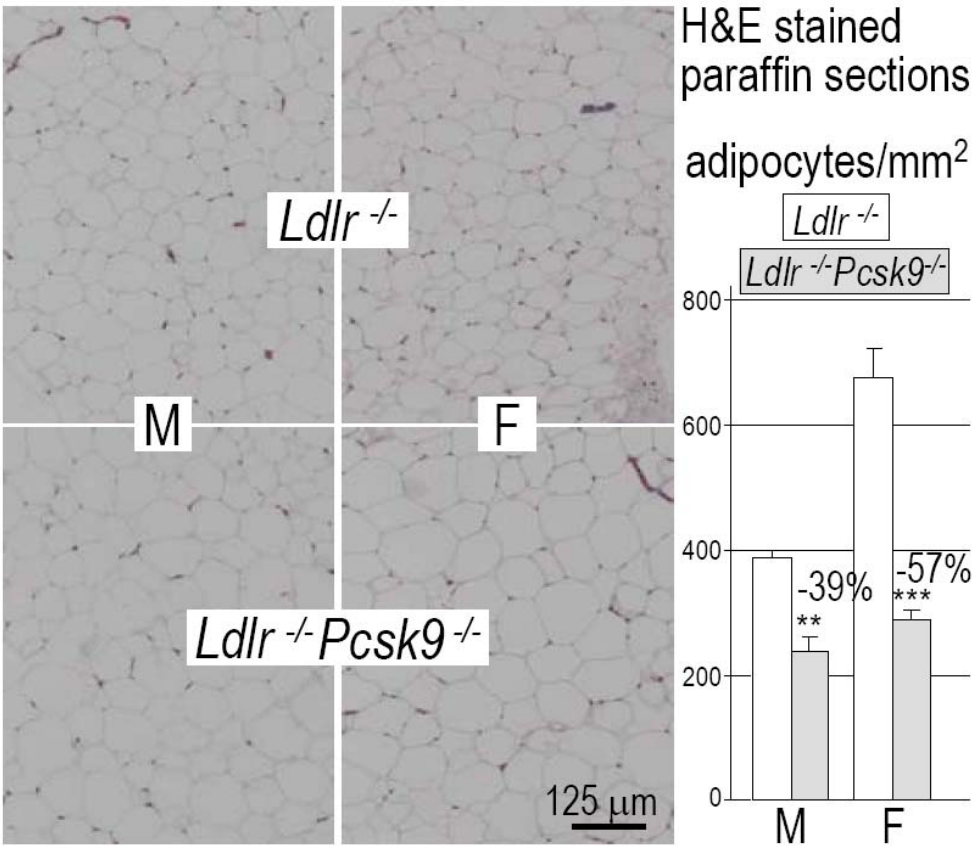

Figure 4. PCSK9 deficiency leads to adipocyte hypertrophy in a LDLR-deficient background. The number of adipocytes/mm^2 was determined in paraffin sections of *Ldlr*$^{-/-}$ or *Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$ perigonadal depots from 26 week-old mice, as in Figure 2. n = 4 mice per gender and genotype. Average ± SEM; **, $P \leq 0.005$; ***, $P \leq 0.0005$.

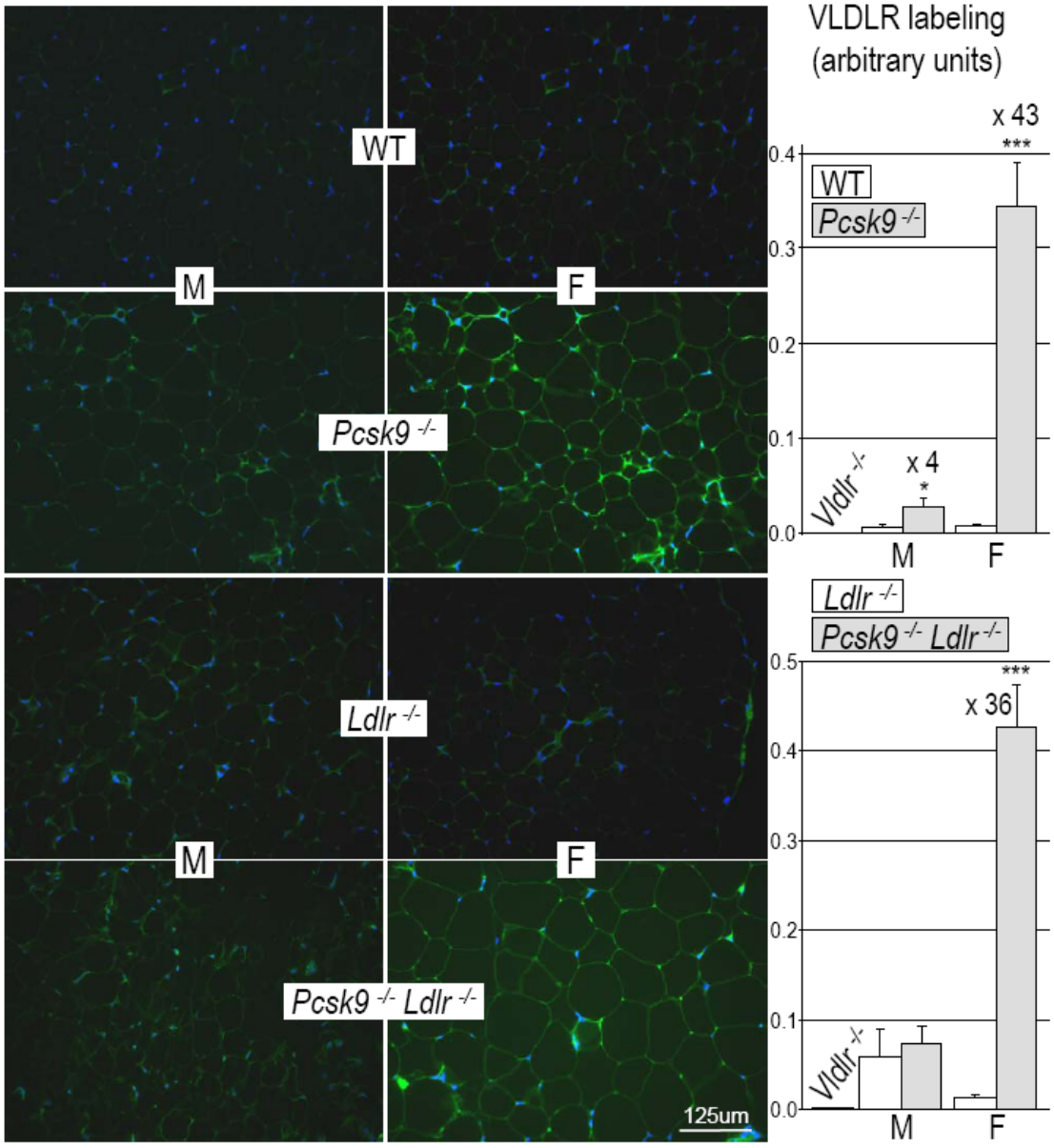

Figure 5. VLDLR protein levels are higher in *Pcsk9*$^{-/-}$ perigonadal fat. Paraffin sections of 26 week-old WT and *Pcsk9*$^{-/-}$ mice, or *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$ mice were incubated with a VLDLR antibody visualized using Alexa488-labeled secondary antibody (green labeling). The VLDLR labeling was expressed in arbitrary units (*see* Methods). n = 4 to 8 mice per point. Average ± SEM; *, $P \leq 0.05$; ***, $P \leq 0.0005$.

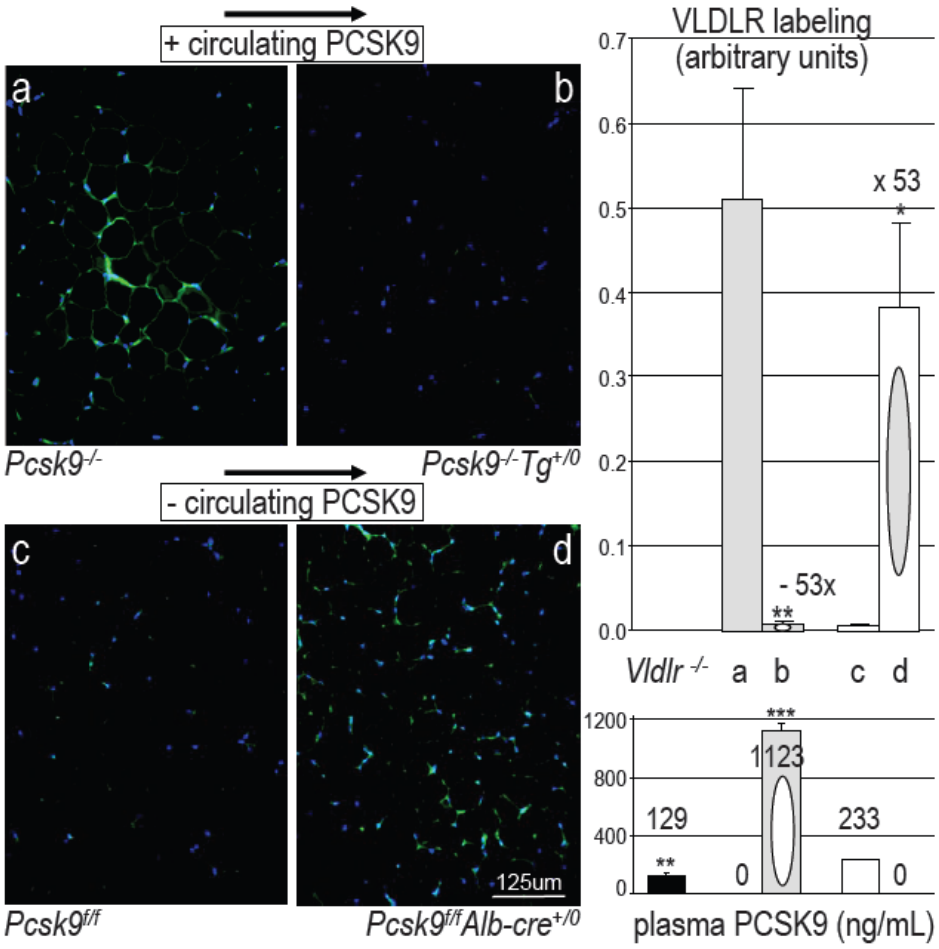

Figure 6. Adipose VLDLR protein is regulated by circulating PCSK9. VLDLR immunohistochemistry was performed on female perigonadal sections from either *Pcsk9*$^{-/-}$ littermates that did not or did receive a transgene expressing PCSK9 in the liver (top panels a and b; + circulating PCSK9) or from *Pcsk9*$^{flox/flox}$ (equivalent to WT) littermates that did not or did receive a transgene expressing *Cre* inactivating specifically the *Pcsk9* gene in hepatocytes (lower panels c and d; - circulating PCSK9). Average VLDLR labeling and plasma PCSK9 levels for these mice are shown, as well as for *Vldlr*$^{-/-}$ mice as negative controls. n = 4 to 5 mice per genotype; average ± SEM; *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$.

