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Prevention of Cardiometabolic Disease in Familial Hypercholesterolemia

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RÉSUMÉ

L'hypercholestérolémie familiale (FH) est un désordre lipidique associé aux maladies cardiovasculaires les plus fréquentes. La FH est causée par des mutations dans les gènes *LDLR*, *APOB* et *PCSK9*. Toutefois, chez 20% des patients souffrant de FH, aucune mutation dans ces gènes n'a été détectée et ceci suggère que d'autres gènes seraient à l'origine de la FH. Actuellement, le seul traitement de la FH est une thérapie aux statines. En général les statines sont bien tolérées, cependant, une monothérapie ne permet pas d'atteindre des niveaux thérapeutiques acceptables et dans bien des cas, une thérapie combinée devient nécessaire. De plus, l'intolérance aux statines est présente dans environ 12% des patients. Dans les trois dernières décennies, la survie des patients avec la FH a augmentée de façon notoire mais on observe aussi l'apparition d'une calcification vasculaire sévère chez certains d'entre eux. Il est donc primordial de développer des nouvelles approches thérapeutiques afin de prévenir ces complications tardives.

Dans cette thèse doctorat, nous présentons l'étude d'une famille avec un phénotype de FH sévère non causé par des mutations dans les gènes *LDLR*, *APOB* et *PCSK9*. Par des études biochimiques et par séquençage d'ADN utilisant les technologies de nouvelle génération (NextGenSeq), nous avons découvert une mutation dans le gène de l'*APOE* (Leu167del). Ceci nous permet de proposer le gène codant pour l'*APOE* comme le 4e locus responsable de la FH (FH4). Par la suite, nous avons effectué deux études de cohortes chez les patients atteints de FH. Premièrement, dans l'étude JUPITER, nous avons démontré que la rosuvastatin augmente les niveaux sanguins de la protéine PCSK9 et ceci limiterait l'efficacité du traitement aux statines. Nous avons aussi étudié l'influence du mutant naturel R46L (perte de fonction de la PCSK9) dans la réponse aux statines. Deuxièmement, nous avons examiné les effets de la perte de fonction de la PCSK9 sur le profil cardiométabolique au sein d'une population pédiatrique. Nous avons déterminé que le génotype de l'*APOE* est déterminant dans ce profil cardiométabolique. Enfin, nous avons étudié la calcification vasculaire chez les patients atteints de FH. Cette calcification vasculaire progresse de façon indépendante des niveaux de cholestérol sérique et n'est pas associée aux anomalies de l'homéostasie du calcium. En utilisant des modèles murins, nous avons démontré que les souris *Ldlr*$^{-/-}$ et Tg(*Pcsk9*) développent des calcifications vasculaires semblables à celles observées chez l'homme.

De plus, nous avons confirmé l'implication de la voie de signalisation LRP5/Wnt dans la pathophysiologie de la calcification artérielle. Avec une étude interventionnelle, nous avons trouvé que l'inhibition de l'interleukine 1β (IL-1β) diminue fortement l'apparition de calcifications vasculaire dans notre modèle murin.

En conclusion, nos études ont permis l'identification d'un nouveau gène impliqué dans la FH, ont démontré aussi que les statines augmentent les niveaux sériques de PCSK9 et que la perte de fonction de la PCSK9 altère le profil cardiométabolique. Enfin, nous avons établi que la calcification vasculaire représente une complication tardive chez les patients atteints de FH et que, dans notre modèle murin, la calcification vasculaire peut être retardée par l'inhibition d'IL-1β. Ces découvertes peuvent avoir d'importantes répercussions cliniques chez l'humain.

Mots-clés: FH, ADH, LDLR, PCSK9, APOE, IL-1β, Calcification, la prévention

ABSTRACT

Familial Hypercholesterolemia (FH) is the most common lipoprotein disorder associated with premature cardiovascular disease. Mutations in the *LDLR*, *APOB* and *PCSK9* genes cause the FH phenotype, but in ~20% of FH patients, no mutations in these genes are identified, suggesting that mutations in other genes cause FH. Treatment with statins has been the cornerstone of therapy. While statins are generally well tolerated, statin intolerance is found in approximately ~12% of patients. Furthermore, statin use may not allow reaching LDL-C goals and combination therapy is often required. Nevertheless, survival of FH patients over the past 3 decades has improved significantly. As FH patients live longer, severe vascular calcifications have been described as a late complication in these patients. Given the increased survival rate and late complications, novel approaches and therapies are needed.

In the present thesis we examined a kindred with a severe FH phenotype, where sequencing of candidate genes failed to identify a causal mutation. Through biochemical analysis and next-generation exome sequencing we report a mutation (Leu167del) within the *APOE* gene that identifies the 4th locus causing FH (FH4). Next, we performed two cohort-based studies. Firstly, in the JUPITER trial we report that 20mg rosuvastatin treatment increases PCSK9 levels by ~30%, thereby possibly limiting the efficacy of statin therapy. Then we show the effect of a loss-of-function (LOF) mutation of *PCSK9*, p.R46L, on the response to rosuvastatin. Secondly, we report that two *PCSK9* gene variants, p.R46L and insLEU, were more frequent in French Canadian individuals. We also report that the *APOE* genotype determine the metabolic risk profile in these mutations. Finally, we studied vascular calcifications in FH individuals. These calcifications appear to progress independently of cholesterol levels and are not associated with disturbances in calcium homeostasis. Using mouse models, we show that *Ldlr*$^{-/-}$ and Tg(*Pcsk9*) mice develop aortic calcifications similar to that observed in humans. Furthermore, the involvement of the LRP5/Wnt pathway in the pathogenesis of calcification is illustrated. In a proof-of-concept experiment, inhibiting the upstream pro-inflammatory cytokine IL-1β attenuates calcification in mice.

In conclusion, we have contributed to the identification of a novel locus responsible for FH, reported the increase in PCSK9 levels with a statins treatment and the associated altered cardiometabolic profile in *PCSK9* LOF. Finally, we demonstrated that vascular calcifications represent a severe complication of FH that can be prevented by inhibiting IL-1β in a mouse model. The latter novel approach may have an important translational application in human.

Keywords: FH, ADH, LDLR, PCSK9, APOE, IL-1β, Calcification, Prevention

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LIST OF ABBREVIATIONS

ADH	*Autosomal dominant hypercholesterolemia*
AnxA2	*Annexin A2*
AoCS	*Aortic calcium score*
Apo	*Apolipoprotein*
APOB	*Apolipoprotein B gene*
APOE	*Apolipoprotein E gene*
apoE	*Apolipoprotein E*
apoER2	*Apolipoprotein E receptor 2*
ARH	*Autosomal recessive hypercholesterolemia*
ASO	*Antisense oligonucleotide*
CVD	*Cardiovascular disease*
CE	*Cholesteryl ester*
CETP	*Cholesteryl ester transfer protein*
CM	*Chylomicron*
CRP	*C-reactive protein*
DC	*Dendritic cells*
DMARD	*Disease-modifying antirheumatic drug*
EC	*Endothelial cell*
EGF	*Epidermal growth factor-like repeat*
ELISA	*Enzyme-linked immunosorbant assay*
ER	*Endoplasmic reticulum*
FFA	*Free fatty acid*
FH	*Familial hypercholesterolemia*
FPLC	*Fast protein liquid chromatography*
GOF	*Gain-of-function*
HCV	*Hepatitis C Virus*
HDL	*High-density lipoprotein*
HDL-C	*High density lipoprotein cholesterol*
HepG2	*Hepatocellular (Liver)carcinoma cells*
HF	*Heart failure*
HMG-CoA	*Hydroxymethylglutaryl coenzyme A reductase*
HSPG	*Heparin sulfate proteoglycans*
IDL	*Intermediate-density lipoprotein*
IL	*Interleukin*
IL-1	*Interleukin-1*
IL-1β	*Interleukin-1 beta*
IL-1Ra	*Interleukin-1 receptor antagonist*
IL-6	*Interleukin-6*
IHC	*Immunohistochemistry*
KO	*Knockout*
LDL	*Low-density lipoprotein*
LDLR	*Low-density lipoprotein receptor*
Ldlr (KO)	*Ldlr gene knockout in mice*
Ldlr $^{-/-}$	*Ldlr gene knockout in mice*
LDLRAP1	*LDLR adaptor protein-1*

LNA	*Locked nucleic acid*
LNP	*Lipid nanoparticle*
LOF	*Loss-of-function*
Lp(a)	*Lipoprotein little a*
LPL	*Lipoprotein lipase*
LPS	*Lipopolysaccharide*
LRP	*LDL-receptor-related protein*
mAb	*Monoclonal antibody*
MI	*Myocardial infarction*
micro-CT	*Micro computed tomography*
mRNA	*Messenger RNA*
NARC-1	*Neural apoptosis-regulated convertase 1*
NLRP3	*NOD-like receptor family pyrin domain containing 3*
Ox-LDL	*Oxidized-low-density lipoprotein*
PACE	*Paired basic amino acid cleaving enzyme*
PC	*Proprotein convertase*
PCR	*Polymerase chain reaction*
PCSK	*Proprotein convertase subtilisin/kexin*
PCSK9	*Proprotein convertase subtilisin/kexin type 9*
***Pcsk9* (KO)**	*Pcsk9 gene knockout in mice*
***Pcsk9* (Tg)**	*Pcsk9 gene transgenic in mice*
Pcsk9 $^{-/-}$	*Pcsk9 gene knockout in mice*
PPAR	*Peroxisome proliferator activated receptor*
qPCR	*Quantitative polymerase chain reaction*
RA	*Rheumatoid arthritis*
RCT	*Reverse cholesterol transport*
RF	*Rheumatoid factor*
siRNA	*Small interfering RNA*
SKI-1	*Subtilisin kexin isozyme-1*
SNP	*Single nucleotide polymorphism*
SP	*Signal peptide*
SR-BI	*Scavenger receptor class B type I*
SREBP	*Sterol regulatory element binding protein*
TC	*Total cholesterol*
Tg	*Transgenic*
TG	*Triglycerides*
TGN	*Trans-Golgi network*
TNF	*Tumor necrosis factor*
VCAM-1	*Vascular cell adhesion molecule 1*
VLDL	*Very-low-density lipoprotein*
VLDLR	*VLDL receptor*
WD	*Western diet*
WHO	*World Health Organization*
WT	*Wild type*

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

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CHAPTER 1

"LITERATURE REVIEW"

INTRODUCTION

CHAPTER 1: LITERATURE REVIEW

1. Prevention of Cardiovascular Disease (Introduction)

The American cardiovascular disease (CVD) epidemic began after the Second World War, often affecting businessmen in their fifties. At that time, CVD was attributed to work-related stress in these otherwise healthy men (Keys *et al.* 1963). Ancel Keys, a biochemist from the University of Minnesota also known as "The Father of the Low Fat Diet", was among the first to suggest that dietary fats might be the cause of this epidemic. In 1952, Keys and his colleagues conducted the pivotal Seven Countries Study (Circulation *et al.* 1970, Keys *et al.* 1980), which enrolled 12,000 participants and was the largest study at that time. From this large study, Keys and his colleagues reported a correlation between fat consumption and rates of heart disease. Thus, as a preventive measure to decrease CVD, the authors advocated the reduction of fat consumption to <39% of total caloric intake. This movement launched the notion of CVD prevention, while later the Framingham Heart Study (Kannel *et al.* 1961) associated cholesterol levels to CVD. From then on, the American Heart Association (AHA) advocated lower dietary fat intake for men with high total cholesterol levels.

Donald S. Fredrickson was later responsible for developing a classification system for dyslipidemia (abnormal amount of lipids in the blood) based on electrophoretic lipoprotein patterns and clinical features (Fredrickson *et al.* 1965). This system was the first to differentiate type III Familial Dysbetalipoproteinemia from type IIa Familial Hypercholesterolemia (FH) (Levy *et al.* 1967). Fredrickson's classification did not address the mode of inheritance, secondary sources of dyslipidemia or plasma high-density lipoprotein cholesterol (HDL-C) levels. Nevertheless, it was adopted by the World Health Organization (WHO) as an international standard and thus brought global attention to lipoprotein and lipid disorders (Fredrickson *et al.* 1971). Fredrickson was also the leading investigator of the group that conducted the landmark Coronary Primary Prevention Trial (CPPT) (The Lipid Research Clinics 1984). When this study began in 1973, epidemiological studies had not yet associated increased low-density lipoprotein cholesterol (LDL-C) levels and CVD. Indeed, CPPT was the first study to conclusively establish that lowering LDL-C levels pharmacologically results in CVD reduction (Gotto *et al.* 2002).

According to the World Heart Federation 17.1 million lives are now claimed annually by the global burden of heart disease and stroke, 82% of which are in the developing world (www.world-heart-federation.org). This alarming statistic is sad, as simple preventive measures can effectively reduce this pandemic. These include low-cost non-pharmacologic measures such as: cessation of smoking, healthy eating, regular physical activity and weight loss. However, such strategies are ineffective in cases of premature CVD (defined as the occurrence of CVD before age 55-60 in men and 65 in women). Prevention of premature CVD requires initiation of pharmacological therapy early on, since the underlying cause is typically a combination of genetic predisposition and unhealthy lifestyle. The prevalence of dyslipidemia is higher in individuals with premature CVD compared to age-matched controls without CVD (Genest *et al.* 1992, Roncaglioni *et al.* 1992) (**Figure. 1**).

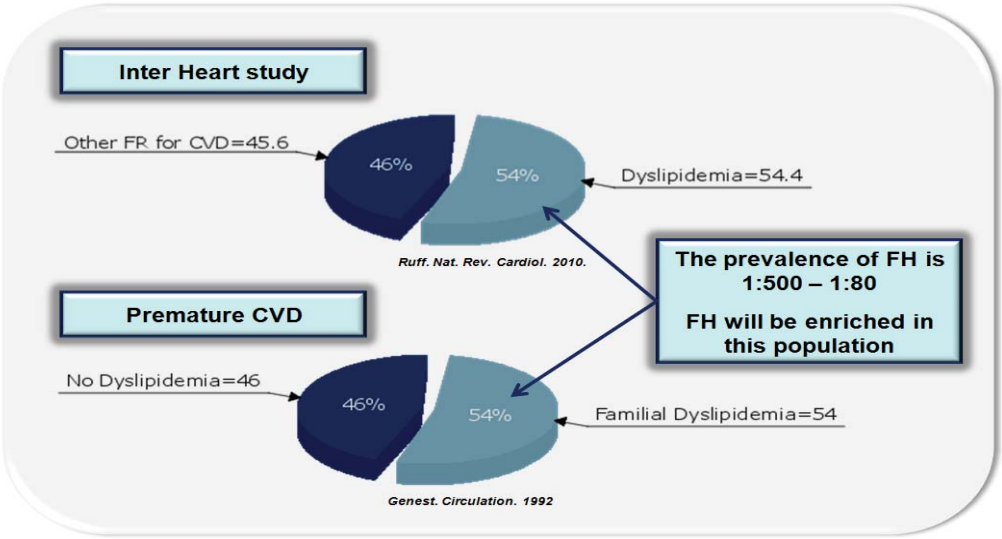

Figure 1: Dyslipidemia is a major risk in CVD. As shown in the chart, dyslipidemia accounts for 54% of the attributable risk for developing CVD in the INTER-HEART study (Yusuf *et al.* 2004 and Ruff *et al.* 2010). In addition, subjects with premature CVD have a 54% chance of having CVD if they had familial dyslipidemia (Genest *et al.* 1992). Given that the prevalence of FH is 1:500 to 1:80 in some subpopulations, it is reasonable to assume that a significant proportion of dyslipidemias will be FH and therefore represent an unmet opportunity in CVD prevention (adopted from both Ruff and Braunwald *et al.* 2010 and Genest *et al.* 1992).

The INTER-HEART study (a global heart study designed to assess the significance of CVD risk factors in different populations) reported that dyslipidemia (high apoB:apoA1 ratio) had the highest attributable risk (54%) for the development of myocardial infarction (MI) (Yusuf *et al.* 2004) (**Table 1**).

Table 1: Risk of CVD associated with risk factors in the general populations

Risk factor	**Odds Ratio (Yusuf *et al* 2004)**	**Attributable risk % (Ruff and Braunwald 2010)**
Diabetes mellitus *	8.08	12.3
No exercise	0.72	25.5
No fruit and vegetable intake	0.70	12.9
No alcohol intake	0.79	13.9
Hypertension	2.48	23.4
Psychosocial factors	2.51	28.8
Abdominal obesity *	2.24	33.7
Current smoking *	2.27	36.4
Dyslipidemia* (high apoB:apoA1 ratio)	**3.87**	**54.1**

* Risk factors for both atherosclerosis and arterial calcification, Apo: apolipoprotein (adopted from Yusuf et al. 2004 and Ruff and Braunwald 2010).

Familial Hypercholesterolemia (FH) also referred to as Autosomal Dominant Hypercholesterolemia (ADH), is a prevalent type of dyslipidemia in the general population. FH and ADH are used interchangeably, ADH being the newer and less used term after the discovery of non-classical genes associated with the same phenotype.

The prevalence of FH in the population is most often quoted as affecting 1:500 individuals worldwide (Goldstein *et al*. 2001). Thus, FH puts an individual at risk for premature CVD. Since early detection and treatment can improve outcomes (Nordestgaard *et al.* 2013) it is imperative that FH be indentified early in life, yet presently it is under-diagnosed. Therefore the majority of national guidelines have recognized that FH and other high risk CVD conditions such as advanced diabetes, chronic kidney disease and chronic inflammation require early intervention (Anderson *et al.* 2013, Stone *et al.* 2013). In these guidelines, patients are stratified according to gender and family history as either high- or moderately high-risk. Regardless of the level of CVD, physician must actively treat patients with such risk factors; the initial preventive measure is the prescription of a cholesterol lowering medication.