5.1.9. Supplemental material

Available at: http://atvb.ahajournals.org

The proprotein convertase of the subtilisin/kexin type PCSK9 regulates triglyceride metabolism via VLDL receptor degradation

By: Roubtsova *et al*.

Online Methods

Paraffin section analysis and immunohistochemistry

For adipocyte counts, paraffin sections of perigonadal depots (5 µm thick) were stained with hematoxylin and eosin (Sigma Aldrich, Oakville, ON), rinsed with xylene and mounted in Entellan New from Merck (Darmstadt, Germany). Adipocytes were counted in 3 random fields on a selected section per mouse, and 4 or more mice were analyzed by gender and genotype.

For VLDLR visualization, unparaffinized sections were incubated overnight at 4°C with a mouse VLDLR antibody (1:300; R&D systems) under non-permeabilizing conditions, and then one hour at room temperature with Alexa Fluor 488-labeled anti-goat IgGs (1:150; Invitrogen, Burlington, ON). Nuclei were counterstained with Hoechst dye (Sigma). Images were acquired using a Retiga EXi camera (Q-Imaging) mounted on a Leica DMRB microscope. Normalized total grey values (G_{VLDLR}) from VLDLR labeling monochromatic images (8 bits - 256 greyscale values) were obtained using Matlab R2010a (The MathWorks Inc.) by summing grey pixel values (threshold to the negative control value) and normalizing to the highest value.

Intragastric Fat Administration

After an overnight fast (16 h), olive oil containing 10 µCi of ^{3}H-oleate was administered by gastric gavage[1]. Briefly, after sacrifice, tissues were excised and weighed. To measure ^{3}H-oleate incorporation in tissular neutral lipids, the latter were extracted from tissue fragments of 10 to 50 mg and counted directly[1]. To assess the *ex vivo* triglyceride

synthesis, small tissue pieces (~15 µg) were incubated with ^{14}C-glucose as previously reported[2]. After lipid extraction, triglycerides were measured and expressed as pmol/mg protein[2].

Quantitation of plasma PCSK9 by ELISA

Mouse plasma were collected in EDTA coated Microtainer tubes (BD Biosciences, Fisher, ON) and 3 µL were used to measure PCSK9 using the CircuLex mouse/rat PCSK9 ELISA (MBL, Woburn, MA), according to the manufacturer's recommendations.

Statistical analysis

Excel software was used for SEM and Student's t-test calculations.

References

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Methods for Online Figures

Animals and treatments

Mice were fed a Western diet TD.88137 that was obtained from Harlan Teklad (Madison, WI).

Quantitative RT-PCR

Total RNA from liver and perigonadal fat was extracted with TRIzol (Invitrogen). cDNA was generated from 250 ng of total RNA using a SuperScript II cDNA reverse

transcriptase (Invitrogen). cDNA amplification by quantitative PCR was done in a final volume of 10 µL using SYBR green SuperMix from Quanta (Gaitherburg, MD). cDNA quantitation of each gene of interest was performed using specific primers (*see* the Table below) with normalization to the TATA box protein (TBP) cDNA amplification and expressed as arbitrary units.

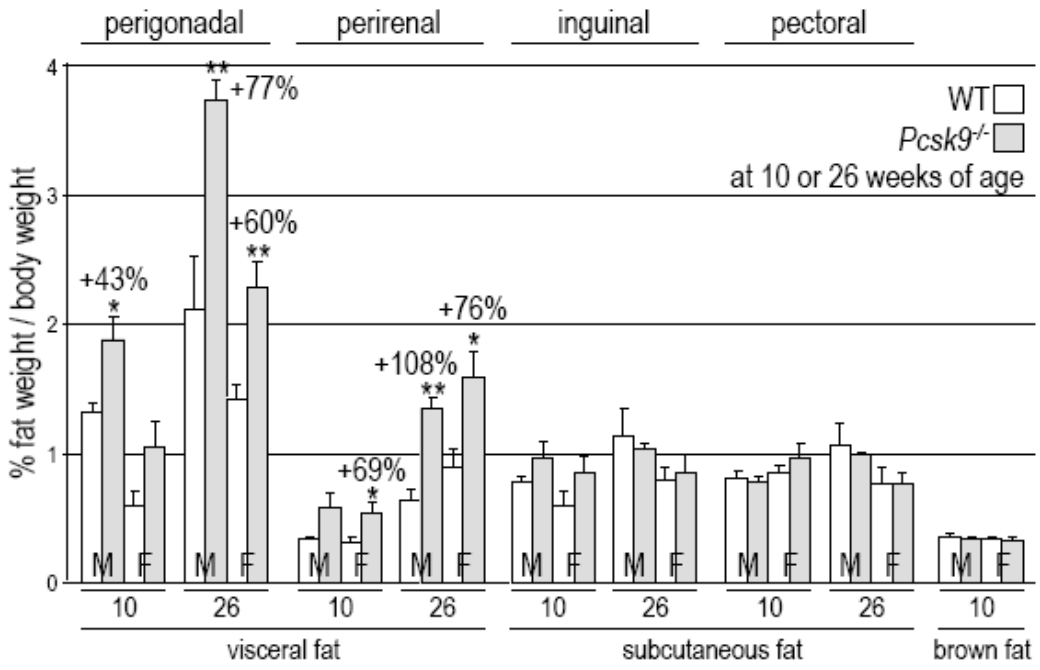
Specific mouse cDNAs were amplified using the following primers:

cDNA	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
TBP	GCTGAATATAATCCCAAGCGATTT	GCAGTTGTCCGTGGCTCTCT
PCSK9	CAGGGAGCACATTGCATCC	TGCAAAATCAAGGAGCATGGG
VLDLR	GGACATTGCAGCTCAGAAGCT	CATCAATTGAGGCACTGAAGATG
FAT/CD36	TGGTGATGTTTGTTGCTTTTATGAT	GCTCATCCACTACTTATTTTCCATTCT
LPL	GTGGCCGAGAGCGAGAAC	CCGTGTAAATCAAGAAGGAGTAGGTT
LDLR	GGAGATGCACTTGCCATCCT	AGGCTGTCCCCCCAAGAC

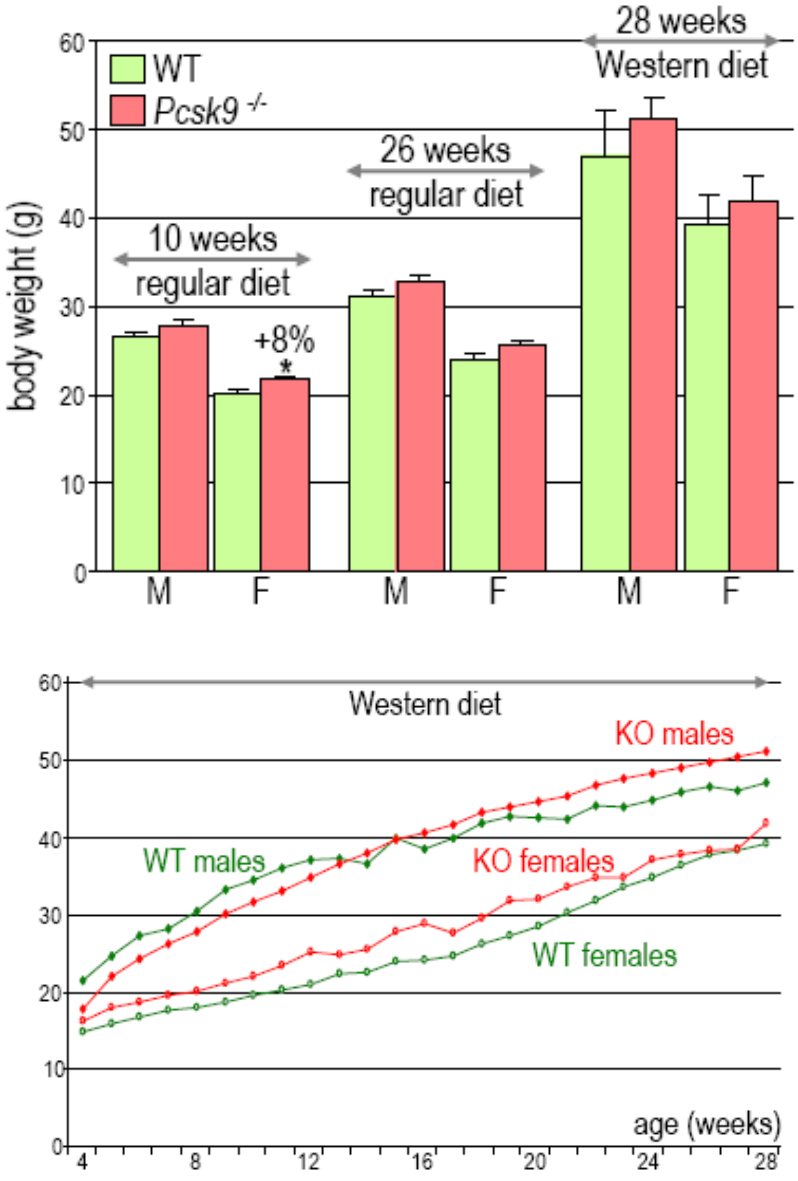
Plasma cholesterol and triglyceride measurements

Plasma was obtained by centrifugation (15 minutes at 850g at 4°C) of 250 µL of blood and kept at –20°C until assay. Total cholesterol and triglycerides were measured using the Infinity Thermo Electron Corporation (Melbourne, Australia) and Wako pure chemical industry (Osaka, Japan) kits, respectively. For lipoprotein profiles, plasma from 3 mice (300 µL) were pooled and analyzed by FPLC on a Superpose 6 column (Pharmacia, Sweden) at a flow rate of 0.5 mL/min. Cholesterol and triglycerides were then measured as above. After the fat load by gavage, triglycerides were measured in the plasma using a colorimetric enzymatic kit from Roche Applied Science.