In some cases, cholesterol lowering medications are either contraindicated (liver toxicity), associated with adverse effects (drug intolerance, myopathy, rhabdomyolysis), or do not allow the patient to reach target levels, as determined by the current guidelines (Anderson *et al.* 2013, Stone *et al.* 2013). Thus, new therapeutic approaches are required to decrease CVD and associated atherosclerosis (hardening of the arteries). Additionally, the development of a national FH registry would facilitate a targeted cascade screening of families. In targeted cascade family screening, undiagnosed family members of the index FH patient (proband) are screened for FH. Cascade family screening also provides an opportunity to implement the best medical practices, and to better understand the underlying basic biology of the disease. For example, new mutations may be identified through index cases and confirmed by family screening leading to the identification of new therapeutic targets. This was how pro-protein-convertase-subtilisin-kexin-9 (PCSK9) was linked to FH, thereby offering a new treatment targeting PCSK9. Furthermore, the measurement of serum levels of PCSK9 can be used as a tool in cascade family screening to identify likely FH individuals, and as a criterion for placing FH patients on PCSK9 inhibitor (blocking monoclonal antibody) regiments. The fascinating role of PCSK9 in lipid metabolism is assisting in the evolution of a new category of pharmacological approaches to lower cholesterol (discussed further in Part 3 of this Chapter).

The first chapter of this thesis is divided into 5 parts:

- ***Part 1:*** Prevention of Cardiovascular Disease (Introduction), see above.
- ***Part 2:*** Overview of Familial hypercholesterolemia. Including a description of the use of registries to discover new mutations (*APOE* gene, 4th loci in FH) that would assist in cascade family screening to prevent CVD (related to **Chapter 2**).
- ***Part 3:*** Familial hypercholesterolemia and PCSK9. Including a description of PCSK9, the 3rd locus in FH and approaches for screening different populations using PCSK9 levels to identify natural loss-of-function (LOF) mutations (related to **Chapter 3** and **Chapter 4**).
- ***Part 4:*** Familial Hypercholesterolemia and calcified atherosclerosis. This section includes the biology and molecular mechanism of aortic calcification in FH and the associated subendothelial inflammation (related to **Chapter 5** and **Chapter 6**).
- ***Part 5:*** Hypothesis and objectives.

Finally, I will discuss and summarize the overall progress (**Chapter 7**).

2. Familial Hypercholesterolemia (FH) Overview

2.1. Familial hypercholesterolemia definition, signs and symptoms

The catalogue assignment number for FH at the Mendelian Inheritance in Man (MIM) system is [MIM:143890] and may also be referred to as essential hyperlipoproteinemia, type IIa hyperlipoproteinemia and hypercholesterolemic xanthomatosis. FH is a genetic disorder where an affected individual has abnormally elevated LDL-C levels; above the 95th percentile. Before the genes that are responsible for the disease were identified, the mode of FH inheritance was first described by Khachadurian in 1964. FH shows a Mendelian pattern of inheritance; it can be inherited in an autosomal dominant fashion or an autosomal recessive fashion. Autosomal dominant inheritance is the most prevalent form of FH. In FH, the abnormal elevation of LDL-C is primarily attributable to mutations in the low-density lipoprotein receptor gene (*LDLR*) (Goldstein *et al*. 1974, Brown *et al*. 1974). High levels of LDL-C increase the risk of atherosclerosis that results in narrowing and hardening of the arteries at an early age seen in many patients with FH. Given the genetic background, FH disease begins at birth leading to lifelong LDL-C elevation and can cause premature CVD, myocardial infarction (MI) or even death.

Many attempts have been made to develop a unified definition and diagnostic criteria of FH. There are at least three sets of genetically and statistically trusted criteria that are most commonly used: the Dutch (Civeira *et al.* 2004), the UK (Simon Broome *et al.* 1991) and the MedPed criteria (Williams *et al.* 1993). Below is an illustration of the Dutch Lipid Network criteria (Nordestgaard *et al.* 2013) (**Table 2**).

Table 2: Dutch Lipid Clinic Network criteria for diagnosis of FH in adults

Group 1: Family history	Group 2: Clinical history	Group 3: Physical exam	Group 4: Biochemical	Group 5: Molecular test
1: 1st degree relative with known premature coronary heart disease (CHD) **OR**	**2:** Subject has premature CHD	**6:** Tendon xanthoma	**8:** >8.5 mmol/L (325 mg/dL)	**8:** mutation in *LDLR*, *APOB*, or *PCSK9* genes
1: 1st degree relative with known LDL-C >95th %	**1:** premature cerebral or PVD*	**4:** Corneal arcus in a person >45 Y	**5:** >6.5–8.4 (251–325)	
2: 1st degree with xanthoma +/- corneal arcus **OR**	Add the pints in each group to obtain a score: - Definite FH if the subject scores 8. - Probable FH if the subject scores 6 to 8. - Possible FH if the subject scores 3 to 5. - Unlikely FH if the subject scores 0 to 2.		**3:** >5.0–6.4 (191–250)	
2: Child(ren) ,18 years with LDL-C >95th %			**1:** >4.0–4.9 (155–190)	

* PVD: peripheral vascular disease (adopted from Nordestgaard *et al.* 2013).

FH diagnosis requires plasma LDL-C levels to be elevated by 2-fold (likely heterozygous FH) or 3- to 5-fold (likely homozygous FH) above the reference levels of a control population adjusted for age and sex. In FH patients, serum triglyceride (TG) concentrations remain within a normal range (Rader *et al.* 2003). Limited workup should be performed to rule out secondary hypercholesterolemia that can mimic the FH phenotype. Essential tests to rule out diabetes, hypothyroidism, hepatic disease and renal disease are usually adequate (Awan *et al.* 2009). Other tests that are done to confirm an FH diagnosis include: (i) genetic screening for mutations known to be associated with FH and (ii) use of fibroblast cultures to determine LDLR levels and/or activity.

Signs of FH may include: fatty skin deposits called xanthomas over the hands, elbows, knees, ankles; xanthelasmas or fatty deposits near the eyelid (**Figure. 2**). In some cases, the systolic murmur (loud sound heard during a heartbeat) of aortic stenosis and carotid artery stenosis reflects valve calcification and severe atherosclerosis, respectively. Angina (pain in the chest) or other signs of coronary artery disease may be present at a young age.

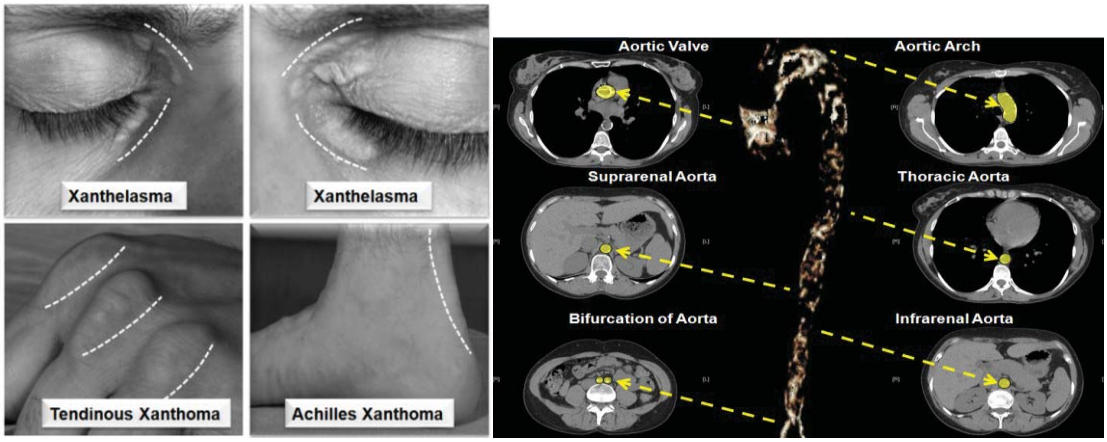

Figure 2: Signs and symptoms of FH. The panel on the left shows the skin manifestations of FH including xanthelasmas around eyes, tendinous xanthomas in hands and Achilles tendon xanthoma in a man in his 40's. The lesions are demarcated with a dash line (photos are related to the patient in chapter 2). The panel on the right shows a computed tomographic (CT) scan of a woman in her 40's with homozygous FH. In the center is the CT scan reconstruction (3D) of her aorta showing calcified lesions and on the sides are horizontal CT scan cuts corresponding to the same lesions indicated by dash arrows (adopted from Awan *et al.* 2008 and 2013).

2.2. Molecular genetics of familial hypercholesterolemia

FH is a genetic disease with a Mendelian pattern of inheritance, where affected individuals have elevated total cholesterol predominantly due to elevation in LDL-C levels. The genetic basis of FH is primarily a result of mutations in the *LDLR* gene (Goldstein *et al.* 1974, Brown *et al.* 1974, Asselbergs *et al.* 2012); however, new genes are still being shown to be associated in the broad-spectrum of FH (i.e., Autosomal Dominant Hypercholesterolemia, ADH). The *LDLR* is located on chromosome 19 (FH1, 19p13.1-13.3) (Lindgren *et al.* 1985) and many mutations have been identified in this gene (Heath *et al.* 2000, Villeger *et al.* 2002). More than 1000 mutations in the *LDLR* gene (Guardamagna *et al.* 2009) with the associated endocytosis pathway have been identified to date. They are classified into the following groups (Goldstein *et al.* 1987, Hobbs *et al.* 1990) (**Table 3**):

Table 3: Broad classes of mutations in the LDL receptor (*LDLR*)
1) A null mutation: LDLR is either not synthesized in Endoplasmic Reticulum (ER) or fails to mature in Golgi Body. For example, the 15kb or larger deletion in the promoter and first exon yield no protein. This null mutation is a common mutation in French-Canadians (Awan *et al.* 2008).
2) Defective binding: LDLR is made and reaches the cell surface but cannot bind its apoB ligand, resulting in LDL not being internalized nor cleared from the blood. This type of defect can also be mimicked by mutations in the *APOB* gene encoding apoB. Interestingly, apoE also binds LDLR and thus the LDLR is often called apoB/E receptor (discussed further in Chapter 2)
3) Defective recycling: LDLR reaches the cell surface (newly synthesised each time) and cannot recycle back once internalized. This type of defect can also be mimicked by gain-of-function mutations in the PCSK9 gene which targets intra-cellular LDLR for degradation and prevents its recycling (Seidah *et al.* 2003).
4) Defective internalization: LDLR can bind the ligand but does not cluster in the coated pits, thereby minimizing LDL internalization. Mutations in autosomal recessive hypercholesterolemia gene (LDLRAP1 or ARH) can cause defective internalization (Anderson *et al.* 1977, Zuliani *et al.* 1999, Garcia et al. 2001, Arca *et al.* 2002).

Sources are indicated in each class

Similarly, mutations in 2 other genes are responsible for causing FH. One of these is the apolipoprotein B-100 gene (*APOB*), located on chromosome 2 (FH2, 2p23-24) (Knott *et al.* 1985, Law *et al.* 1985). *APOB* encodes for the protein ligand of the LDL particles (Innerarity *et al.* 1990). In contrast to *LDLR*, only a small number of loss-of-function (LOF) mutations have been identified in *APOB*. The third gene found to be associated with FH, is the *PCSK9* gene identified on chromosome 1 (FH3, 1p32) (Seidah *et al.* 2003, Abifadel *et al.* 2003). In one FH cohort, it was estimated that 64% of mutations were associated with the *LDLR*, 16% with *APOB* and 2% with *PCSK9* (Abifadel *et al.* 2003, Seidah 2009). There are still 17% unknown mutations that cannot be accounted for. Thus, identifying new mutations and novel genes associated with FH will bring important insights to the field.

The carrier frequency of mutations leading to FH in the general population is 1:500 (Brown *et al.* 1983); however, this frequency increases in certain French-Canadian subpopulations who may suffer from the "founder effect" of the first French settlers. This founder effect may be explained by 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 *et al.* 2001). This phenomenon may be observed not only in the French-Canadian population but in similar culturally isolated environments in South-Africa, as well as in some regions of Italy, Scandinavia and Lebanon (Khachadurian 1964).

Homozygous FH occurs when a child inherits the same mutation from both parents. When this rare form of FH occurs (1 in a million), the increase in cholesterol levels is much greater than in the heterozygous form (Awan *et al.* 2008). The homozygous form of undiagnosed and untreated FH can lead to heart attack and death within the first decade of life.

From a clinical perspective, human mutations in FH are of two categories depending on residual LDLR activity (Rader *et al.* 2003), either less than 2% activity (receptor-negative, poor response to statin) or 2-25% activity (receptor-defective, good response to statin). The latter category is the one that would respond to PCSK9 inhibition (Stein 2013) since PCSK9 function is mediated through LDLR. Mutations in PCSK9 causing FH are usually on the background of an active LDLR and will be discussed further in Part 3

Familial hypercholesterolemia molecular diagnosis - Individuals with clinical signs and symptoms of FH are referred to specialized lipid centres where they can be offered a sensitive lipoprotein testing that includes lipoprotein fractionation by Fast Protein Liquid Chromatography (FPLC) and molecular testing. A pedigree is constructed around the proband (with a premature CVD; for example) and detailed lipid profiles and histories of CVD from close family members are carried out to identify affected subjects. A candidate gene approach usually is used to sequence exons of the *LDLR*, *APOB* and *PCSK9* genes, as well as genes involved in Autosomal Recessive Hypercholesterolemia (*ARH*; also called *LDLRAP1*), and recently the *LIPA* gene that regulates cholesterol ester accumulation (Stitziel *et al.* 2013). In many clinical cases, FH patients fail to show a mutation in these genes.

In addition, candidate genes are reported to be negative in many clinically diagnosed FH cohorts who are screened for mutations. Thus, a search for common copy number variants (CNV) at the 5' end of *LDLR* is usually performed if the individual is of French-Canadian heritage. One study showed that exon-by-exon sequencing analysis diagnosed only two thirds (67%) of the FH patients (Wang *et al.* 2005). However, using the Multiplex Ligation-Dependent Probe Amplification (MLPA) technique to detect CNV, additional 9% mutations were detected in the negative patients (Wang *et al.* 2005). This suggests that heterozygous *LDLR* CNV's are associated with more severe phenotypes and they are usually missed by exon-by-exon sequencing (Hegele *et al.* 2006). This suggests that mutations in *LDLR* represent ~76% of those associated with FH. With the estimated 14% mutations in *APOB* contributing to FH (Seidah 2009), this leaves ~10% contributions to FH by mutations in other genes. Thus, more mapping studies to look for novel genes (discussed further in Chapter 2) are needed to complete the list of genes in the FH spectrum.

Whole exome sequencing with next-generation sequencing technology is a powerful approach (Marduel *et al.* 2013, Reddy *et al.* 2012). This involves a large-scale automated genome analyser that screens the entire coding genome region for mutations. However, this method identifies a very large number of variants (typically $>10^5$) that are non-deleterious. These include common variants (i.e., genetic variants with a minor allele frequency of > 5%), intronic variants and variants that do not fit a Mendelian pattern of inheritance. Therefore, genetic filters are used to remove variants that are unlikely to be causal. This includes identified SNPs in non-coding regions and genetic variants that are not expected to impart functional defects through *in silico* analysis using genetic software; PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and SIFT (http://www.jcvi.org). Once a novel candidate is identified, we evaluate the mutation *in vivo* or *in silico* to understand its impact. The latter involves computer prediction, using modeling software and algorithms to identify and illustrate the impact of a mutation on a protein function, thus the name *in silico* experiments. For example; the Protein Variation Effect Analyzer (PROVEAN) server is an *in silico* tool that is reserved for variants with more than one SNP change. This tool is able to reliably predict if a given genetic variant is deleterious to the protein structure and assign a probability score (http://provean.jcvi.org/index.php) (PROVEAN 2013).

Afterwards, using the template-based protein structure modeling I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER), or similar software available online, we can predict *in silico* the effect of the variant on the 3-dimensional structure of the protein. This important step allows the visualization of the protein structure and the mutations site with programs like PyMol (http://www.pymol.org). PyMol is a computer program that allows the 3-dimensional viewing of both a mutated protein generated as mentioned above and a crystallized wild-type protein side by side and thus permits comparative studies. These tools globally assist in predicting the effect of a given genetic variant on the protein structure. However, the ultimate functional test is the LDLR binding assay. Once a new mutation is identified from genetic screening and is suspected to be causal, this candidate gene must be sequenced in affected and unaffected family members (Sanger *et al.* 1977) to confirm association of the mutation with disease. Sanger sequencing is the gold standard for detecting genetic variants; even variants identified by next generation sequencing technologies must be confirmed by Sanger sequencing. The new mutation is finally sequenced independently in a series of individuals with a clinical diagnosis of FH but without (negative) a known mutation in *LDLR*, *APOB* or *PCSK9* to determine the prevalence of this mutation. If analyses from sequencing results show that this new mutation segregates very well with FH, the gene, in which this new mutation is located, can be classified as a new causal gene for FH, in addition to the three known FH-causing genes.

Familial hypercholesterolemia and GWAS studies - The Global Lipid Genetic Consortium is an international, collaborative, meta-analytical study that used the Genome Wide Association Study (GWAS) approach to identify common loci that are associated with lipid levels in the human population. The GWAS study by the Global Lipid Genetic Consortium (GLGC) identified 95 loci that affect lipid levels in the human population. The GLGC has shown that 37 of the 95 SNPs affect LDL cholesterol levels in the general population (Teslovich *et al.* 2010). This is of significant value since a percentage of FH patients who do not have mutations in any of the known FH-causing genes, thus referred to as mutation negative FH, and may actually be associated with these SNPs.

Recent work by Talmud *et al* to identify an alternate molecular basis for FH, studied mutation negative FH patients and found that FH could be polygenic and not of conventional Mendelian inheritance. In this work, they tested for significantly greater

association of 12 LDL-C SNPs in mutation negative FH patients relative to FH patients with known FH causing mutations (i.e. Mutation positive FH patients) (Talmud *et al.* 2013). The work of Talmud *et al* showed that FH can also have an alternate genetic origin such as a polygenic etiology because the accumulation of those 12 SNPs was significantly greater in mutation negative FH patients than in mutation positive FH patients. These SNPs include two mutations in apoE and many apoE isoforms. GWAS studies have shown that apoE is strongly linked to LDL-C levels (Asselbergs *et al.* 2012). Furthermore, the first to report a strong genetic link between an *APOE* mutation and FH in a large family was Marduel *et al.*

2.3. Familial hypercholesterolemia, lipid metabolism and atherosclerosis

Prognosis of FH depends largely on the severity of the mutation, the impact of the mutation on *LDLR* function, how early a diagnosis of the disease is made and the start and intensity of treatment. Treatment may significantly delay atherosclerosis and heart attack, but does not eliminate the underlining condition. Risk of death generally varies among FH, but individuals who inherit two copies of a defective gene (homozygotes) have a poorer outcome. Homozygous FH causes early heart attacks, is resistant to treatment and may cause development of aortic calcification (Awan *et al.* 2008). Late complications include aortic valve stenosis that may require urgent replacement.