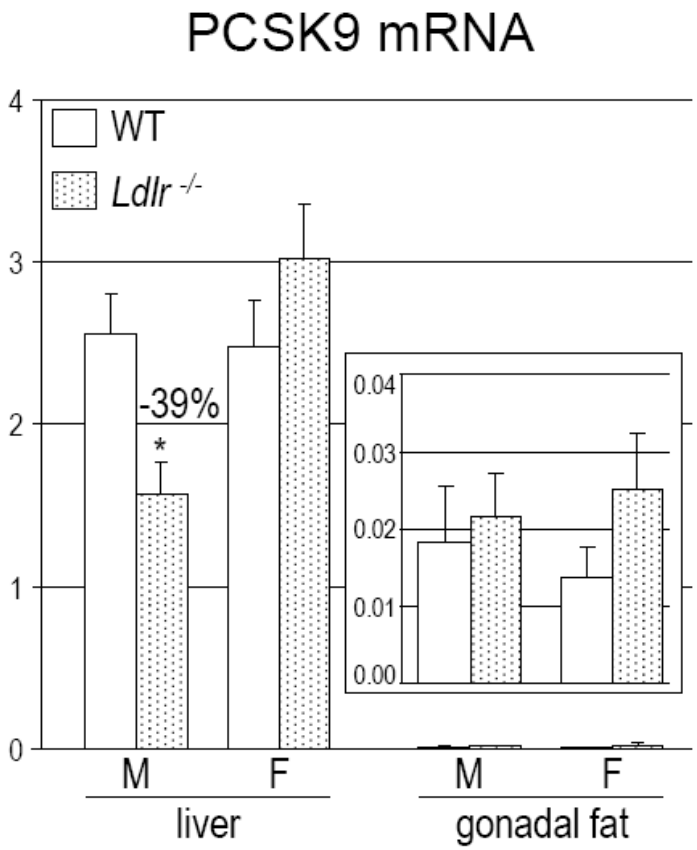
Online Figure legends



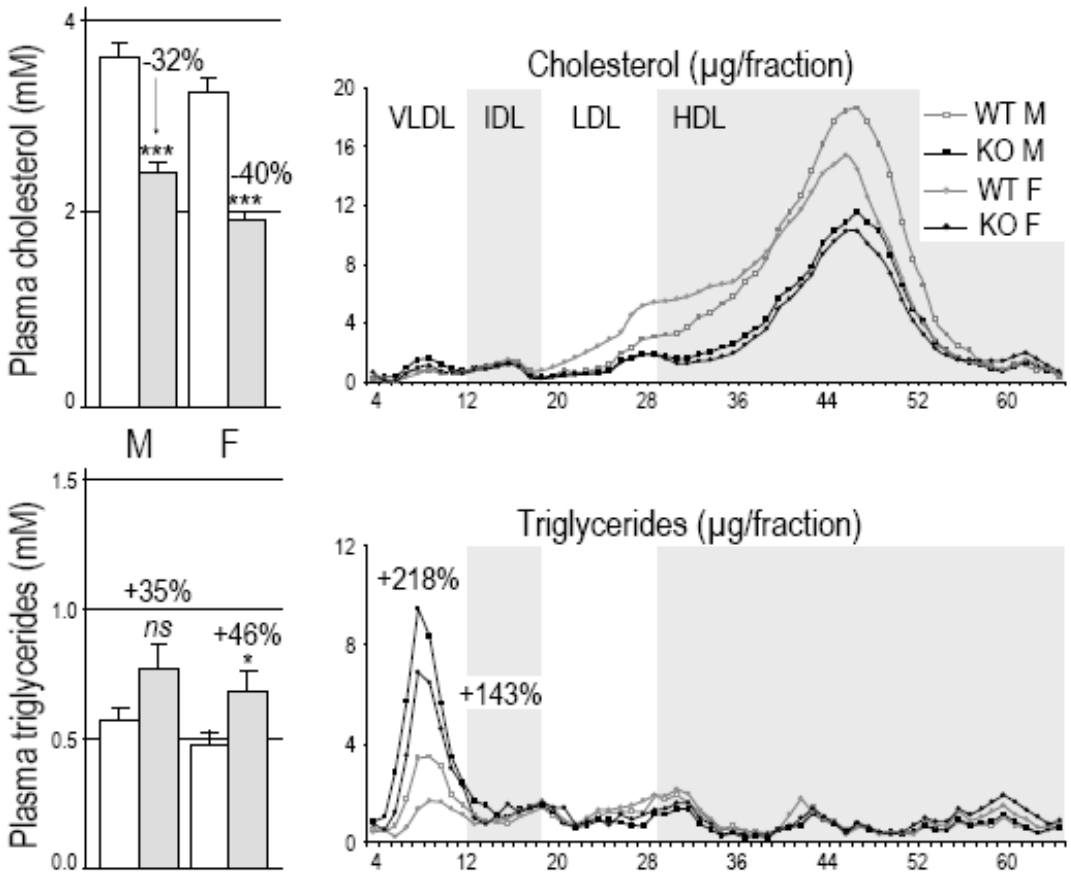
Online Figure I. *Pcsk9*$^{-/-}$ mice accumulate fat in visceral adipose tissue. Visceral (perigonadal and perirenal), subcutaneous (pectoral and inguinal) and brown fat was dissected out from 10 and/or 26 week-old males (M) and females (F) and its weight expressed as the percentage of body weight. n = 4 to 15 mice per gender and genotype. Average ± SEM; *, $P \leq 0.05$; **, $P \leq 0.005$.



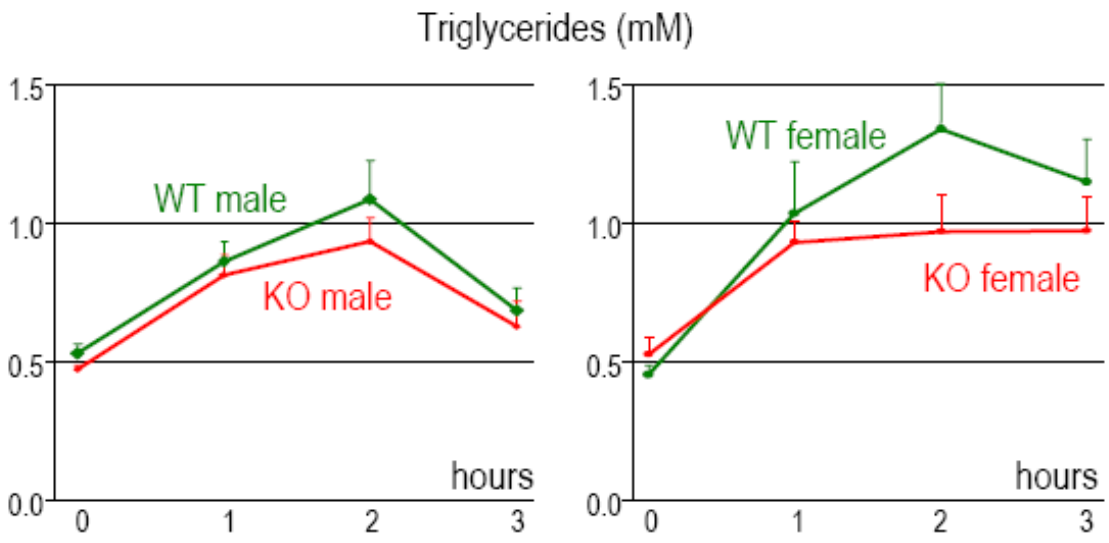
Online Figure II. The body weight of *Pcsk9*$^{-/-}$ mice is moderately increased. WT and *Pcsk9*$^{-/-}$ males (M) and females (F) were fed either a regular diet and weighted at 10 or 26 weeks of age (n = 14 to 15 mice per genotype and gender), or a Western diet (for 25 weeks after weaning) and weighted weekly up to 28 weeks of age (one of three experiments, n = 5 mice per genotype and gender). Mean ± SEM; *, $P \leq 0.05$.



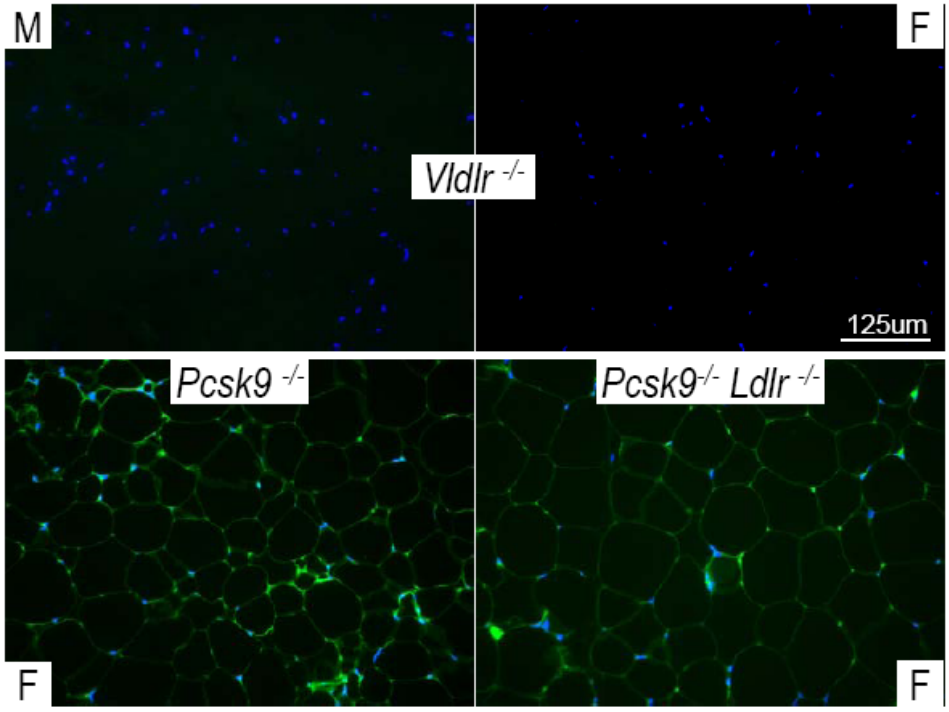
Online Figure III. PCSK9 mRNA is poorly expressed in perigonadal depots. Quantitative RT-PCR using PCSK9 specific primers detected 140 and 180-fold less PCSK9 mRNA in perigonadal fat than in the liver in WT (open bars) males and females, respectively. These levels were not significantly affected in *Ldlr*$^{-/-}$ mice (dotted bars; see enlargement in the inset), except in the male liver (-39%). Average ± SEM; *, $P \leq 0.05$; **, $P \leq 0.005$.



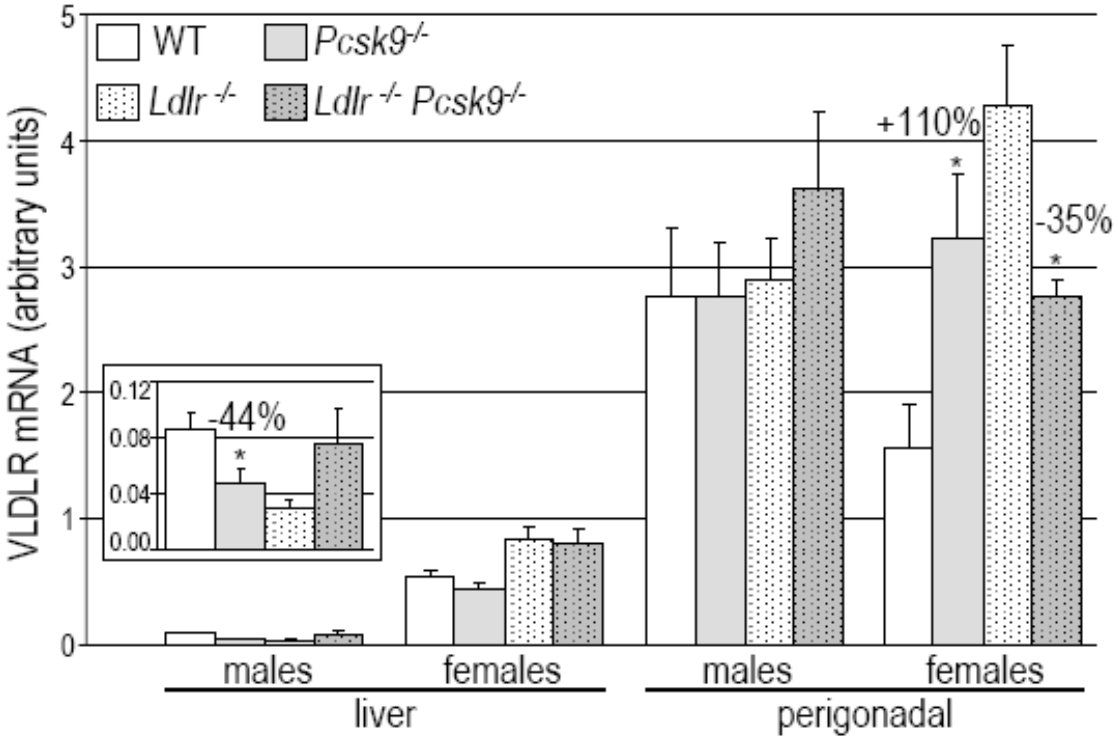
Online Figure IV. Circulating and liver cholesterol and triglycerides levels are affected in *Pcsk9*$^{-/-}$ mice. While circulating cholesterol levels decreased in KO mice, circulating triglycerides levels tended to increase in 6 month-old mice fasted for 3 hours; n = 11 mice. Average ± SEM; *, $P \leq 0.05$; ***; $P \leq 0.0005$).



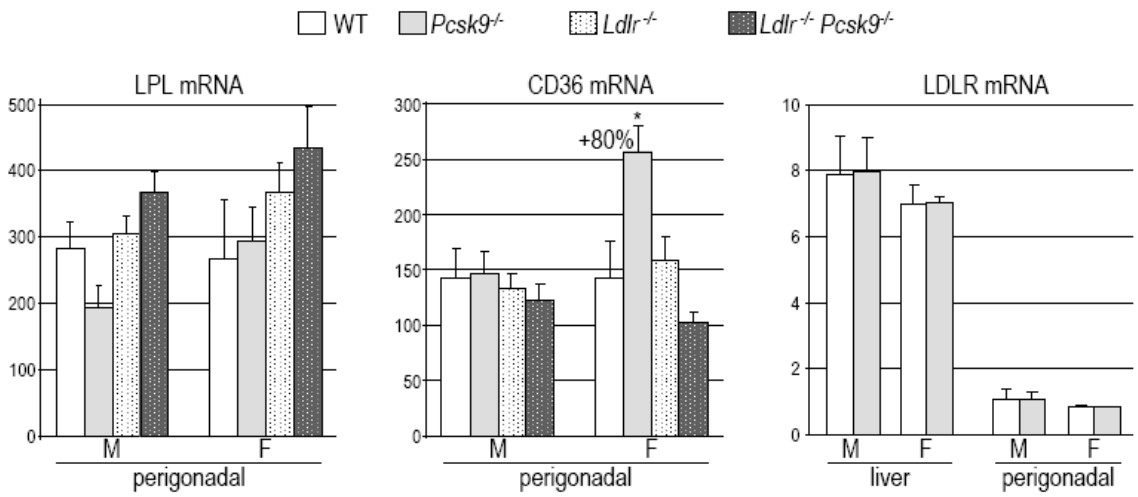
Online Figure V. Clearance of postprandial triglycerides in *Pcsk9*$^{-/-}$ mice was maintained. Concentrations of plasma triglycerides in millimoles/liter (mM) at 0, 1, 2 or 3 hours following gavage with 200 µL of olive oil were obtained in males (left panel) or females (right panel).



Online Figure VI. VLDLR labeling is highly specific. Control *Vldlr*$^{-/-}$ (top panels) and *Pcsk9*$^{-/-}$ or *Pcsk9*$^{-/-}$ *Ldlr*$^{-/-}$ (lower panels) perigonadal paraffin sections were simultaneously incubated with VLDLR antibody (*see* Methods). While a strong labeling was observed in the sections of PCSK9-deficient mice, virtually no labeling was detected in *Vldlr*$^{-/-}$ sections.



Online Figure VII. VLDLR mRNA is modestly regulated in *Pcsk9*$^{-/-}$ mice. Quantitative RT-PCR was performed to assess the VLDLR expression in the liver and perigonadal depots of WT, *Pcsk9*$^{-/-}$, *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$ mice. Values for male livers are expanded in the inset. n = 7 to 9 mice per point. Average ± SEM; *, $P \leq 0.05$.



Online Figure VIII. LPL, CD36 and LDLR mRNA levels are almost not affected by PCSK9 and/or LDLR deficiencies. Quantitative RT-PCR was performed to assess lipoprotein lipase (LPL), CD36 and LDLR expression in perigonadal depots of WT, *Pcsk9*$^{-/-}$, *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Pcsk9*$^{-/-}$ mice. n = 5 to 9 mice per point. Average ± SEM; *, $P \leq 0.05$.

CHAPTER 4

CLOSING REMARKS

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 4:
GENERAL DISCUSSION AND CONCLUSIONS

6. General discussion

The purpose of these two studies was to gain insight into the yet undisclosed biology of PCSK9. Although PCSK9 is considered a member of the PC family that activates or inactivates proprotein, nothing is known about its ultimate substrate. Apart from cleaving itself and mysteriously targeting the LDLR to the degradation pathway, less is known about other functions in non-hepatic tissue. One way of exploring the role of PCSK9 is by examining the state of protein deficiency or overexpression in an animal model. In this thesis, we investigated both aspects:

- First, overexpressing *Pcsk9* gene in mice leads to a phenotype of hypercholesterolemia and atherosclerotic calcification in aortic wall, which was accelerated by a Western diet.
- Second, the lack of *Pcsk9* gene in mice leads to a phenotype of mild visceral fat accumulation. The VLDLR accumulates in *Pcsk9*$^{-/-}$ fat tissue enhances TG trapping in adipose tissue.

In both models we address medical concerns that will confront health professional treating patients harboring mutations in PCSK9 gene.

In the case of a gain-of-function mutation in PCSK9, patients will develop familial hypercholesterolemia. If they survive coronary artery disease, they will encounter morbidities associated with premature calcification of the aorta, such as hypertension or embolizing calcified lesions. Conventional treatment modalities may lower cholesterol level and prevent coronary artery disease but might not necessary stop arterial calcification and aortic valve calcification. Furthermore conventional treatment may cause serious side effect or might be contra indicated in some medical conditions (*Awan et al 2009*). By using animal models, we could therefore test single and/or combination therapies to treat arterial calcification like; vitamin K, estrogen, selective estrogen receptor modulators, calcium channel blockers chelating and demineralization therapy (*Wallin et al 2001*). Following application of a new treatment combination we could then use the micro-CT technique we developed to evaluate each treatment modality. This is

the first study to characterize and utilize micro-CT in determining aortic calcification in *Ldlr*$^{-/-}$ and PCSK9 *Tg* mice which will lead to major impacts in biomedical research.

Hence, the evolution of vascular calcification appears to be, at least in part, independent of LDL-C levels (*Awan et al 2008*) and once osteoblast-like cells differentiate; the process may become only poorly reversible. Prospective from our studies in human and animal models have advanced the understanding of arterial calcification in the aorta in the following ways:

1. Long-term clinical data in human indicate that despite marked LDL-C lowering, arterial calcification progress in an age-dosage manner and contribute to significant life long morbidity and associate with important complications when surgical procedures are needed (*Grenon et al 2007*). Consequently, experts in the field have noticed that once calcified, tendinous xanthomas fail to regress. From our mice models we have simulated FH homozygote and heterozygote by using LDLR-deficient and PCSK9 *Tg* mice. Then we confirmed the age- and gene-dosage effects we see in human, and confirmed that calcification do not correlate with circulating cholesterol levels. Therefore early screen and detection with a sensitive method like CT and micro-CT scan will help disease understanding, evaluation of treatment response and planning future surgical interventions.

2. Arterial calcification stands in sharp contrast with disturbances in calcium and phosphate metabolism of end-stage renal failure. Even when extracorporeal LDL-C filtration techniques (LDL aphaeresis) are used to normalize plasma LDL-C, patients do not suffer calcium levels alterations (*Awan et al 2008*). In arterial calcification, bone and calcium homeostasis do not appear to be significantly disturbed in LDL-deficient patients as seen in a recent publication (*Awan et al 2010*). In the same study we reported that despite apparently normal levels of calcium, phosphate and bone mineral density, a negative correlation between osteocalcin levels and AoCS was found. This suggests an alteration in osteoblast function that may further fuel extra-skeletal tissue with the necessary elements to start calcifying. Therefore it would be of interest to examine bone metabolism in our established animal models.

3. LDLR-deficient mice developed calcium deposition in the tunica intima space of the aorta similar to human data (*Awan et al 2008*). This implies that early sub-endothelial damage caused by foam cell formation during the atherosclerosis process results in accumulation of structural matrix (collagen) for which calcium is deposited within. It is

thought that the structural matrix is laid down by differentiated local or stromal cells that acquire an osteoblast-like phenotype. A proposed unifying hypothesis links atherosclerosis risk factors, inflammatory mediators with the activation of the Wnt/Lrp5 and Runx2/Cbfa1 pathways and osteoblast differentiation (*O'Brien 2006, Shao et al 2010*) **(Figure 7)**. Therefore it would be beneficial to develop lifelong treatment to prevent early intimal injury and prevent the osteoblast differentiation processes.