Herein, I will present the biochemical pathways where excess circulating cholesterol leads to complications. I will illustrate how cholesterol is transported in the blood and the essential role of different biological players, namely, the low-density lipoprotein receptor (*LDLR*), Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) and the widely expressed ligand apoE.

Lipoproteins are macromolecular structures *via* which hydrophobic lipids can be transported through the aqueous environment of the blood. A lipoprotein is defined as a complex of lipid and protein (apolipoprotein), wherein hydrophobic lipids are surrounded by a monolayer of phospholipid and peripherally located apolipoproteins. The amphipathic apolipoprotein interacts with the aqueous environment of the blood via its hydrophilic face while the hydrophobic face is buried in the ‘hydrophobic lipid core’. Lipoproteins can be classified by size and density, and the following are lipoproteins found in the body in the order of decreasing size: chylomicrons, very-low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low-density lipoprotein (LDL),

and high-density lipoprotein (HDL). Lipoprotein particles constantly exchange, transfer and accept free cholesterol, triglycerides (TG) and proteins from other lipoprotein particles and tissues. Lipoproteins are also classified according to their densities and diameters, which reflect their load of cholesterol, TG and apolipoproteins as illustrated in (**Figure. 3**):

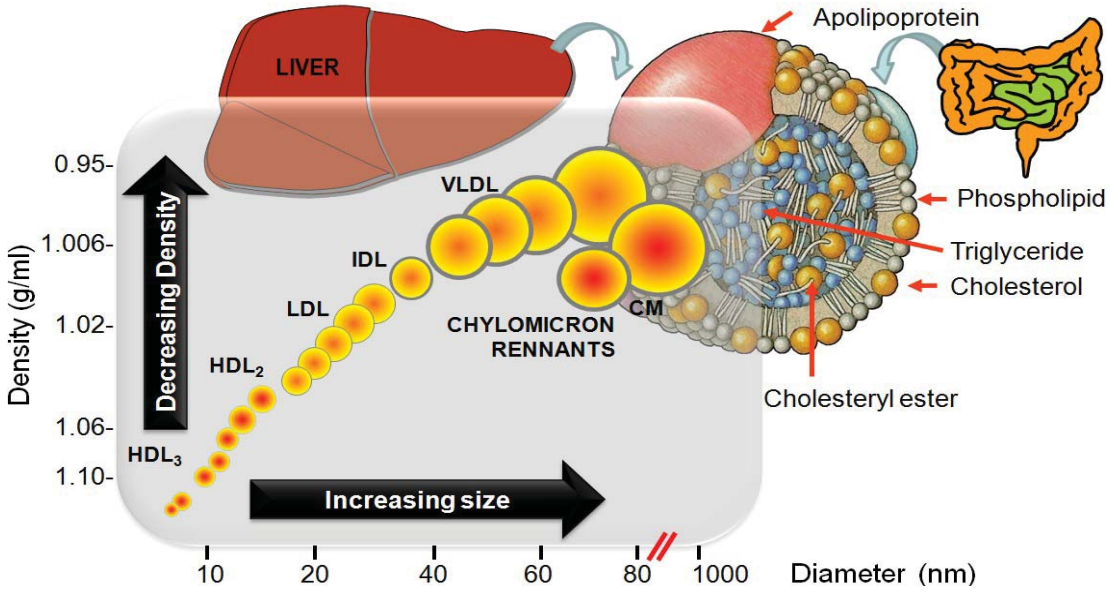

Figure 3: Lipoprotein class and subclass. Lipoprotein are measured by density-gradient ultracentrifugation. A lipoprotein particle is composed of apolipoproteins, phospholipids, triglycerides, cholesterol and cholesteryl ester (adopted from Grundy *et al.* 1990).

A single lipoprotein particle is usually composed of specific combinations of apolipoproteins, free cholesterol, triglyceride (TG), phospholipids and cholesteryl esters. When traveling in the blood, lipoproteins are susceptible to plasma and tissue enzyme actions, thus lipoprotein metabolism/catabolism can be described in three conventional pathways namely; the endogenous pathway, the exogenous pathway and the reverse cholesterol transport pathway (Brown *et al.* 1983, Von Eckardstein *et al.* 2001) (**Figure. 4**).

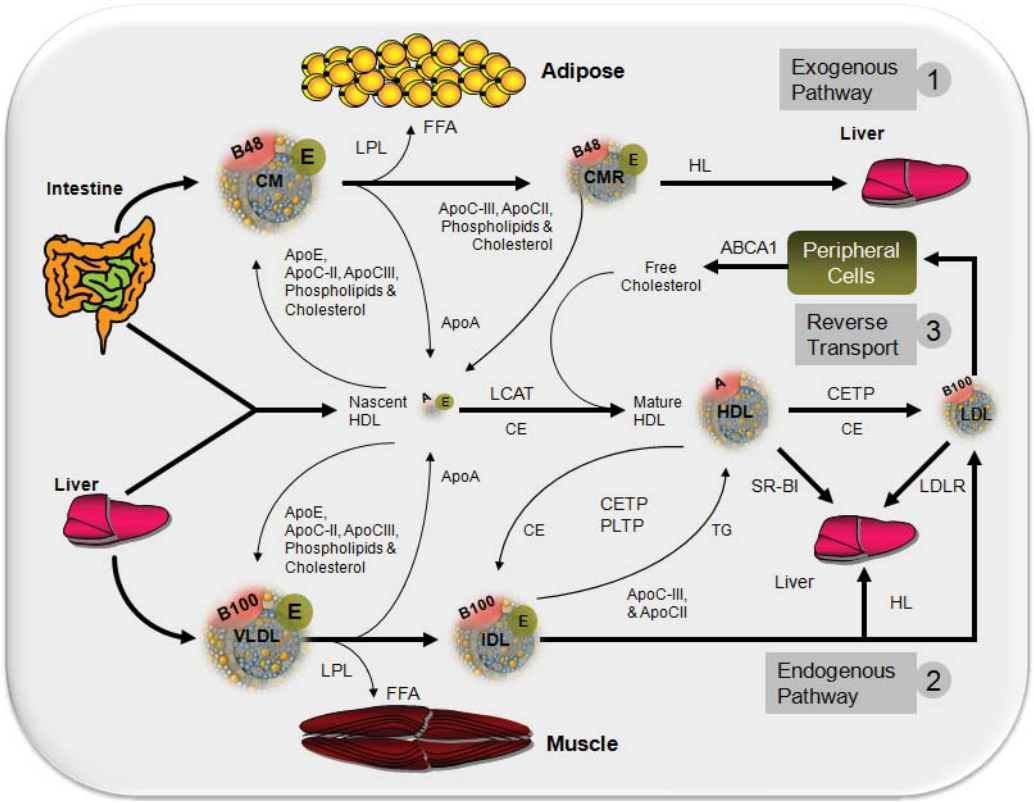

Figure 4: Lipid metabolism and cholesterol transport pathways. VLDL, CM and HDL initiate and mediate the (1) exogenous, (2) endogenous and (3) reverse cholesterol transport processes; respectively. Abbreviation – LDL: low density lipoprotein, VLDL: very low density lipoprotein, HDL: high-density lipoprotein, IDL: intermediate-density lipoprotein, ABCA1: ATP-binding cassette 1, CETP: Cholesteryl ester transfer protein, CM: chylomicron, FFA: free fatty acid, LCAT: lecithin: cholesterol acyl transferase, LPL: lipoprotein lipase, PLTP: phospholipid transfer protein (adopted from Genest and Libby 2011).

The exogenous pathway - In the intestinal epithelium; dietary cholesterol, TG, apolipoproteins (apo) A-I and A-II, A-IV, A-V, apo B-48 and phospholipids are assembled into chylomicron (CM) particles. Postprandially, CM facilitates the transport of these dietary lipids through the aqueous plasma and distributes them to different tissues. Shortly after their secretion into the circulation, an exchange of lipid components and apolipoproteins takes place between CM and HDL. In circulating blood, free cholesterol, apoE, apoC-II, apoC-III and phospholipids are transferred from HDL to CM while apoA's are transferred from CM to HDL. ApoC-II in the chylomicrons activates lipoprotein lipases (LPL) found on the epithelium of muscle and adipose tissues (Saxena *et al.* 1991) and associated with heparan sulfate proteoglycans (HSPG). Thus when a VLDL

particle docks onto a VLDL receptor (VLDLR), the LPL starts to hydrolyze TG in the core of the particle. Newly liberated free fatty acids (FFA) can then enter either adipocytes for storage as TG or enter myocytes for energy utilization (Jensen *et al.* 2003). As a result, CM shrink in size and free cholesterol, apoC-II, apoC-III, and phospholipids are taken back to HDL particles. CM-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). In brief, exogenous source of lipids (i.e. from diet) are transported as CM which circulate and transfer lipids to tissues.

The endogenous pathway - The metabolism of lipids from an endogenous source is referred to as the endogenous pathway or *de novo* pathway. This pathway is the fundamental cause of FH. *De novo* lipids synthesised by the liver are incorporated in nascent VLDL particles for export. These VLDL contains more TG relative to cholesterol in addition to apoB-100, apoE and phospholipids. ApoB-100 and TG are assembled by microsomal TG transfer protein (MTP) enzyme and secreted by hepatocytes into the circulation when the body is fasting. In circulation; apoE, apoC-II and apoC-III are transferred to the VLDL particle from HDL. ApoC-II activates LPL to hydrolyze TG from VLDL and yield denser and smaller intermediate-density lipoprotein (IDL) particles. The liver removes some IDL particles through the apoE receptor (Brown *et al.* 1983). Formation of LDL from IDL is only accomplished after most CE is transferred from HDL to IDL, while apolipoproteins and TG (except apo B-100) are removed from IDL. The LDL particle is now capable of taking cholesterol to hepatocytes and metabolically active tissues like adrenals that are also capable of LDL receptor (LDLR)-mediated endocytosis. The LDLR is mostly expressed at the hepatic cell surface (Brown *et al.* 1983) that recognizes the ligand apoB-100, but also recognizes the apoE carried on VLDL remnants, IDL and some classes of HDL (Innerarity *et al.* 1990, Kane *et al.* 2001). Thus, the LDLR is frequently called apoB/E receptor (Olsson *et al.* 1997). Receptor-mediated endocytosis of the LDL particles by LDLR occurs in most, but not all, cells in clathrin-coated vesicles. This step involves the LDLR adapter protein 1 (LDLRAP1; ARH), hence its absence causes an autosomal recessive form of the disease (Garcia *et al.* 2001, Arca *et al.* 2002). Upon internalization, the low pH in endosomes facilitates the dissociation of the LDLR from its ligand LDL, composed of apoB and lipid (**Figure. 9**, Part 3).

The LDLR then recycles back to the surface to clear more LDL particles from circulation (Goldstein *et al.* 1985). The liver then utilizes this cholesterol and TG to synthesize VLDL particles, which are then exported once again into circulation and/or excreted in the intestine as bile acid salts. Shortly after the LDLR discovery in 1979, Brown and Goldstein won the Nobel Prize for their decisive discovery of the endogenous cholesterol pathway (Brown *et al.* 1983). The transcribed LDLR is a transmembrane glycoprotein (839-amino acids) (Brown *et al.* 1987) (**Figure. 5**). Its C-terminal cytoplasmic domain (50-amino acids) serves to direct the receptor to the coated pits where it meets the LDLR adaptor protein, ARH. Binding ARH helps LDLR cluster in the coated pits and assists endocytosis (Anderson *et al.* 1977, Zuliani *et al.* 1999). In addition to the membrane-spanning region (22-amino acid), the extracellular portion of the receptor contains a region of 13-18 O-linked carbohydrate chains (58-amino acid) and a domain (400-amino acid) that is homologous to the precursor for epidermal growth factor (EGF) (Brown *et al.* 1997). Its N-terminal ligand-binding domain (292-amino acids) is composed of a cysteine-rich sequence where apoB-100 binds the LDLR. The EGF precursor homology domains contain EGF repeats and YWTD spacer regions which are involved in the pH-dependent release of the LDL from LDLR. PCSK9 binds to the EGF-A like domain, thereby negatively regulating the recycling of the LDLR (discussed further in Part 3). LDLR shares structural similarity with members of the LDL receptor superfamily. Sequence alignment revealed that the closest members to the LDLR in structure were the VLDLR (59% identity) and the apolipoprotein E receptor 2 (apoER2) (46% identity) followed by the LDL receptor-related protein 1 (LRP1) (Poirier *et al.* 2009). Mutation in LDLR and/or associated proteins causes FH and depending on the residual LDLR activity, severity of FH may vary. The apoER2, VLDLR and LRP1 proteins all bind apoE carried on many particles (Herz *et al.* 2006).

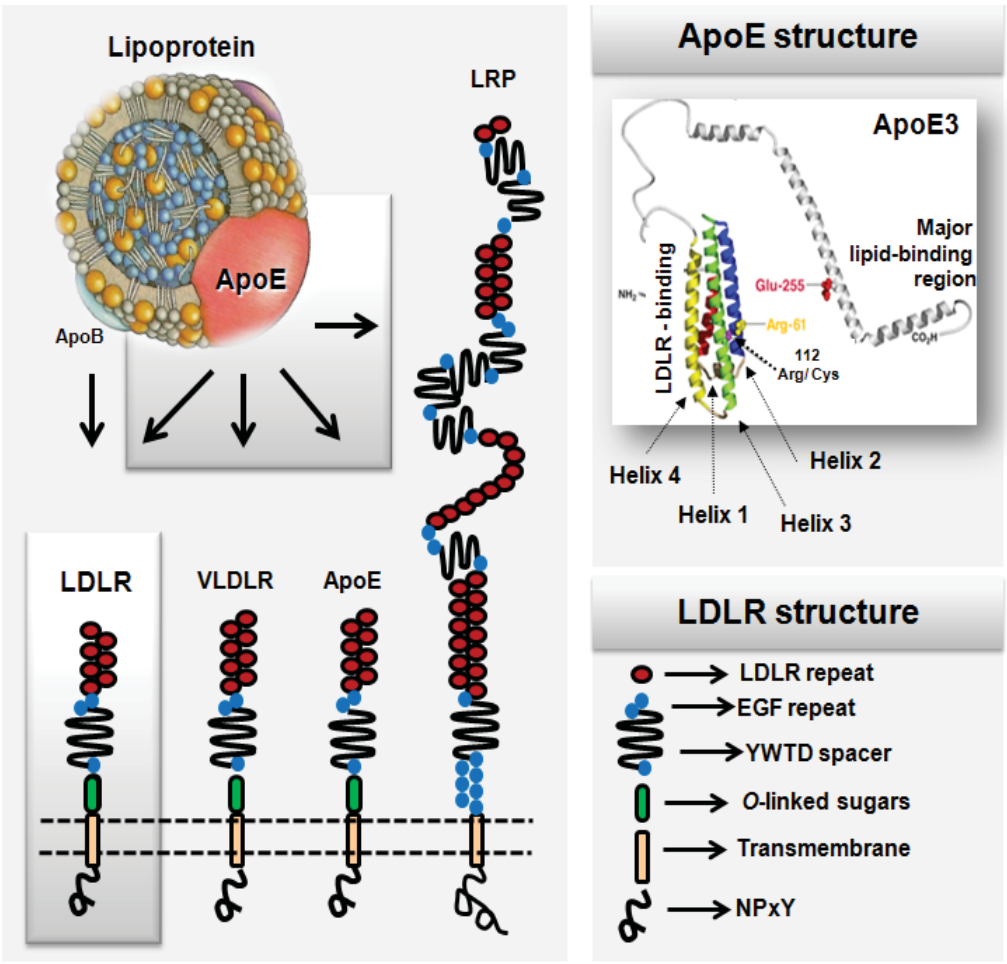

Figure 5: The LDL receptor superfamily and the apoE ligand. The structure of some members of the LDL receptor family is illustrated on the left side. The closest member to the LDLR is the VLDLR and the apoE receptor followed by the LRP. The apoE structure (on the right top corner) is a component of most lipoproteins and binds most of the LDL receptor superfamily members. The right lower corner, shows the structure of the LDLR that is common for all the LDLR family members (adopted from Brown *et al.* 1997 and Protein Data Bank, NMR structure, full length apoE3: 2l7b).

ApoE is a multi-functional glycosylated protein (34 kDa) with two structural domains; the N- and the C-terminal domain. The N-terminal domain contains the receptor-binding region (**Figure. 5**). ApoE is secreted from a variety of tissues, including the liver, adrenals, adipocytes, kidney, brain and macrophages. It is a key component of all lipoproteins, especially TG-rich lipoproteins such as CM, CM-remnants, VLDL and IDL. ApoE participates in their catabolism through interactions with LDLR superfamily

members. Three common polymorphisms in the *APOE* gene, apoE3, E4 and E2 have been recognized, with apoE2 having the least affinity to the LDLR. Homozygosity for the E2 allele occurs in approximately 0.5% of the population and a small percentage of these subjects will develop type III dyslipidemia, characterized by accumulation of remnant lipoprotein particles in plasma and premature vascular disease. A “second hit” is postulated to contribute to the expression of the apoE2/E2 genotype leading to an obvious clinical dyslipidemia (Johansen *et al.* 2011), characterised by tuberous xanthoma and palmar creases, which sometimes can mimic a FH phenotype. Interestingly, rare mutations in *APOE* can lead to a form of lipid glomerulopathy (Tsimihodimos *et al.* 2011).