In the first manuscript without any doubt we show that the LDLR-deficient mice and PCSK9 Tg mice develop significant aortic calcification, but not WT mice fed western diet by micro-CT and conventional histological analysis. This signifies a role of LDLR in aortic calcification. However our data are mostly descriptive and clearly lack mechanistic insight but still represent the first model of aortic calcification that mimics the human disease in FH. The intriguing finding that diet-induce hypercholesterolemia to levels that of homozygous FH alone does not lead to arterial calcification is a key finding that will completely revolutionize the concept of arterial calcification. Therefore we propose that arterial calcification needs multiple factors one for initiation in the form of endothelial insult (oxidized LDL, angiotensin II, and cytokines) and factors one for maintenance in the form of genetic predisposition (lacking a cell surface receptor, gaining or lacking a calcium regulatory protein). We are currently considering a model whereby fibroblasts or myofibroblasts cell-lines on LDLR-deficient background and through a variety of initiating factors that leads to osteoblast dedifferentiate in our future work. In turn, these initiating factors we expect will trigger the bone morphogenic protein 2 (BMP2) activation of Msx2 and the Wnt/Lrp5/β-catenin signaling pathway, as well as the Runx2/Cbfa1 pathway mediated activation of osteoblast transformation. In conjunction it would be of interest to use the *Ldlr*$^{-/-}$ animal model to validate the osteoblast/renal mild dysfunction that we observed in FH patients in our recent publication (*Awan et al 2010*) or test a type of preventive intervention as suggested in the discussion previously. Any of these experiments would be of certain clinical important basically because LDL-C lowering with statins or exetimibe in the prevention of aortic calcification has not met with success. Complexes as it is the mystery of premature arterial calcification is not unfolded completely and many scientific breakthroughs are need to understand and modulate this

process and achieve better patient outcome thus we trust that our model will contribute to identifying of novel therapies.

Regarding the reason why other investigators who have used *Ldlr*$^{-/-}$ mice have not and do not report calcification are due to several factors. The investigators did rarely keep mice for more that a year and/or included high fat diet as a condition for developing atherosclerosis unlike our old mice on show diet. Conventional methods are not sensitive to pick up calcium crystals unlike micro-CT. Old studies reported these arterial calcifications as late passive complication of atherosclerosis and not a dynamic process. Finally the distention between atherosclerotic plaques and atherosclerotic calcification is not often made in the literature. Our study examines aortic calcification, using a novel micro-CT method to better evaluate premature aortic calcification. This is of particular interest since cardiovascular calcification induces vascular morbidities in FH patients that do not seem to be prevented by conventional therapies. Unfortunately we did not provide direct *Ldlr* $^{+/-}$ (heterozygote FH) data as to whether they have the same disease in mice and this is because we believe that the best estimate of the heterozygous state is represented by the PCSK9 *Tg* leading to an accurate intermediate phenotype and that is not compensated as in *Ldlr* $^{+/-}$ mice. This is further supported by western blotting analysis of hepatic LDLR protein in WT, *Ldlr*$^{-/-}$ and PCSK9 Tg showing the intermediated LDLR protein expression in PCSK9 *Tg* (*Zaid et al 2008*).

Another interesting finding is that PCSK9 *Tg* mice presented with slightly higher plasma cholesterol than that of WT mice when fed a regular chow diet did not develop much calcification at 18 month (*n*=2). In part, this problem was resolved when the PCSK9 *Tg* mice (*n*=9) were fed a Western diet for 12 months. However these PCSK9 *Tg* mice presented later with lower plasma triglycerides and more weight again in compare to *Ldlr*$^{-/-}$ mice. This observation became challenging to understand in light of our second paper where we show that PCSK9 degrades not only LDLR but also VLDLR (*Roubtsova et al 2011*). We think that this effect might in part be due to the fact that PCSK9 *Tg* mice reduce LDLR expression to approximately 50% of WT mice and therefore the expected phenotype (i.e. lipid levels) will be less severe than the *Ldlr*$^{-/-}$, however the weight issue is more related to the relative weight loss in *Ldlr*$^{-/-}$ (statistically significant) rather than the weight gain in PCSK9 *Tg* (statistically not significant). This is because *Ldlr*$^{-/-}$ mice are generally sicker (CAD) and when fed Western diet they tend to develop fatty livers that later progress to liver cirrhosis and weight loss.

In the case of loss-of-function mutations in PCSK9 or the future use of PCSK9 inhibitors, the fat accumulation in perigonadal and perirenal pads did not translate to significant weight gain and/or lead to overt diabetes in mice. Therefore we stress again the fact that the site of fat accumulation matters the most. In contrast to harmful visceral obesity in human due to the accumulation of omental fat and pericardial fat that gain easy access to systematic circulation, perigonadal and perirenal fat has poor access to systematic circulation due to the distal location of large vessels. Therefore perigonadal and perirenal fat pads resemble a safe storage of access fat without adipokines, adiponectin, leptin and resistin induction into the systemic circulation. In addition, unpublished data from our lab show no upregulation of inflammatory mediators in PCSK9-deficient mice compare to WT mice. A recent paper indicated that intra hepatic fat accumulation and not the visceral fat accumulation is the cause of insulin resistance (*Fabbrini et al 2009*). However impaired glucose tolerance (*Mbikay et al 2010*) may be associated with perigonadal and perirenal fat accumulation in PCSK9-deficient mice remotely, or due to lipotoxicity induced by higher LDLR levels in β-cells of PCSK9 KO mice (*Mbikay et al 2010*).

This provides compelling rationale to examine the long-term effects of early and sustained intervention in hypercholesterolemia, to prevent or delay atherosclerosis and its complications. A potential therapeutic approach is to develop newer and improved lipid-lowering agents that are easily administered and can be used in combination with current medications. PCSK9 inhibitor may very much fit this definition but safety profile must be examined carefully. Here are some prospective from studying PCSK9-deficiency models to understand the biological effect of a PCSK9- inhibitor:

1. As many medications tend to increase weight it is of concern, as we intend to fight risk factors of atherosclerosis and CVD we do not want to accidentally induce one by our newer medication. The observation of abdominal adiposity that was associated with PCSK9-deficient mice was examined here and a new role of PCSK9 was identified in fat tissue. Therefore, any long-term monitoring of PCSK9-inhibitor use should include weight gain, obesity and an insulin resistance monitoring.

2. The site of obesity or fat accumulation is the main determinant of health related complication. Abdominal obesity or apple-like fat distribution is more related to metabolic syndrome than pear-like fat distribution (*Lebovitz et al 2003*). Abdominal obesity were fat accumulate in omental fat and pericardial fat is more harmful than perigonadal and perirenal fat accumulation. This is because the more the fat pad is proximal to systemic circulation the more likely it is to exhibit insulin resistance. However, at this point we could not exclude the late onset of glucose intolerance (*Mbikay et al 2010*) associated with perigonadal and perirenal fat accumulation in PCSK9-deficient mice. Therefore, more studies are needed to explore this risk.

3. Additional evidence of new PCSK9 biology in fat metabolism in adipose tissue since PCSK9 bind the EGF-like A domain of LDLR, thus may bind similarly VLDLR a close family members. Therefore PCSK9 regulate VLDLR in fat tissue, FFA trafficking and adiposity TG synthesis. Other LDLR family members may lead to more PCSK9 biology role in different tissues.

In the second manuscript we address the conflicting data to wither the PCSK9 bind VLDLR or not. Ex vivo and in vitro studies showed that PCSK9 binds and induces the degradation of the VLDLR protein by assessing the receptor levels in various cell lines overexpressing PCSK9 and direct binding of a synthetic EGF-A repeat to the LDLR (*Poirier et al 2008, Shan et al 2008*). However, leaders in this field, Hobbs and colleagues, had concluded in an earlier study that PCSK9 did not bind the VLDLR, due to amino acid differences in its EGF-A repeat domain (*Zhang et al 2007*). Thus, this issue remains controversial prior to this day. Even though the activity of PCSK9 on VLDLR could be anticipated, we think that the demonstration of its reality, and the description of the adipose phenotypes associated with the absence of a PCSK9 control on the VLDLR protein, constitute a key work to fully understand the pivotal role of PCSK9 in connecting different metabolic pathways. Therefore we do not exclude that PCSK9 regulates (directly or indirectly) other key receptors or targets that may contribute to the amplitude of the phenotypes. However, the phenotypes that characterize *Vldlr*$^{-/-}$ mice and the reported functions of the VLDLR in lipoprotein binding corroborate our data. We are indeed very interested in considering the consequence of the absence of PCSK9 on the FFA uptake machinery; this could be part of a future work.

It seems that FFA catabolism is higher in females specifically in the inguinal pad, raising the question of an activation of PPAR alpha pathway. PPAR alpha may indeed be involved in this 81% increase in FFA catabolism. It is well shown that FFA uptake, oxidation and VLDLR mRNA could be co-regulated. However, it is not obvious without further analyses to determine the contribution of PPAR (peroxisome proliferator-activated receptor) alpha versus that of PPAR gamma or other transcription factors that were shown to regulate these pathways.

However the evidence for PCSK9 targeting VLDLR in our study was the quantification of VLDLR expression by immunohistochemistry. We acknowledge that such experiments are somewhat hard to control, since the signal generated by the antibodies used could also be derived from cross-reacting antigens. We have thus repeated the immunohistochemical analyses of the VLDLR labeling in WT and *Ldlr*$^{-/-}$ backgrounds with the inclusion of negative controls from *Vldlr*$^{-/-}$ mice that we purchased from The Jackson Laboratory. The finding that the LDLR and VLDLR are controlled by the same mechanism (PCSK9) in the same direction therefore is novel. Thus circulating PCSK9 has a pivotal role in coordinating between peripheral fatty acid uptake and liver lipoprotein uptake, via regulating cell surface levels of VLDLR and LDLR resulting in slowing down lipoproteins catabolism, and maintain proper circulating levels of cholesterol and triglycerides. In agreement with its role, PCSK9 is regulated at the transcriptional level by sterols, insulin, glucagon, ethinylestradiol and fasting, but also at the protein level by hepatic furin.

In this study we underline the phenotype of PCSK9 deficiency in females where it is more severe than men, in terms of increase VLDLR protein in fat tissue. However this did not translate into massive fat accumulation. Therefore this work does not suggest that PCSK9 inhibition could lead to harmful increase of visceral fat, in particular in women as the reader might conclude. This is because in the complete absence of PCSK9, likely not achieved by any potential drug, the weight gains are quite low, even when the mice were fed a Western diet and it is localized in fat pads only. In addition, *Fabbrini et al* showed that fat accumulation is more deleterious when it occurs in ectopic sites other than fat pads where they might cause organ dysfunction. Although the experiments performed in the second paper were not designed to evaluate the hyperplasia of the adipocytes, therefore can not be excluded, we provide sound evidences from paraffin blocks containing entire perigonadal pad sections every 200

micrometers with a highly reproducible counts for the number of cells per field, indicating that the size of the adipocytes in *Pcsk9*$^{-/-}$ is quite homogenously hypertrophied throughout the tissue with no evidence of hyperplasia suggest that hyperplasia is likely not a major issue in the *Pcsk9*$^{-/-}$ adipose tissue. The perfect experiment to monitor any hyperplasic trend in *Pcsk9*$^{-/-}$ perigonadal pads therefore would be to dissociate the adipocytes by digestion and to count them.

8. Conclusions

7.1. Article 1

1. We provided mice models of moderate to extensive vascular calcification that replicate the disease observed in humans. These two animal models resembling absolute LDLR-deficiency and low LDLR-availability. The latter phenotype is mimicked by overexpression of PCSK9.
2. We validated a sensitive technique to quantify aortic calcification in murine models using micro-CT. This method proved to be precise, quantifiable and informative.
3. The results of the current study suggest that aortic calcification is associated with ageing and LDLR-deficiency, and is accelerated by a Western diet.
4. PCSK9 *Tg* confirmed the possible role of LDLR in calcium deposition.
5. The two models should facilitate studies on both the pathophysiology of vascular calcification and the efficacy of different treatment regimens.
6. The mechanism of aortic calcification in FH could not be elucidated from this work, but the mouse models have sub-endothelial, tunica intima type of calcification similar to human, suggesting an inflammatory, proliferative and remodeling mechanism.
7. Future therapy would be most likely combination: pyrophosphate inhibitors, chelating therapy, vitamin K, estrogen, selective estrogen receptor modulators and calcium channel blockers

The following figure depicts the pathways that we are planning to test in our animal model in our effort of understand the molecular bases of aortic calcification in FH (Figure 1).

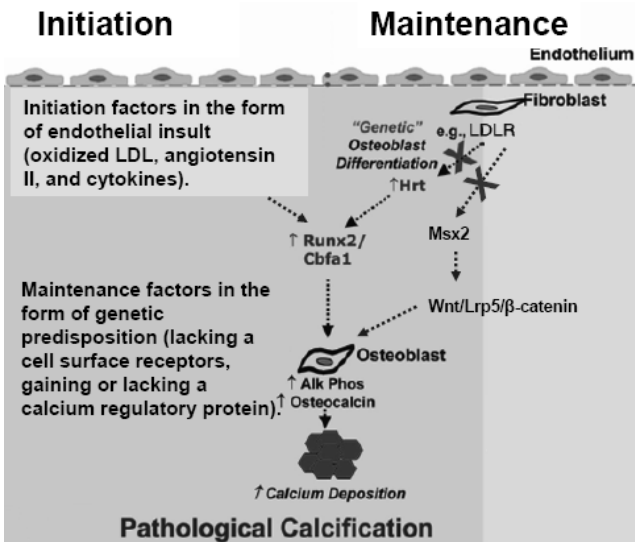


Figure 1.
Future experiments to identify the mechanism of aortic calcification. LDLR-deficiency may trigger Msx2 and the Wnt/Lrp5/β-catenin signaling pathway, as well as the Runx2/Cbfa1 pathway mediated activation of osteoblast transformation.

7.2. Article 2

1. We investigated the role of PCSK9 in adipose tissue metabolism and potential impact of PCSK9 lost-of-function mutations or the effect of a PCSK9 inhibitor on fat deposition.
2. *Pcsk9*$^{-/-}$ was associated with adipocyte hypertrophy, adipocytes increased *in vivo* fatty acid uptake and adipocyte *ex vivo* triglyceride synthesis, in an LDLR independent fashion.
3. By immunohistochemistry we showed that *Pcsk9*$^{-/-}$ males and females exhibit 4- and 40-fold higher cell surface levels of VLDLR in perigonadal depots, respectively.
4. Specific inactivation of PCSK9 in the liver of WT females led to 50-fold higher levels of perigonadal VLDLR.
5. Expression of PCSK9 in the liver of female *Pcsk9*$^{-/-}$ mice re-established both circulating PCSK9 and normal VLDLR levels.
6. PCSK9 regulates VLDLR protein levels in adipose tissue. This regulation is achieved by circulating PCSK9 that entirely originates from the liver.
7. PCSK9 is thus pivotal in fat metabolism: it maintains high circulating cholesterol levels via hepatic LDLR degradation, but also limits visceral adipogenesis likely *via* VLDLR regulation.
8. Since weight gain in PCSK9-deficent mice was minimal, it would be of great interest to examine the residual affect of visceral fat accumulation on the long-term insulin resistance development.

The following figure shows a suggested model of PCSK9 mechanism of action in liver and fat tissue in light of our recent work (Figure 2).

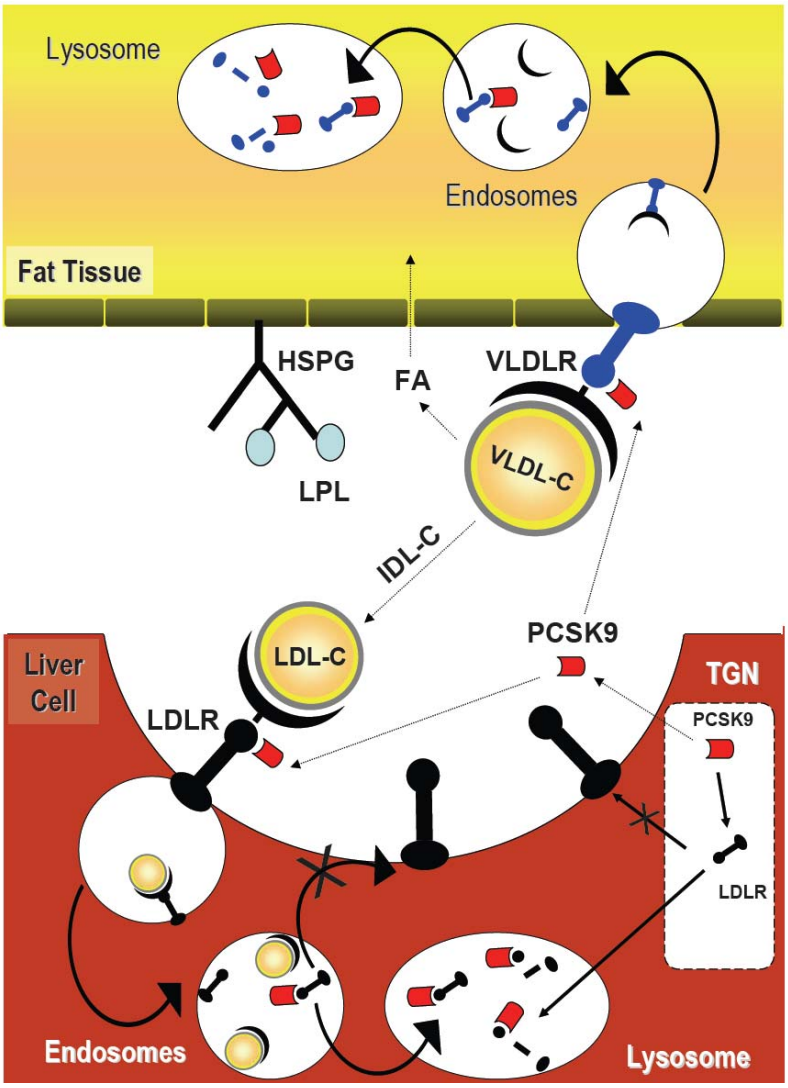


Figure 2.
A model of PCSK9 mechanism of action in different tissue. Both the LDLR and PCSK9 in the liver are regulated by SREBP-2. In the liver PCSK9 targets the LDLR degradation intracellular and extracellular while the circulating PCSK9 originating from the liver distally targets the VLDLR degradation at the site of fat tissue.

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PCSK9 inhibition is a very promising class of drugs that will become a perfect add-on to statin or an alternative to statin in statin intolerant patients due to its unique mode of action. Currently several registered clinical trials using deferent approaches to inhibit PCSK9 reaching phase I and II. With PCSK9 inhibitors assisted for ascending multiple dose, safety, tolerability, pharmacokinetics, and pharmacodynamics it is only a matter of time until they will become part of our practice. Therefore our attempt and many others are focused on the prediction of these future drugs on cardiovascular health.

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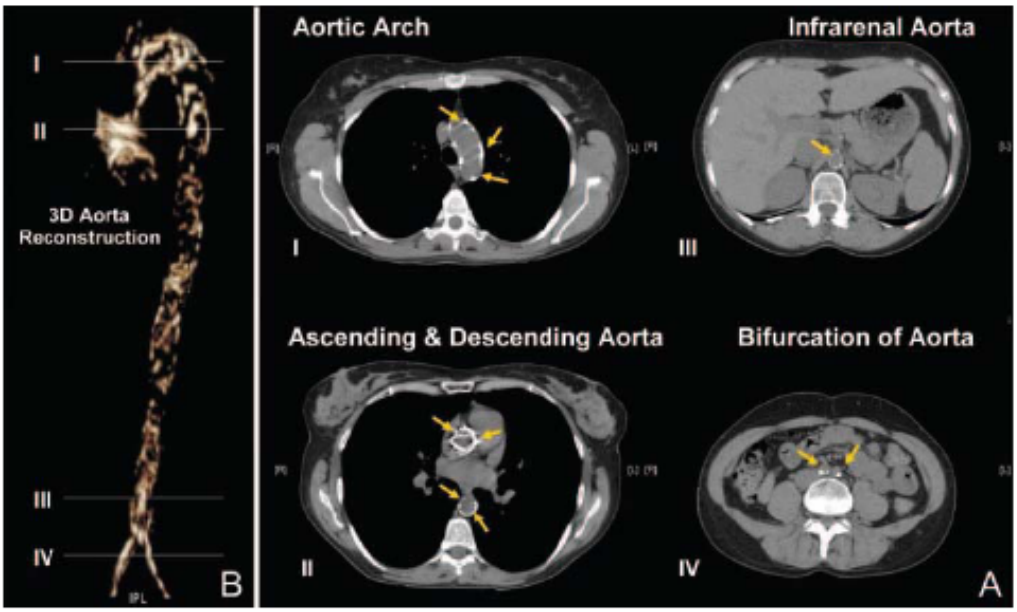
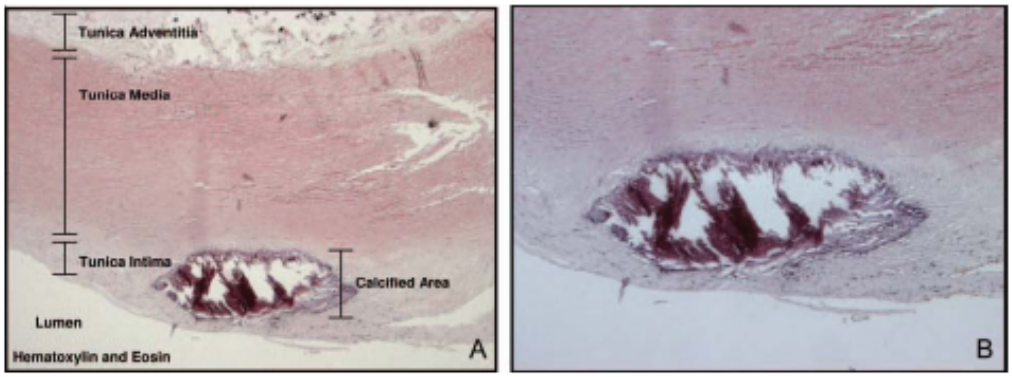
APPENDIX