Reverse cholesterol transport - HDL particles facilitate the return of cholesterol from peripheral cells to the liver and thus are believed to protect the vessels from the harmful effect of excess lipids, hence the name “good cholesterol”. Nascent HDL particles containing apoA1 and apoE are assembled and secreted by both the intestine and liver. Free cholesterol from other lipoproteins or peripheral cells is transferred to HDL and esterified by lecithin cholesterol acyltransferase (LCAT). The cholesteryl esters can be transferred to other lipoproteins such as LDL and IDL to be transported back to the liver or transported by HDL directly to the liver *via* scavenger receptor class B type I (SR-BI) (Von Eckardstein *et al.* 2001). Scavenger cells (e.g. macrophages) in vessels may pick up oxidized LDL (ox-LDL) particles, in case of hypercholesterolemia, leading to atherosclerotic plaque formation (Brown *et al.* 1983, Gregory *et al.* 2001) as we will discuss below.

Pathogenesis of atherosclerosis - The pathogenesis of atherosclerosis is far too complex to be summarized in a thesis introduction; however I will allude to key processes (**Figure. 6**). The remote description of atherogenesis includes foam cell accumulation and plaque formation that may potentially erupt to cause thrombosis. When excess LDL-C stays in the blood too long (as is the case of FH), the LDL is likely to be oxidized. The oxidized LDL (ox-LDL) accumulates between the endothelium and tunica intima layer of the arterial wall. As ox-LDL becomes an immunogen, it triggers an immune response; so in essence, atherosclerosis can be considered as an antigenic immunological response. Furthermore, these ox-LDL particles are then engulfed by macrophages that become activated (**Figure. 6**).

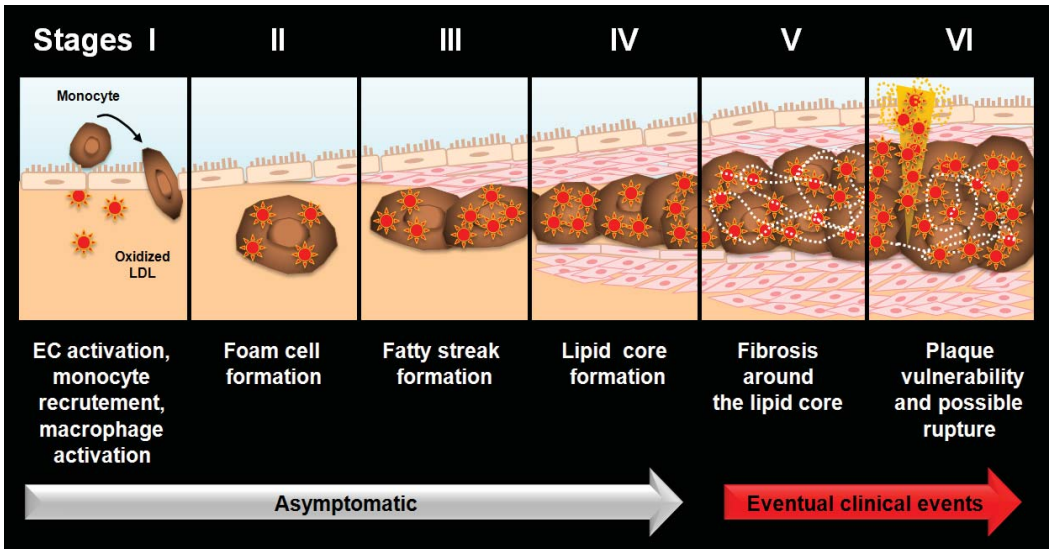

Figure 6: The stages of atherosclerosis development. Stage I: vascular injury leads to endothelial cell (EC) activation, monocyte recruitment into the intima and macrophage activation. Stage II: the earliest atherosclerotic lesion, the fatty streak, is an almost purely inflammatory lesion consisting of monocyte-derived, lipid-laden macrophages (foam cells) and T-lymphocytes. Stage III–IV: the progressive accumulation of lipids (intracellular and then extracellular) forms the lipid core. Stage V: a fibrous cap develops around the lipid core forming an atherosclerotic plaque. Stage VI: activated macrophages secrete enzymes that weaken the fibrous cap leading to plaque rupture, hemorrhage or thrombosis and CVD (adopted from www.r3i.org).

Macrophages engulfing ox-LDL enlarge and are then referred to as foam cells. These foam cells subsequently may undergo apoptosis and become part of fatty streaks and plaques. The plaque formation process stimulates the cells of the vascular bed to differentiate and produce substances that accumulate in the subendothelium (i.e. tunica intima). Notably, calcium deposition and calcified connective tissue start to accumulate. The inner layer of the arterial wall thickens, the artery diameter shrinks and the blood flow starts to be compromised. This plaque formation may lead to decreased blood flow to vital organs like the heart or the brain. Cholesterol-lowering drugs such as statins might regress the atherosclerotic plaques; however calcified plaques (as seen in FH homozygotes) may be more resistant to regression upon statin treatment (Chan KL *et al.* 2009), underscoring a hidden process beyond lipid lowering. Current data implicates cholesterol micro-crystals as being an initiating factor in liberating cytokines (discussed further in Part 4). In Part 4, we go in-depth into the inflammatory component of atherosclerosis and the earlier trials to modulate the immune system to prevent CVD and in Chapter 6 we investigated whether inhibition of the inflammatory response prevents vascular calcification in an animal model.

2.4. Familial hypercholesterolemia disease prevention

Pharmacological intervention in familial hypercholesterolemia - The goal of treatment is to reduce the risk of atherosclerotic plaque formation and consequently heart attacks. These include appropriate physical activity and early reduction of saturated fatty acid intake to less than 7% of daily calories and a cholesterol intake of less than 200 mg/day (Guardamagna *et al.* 2009). Usually diet represents a first line of treatment but it takes several years to see an effect. Counselling is often recommended to help people make changes to their dietary habits. Those who inherit only one copy of a defective FH gene may respond well to dietary changes combined with use of drugs such as statins. However, these environmental changes are not expected to drastically reduce hypercholesterolemia in homozygous FH individuals. Therefore, the first line of therapeutic action for molecularly confirmed homozygous FH is the implementation of a lipid lowering medication. There are several types of drugs available to help lower blood cholesterol levels, and they work in different ways. Some are better at lowering LDL-C; some are good at lowering triglycerides, while others help raise HDL cholesterol. The most commonly used and most effective drugs for treating high LDL-C are the statins: inhibitors of hydroxylmethylglutaryl coenzyme A (HMG-CoA) reductase, a rate limiting step in cholesterol biosynthesis. Statins also induce the cellular production of LDLR; therefore statins are effective mostly when LDLR activity is still preserved in FH subjects. Conventional statin drugs include; pravastatin (Pravachol), lovastatin (Mevacor), fluvastatin (Lescol), simvastatin (Zocor), atorvastatin (Lipitor), pitivastatin (Livalo), and rosuvastatin (Crestor) being the most recent and potent. Other cholesterol-lowering medicines include: Bile acid-sequestering resins (not frequently used due to gastric symptoms, useful in pregnancy), Ezetimibe (inhibits cholesterol absorption from gut, usually in combination with statin or in FH with complete LDLR loss of activity), Fibrates (less effective on LDL-C, but better on TG) and Nicotinic acid (mostly used to raise HDL-C, with subtle effect on LDL-C).

Before the statin era, cholestyramine (bile acid binder) and niacin (vitamin B3 analog) (Grundy *et al.* 1981, Goldstein *et al.* 1987) were among the prescribed options to lower lipids, but intolerability limited wide spread usage. Statins on the other hand represent a class of medication that inhibits HMG-CoA reductase (Goldstein *et al.* 1987), limiting *de novo* cholesterol synthesis. Thus, statins reduce LDL-C by reducing endogenous cholesterol production from the liver; statins also increase the uptake of

circulating LDL particles by increasing the expression of LDLR and PCSK9 regulator as we are going to discuss below (Goldstein *et al.* 1987, Dubuc *et al.* 2004). Statins also have lower side effects which lead to higher patient compliance. Statins not only increase LDLR and decrease cholesterol levels, but they also have been correlated with a reduction in CVD in over 26 randomized clinical trials (Baigent *et al.* 2010). 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 function, coagulation, vasomotor activity and even inflammation to a certain degree (De Lorenzo *et al.* 2006). However, in patients lacking LDLR or having significantly lower LDLR activity, the statins are not efficient in lowering 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 (similar to renal dialysis), is then required (Rader *et al.* 2003). In apheresis, blood or plasma is removed from the body and travels through special filters, where apoB binding beads then remove the extra LDL, and the blood plasma is then returned back into the body. LDL apheresis has allowed homozygous FH individuals with no LDLR activity to live beyond their short life expectancy; however they are not free of disease, as they experience premature arterial calcification as a late complication of the underlying disease (Awan *et al.* 2008). New to the lipid field are sequence-specific antisense oligonucleotide against apoB mRNA (Mipomersen) and the synthetic inhibitor of Microsomal TG transfer protein MTP (Lomitapide). The MTP antisense drug down regulates the MTP enzyme in a post translational fashion leading to less TG and apoB assembly and thus lowers hepatocyte output of VLDL into the plasma. Mipomersen and Lomitapide have been tested in phase III clinical trials in patients with FH, but discouraging results in the form of liver toxicity are reported (Cuchel *et al.* 2007), underscoring the importance of cardiometabolic screening. The latest and highly anticipated drugs, PCSK9 inhibitors, are designed to block PCSK9 from binding to LDLR, thus allowing LDLR to lower cholesterol and will be discussed further in Part (3) of this Chapter.

Cardiometabolic disease prevention in familial hypercholesterolemia - A diet low in cholesterol and saturated fat would not prevent disease in FH without a statin based therapy. In statin-resistant individuals with a lack of LDLR activity, a combination therapy that includes a potent statin and ezetimibe is required; for some severe homozygotes

LDL-apheresis may be the only remedy. However, cardiometabolic burden associated with homozygotes that receive apheresis on chronic bases include calcification and anemia (Stefanutti *et al.* 1995). Genetic counselling is an option for those who have a family history of this condition, particularly if both future parents carry the defective gene; genetic counselling is also an option for individuals who have signs of xanthoma and xanthelasma. In essence, prevention of a cardiometabolic disease refers to the optimal care that takes into account all metabolic conditions that are associated with the underlying disease as well as diseases that may result from treatment with drugs. For instance, atherosclerosis and arterial calcification may lead to high blood pressure as the aortic wall loses compliance; chronic kidney disease may result from renal artery stenosis (Fantus *et al.* 2013). Interestingly, FH patients do not suffer from bone metabolic dysregulation even though the aorta might calcify completely on lipid treatment as one study suggested (Awan *et al.* 2010). As alluded to earlier, there are cardiometabolic diseases that are treatment-associated, like statin-induced muscle toxicity (myositis, rhabdomyolysis), renal toxicity (elevated creatinine), liver toxicity (elevated alanine aminotransferase or ALT), glucose intolerance or even remote diabetes (Ridker *et al.* 2008). Thus complete assessment and follow-up is required.

Familial hypercholesterolemia screening - Screening individuals for a mutation known to be common in a family or common in an isolated sub-population like French-Canadians has proven to be both life saving and cost-effective (Wonderling *et al.* 2004, Nherera *et al.* 2011). This usually is triggered when an index case is brought to medical attention or when surveying high risk populations. After identifying the DNA mutation in FH individuals, the hope is to stop CVD events (primary prevention) in that family or sub-population. For index cases identified after a CVD event, the goal is to prevent a subsequent event (secondary prevention) with aggressive treatment and close follow-up. Various mutation screening methods are used in different countries, including direct sequencing (Defesche *et al.* 2010), diagnostic criteria (Civeira *et al.* 2008) or PCR melting analysis (Laurie *et al.* 2009) and Denaturing High Performance Liquid Chromatography (DHPLC). Today most screening strategies cover the candidate genes; *LDLR, APOB* and *PCSK9*. A more novel screening strategy has been implemented in Iceland, whereby ancestors of FH probands were traced and the oldest in every pedigree was screened for the Icelandic common *LDLR* mutations (Thorsson *et al.*

2003). This genealogical tracing might be superior to the conventional first-degree relative approach in founder populations.

Familial hypercholesterolemia registry - FH is commonly referred to as the missed opportunity in preventive medicine (Watts *et al.* 2007). With an incidence of 1:500 or even more in some areas, the benefit of early diagnosis and treatment will translate into decreased mortality and morbidity in a short time. Many campaigns from the National Lipid Association (Goldberg *et al.* 2011) is calling for a "FH Awareness Day" so that medical practitioners recognize that they are missing one of the more common genetic diseases while concentrating on very rare ones, including Cystic Fibrosis and Phenylketonuria. A national registry is being called for in Canada (Saraf *et al.* 2013). A registry can be defined as an official national record keeping of individuals with FH across the borders. Building a national FH registry would require cascade family screening of each FH proband or index case (as we will discuss in Chapter 2), which will, as a result, reduce national mortality rates and have a better economic outcome. An article by Al-Saraf *et al* recently pointed out the importance of an FH registry in Canada and the need to devise a system where FH can be systematically detected in the population. Lack of a national registry led to FH being diagnosed in only 15% of Canadians who have FH; in other words 85% of Canadians do not know that they have the life-threatening disease (Al-Sarraf *et al.* 2013). Countries that have a national FH registry, with Netherlands having the best national FH registry in the world, identifies 8 new FH cases per family using cascade screening (Al-Sarraf *et al.* 2013). Thus, these real examples should fuel the Canadian government to quickly make a national FH registry an imperative. To simplify this further, entry to FH registries should include the first and at least one of the following:

- Lipid Profile (isolated increase in LDL-C/apoB) and at least one of:
  - Family history of premature coronary artery disease
  - Clinical stigmata (Xanthoma/Xanthelasma)
  - Molecular diagnosis/mode of inheritance
  - Functional analysis of LDLR (skin fibroblast)

Simplified suggested criteria for FH registry

In Chapter 2, we present a case directly related to FH registry at the McGill University Health Centre that was referred to a specialized lipid clinic, where advanced molecular testing and cascade family screening were preformed. As a result, we identified a novel mutation in the *APOE* gene responsible for FH. The latter mutation was used to screen family members and successfully could identify asymptomatic carriers for FH that might benefit from early diagnosis and follow-up. In summary, lipoproteins and lipoprotein disorders play an important role in the onset and progression of FH disease. Unrecognised FH and cardiometabolic related risks will prolong the period of elevated cholesterol exposure in the individuals, thus leading to atherosclerosis and an acute CVD event. Suboptimal treatment in FH will lead to advanced subendothelial inflammation, atherosclerotic calcification, hypertension, renal failure and stroke. Mistreated FH without close follow-up and specialized care may result in insufficient LDL-C reduction, statin-related muscle, liver and renal toxicity. In addition, available treatments to date, including statins and LDL- apheresis, are not sufficient to eliminate cardiovascular risk and so the search for newer and safer cholesterol-lowering agents continues. Therefore, classes of drugs, such as the PCSK9 inhibitors are viable candidates if proven safe and thus we will discuss the PCSK9 biology below.

3. Familial Hypercholesterolemia and PCSK9

In the 1970's, it was established that bioactive proteins, including hormones and enzymes, were secreted as inactive precursors and processed to a bioactive form, as illustrated by the activation mechanism associated with pro-insulin being converted to the bioactive insulin (Bailyes *et al.* 1991). Conversion from an inactive precursor is catalyzed by a special group of enzymes known as proprotein convertases. Proprotein convertases (PCs) intracellularly cleave precursors in both the constitutive and secretory pathway to produce a mature protein. In the 1990's, eight of these mammalian proprotein convertases were discovered and shown to be responsible for the tissue-specific processing of secretory precursors (Seidah 1999, Fugère *et al.* 2002) (**Figure. 7**).

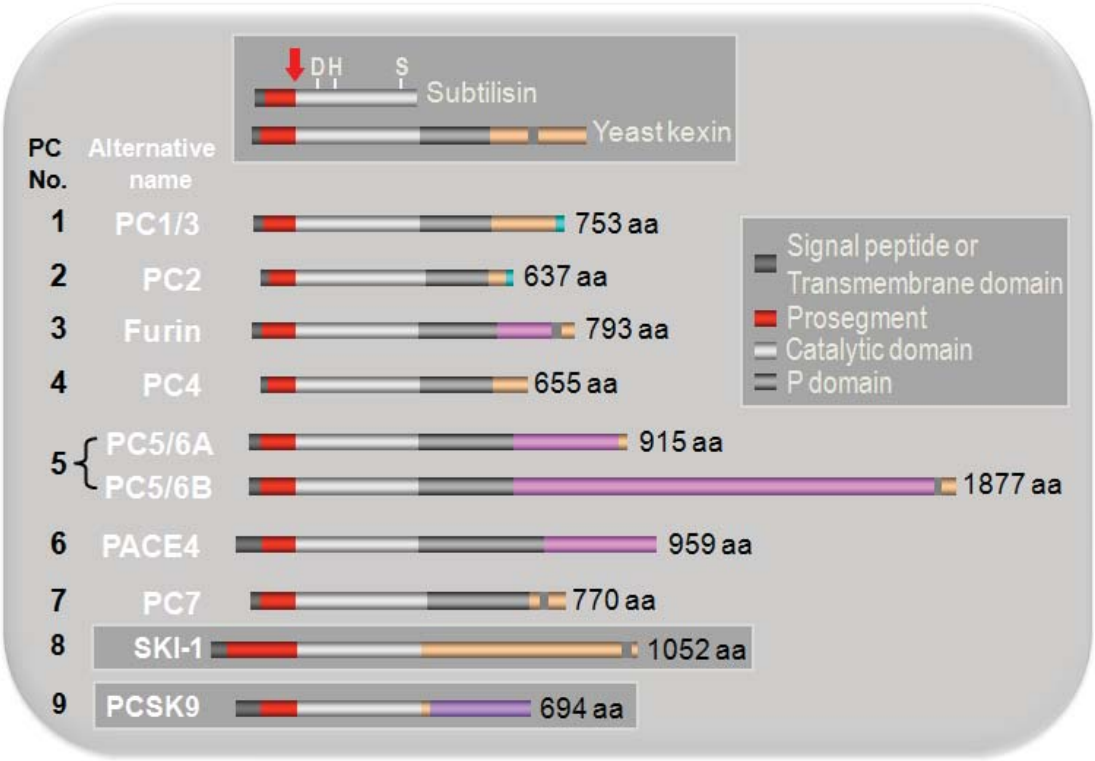

Figure 7: Primary structures of the human proprotein convertases. The kexin-like basic amino acid specific proprotein convertases (PC 1-7), pyrolysin-like subtilisin kexin isozyme 1 (SKI-1) and proteinase K-like proprotein convertase subtilisin kexin 9 (PCSK9) are grouped to emphasize their distinct subclasses. The various domains are emphasized, along with the primary (depicted using arrows) processing sites. PCSK9 has a Cys-His-rich domain (indicated by a lavender color) that is required for the trafficking of the PCSK9–LDLR complex to endosomes and lysosomes (adopted from Seidah and Prat 2012).

Substrates of these proprotein convertases include: hormones, growth factors, receptors, transcription factors and surface glycoproteins. Proprotein convertases are known calcium-dependent serine endoproteases. The family of these proprotein convertases comprises 9 members coded by genes assigned by the letters 'PCSK' (Pro-protein Convertase Subtilisin Kexin) and numbered from 1 to 9 (Seidah *et al.* 2008). PCSKs are critical to several physiological processes depending on their site of action, and their protease activities result in activation/inactivation events, some of which indirectly impact cardiovascular health (Seidah *et al.* 2007). All PCSKs contain an N-terminal signal peptide (SP), a prosegment domain, a P domain and a catalytic domain that is highly conserved across species. The ~80-90 amino acids prosegment domain is cleaved first by an autocatalytic event in the endoplasmic reticulum (ER). The prosegment domain acts both as an intracellular chaperone to ensure proper folding and as a competitive inhibitor of PCSKs' action. The competitive inhibitory effect seems to prevent PCSKs from being inadvertently activated during synthesis. The second round of cleavage occurs in the prosegment domain and results in its release from the catalytic domain and activation (Bergeron *et al.* 2000, Seidah *et al.* 2008).

The eighth member of the PCSK family, PCSK8, is known to cleave membrane-bound transcription factors including sterol regulatory element binding proteins (SREBP), in the luminal domain of the Golgi apparatus (Horton *et al.* 2002). In the absence of sterols, the SREBP are further cleaved by site-2 protease (S2P) that results in the release of their DNA-binding domain which becomes soluble and translocates to the nucleus (Horton *et al.* 2002). The activated SREBP then binds to specific sterol regulatory element sequences to upregulate the synthesis of enzymes involved in sterol/lipid biosynthesis. Sterols inhibit the additional cleavage of SREBP through a negative-feedback-loop and cholesterol synthesis is tapered down. Thus, SREBPs are named master regulators of lipid homeostasis (Eberlé *et al.* 2004).

The last member of the PC family is PCSK9. To date, the only confirmed biological property of PCSK9 is the protein-protein interaction (PPI) with LDLR, and possibly apoB, leading to its degradation (Seidah *et al*. 2003, Lambert *et al.* 2006). Disrupting this PPI between PCSK9 and LDLR prevents LDLR degradation, lowers cholesterol and is thought to protect from atherosclerosis. A third of the adult population in the United States suffers from elevated LDL-C and as a consequence is at risk for CVD [CDC. MMWR. 2011;60(4):109–14]. Furthermore, cholesterol lowering treatment based solely on statins has proven futile in a significant number of patients. Developing PCSK9 inhibitors as a new class of cholesterol lowering drugs has emerged recently. Within less than a decade, inhibitors of PCSK9 have been translated into a potential clinical application. After this discovery, there are at least a dozen potential drugs at different stages of clinical trial. All these potential drugs are awaiting endpoint data that fully describes their safety and utility.

3.1. PCSK9 history

The discovery of PCSK9 came about when a search for novel members in the proprotein convertase (PC) family was conducted; around the era of the human genome project. Degenerate oligonucleotides and the polymerase chain reaction (PCR) were used to amplify the catalytic subunit of various PCs, including the 8th member SKI-1/S1P. This led to the identification and biochemical characterization of the 9th member of the PC family, previously called Neural Apoptosis-Regulated Convertase 1 (NARC-1) (Seidah *et al*. 2003). NARC-1 shows sequence similarity across many species (http://www.ncbi.nlm.nih.gov/), and since it shares many structural features of subtilases it qualifies as a member of the PC group. NARC-1, now renamed PCSK9 (Abifadel 2003), was proposed to be involved in a wide spectrum of biological processes, including neurogenesis, hepatogenesis, enterogenesis and nephrogenesis (Seidah *et al*. 2003). The expression of *Pcsk9* in the liver was the most robust, signifying a role therein yet to be established for this protein. The history of PCSK9, from discovery to application, is shown in (**Figure. 8**):

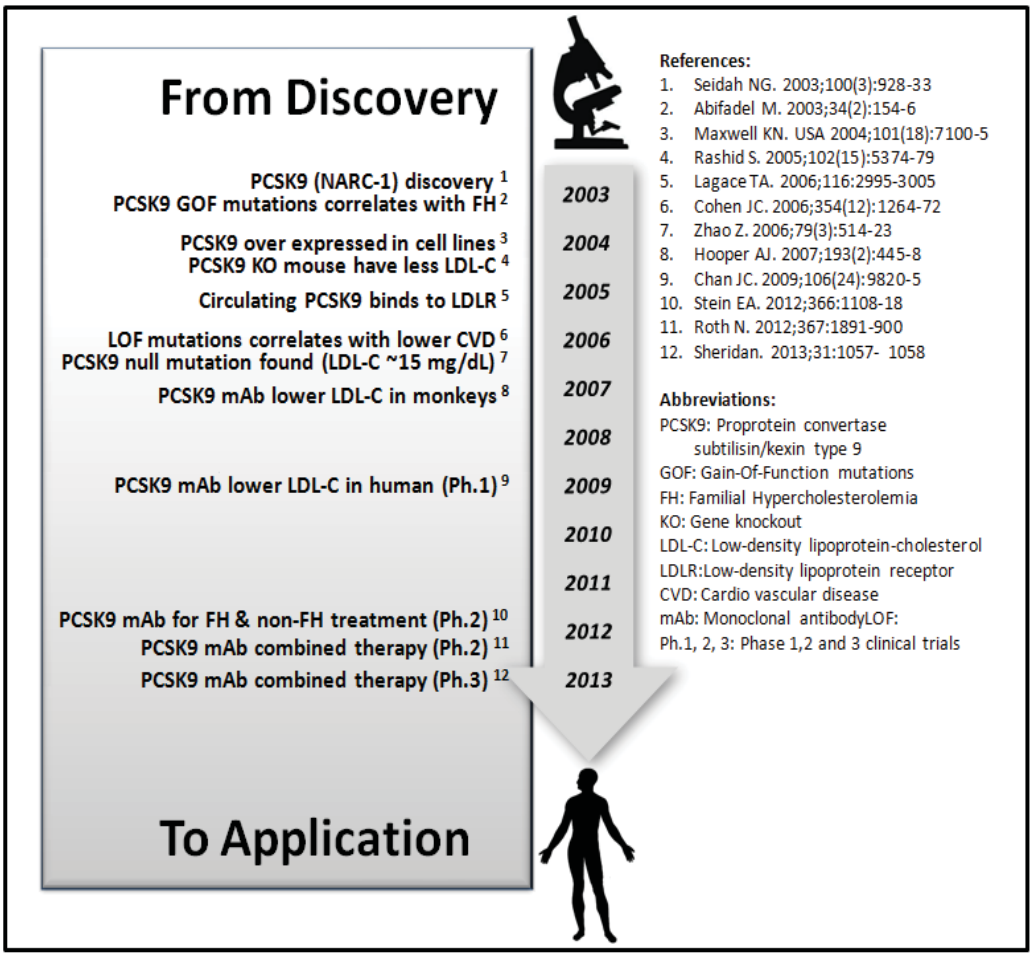

Figure 8: PCSK9 history at a glance (Reference on the top right corner).

Before the discovery of PCSK9, FH had only two loci linked to it: Chr19 p13.1-13.3 (*LDLR*) and Chr2 p23-24 (*APOB*). A fruitful collaboration between Canadian scientists and a French group studying a cohort of non-LDLR/non-apoB hypercholesterolemic families confirmed that the newly discovered *PCSK9* gene was indeed the culprit of the disease that they were looking for around Chr1p34.1-p32 (Varret *et al.* 1999). Thus, it was designated as the third locus implicated in FH (Abifadel *et al.* 2003). This sparked a worldwide race to identify gain-of-function (GOF) mutations causing hypercholesterolemia as well as loss-of-function (LOF) mutations compatible with hypocholesterolemia (low cholesterol in the blood) (Cohen *et al.* 2005). The Caucasian *PCSK9* p.R46L variant was not only associated with hypocholesterolemia but

also showed a remarkable 47% reduction in CVD (Cohen *et al.* 2006). The African nonsense mutation had an even more remarkable 88% reduction in CVD risk compared to non-carriers. Additionally, two complete loss-of-function mutations causing severe hypocholesterolemia were found in apparently healthy individuals. The loss-of-function (LOF) mutations were (i) a compound heterozygous LOF mutation and (ii) a homozygous LOF mutation that leads to almost undetectable levels of PCSK9 (Zhao *et al.* 2006, Hooper *et al.* 2007). The discovery of *PCSK9* LOF mutations leading to hypocholesterolemia in healthy individuals represented a potentially novel therapeutic target for the pharmaceutical industry. Being healthy and hypocholesterolemic due to a LOF mutation in *PCSK9* was thus demonstrated for the very first time in humans.

Interestingly, the enzymatic activity of PCSK9 is solely used to cleave itself, which in turn prevents it from its own proteolytic activity after the prosegment occupies the catalytic pocket (**Figure. 9**). The evidence suggests that *PCSK9* expression enhances LDLR degradation through a pathway that involves endosomal/lysosomal shuttling. However, its mechanism remains to be fully elucidated (Canuel *et al.* 2013). Nevertheless, the downstream effects of a GOF mutation in *PCSK9* are mediated by increased LDLR removal, adversely leading to hypercholesterolemia. The downstream effects of *PCSK9* LOF mutations lead to considerably increased accumulation of LDLR at the cell surface, leading to more LDL-C clearance and consequent hypocholesterolemia.

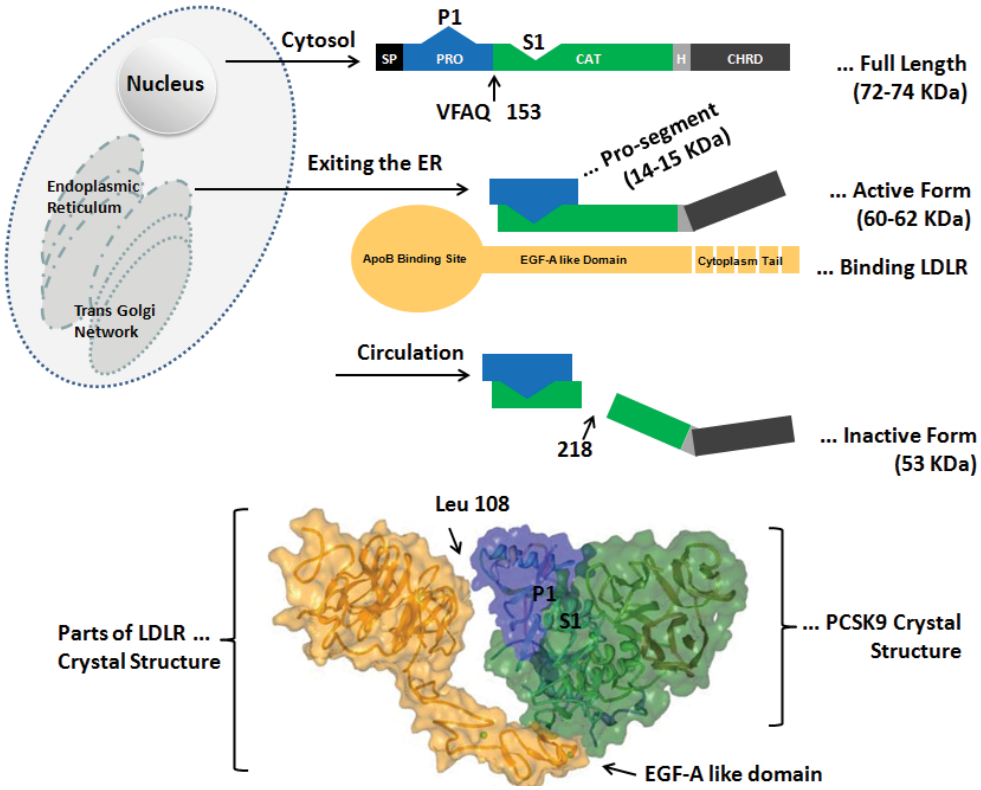

Figure 9: PCSK9 domains, biology and crystal structure. PCSK9 is a protease that is synthesized as an enzyme precursor. Following synthesis in ER, PCSK9 undergoes autocatalytic cleavage, which is required before exiting the ER. The various domains and N-glycosylation positions are emphasized, along with the primary (VFAQ $_{153}$ ↓, black arrows) as well as the secondary autocatalytic processing sites (at position 218, black arrows). Although cleaved, the pro-segment and catalytic domains of PCSK9 remain together. PCSK9 binds to the LDL receptor, near the EGF-A-like domain, as shown by the crystal structure of both (Source candidate private collection and Protein Data Bank, structure of PCSK9 binding LDLR: 2W2M).

3.2. PCSK9 Cellular Biology

PCSK9 biosynthesis - The 25-kilobase *PCSK9* is located on the small arm of chromosome 1, region p32 in humans and contains 12 exons and 11 introns (Seidah *et al.* 2007, Davignon *et al.* 2010) (**Figure. 9**). This novel proprotein convertase showed atypical biology that made it a non-classical convertase. PCSK9 is a typical PCSK in that it has a serine protease-like structure of the bacterial subtilase family, but it is different in that it also shares structural similarity with the fungal proteinase K-like enzymes. Full length PCSK9 is characterized by 9 leucine residues within the signal peptide domain that guides the synthesized protein to the ER. As a PC, it has all the domains, namely

the signal peptide (SP), prosegment (PRO) and a catalytic domain (CAT), but not a P-domain. Instead, it contains a C-terminal region characterized by a Cysteine/Histidine-Rich Domain (CHRD) (**Figure. 9**). A hinge region (H) also is present between the catalytic pocket and the CHRD; the function of the hinge region is poorly understood. Once in the ER, the SP domain of PCSK9 is cleaved and shortly after the 692 amino acid pro-protein undergoes autocatalytic processing at position VFAQ $_{152}$ –SIP. This autocatalytic event deprives mature PCSK9 of its proteolytic activity since the prosegment occupies the catalytic site. This step is necessary for exiting the ER (Seidah *et al.* 2008). Mature PCSK9 together with the prosegment (62 kDa + 15kDa) bind non-enzymatically (PPI) LDLR. The crystal structure of PCSK9 was determined in 2007 (Cunningham *et al.* 2007, Piper *et al.* 2007) (**Figure. 9**). Sequence analysis of the catalytic domain shows that 3 conserved residues; Asp186, His226 and Ser386 are involved in the active site and mutations at these sites makes the protein inactive (Piper *et al.* 2007).The PCSK9 S1 pocket that recognizes the P1 cleavage site of its prosegment is shallower in comparison with other PCs (**Figure. 9**). This is due to a glycine residue in the S1 pocket of PCSK9, *versus* alanine in other PCs, thereby raising the floor of the catalytic pocket (Piper *et al.* 2007). Mature PCSK9 interacts with the EGF-A domain of the LDLR through a region near the PCSK9 catalytic domain (Kwon *et al.* 2008). This interaction of PCSK9 with LDLR targets LDLR for degradation by an unknown mechanism: one hypothesis is that a third protein escorts the entire complex to the degradation pathway. Substituting certain domains in the CHRD or removing them from this region has shown that the M2 module (one of 3 distinct subdomains) of the CHRD is essential for the extracellular activity of PCSK9 on cell surface LDLR (Saavedra *et al.* 2012) (**Figure. 9**). Finally, the mature protein is post-translationally modified by Asn-glycosylation, Ser-phosphorylation and Tyr-sulfation (Seidah 2009).

PCSK9 cellular activity - Recent studies showed that PCSK9 is secreted mostly by the liver (Roubtsova *et al.* 2011). Unlike other PCs, no substrate has been identified for PCSK9 other than itself (Zaid *et al.* 2008). In blood, a significant percentage of circulating PCSK9 (40%) is carried on the LDL-C particle and is postulated to bind apoB (Kosenko *et al.* 2013, Tavori *et al.* 2013). The PCSK9-prosegment complex binds in a non-enzymatic fashion to the EGF-A like surface of the LDLR (Zhang *et al.* 2007) (**Figures. 9 and10**):

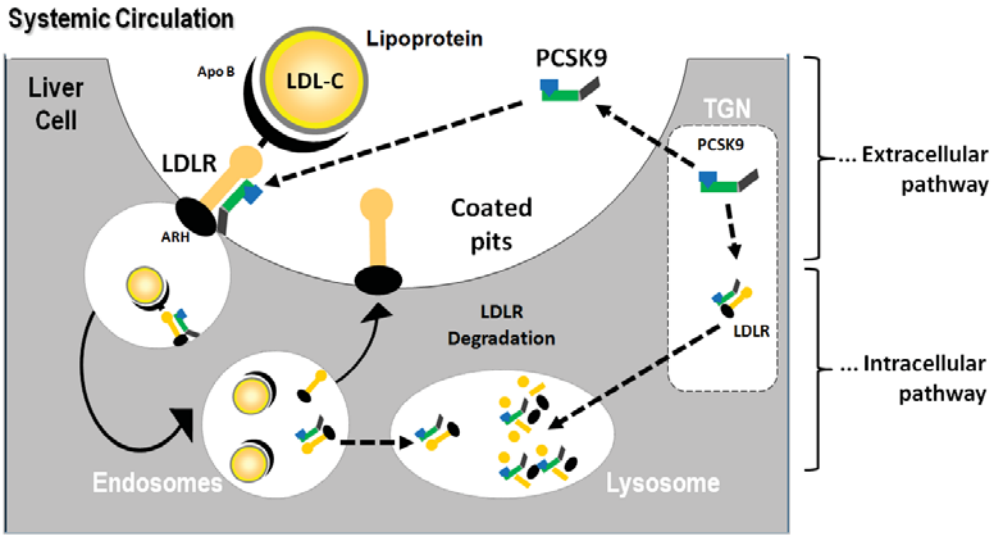

Figure10: LDLR recycling and PCSK9 pathways. Proprotein convertase subtilisin/kexin 9 (PCSK9) undergoes autocatalytic cleavage to exit the ER. Upon reaching the trans-Golgi network (TGN), PCSK9–prosegment complex can either be sorted directly to lysosomes as a complex with the low-density lipoprotein receptor (LDLR) (intracellular pathway), or it is secreted and then internalized with the LDLR into clathrin-coated endosomes for lysosomal targeting and degradation, which requires the binding of the cytosolic adaptor protein autosomal recessive hypercholesterolemia protein (ARH) (extracellular pathway). Therefore, in the absence of PCSK9, the endocytosed APOB–LDLR complex dissociates and the LDLR is recycled back to the cell surface. In the presence of PCSK9, the LDLR is not recycled, and (by a currently undefined mechanism) the PCSK9–LDLR tight-binding complex is sent to lysosomes for degradation (Source candidate private collection, adopted from Seidah and Prat 2012).