APPENDIX I :



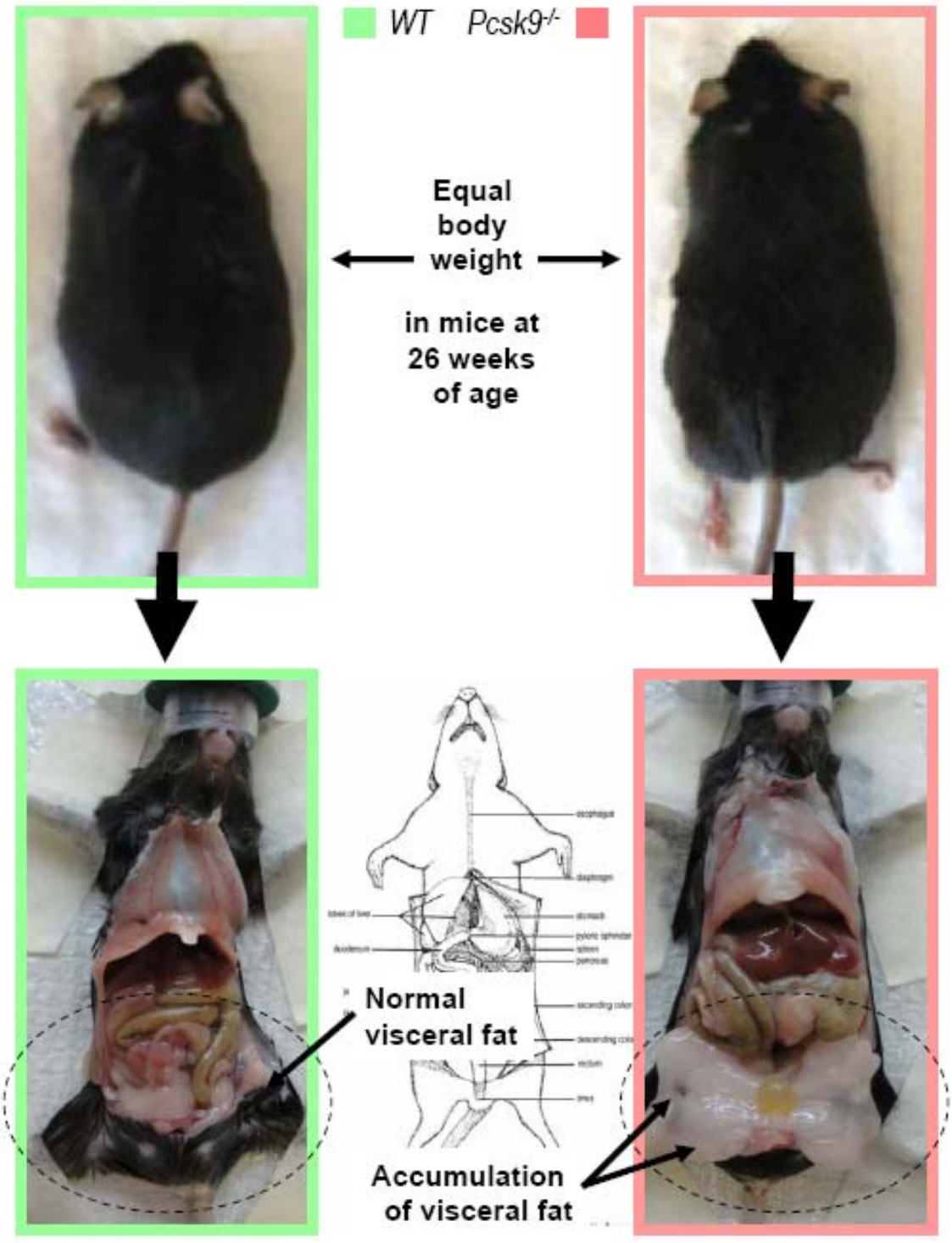
A, Representative computed tomographic scans of the aorta (indicated by arrows). B, Computerized reconstruction of calcifications



Calcification within the Tunica Intima of the ascending aorta from (patient R), taken at the time of arotic valve replacement. A, 60 magnification; B, 100 magnification. Hematoxilin and eosin stain.

Awan Z, et al. Vascular calcifications in homozygote familial hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2008 Apr;28(4):777-85. Epub 2008 Jan 31. PubMed PMID: 18239150.

APPENDIX II:


WT
Pcsk9-/-
Equal body weight
in mice at 26 weeks of age
Normal visceral fat
Accumulation of visceral fat

APPENDIX III:

IRCM
institut de recherches cliniques de Montréal

PROTOCOL NO : 2006-15
(exp 09-2009)

ANIMAL CARE COMMITTEE REPORT

The Animal Care Committee established by the IRCM has examined the application for research funds entitled:

Étude du rôle physiologique et physiopathologique de la porprotéine convertase PCSK9

Applicant: **Dr. Nabil G. Seidah**

Submitted to: **Canadian Institutes of Health Research**

and has found that the proposed research involving animals meets the standards of the Canadian Council on Animal Care, and that the facilities in which the animals will be housed and used comply with CCAC requirements.

Christian F. Deschepper
Chair, Animal Care Committee

Approval: 2006-12
Expiry: 2009-09

110, avenue des Pins Ouest, Montréal (Québec) Canada H2W 1R7
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IRCM
institut de recherches cliniques de Montréal

La formation et la recherche

Marie Kmita, Ph.D.
Directrice, Unité de génétique et développement
Research Unit Director Genetics and Development

Le 24 mars 2010

Dr Nabil G. Seidah
Biochimie neuroendocrinienne

Objet : **Protocole no 2010-01 intitulé « Étude du rôle physiologique et physiopathologique de la proprotéine convertase PCSK9. »**

Cher Collègue,

Le renouvellement du protocole 2006-15 (2010-01) mentionné en titre, que vous avez soumis au Comité de protection des animaux, a été approuvé.

Prière de noter que la période d'approbation pour l'utilisation des animaux a débuté le 16 février 2010 et se terminera le 30 septembre 2012.

Veuillez agréer, Docteur Seidah, l'expression de mes sentiments les meilleurs.

Marie Kmita, Ph.D.
Présidente
Comité de protection des animaux

Affilié à l'Université de Montréal

110, avenue des Pins Ouest, Montréal (Québec) Canada H2W 1R7
Téléphone : (514) 987-5749
Télécopieur : (514) 987-5740
Courriel : mariekmita@ircm.qc.ca

Projet no	2010-01
Date d'approbation	
Date de fin d'approbation	

Protocole d'utilisation des animaux d'expérimentation

N.B. : S'il s'agit d'un amendement, réutilisez le protocole original avec la fonction *Track Changes / Suivi des modifications* et cochez la case appropriée

[ ] **Amendement**
[ ] **Nouvelle Application**
[x] **Renouvellement de projet no :**

1. Information sur le chercheur

Directeur/Directrice d'unité de recherche à l'IRCM (responsable de la demande) : Nabil G. Seidah

Unité de recherche : Biochimie neuroendocrinienne

Autre personne de l'unité de recherche responsable de la conduite du projet, le cas échéant : Annik Prat

Titre du projet : Étude du rôle physiologique et physiopathologique de la proprotéine convertase PCSK9

2. Source de financement

Organisme(s) subventionnaire(s) : CIHR (MOP 82946)

Subvention demandée [ ]
Subvention obtenue [x] **Date de début :** Oct 2007 **Date de fin :** Sep. 2012

3. Urgence (personnes à rejoindre en cas d'urgence)

Nom : Rachid Essalmani Tél. (labo): 514-987-5607 Tél. personnel (urgence) :514-337-9331

Certification

Les informations dans cette demande sont exactes et complètes. Je m'engage à suivre les procédures et règles émises par le Comité d'éthique animale, ainsi que celles décrites dans le "Manuel sur le soin et l'utilisation des animaux d'expérimentation" préparé par le Conseil Canadien de Protection des Animaux (CCPA). Je devrai demander l'approbation du Comité d'éthique animale avant d'effectuer des changements aux procédures décrites dans cette demande.

Signature du directeur de l'unité de recherche		Date	

Approbation

Président Comité d'éthique animale			Date	
Période d'approbation pour l'utilisation des animaux	Début		Fin	

4. Personnel de recherche et qualifications

a) Personnel spécialisé de l'IRCM

Si votre protocole fait appel à la contribution de personnel spécialisé de l'IRCM, veuillez indiquer ci-dessous les procédures qui seront prises en charge.

Service	Procédure(s)
Santé animale	**Injection de cellules tumorales en sous cutané ou dans la veine caudale** **Gavage de souris**
Physiologie animale	
Micro-injection	

b) Personnel du laboratoire

Veuillez noter que tout le personnel ayant à travailler avec des animaux doit avoir suivi le cours de formation interne qui comprend une partie théorique et une partie pratique.

Indiquez le nom de toutes les personnes qui travailleront avec des animaux dans ce projet (chercheur principal compris) et leur classification d'emploi (chercheur, technicien, assistant de recherche ou étudiant). Le chercheur principal certifie que tout le personnel énuméré dans cette section a reçu la formation *interne* nécessaire afin d'exécuter les procédures décrites dans le protocole.