Once PCSK9 interacts with LDLR, the whole complex is directed to the endosomal/lysosomal pathway (**Figure. 10**) for degradation as shown by using a DiI-LDL tracing experiment (Qian *et al.* 2007).

Deletion analysis revealed that an N-terminal region of the PCSK9 prosegment domain (31–52 aa) inhibits LDLR binding (Benjannet *et al.* 2010, Kosenko *et al.* 2013). The CHRD domain of PCSK9 is not ordered in most crystal structures, likely due to its high mobility; nevertheless module M2 of the CHRD, as previously mentioned, is essential for extracellular activity of PCSK9 on cell surface LDLR (Saavedra *et al.* 2012), but not required for secretion or autoactivation (Zhang *et al.* 2008). PCSK9's journey through the secretory pathway is also conditional (**Figure. 10**), hence a low pH in endosomal compartments facilitates PCSK9 binding to the LDLR (Benjannet 2004, Fisher *et al.* 2007). A pH change to a more acidic pH ~5.5-6.0 leads to a ~2.5-fold

increase in PCSK9 activity on total and cell surface LDLR; thus pH-dependent activation of PCSK9 is optimal in the acidic endosomes (Zhang *et al.* 2007, Benjannet *et al.* 2010).

Evidence exists for an intracellular pathway that targets LDLR directly to endosomes/lysosomes without it being secreted (Poirier *et al.* 2009). Thus, there are two proposed sites where PCSK9 can interact with LDLR: on the cell surface of hepatocytes (extracellular route, major) (Lagace *et al.* 2006) and in the TGN (intracellular route, minor) (Nassoury *et al.* 2007, Poirier *et al.* 2009) (**Figure. 10**). This may explain why circulating levels of PCSK9 do not correlate well with LDL-C levels in population studies (Awan *et al.* 2012).

Other members of the LDLR superfamily sharing homology in the EGF-A like domain have also been reported to be targeted by PCSK9. VLDLR, apoER2, LRP-1, intestinal cell surface receptors like NPC1L1, CD36 and other receptors used by viruses, like CD81, are shown to be PCSK9 targets. A similar degradation pathway thus is expected to down regulate expression of these receptors (**Table 4**):

Table 4: Evidence of PCSK9 targeting receptors and proteins

PCSK9 Targets	Evidence	Consequences	Reference
LDLR	*in vivo*	LDL-C regulation	Maxwell *et al.* 2004
VLDLR and apoER2	*in vitro*	TRL regulation	Poirier *et al.*2008 Shan *et al.* 2008
CD81	*in vitro*	Hepatitis C virus receptor. RSV, Rhino virus & VSV receptor.	Labonté *et al.* 2009
VLDLR	*in vivo*	TRL regulation	Roubstova *et al.* 2011
LRP-1	*in vitro*	Numerous regulation	Canuel *et al.* 2013
NPC1L1 and CD36	*in vitro*	Intestinal lipid transport regulation	Levy *et al.* 2013
CD36	*in vivo*	Intestinal lipid transport regulation	(unpublished data)
apoB	*in vivo*	Raises ApoB production	Ouguerram et al. 2004
Lp(a)	Observational	Lower levels with mAb PCSK9	Desai et al. 2013

Source indicated in each. mAb: monoclonal antibody. RSV: respiratory syncytial virus. VSV: vesicular stomatitis virus. TRL: TG-rich lipoproteins.

One study has linked the GOF *PCSK9* phenotype to an increase in ApoB production. ApoB overproduction would drive hepatic VLDL production, as seen in fasted mice (Lambert *et al.* 2006). However, this is secondary to intracellular depletion of lipids and activation of SREBP (Ouguerram *et al.* 2004, Park *et al.* 2004, and Lanlanne *et al.* 2005). Consistent with this, LOF in *PCSK9* was associated with low LDL-C and lowered production of apoB in primary hepatocytes after *PCSK9* silencing (Rashid *et al.* 2005).

PCSK9 is upregulated by SREBP in response to a low intracellular lipid pool and also by statin treatments and downregulated by PPARα agonists (e.g., fenofibrate)

(Lambert *et al.* 2006). Additional regulation of *PCSK9* is believed to be mediated by the hepatocyte nuclear factor 1α (HNF1α). PCSK9 has been documented to undergo a Furin (or PC5/6A) cleavage inactivation at position 218 (human nomenclature) of the catalytic domain (Benjannet *et al.* 2006, Essalmani *et al.* 2011) (**Figure. 9**). One study had indicated that circulating furin-cleaved PCSK9 is capable of regulating LDLR but less efficiently than the intact PCSK9 (Lipari *et al.* 2012). However, the complete role of the furin-cleaved PCSK9 form is not known.

3.3. PCSK9 animal models

Hypercholesterolemia and atherosclerosis (Transgenic models) - The *PCSK9* gene sequence is conserved in many species including; chimpanzee, rhesus monkey, mouse, rat, chicken and zebrafish (http://www.ncbi.nlm.nih.gov/). However, adenoviral-mediated expression of *PCSK9* was first demonstrated in mice models and these models resembled an intermediate *LDLR* knockout phenotype (Maxwell *et al.* 2004). The effects of PCSK9 are mostly exerted in a paracrine fashion as shown by earlier parabiosis experiments (Lagace *et al.* 2006). As expected, transgenic mice expressing PCSK9 are viable, fertile and develop severe hypercholesterolemia. *Pcsk9(Tg)* mice on an *Apoe*(KO) background display an increase in plaque size as well as accelerated atherosclerosis when maintained on a regular diet. However, a protective effect was found when crossing *Apoe*(KO) mice with *Pcsk9*(KO) mice (Denis *et al.* 2012). Advance calcified atherosclerotic plaque takes place in *Pcsk9(Tg)* mice to a lesser degree (and takes longer to occur) in comparison to *Ldlr*(KO) mice (Awan *et al.* 2011) (**Figure 11. A, B and C**):

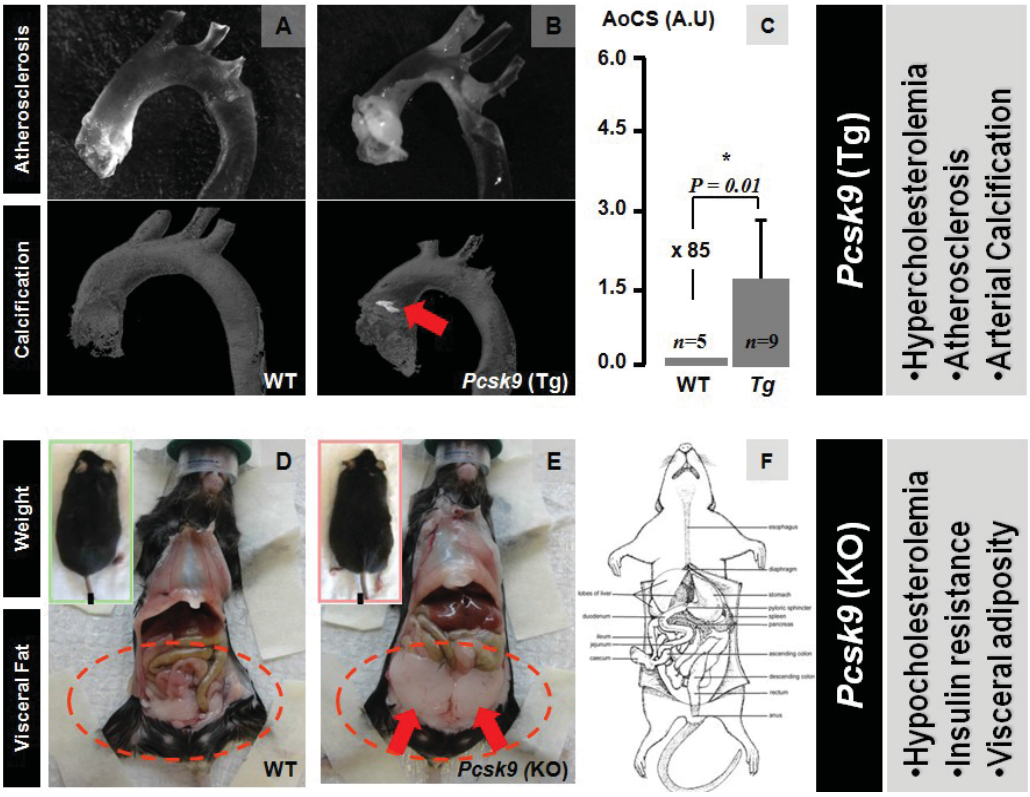

Figure 11: PCSK9 animal models. (A) and (B) are representative wild type (WT) and *Pcsk9* transgenic (Tg) mice; respectively. Aortas are seen after fine dissection (top panel), and a corresponding 3D reconstructed image by micro-CT (bottom panel); Atherosclerosis and aortic calcification are seen in *Pcsk9(Tg)* but not WT after 12 months on a Western diet (Arrows indicate site of calcification). (C) Aortic calcium score (AoCS) histogram of WT mice compared to *Pcsk9(Tg)* mice. (D) and (F) are representative WT and *Pcsk9*(KO) mice; respectively. Abdominal incision is made to expose the perigonadal fat. Visceral adiposity is seen in *Pcsk9*(KO) but not in WT at 6 months. Arrows indicate site of perigonadal fat. (C) Diagram showing the site of abdominal incision (adapted from Awan *et al* 2011, Roubtsova *et al.* 2011 and candidate private collection).

Finally, hypercholesterolemia and atherosclerosis were recapitulated in a pig model of a PCSK9 gain-of-function (GOF) mutant. Following liver-specific expression of human GOF p.D374Y, pigs displayed reduced levels of hepatic LDLR, impaired LDL clearance, severe hypercholesterolemia and eventually developed spontaneous progressive atherosclerotic lesions that could be visualized by non-invasive imaging. Based on these observations several conclusions were made: first, an inhibitor of PCSK9 would be a potential therapeutic modality; second, the use of these models will advance the translational research aspect of treating atherosclerosis from animals to human (Al-Mashhadi *et al.* 2013).

Hypocholesterolemia and fat metabolism (Knockout models) - One of the standard experiments to understand the function of a given gene is to delete the gene in question from the genome and observe the consequences of such deletion. Knocking down PCSK9 in zebrafish resulted in loss of hindbrain-midbrain boundaries and embryonic death in less than 4 days (Poirier *et al.* 2006) hence the earlier name NARC1. This result signified that PCSK9 is essential in some lower vertebrates, but not in mice or humans. To further investigate the effect of PCSK9 inhibition, conditional knockout (KO) of the *Pcsk9* gene have been generated in mice on a C57BL/6 background, as previously described (Zaid *et al.* 2008). Total inactivation of *Pcsk9* by disrupting the proximal promoter and exon 1 led to a viable and fertile mouse with severe hypocholesterolemia (Zaid *et al.* 2008). A mouse with a total *Pcsk9* gene (KO) is apparently normal with a 40% drop in total cholesterol and 80% drop in LDL-C. Total and liver specific (KO) mice exhibit a ~42% and ~27% decrease in circulating cholesterol, respectively. This observation confirmed that PCSK9 produced in the liver was responsible for more than two thirds of the phenotype (Zaid *et al.* 2008). By examining the outcome of crossing multiple mouse models, it is now believed that most circulating PCSK9 comes from the liver as seen from total KO expressing *PCSK9* in the liver which recovers 80-90% of wild type mice PCSK9 levels (Roubtsova *et al.* 2011). Some data suggested that local and not circulating PCSK9 regulates cholesterol levels, because mice did not reach the total cholesterol levels observed in *Ldlr*(KO) mice. In *Pcsk9*(KO) mice, LDLR accumulates at the hepatocyte surface as determined by immunohistochemistry (IHC) (Roubtsova *et al.* 2011), leading to more LDL clearance from the blood and lower cholesterol levels. However, with lipidomic analysis of lipid subspecies, *Pcsk9* inactivation or (KO) changes the composition of lipid classes in comparison to wild type (WT) mice. KO mice express a higher level of plasma sphingomyelin and ceramide, known risk factors for coronary artery disease (Jänis *et al.* 2013).

As mentioned above, PCSK9 may target other receptors. VLDLR is up-regulated in perigonadal depots of *Pcsk9*(KO) mice, specifically females. As a consequence, adipocyte tissue becomes hypertrophied after the excessive free fatty acid (FFA) internalization due to the accumulation of VLDLR in the adipocytes of these *Pcsk9*(KO) mice (Roubtsova *et al.* 2011) (**Figure 11. D, E and F**). Conversely, plasma triglycerides were slightly increased in *Pcsk9*(KO) males (+35%; not significant) and females (+46%;

P=0.03). Furthermore, *in vivo* studies had shown that PCSK9 deficiency was associated with a 2-fold decrease in postprandial triglyceride levels, suggesting improved TG clearance and a role for PCSK9 in TG metabolism (Le May *et al.* 2009). PCSK9 is thus essential in fat metabolism as it maintains high circulating cholesterol levels via hepatic LDLR degradation, but at the same time restricts visceral adipogenesis possibly via adipose VLDLR degradation (Roubtsova *et al.* 2011).

3.4. PCSK9 in extrahepatic tissue

Adipocytes and muscles - PCSK9 is expressed in many tissues, but abundantly in liver, small intestine, and kidneys (Seidah *et al.* 2003, Zaid *et al.* 2008). However, the majority of PCSK9 effects on cholesterol are exerted by circulating levels produced by the liver. Since human PCSK9 targets human VLDLR *ex vivo* (Poirier *et al.* 2008) and binds mouse VLDLR *in vitro* (Shan *et al.* 2008), PCSK9 has the capacity to target human VLDLR *in vivo*. Indeed, visceral fat accumulates in *Pcsk9*(KO) mice (**Figure 11. D, E and F**) due to the high uptake of chylomicrons and VLDL-C in tissues that predominately express VLDLR (such as adipose tissue, heart and muscles). Lack of PCSK9 increases the surface expression of VLDLR and this facilitates triglyceride hydrolysis and free fatty acid uptake in the visceral adipose. In theory, skeletal muscles and striated muscles of the heart should be affected too, but muscles burn fat continuously while adipose tend to store energy in the form of fat droplets for later usage. Thus, LOF PCSK9 variants in humans are suggested to affect postprandial lipemia, which further establishes the association between serum PCSK9 and lipids (Mayne *et al.* 2013). However, the capacity of LOF subjects to clear fat in their adipose could be different from individual to individual, such that visceral fat may be metabolically harmful to some. At least in mice, *Pcsk9*(KO) was not associated with cytokine induction due to visceral adiposity (unpublished data).

Adrenals and kidney - The adrenals are metabolically active organs like the liver and thus require synthesis and uptake of cholesterol for proper function. It was found that by blocking Annexin A2, a natural inhibitor of PCSK9 (discussed below), plasma PCSK9 levels double and LDLR levels decrease by a 50% in extrahepatic tissues such as adrenals and the colon.

This translates to higher LDL-C and suggests a potential cholesterol regulatory role yet to be identified in the adrenals (Mayer *et al.* 2008). In addition, transgenic mice expressing human *PCSK9(Tg)* in the kidney showed degraded LDLR in the liver. However, adrenal LDLR levels were not affected, and the authors attributed this to poor PCSK9 retention in adrenals. Nevertheless, human *PCSK9* expression in these mice increased the hepatic secretion of apoB-containing lipoproteins in an LDLR-independent fashion and thus a mutual regulation of PCSK9 by the liver and the kidney is thought to exist (Tavori *et al.* 2013). Interestingly, silencing PCSK9 using locked nucleic acid (LNA) led to an adverse kidney toxicity (van Poelgeest *et al.* 2013), thought to be technically related. However, given the accelerated renal disease, one would not rule out an alternative hypothesis by which LNA may have mediated an acute cholesterol influx in the proximal convoluted tubules of the kidney. This is especially relevant given that a phase I clinical trial did not report kidney complications (Fitzgerald *et al.* 2013).

Small and large intestine - Findings from examination of cellular and tissue expression in rat, mouse and human showed that not only are the liver and cerebellum rich in PCSK9, but so are the intestines, especially the ileum (Seidah *et al.* 2003). The relationship between the small intestine and PCSK9 was recently discussed, as well as its implication in trans-intestinal reverse cholesterol excretion (TICE). It is possible that PCSK9 regulates LDLR at the intestinal surface the same way it does in hepatocytes. However, *Ldlr(*KO) mice exhibited an increase in intestinal LDL uptake, contrary to what is observed in the liver, and tended to have higher TICE independent of the LDLR. The authors suggested that there might be at least 2 mechanisms contributing to TICE, one involving LDL receptor and a second unknown mechanism. More experiments are needed to explain some of the missing links in the TICE model (Le May *et al.* 2013). Interestingly, the phenotype of the *AnxA2*(KO) mouse suggests that AnxA2 acts as a natural regulator of LDLR degradation, primarily in extrahepatic tissues and especially in the intestine (Seidah *et al.* 2012).

Pancreas - It was reported that PCSK9 was expressed in pancreatic delta-cells and that PCSK9 co-localized with somatostatin in human pancreatic islets. In one study, changes in insulin secretion were not seen in young *Pcsk9*(KO) mice under glucose challenge (Langhi *et al.* 2009). Later studies have showed that compared to controls,

older male *Pcsk9*(KO) mice express more LDLR in insulin-producing β-cells, secreted less insulin and were hyperglycemic as well as glucose-intolerant.

In addition, *Pcsk9*(KO) mice islets exhibited signs of malformation, apoptosis and inflammation (Mbikay *et al.* 2010). In view of these discordant reports, it is thought that circulating PCSK9 may be necessary to protect the pancreatic islets by limiting the role of LDLR in internalizing excessive LDL-C. Therefore, a concern is that by losing this protective mechanism, lipotoxicity-induced diabetes may occur when PCSK9 inhibitors are given (discussed below) (Cnop *et al.* 2002, Roehrich *et al.* 2003, Ishikawa *et al.* 2008). Thus, more experiments are needed to address this issue.

3.5. PCSK9 in other pathological conditions

Liver regeneration - Due to the wide expression of PCSK9, investigators are currently examining the role of PCSK9 in many organs and in different pathological conditions. Given that PCSK9 is mainly produced by the liver, any liver condition is expected to influence the production of PCSK9. Studies in mice have shown that conditional knockout in livers have led to delay in liver regeneration and formation of liver lesions (Zaid *et al.* 2008). The regenerating liver in *Pcsk9*(KO) mice exhibited necrotic lesions but when a high-cholesterol diet was supplemented after partial hepatectomy, the lesions where absent indicating an indirect role of PCSK9 in hepatic regeneration (Zaid *et al.* 2008). This data indicate that upon hepatic damage, patients lacking PCSK9 could be at risk. However, under normal circumstance lipid accumulation in hepatocytes of these mice was markedly reduced under both regular and high-cholesterol diets. This indicates that PCSK9 deficiency confers resistance to liver steatosis (fatty liver disease) (Zaid *et al.* 2008). So, PCSK9 inhibition protects against steatosis, but puts one at risk if the liver receives an insult (liver surgery).

Insulin resistance - Adult mice deficient in PCSK9 exhibit impaired glucose tolerance and may be at risk for diabetes mellitus as stated above (Mbikay *et al.* 2012). However, the mechanism is largely unknown. In one population study, it was shown that *PCSK9* LOF p.R46L variants have a significant increase in markers of insulin resistance: namely, insulin and HOMA-IR (homeostasis model assessment of insulin resistance) in individuals carrying apoE2 isoforms in comparison to other apoE isoforms (Awan *et al.* 2013). This indicates that there is an *APOE* and *PCSK9* gene interaction that regulates

insulin resistance. There is additional evidence that another *PCSK9* LOF p.Q152H induces insulin tissue resistance (Unpublished data).

On the contrary, when rats were made diabetic by streptozotocin (STZ) injection, qPCR and Western blot analyses showed that there was an 81% reduction in hepatic LDLR mRNA but no change in LDLR protein (Niesen *et al.* 2007). Interestingly, PCSK9 protein levels were decreased to almost undetectable levels and there was a 72% reduction in *PCSK9* mRNA levels. This indicates that the rate of LDL receptor recycling is impaired in diabetic rats and thus the diabetic induced dyslipidemia.

Cancer metastasis - PCs are known to play a significant role in tumor progression, notably furin (PCSK3) and PACE4 (PCSK6) (Bassi *et al.* 2000). Furthermore, growing cancer cells need a constant source of nutrition to survive and lipids in particular to meet the need to form new cell membranes. A tissue *PCSK9* expression analysis in tumor vs. normal tissue was conducted to select candidate malignancies associated with PCSK9 activity. It was found that *PCSK9* is upregulated in human primary renal cell carcinoma, various head and neck cancers, germ cell tumors, esophageal adenocarcinoma and cervical cancer, but not elevated in breast carcinoma or in prostate carcinoma (unpublished data). Intrasplenic injection of B16 melanoma cells to recipient *Pcsk9*(WT) and *Pcsk9*(KO) mice revealed more than a 50% decrease in cellular invasion of cancer in the absence of PCSK9, a protection that was lost upon feeding the mice with a high fat diet, which enhanced cancer spread (Sun *et al.* 2000). Thus, the absence of PCSK9 can be protective against melanoma invasion at least in animal models and could open the door to novel applications of PCSK9 inhibitors/silencers in cancer/metastasis. Of note, it was reported in a thesis report that *PCSK9* expression was significantly downregulated in hepatocellular carcinoma (HCC) and associated with poorer survival after transplantation (Bhat *et al.* 2010). This may imply that more aggressive HCC tumors alter their local microenvironment by modulating *PCSK9* expression, thus enabling energy supply to fuel tumor growth. These findings, if confirmed with mRNA studies, could have interesting therapeutic implications.

Viral infections - Most infectious hepatitis C virus (HCV) particles interact with a number of putative HCV receptors, including CD81, scavenger receptor class B type I (SR-BI) and claudin-1 (Andre *et al.* 2005, Meredith *et al.* 2012). Circulating HCV particles are associated with VLDL and LDL of infected patients, suggesting that LDLR is critical

for hepatic viral infection. Recently, it was shown that liver expression of CD81 is markedly increased in *Pcsk9*(KO) mice and that the HCV receptor CD81 protein is downregulated independently of LDLR (Labonte *et al.* 2009). Therefore, it was proposed that the plasma level and/or activity of PCSK9 could modulate HCV infectivity in humans. In summary, LDLR and CD81, two HCV entry receptors are dose-dependently down-regulated by PCSK9, resulting in the reduction of the cellular infectivity of HCV in mice. Although never shown in human, PCSK9 has the potential to protect humans against HCV. Thus, caution must be exercised when administering PCSK9 inhibitors to subjects that could be potentially infected with HCV or other viruses that use one or more of the LDLR superfamily members as entry receptors (Finkelshtein *et al.* 2013).

Inflammation - Pcsk9 gene expression was upregulated in a concentration-dependent manner *in vitro* after incubation with oxidized LDL (ox-LDL) (Wu *et al.* 2012). In addition, ox-LDL upregulated the expression of inflammatory cytokines; interleukin-1α (IL-1α), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα) in macrophage precursor cells that also exhibited an ox-LDL dose-dependent upregulation in *PCSK9* expression (Tang *et al.* 2012). In mice, lipopolysaccharide (LPS)-induced inflammation, stimulates *Pcsk9* expression (Feingold *et al.* 2008). High levels of PCSK9 accelerate atherosclerosis by promoting inflammation, endothelial dysfunction, and hypertension by mechanisms independent of the LDLR pathway (Urban *et al.* 2011). Furthermore, *Pcsk9(Tg)* mice develop advanced atherosclerosis (Denis *et al.* 2012) and aortic calcification (Awan *et al.* 2011). However, under stimulation, *Pcsk9(Tg)* mice induce half the expression of interleukin-1β (IL-1β) seen in total *Ldlr*(KO) mice, possibly contributing to the intermediate atherosclerosis phenotype seen in *Pcsk9(Tg)* compare to *Ldlr*(KO) mice (Chapter 6). Whether such inflammation is related to the amount of accumulated ox-LDL or PCSK9 levels in these models is not clear. In contrast, conflicting data exists in *Pcsk9*(KO) mice. Liver specific *Pcsk9*(KO) that are resistant to melanoma invasion exhibit higher levels of the inflammatory cytokine TNFα (Sun *et al.* 2012), while total *Pcsk9*(KO) mice accumulate visceral fat without a change in TNFα (unpublished data). It must be emphasized that mice and human do not always exhibit similar inflammatory responses, as evidenced in a recent human study. In the latter, both *PCSK9* LOF and *APOE* LOF significantly elevates insulin and leptin levels, underscoring chronic inflammation of adipose tissue (Awan *et al.* 2013). Thus, further research is needed to

better understand the tissue- and species-specific association of PCSK9 with inflammation (Urban *et al.* 2011).

3.6. PCSK9 physiological variations

Variation in PCSK9 measurements - Before discussing the physiological variations in PCSK9 levels, it is important to note that PCSK9 exhibits a wide interindividual variation (Dubuc *et al.* 2010). Therefore, levels vary considerably among human subjects and exhibit a skewed distribution toward higher values in both men and women (Dubuc *et al.* 2010). However, the absolute values are mainly dependant on variations in the detection methods and the type of antibody used (Cariou *et al.* 2011) (**Table 5**).

Table 5: Physiological condition associated with PCSK9 levels
Exhibits a diurnal rhythm (parallels cholesterol biosynthesis) (Persson *et al.* 2012)
Exhibits a pubertal rhythm (growth hormone secretion) (Baass *et al.* 2009)
Exhibits stability over time as a biomarker (during hormonal stability) (Awan *et al.* 2012)
Exhibits a right-word skewed distribution (Lakoski *et al.* 2009, Awan *et al.* 2012)
Higher in familial hypercholesterolemia (LDLR dependent mechanism) (Huijgen *et al.* 2012)
Higher with certain drugs: statin, fibrate (SREBP, PPARa) (Dubuc *et al.* 2004, Kourimate *et al.* 2008)
Higher in females than males (suggesting sex hormonal regulation) (Awan *et al.* 2012)
Higher in premenopausal than postmenopausal women (central regulation) (Lakoski *et al.* 2009)
Higher in pregnancy and lower in cord blood (parallels cholesterol demand) (Peticca *et al.* 2013)
Higher postprandial (suggesting insulin regulation) (Costet *et al.* 2006)
Higher with inflammation (LPS stimulation) (Feingold *et al.* 2008).
Lowered by estrogens induction therapy (Persson *et al.* 2012)
Lowered by fasting over 16 hours (Costet *et al.* 2006)
Lowered in response to acute stress (suggesting inflammation mechanism) (unpublished data)

Source are indicated in each

Variation with age, gender and pregnancy - In a cross-sectional pediatric study of ~1700 subjects, plasma PCSK9 decreased with age in boys, whereas the inverse was true for girls. In girls of the same age, mean TC levels are higher in 9- and 16-year olds than in 13-year olds and there is an inverse effect on mean plasma PCSK9 concentrations. It is believed that this follows a growth hormone pattern (Baass *et al.* 2009). In the multi-ethnic Dallas Heart Study carried out in 3138 patients between 2000 and 2002, plasma PCSK9 levels were significantly higher in women (n=1863) than in men (n=1489). This difference persisted after adjusting for most variables (Lakoski *et al.* 2009). In the same study (n=3138), premenopausal women had considerably higher levels of plasma PCSK9 than postmenopausal women. Estrogen treatment did not significantly affect fasting PCSK9 levels in postmenopausal women. In comparison,

there was no difference in plasma PCSK9 levels in men over 50 years old in comparison to men under 50 years old. Similar findings were obtained in postmenopausal women and older men with a 9-14 % higher level observed in female (Awan *et al.* 2012). Finally, serum PCSK9 levels were increased in pregnancy at term. However, recent data also showed that the human fetus has a ~2-fold lower PCSK9 level than the mother, which may be necessary to provide LDL-C to the growing fetus (Peticca *et al.* 2013).

Variation with cardiometabolic markers - In the paediatric population mentioned above, PCSK9 was associated with fasting insulinemia, which suggests that PCSK9 could play a role in the development of dyslipidemia associated with the metabolic syndrome (Baass *et al.* 2009). There were statistically significant positive associations between PCSK9 and fasting glucose and HOMA-IR (homeostasis model assessment of insulin resistance). PCSK9 levels correlated with multiple demographics, lipid and carbohydrate metabolic variables. The highest constant correlation is with total cholesterol and LDL-C. However, PCSK9 has a positive correlation between TC, LDL-C, apoB, and infrequently TG, and a weak correlation with HDL-C in most population studies. The next highest correlation after cholesterol and LDL-C is with triglycerides, suggesting a link to the metabolism of triglyceride-rich lipoproteins. The correlation with serum glucose, insulin and HOMA raises the possibility of a causative role of PCSK9 in the metabolic syndrome (Baass *et al.* 2009). In a randomized trial, variations among those taking statins was wide, nevertheless a significant relationship was observed between the magnitude of LDL-C reduction and the increase in PCSK9 concentrations. This relationship was seen in both an analysis of quintiles of LDL-C reduction and when the LDL-C change was treated as a continuous variable (Chapter 4).

Variation with diet and medication - It was found that a 5-week Mediterranean diet in normal subjects can lower plasma LDL-C and PCSK9 by as much as ~10% and ~15%, respectively (Richard *et al.* 2011). Moreover it was shown that PCSK9 transcription could be suppressed by fasting and induced by insulin, likely by activating liver X receptor and SREBP (Costet *et al.* 2006). Like the *LDLR* gene, *PCSK9* is also regulated by intracellular sterol depletion and with statins. Thus, statins, including atorvastatin, upregulated LDLR and *PCSK9* mRNA expression in hepatic cell lines

(Dubuc *et al.* 2004). This suggested that statins would have a higher capacity to decrease LDL-C if not for the associated rise in PCSK9 level following statin treatment.

Indeed, statin treatment of *Pcsk9*(KO) mice results in a greater reduction in LDL-C than WT mice, signifying a hypersensitive state (Rashid *et al.* 2005). This was confirmed in individuals with FH harboring the common R46L LOF variant that seems to be more responsive to statins (Berge *et al.* 2006). While statins directly increase *PCSK9* mRNA expression, PPARα agonists, such as fibrates, indirectly affect *PCSK9* expression through modulation of cholesterol levels (Mayne *et al.* 2008). In a randomized trial, while statins were shown to increase the levels of circulating PCSK9, the cholesterol absorption inhibitor ezetimibe had no effect on PCSK9 (Berthold *et al.* 2013). Finally, in a study where women underwent *in vitro* fertilization, high estrogens resulted in a reduction in VLDL, LDL and PCSK9 levels (Persson *et al.* 2012). However this might have been an effect of the 3-fold increase in growth hormone (GH) as a response to induction of ovulation. Thus, GH my negatively impact PCSK9 to favour increased uptake of circulating cholesterol by growing cells.

3.7. PCSK9 genetic variations

Family studies of coronary heart disease patients led to the mapping of *PCSK9* gene to FH (Abifadel *et al.* 2003). This was supported by GOF mutations exhibiting high serum levels of LDL-C. In addition, the LOF mutations were found to lower LDL-C and protect against coronary heart disease (Cohen *et al.* 2005). Until now, one compound heterozygote LOF mutation and one homozygote mutation have been reported (Zhao *et al.* 2006, Hooper *et al.* 2007). One of the individuals harbouring these mutations, a 32-year-old fertile apparently healthy female, had been described with undetectable circulating PCSK9 resulting in an eighty percent reduction in LDL-C (Zhao *et al.* 2006). She is now 40 and all eyes are on her hoping that she will provide human confirmation that lowering lipids is better and PCSK9 inhibition is a safe modality. The function of PCSK9 is to enhance the elimination of LDLR, thus *PCSK9* genes that increase the expression or affinity toward LDLR lead to cholesterol accumulation and vise versa. Gain or loss of function mutations have been reported in all domains of *PCSK9*.

A continuously updated list of all natural mutations of *PCSK9* is found at (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/index.php?select_db=PCSK9).

Variations in determining PCSK9 levels - By screening the *PCSK9* gene in individuals with the FH phenotype, but negative for *LDLR/APOB* mutations, FH causes attributed to *PCSK9* GOF mutations are estimated to be 2% of all FH patients (Abifadel *et al.* 2003, Seidah *et al.* 2009). However, since the LDLR is the major route for PCSK9 uptake, its circulating levels are also higher in FH patients with a defective LDLR mutations, which may contribute to the wide spectrum of FH and the variation seen in PCSK9 levels (Huijgen *et al.* 2012). Plasma PCSK9 levels are increased not only in FH but also in Familial Combined Hypercholesterolemia (FCHL) patients (Brouwers *et al.* 2013). *PCSK9* GOF mutations were associated with increased severity of coronary atherosclerosis in patients with polygenic hypercholesterolemia (Chen *et al.* 2005). These findings suggest that variations in the *PCSK9* gene may alter the lipid clinical phenotype of patients having FH.