Nom	Poste	Expérience antérieure	Date de formation à l'IRCM
Claudia Toulouse	Technicienne SPF	Personnel de Santé animale (fait des sc et iv)	Formation à l'embauche
Rachid Essalmani	Chercheur associé	Très expérimenté (UQUAM, INRA en France et à l'IRCM)	2 Octobre 2009
Anna Roubtsova	Technicienne	Très expérimentée (médecin de formation)	29 Septembre 2009
Maxime Denis	Stagiaire post-doctoral	Expérimenté	2 Octobre 2009
Edwige Marcinkiewicz	Technicienne	Très expérimentée (depuis plus de 20 ans à l'IRCM)	2 Octobre 2009
Estelle Rousselet	Stagiaire post-doctoral	Très expérimentée (habilitée par U. McGill, diplômée en expérimentation (France, INSERM)	29 Septembre 2009
Xiaowei Sun	Étudiante en doctorat	Expérimentée	29 Septembre 2009
Zuhier Awan	Étudiant en Maîtrise	Expérimenté (médecin de formation)	29 Septembre 2009
Gaétan Mayer	Stagiaire post-doctoral	Expérimenté	29 Septembre 2009

5. Modification(s) à apporter

Les modifications suivantes ont été demandées lors de la révision de votre protocole. Veuillez apporter ces changements à votre demande originale avec la fonction *Track Changes/Suivi des modifications* et resoumettre deux copies de votre protocole (l'une avec les commentaires indiqués et l'autre en version finale signée). Vous devez vous conformer aux changements recommandés afin de recevoir l'approbation du Comité.

6. Sommaire

OBJECTIFS GÉNÉRAUX DU PROJET - Texte vulgarisé (modèle : sommaire vulgarisé tel que dans les demandes des IRSC)

PCSK9 appartient à la famille des proprotéines convertases et est liée l'hypercholestérolémie familiale. Son gène est le troisième à être associé à cette maladie, après ceux du récepteur des

«low density lipoprotein» (LDLR) et de l'apolipoprotéine B (apoB). Alors que certaines mutations dans le gène de PCSK9 conduisent à une hypercholestérolémie familiale, d'autres responsables de la perte de fonction de la protéine se traduisent au contraire par une hypocholestérolémie sévère. PCSK9 favorise l'élimination du LDLR par la voie endosomale/lysosomale, sans l'intervention de son activité enzymatique. L'absence de PCSK9 favorise donc une activité maximale du LDLR et se traduit par une chute de 80% du cholestérol associé aux LDL. De nombreux laboratoires se sont attachés au développement d'inhibiteurs de PCSK9, susceptibles de substituer les statines.
Nous voulons comprendre comment l'interaction entre PCSK9 et le LDLR conduit ce dernier vers la voie de dégradation. Connaître aussi le rôle de PCSK9 circulante, qui est essentiellement d'origine hépatique, dans ce mécanisme. Nous avons récemment montré que l'annexine A2 lie fortement PCSK9 et inhibe son activité sur le LDLR. Nous souhaitons aussi explorer les rôles de PCSK9 autres que ceux liés directement au contrôle du niveau de LDLR. Par exemple, l'absence de PCSK9 semble accompagnée de changements importants dans le métabolisme des triglycérides et d'une intolérance au glucose.

OBJECTIFS SPÉCIFIQUES DU PROJET (distinct des procédures) – Texte vulgarisé

Nous disposons d'outils développés ici, à l'IRCM : des souris portant des allèles *Pcsk9* inactivés (*Pcsk9*$^{-/-}$) ou conditionnels (*Pcsk9*$^{flox/flox}$), permettant d'inactiver le gène dans un tissu de notre choix, et des souris transgéniques (Tg(Apoe-PCSK9)) qui surexpriment PCSK9 dans le foie. Pour étudier les fonctions physiologiques de PCSK9 autres que celles liées au contrôle du LDLR, et son impact sur le développement de l'athérosclérose, nous avons engendré des souris ayant un fond WT (C57BL/6), *Ldlr*$^{-/-}$ ou *Apoe*$^{-/-}$ (voir section 8 pour soins particuliers).

Nos objectifs sont les suivants:

1 – Caractériser nos modèles murins PCSK9 sur fond WT, *Ldlr*$^{-/-}$ ou *Apoe*$^{-/-}$
Les teneurs en lipides du plasma, du foie ou autre tissu sont mesurées sur des groupes de 10 souris. Les saignées sont faites à l'âge de 3 mois (250 µL) après 4 à 16 heures de jeûne. La capacité de clearance des triglycérides et du glucose sera analysée si besoin.
La contribution de PCSK9 du foie et de l'intestin dans la régulation du cholestérol sera analysée à l'aide de souris *Pcsk9*$^{flox/flox}$ ± *Tg(Alb-cre)* ou ± *Tg (Vil-cre)* qui ont des hépatocytes ou entérocytes déficients en PCSK9, respectivement.

2 – Mécanisme de la dégradation du LDLR induite par PCSK9
Des cultures primaires d'hépatocytes WT, *Pcsk9*$^{-/-}$, TgPCSK9 seront effectuées afin de comprendre comment le LDLR disparaît de la surface en présence de PCSK9 et comment l'absence ou la surexpression de PCSK9 affecte l'expression génique ou protéique (µarrays et protéomique). Ponctuellement, d'autres souris modèles seront utilisées (fond *Ldlr*$^{-/-}$ …).

3 – Inhibition de PCSK9 *in vivo* par l'annexine A2
Nous surexprimerons l'annexine A2 dans le foie et vérifierons si l'inhibition anticipée de PCSK9 conduit à une augmentation du LDLR et donc à une diminution du LDL-cholestérol. Ceci validera le rôle *in vivo* de l'annexine A2 et établira une base solide pour développer un inhibiteur de PCSK9. Plusieurs types d'injections dans la veine caudale sont envisagés : (i) d'annexine A2 purifiée (ou alternativement de PCSK9 purifiée), (ii) d'ADN recombinant codant pour l'annexine A2 ou (iii) d'adénovirus défectifs codant pour l'annexine A2 (préparés par notre collaborateur, le Dr Weijun Jin).

4 – PCSK9 et la régénération du foie
Nous comparerons la capacité de souris *Pcsk9*$^{+/+}$ et *Pcsk9*$^{-/-}$ à régénérer leur foie après une hépatectomie partielle. Nous savons déjà que l'absence de PCSK9 entraîne des lésions irréversibles dans le foie en régénération. Les mêmes expériences conduites dans des souris déficientes en LDLR nous permettront d'analyser la contribution de l'hypocholestérolémie à ce phénomène. Toutes les souris *Ldlr*$^{-/-}$ ont en effet un excès de cholestérol circulant, que PCSK9 soit présente ou non. Ce travail a déjà été partiellement accompli et l'analyse de quelques souris reste encore à faire.

5 – PCSK9 et le développement de l'athérosclérose
La souris *Apoe*$^{-/-}$ est un modèle d'étude de l'athérosclérose reconnu. Nous observerons l'impact de l'absence ou de la surexpression de PCSK9 sur le développement des plaques. Les souris seront analysées après 6 mois et 12 mois de diète normale ou de type « Western ». Des traitements utilisant des inhibiteurs de PCSK9 (composé sélectionné, anticorps monoclonal ou antisenses), en combinaison ou non avec des statines qui inhibent la synthèse cellulaire du cholestérol, pourront aussi être testés.

6 – PCSK9 et la tumorigenèse
Le développement de métastases hépatiques chez des souris *Pcsk9*$^{+/+}$, *Pcsk9*$^{-/-}$ et TgPCSK9 sera analysé après injection de cellules tumorales (mélanomes B-16 et CT-26) par la veine porte, en utilisant un protocole similaire à celui que nous avons décrit dans J Biol Chem (2001) 276:30686 et Cancer Res (2003) 63:1458.

7 - PCSK9 et l'ischémie cérébrale transitoire
Dans le cerveau, PCSK9 est présente à des périodes cruciales pour la neurogenèse et la différentiation cellulaire lors du développement. Le but est d'induire une ischémie cérébrale dans des souris *Pcsk9*$^{+/+}$ et *Pcsk9*$^{-/-}$ pour étudier le rôle de PCSK9 dans la récupération neuronale.

8 – Obtention d'anticorps anti-PCSK9 de souris
Nous avons acquis un appareil à tatouer susceptible d'améliorer la réponse immunitaire en permettant l'injection d'un ADN plasmidique codant pour PCSK9 dans le derme.

7. Description des animaux

Le CCPA a émis le principe des «**trois R**» pour Remplacement, Raffinement et Réduction.

Que signifient les mots « Remplacement », « Réduction » et « Raffinement » ?
Tel qu'expliqué, le mot « alternatives » est utilisé pour signifier tout changement à des procédures existantes qui a pour effet le remplacement des animaux, la réduction du nombre d'animaux utilisés ou le raffinement des procédures dans le but de réduire le nombre d'animaux utilisés ou de les remplacer, ou encore de réduire la douleur, le stress ou la détresse des animaux.

Le Remplacement signifie, en règle générale, l'utilisation d'un système inanimé à titre d'alternative (ex. : un programme ou modèle informatique, un mannequin). Il peut également s'agir du remplacement des animaux doués de sensations (en général des vertébrés) par des animaux moins sensibles (en général des invertébrés tels que des vers, des bactéries ou autres). Il peut également s'agir de l'utilisation de cultures de cellules et de tissus. Les cellules ont une source, et cette source est souvent animale.

La Réduction signifie l'utilisation de moins d'animaux sans qu'il n'y ait perte d'information utile. Cela peut résulter d'une réduction du nombre de variables grâce à une bonne planification du protocole expérimental, d'une utilisation d'animaux génétiquement homogènes ou d'un contrôle rigoureux des conditions de l'expérience.

Le Raffinement signifie un changement dans au moins un des aspects de l'expérience avec pour effet la réduction du nombre d'animaux ou leur remplacement, ou encore la réduction de toute douleur, détresse ou stress que pourrait vivre les animaux. L'établissement de points limites précoces dans le cas d'études pouvant potentiellement provoquer de la douleur ou de la détresse chez un animal est un exemple de raffinement.

IRCM
Institut de recherches cliniques de Montréal

Training Certificate

IRCM – Animal Health Services

We certify that *ZUHIER AWAN* has successfully passed the exam for the theory part of the Animal User Training Program on the **14th** day of the month of **July 2009**.

List of Modules:

MODULE 1 : CONTROL AND REGULATIONS
MODULE 2 : PROCEDURES AND SECURITY
MODULE 3 : THE ANIMAL AND THE ENVIRONMENT
MODULE 4 : THE ANIMAL AND RESEARCH

Ovidiu I. Jumanca, DVM, Cert LAM
Veterinarian
Director, Animal Facilities and Animal Experimentation
IRCM – Institut de recherches cliniques de Montréal

This certificate is valid for a period of five years

Le centre universitaire de santé McGill (CUSM)
McGill University Health Center (MUHC)

Service de radioprotection
Radiation Protection Service

Ceci certifie que :
This is to certify that :

Zuhier Awan

A réussi avec succès la formation du CUSM en radioprotection à l'intention des travailleurs du secteur des radioisotopes.
Has successfully completed the MUHC radiation Safety Training for Radioisotope Workers

Date of training: 2/13/2009

RSO's signature:

Service de la radioprotection
Radiation Protection Service