After the first report of *PCSK9* gene implementation in FH in 2003, several additional mutations were reported. Residents in Japan, US and Finland were among the first to confirm mutations associated with FH and higher levels of PCSK9 (**Figure. 12**). However, not all *PCSK9* mutations were associated with LDL-C changes in large cohorts (Kotowski *et al.* 2006). One of the first mutations identified was p.D35Y and later p.L108R with moderate changes in PCSK9 levels. Both mutations occur in the PCSK9 prosegment and thus it is thought to enhance the interaction of the prosegment of PCSK9 with the beta-barrel domain of the LDLR (Benjannet *et al.* 2012). However, as a general rule, the higher the PCSK9 levels the more it is indicative of a GOF and FH (e.g., p.L108R, p.S127R, p.F206L, p.R218S, p.D374Y, p.R469W, p.L474V, p.R496W, p.H553R) and lower PCSK9 levels are more indicative of a LOF and hypocholesterolemia (e.g., insLeu 10L, p.R46L, p.G106R, p.Y142X, p.Q152H, p.Q219E, p.C679X) especially when the mutation is a nonsense mutation that leads to a truncated protein. The p.C679X mutation introduces a stop codon and this leads to a misfolded protein that fails to exit the ER (Peterson *et al.* 2008). The p.R46L is a common LOF mutation most likely due to an alteration in phosphorylation of an adjacent serine (Dewpura *et al.* 2008). LOF mutations are more frequent than GOF mutations; the carrier rate of p.R46L in French-Canadians is higher (4.8%) than the general population

(2.4%), perhaps to counter the founder effect of FH gene enriched in this genetic pool (Chapter 3). p.R46L is the second most common variant in the French-Canadian population. However, the most common *PCSK9* LOF mutation in the French-Canadian population is the insertion of an extra leucine (L10) in the SP region with a carrier rate of 24%. Many *PCSK9* mutations were recently summarized (Davignon *et al.* 2010) and presented in **Figure 12**:

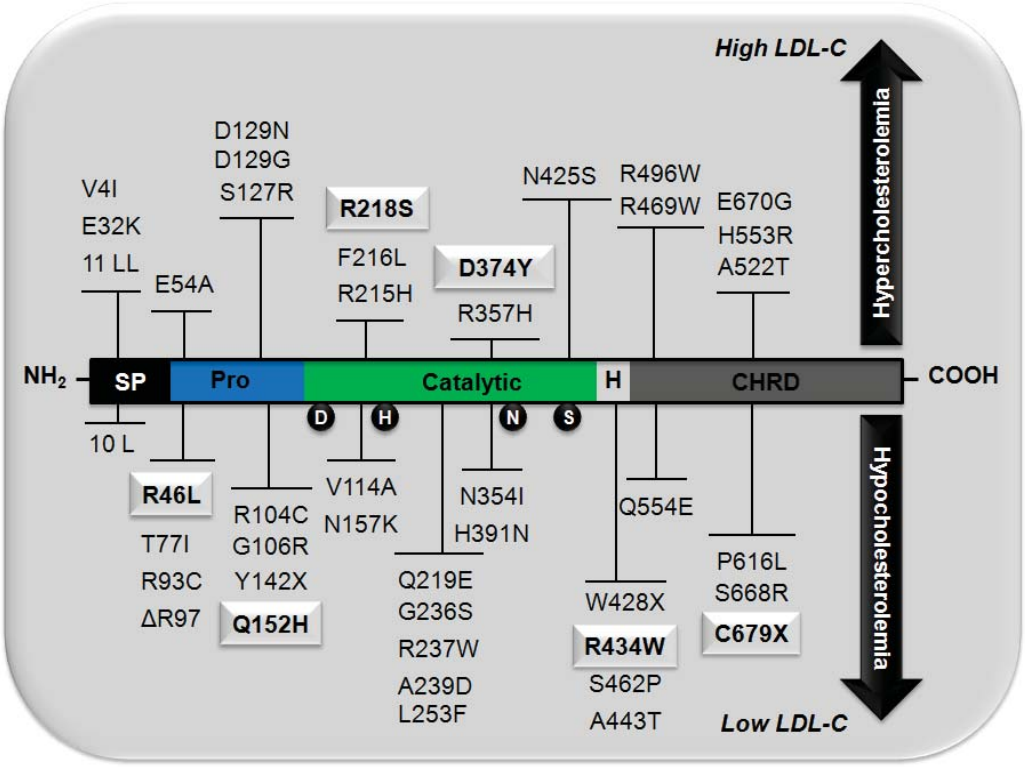

Figure 12: The PCSK9 protein and mutations impacting plasma LDL-C level. The primary structure and domains of PCSK9 (SP: signal peptide, pro: pro-segment domain, catalytic: catalytic domain, H: hinge region and CHRD: Cysteine-Histidine rich domain). Key residues, aspartic acid (D), histidine (H), asparagine (N) and serine (S) are depicted. 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 (adopted from Davignon *et al.* 2010).

Finally, *PCSK9* GOF and LOF mutations are synergistic and additive in their final impact on the cholesterol phenotype (Zhao *et al.* 2006). Furthermore, *PCSK9* and *LDLR* gene mutations exert an additive effect on the FH phenotype. Interestingly *PCSK9* and *APOB* genes were sequenced in the pedigree of 2 subjects with homozygous FH who

were heterozygous for *LDLR* gene mutations. LDL-C levels were ~50% higher in double heterozygotes than those found in simple heterozygotes for two *LDLR* mutations (Pisciotta *et al.* 2006). Therefore, rare missense mutations in *PCSK9* may worsen the clinical phenotype of patients carrying *LDLR* mutations. On the other hand, *APOE* genotype will influence the lipid phenotype of *PCSK9* mutations as suggested in Chapter 3, underscoring the importance of gene-gene interaction in *PCSK9.*

Variations with exceptional mechanism - Some mutations allowed a better understanding of the biosynthesis and secretion of PCSK9 biology and are worthy of highlighting (**Figure. 12**). (i) The French-Canadian LOF p.Q152H mutation prevents the autocatalytic processing of proPCSK9, resulting in a dominant negative form of the protein that in a heterozygote state reduces the circulating levels of PCSK9 and LDL-C by as much as ~80% and ~50%, respectively (Mayne *et al.* 2011). This mechanism was later confirmed following an exhaustive analysis of all possible Gln152 mutations (Benjannet *et al.* 2011). (ii) Intriguingly, the GOF p.R218S mutation significantly decreases PCSK9 catabolism allowing it to circulate longer and negatively impact LDLR to promote high cholesterol (Allard *et al.* 2005). This is likely due to the resistance of this mutant for furin inactivation (Benjannet *et al.* 2006). Conversely, the LOF p.A443T (Kotowski *et al.* 2006) mutation likely results in a novel PCSK9 O-glycosylation site that favours furin-induced degradation of the protein (Benjannet *et al.* 2006). (iii) The most severe Anglo-Saxon GOF mutation p.D374Y (Timms *et al.* 2006), which despite lower circulating levels results in a 10-25 fold higher binding affinity of PCSK9 to the LDLR (Cunningham *et al.* 2007). (iv) A few variations in the PCSK9 have also been reported in the hinge region (**Figure. 12**), for example the LOF p.R434W mutation results in lower secretion levels of PCSK9 and reduced circulating LDL-C levels, likely due to a negative effect on the folding of the protein in the ER (Dubuc *et al.* 2010). (v) On the opposite side, the *LDLR* GOF p.H306Y mutation in the EGF-A domain associated with PCSK9 binding, results in an enhanced PCSK9-mediated cellular degradation (McNutt *et al.* 2009).

3.8. PCSK9 therapeutic approaches

Berberine (BAR) is an alkaloid compound that decreases PCSK9 gene expression by reducing HNF1α mRNA levels (Cameron *et al.* 2008, Li *et al.* 2009). In addition, berberine also increases *LDLR* mRNA stability (Abidi *et al.* 2005) and based on these properties it has been proposed that it could be used as a monotherapy or in combination with statins to treat hypercholesterolemic patients (Kong *et al.* 2004, Cameron *et al.* 2008). In an animal model of LPS-induce inflammation, BAR treatment reduced the expression of hepatic PCSK9 and decreased the plasma levels of TC, TG, LDL-C, IFNγ, TNFα and IL-1α while increasing HDL-C level and *LDLR* expression (Xiao *et al.* 2012). Thus, both lipid and inflammatory lowering effect are demonstrated by Berberine therapy.

Oncostatin M (OM) is a pleiotropic cytokine that induces *LDLR* gene transcription resulting in an enhanced LDL uptake in liver cells and a reduction of plasma LDL-C as seen in hypercholesterolemic hamsters (Cao *et al.* 2011). With OM, a rapid increase in *LDLR* mRNA levels occurs while *PCSK9* mRNA steadily decreases in treated cells. Using siRNA, it was possible to identify a cytokine-triggered regulatory network for *PCSK9* expression implementing JAKs signaling pathway. Therefore, OM is a proinflammatory cytokine (IL-6 family) that activates *LDLR* transcription through binding to transcription factors on a sterol-independent regulatory element of the *LDLR* promoter. However, lowering PCSK9 and inducing inflammation is not a suitable therapeutic approach in the context of CVD protection.

Annexin-A2 (AnxA2) binds the C-terminus of PCSK9 protein identified by our group (Mayer *et al.* 2008). AnxA2 was strongly expressed in lungs, aorta, heart, adrenals and small intestine. Immunofluorescence staining demonstrates a partial co-localization of PCSK9 with AnxA2 at the plasma membrane of cells *in vitro*. AnxA2 inhibits PCSK9-induced LDLR degradation and peptides from the R1 domain of AnxA2 act as PCSK9 inhibitors. Expression analysis showed that surface LDLR doubles in *AnxA2*(KO) mice while decreasing by 50% in extrahepatic tissues such as adrenals and colon that translated to higher LDL-C. This effect occurs without significant changes in VLDLs or HDLs, thus AnxA2 peptides block the PCSK9≡LDLR interaction and may represent a promising therapeutic approach (Mayer *et al.* 2008). One possible mechanism is through binding the CHRD domain of PCSK9 and inducing a conformational change in PCSK9

such that the interaction with the LDLR is compromised. This suggested allosteric model is supported by the observation that blocking CHRD, using a monoclonal antibody (mAb), can also inhibit ~50% of PCSK9-induced LDLR degradation. Indeed, a human AnxA2 V98L variant was associated with reduction in PCSK9 both in healthy and hypercholesterolemic subjects treated with statins (Seidah *et al. 2012*).

On a separate note, companies are trying to develop drugs using the knowledge gained from *PCSK9* LOF mutations. Monoclonal antibodies, antisense approaches and small molecules are among the approaches to modulate PCSK9. As previously mentioned, PCSK9 favors the post-translational degradation of the LDLR, therefore decreasing its capacity to lower LDL-C. This makes PCSK9 a promising therapeutic target (Seidah *et al.* 2007) and several approaches to inhibit PCSK9 are in active clinical or preclinical trials (**Figure. 13**):

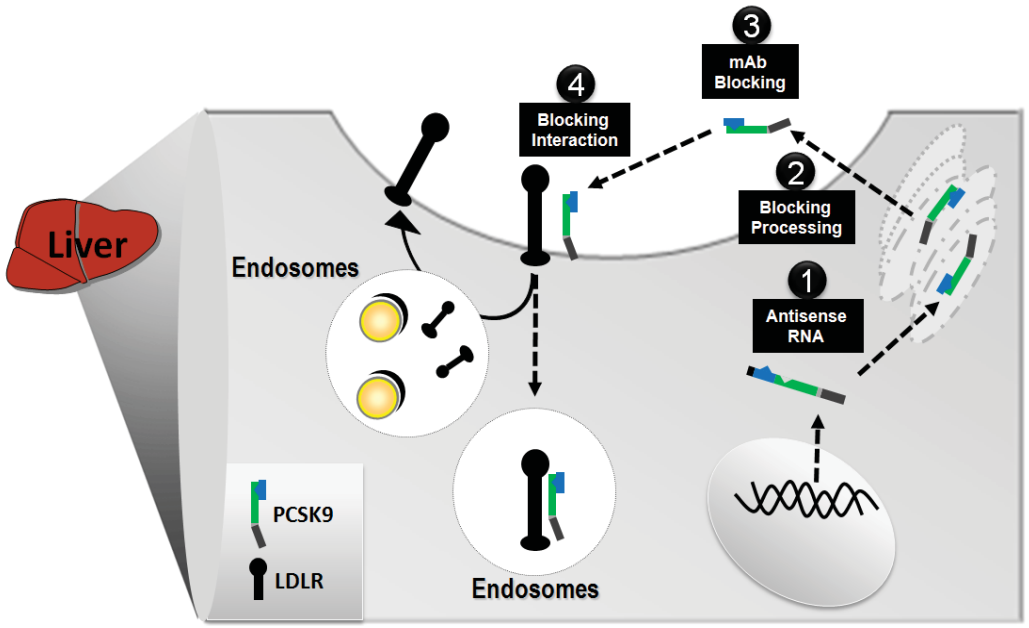

Figure 13: Novel pharmacological approaches to treat hypercholesterolemia. After the cholesterol particle binds LDLR, the PCSK9 can also bind the LDL receptor thus redirecting the complex to lysosomal degradation compartment and prevented the recycling of the LDLR. If an internalized LDL receptor is not bound to PCSK9, the receptor is recycled back to the cell surface, where it continues to remove LDL-C from the blood. A number of companies are developing compounds that suppress PCSK9 activity, the numbers in black circles represent the following approaches: (1) Inhibit PCSK9 expression (Antisense drugs: LNA-ASOs and LNP or siRNAs). (2) Inhibit PCSK9 self autocleavage and secretion (Small molecules inhibitors). (3) Neutralizing circulating plasma PCSK9 activity (mAbs or enhance inactivation of furin). (4) Disrupt PPIs and inhibit PCSK9 binding to the LDLR surface (small domain or EGF-A mimetic), (Source candidate private collection, adopted from Seidah and Prat 2012).

The best approach to date has been monoclonal and polyclonal antibodies. Monoclonal antibodies (mAb) targeted against PCSK9 showed remarkable cholesterol lowering in monkeys as well as an amazing 80% reduction in LDL-C levels for more than a week (Chan *et al.* 2009). Furthermore, there is a synergistic effect of mAb and statins on the reduction of LDL-C levels. This suggests that a PCSK9 mAb in combination with a statin would lead to a greater reduction of hypercholesterolemia (Chan *et al.* 2009).

Indeed, phase I and II clinical trials in humans have been conducted by many pharmaceutical companies including Sanofi/Regeneron and Amgen. In phase I trials, safety and efficacy were confirmed. In phase II trials, both companies report LDL-C reductions varying between 60-70% upon subcutaneous injection of ~140-150 mg of the mAb every 2 weeks, with no significant elevation in liver enzymes (Seidah 2013, Stein 2013). However, the reduction is dependent on the frequency and dose. **Table 6** summarizes some of the clinical trials outcomes:

Table 6: Selected PCSK9-targets in development

Approach	Company	Stage	Comments
mAb-REGN727 (SAR236553)	SA-Regeneron	Ph 3 Odyssey	~22,000 subjects injected (by 2018)
mAb-REGN727 (SAR236553)	SA-Regeneron	Ph 2	40-72% LDL-C↓ vs 17% Atorvastatin alone
mAb-AMG145	Amgen	Ph 2/3	Subcut. injections/4 wks; 43-55% LDL-C↓ at wk 12
mAb-RN316 (PF-04950615)	Pfizer-Rinat	Ph 2	Completed
LGT209	Novartis	Ph 2	Completion: 2015
RG7652	Roche/Genentech	Ph 2	Completion: 2015
mAb	Merck	In develop.	WO-patent appl. 2010
Ad. BMS-962476	BMS-Adnexus	Ph 1	Completion: 2013
siRNA	Alnylam	Ph 1	Cationic lipidoid formula
Small molecule	Shifa Biomedical Corp	Pre-Clinical	In preparation for Ph 1
	Serometrix	In develop.	
	Neurochem/Amorchem	In develop.	

LDL-C, low-density lipoprotein cholesterol; LNA, locked nucleic acid; mAb, monoclonal antibody; PCSK9, proprotein convertase subtilisin/kexin 9; siRNA, small interfering RNA (adopted from Seidah and Prat 2012*).*

Conversely, the antisense small interfering RNA (siRNA) (Frank-Kamenetsky *et al.* 2008) and locked nucleic acid (LNA) approaches had less successful starts (Gupta *et al.* 2010). Phase I clinical trials with siRNA targeting PCSK9 have been recently published with encouraging efficiency in healthy volunteers (Fitzgerald *et al.* 2013). On the other hand, locked nucleic acid antisense oligonucleotides (LNA ASO) that target both human and mouse PCSK9, though efficient in reducing PCSK9 (Gupta *et al.* 2010), cause kidney toxicity and this has resulted in LNA-targeted PCSK9 treatment to be terminated from further clinical trials (van Poelgeest *et al.* 2013).

Finally, the development of conventional small-molecules to inhibit PCSK9 continues to present challenges (Hedrick *et al.* 2009). None of the published therapeutic anti-PCSK9 approaches used a small-molecule inhibitor, possibly due to the relative flatness of the PCSK9≡LDLR interface (Seidah *et al.* 2012).

The approach that will move to routine clinical application will depend on long-term safety data (phases III clinical trials), ease of administration and price (Davignon *et al.* 2010). While injections are not particularly attractive for long-term treatment, such treatments would likely be embraced by patients suffering from side effects with current lipid lowering agents or in high risk subjects striving to achieve lower LDL-C (Anderson *et al.* 2013, Stone *et al.* 2013). For example, homozygous FH patients, generally have initial LDL-C levels around 3-4 times higher than those of the general population. Thus, they are usually unable to achieve a 50% reduction using available oral agents and thus require LDL apheresis, a form of dialysis eliminates LDL-C from the blood (Sachais *et al.* 2005).

In summary, with the discovery of PCSK9 in 2003 the lipid field took a sharp turn, with PCSK9 inhibitors becoming an unquestionable therapeutic reality. The molecular basis for PCSK9 action supports a model in which PCSK9 is self-cleaved, secreted, and tightly bound to the EGF-A like domain of LDLR. This reduces LDLR recycling, and down-regulates LDLR activity, thereby increasing the levels of LDL-C in the blood. Thus, PCSK9 plays a key role in cholesterol homeostasis. Humans with *PCSK9* GOF mutations leading to high levels of PCSK9 and increased levels of plasma LDL-C are at significantly increase risk of CVD. Humans with LOF mutations have reduced levels of plasma PCSK9 and LDL-C and are at a significantly lower risk of developing a CVD during their lifetime.

In addition, PCSK9 exhibits pleiotropic metabolic effects that need to be further explored. Statin therapy results in increased plasma PCSK9 levels but lower overall LDL-C levels. This suggests that lowering PCSK9 levels may enhance the efficacy of statins to reduce LDL-C. Animal models have proven invaluable to screen a new drug modality, as atherosclerosis and vascular calcification are enhanced in *Pcsk9(Tg)* mice and reduced in *Pcsk9*(KO) mice. In humans, loss of one copy of *PCSK9* prevented 88% of cardiovascular events in the ARIC trial that followed more than 3000 individuals over 15 years. Thus, PCSK9 is a clear target for the development of new lipid lowering therapies. Long-term studies will establish whether the beneficial effects of PCSK9 inhibition on LDL-C levels directly translates into safe and effective CVD risk reduction.

Despite its apparent safety, there are concerns about the inhibition of PCSK9, since we still know little about its global physiological functions. Nevertheless, it has been such a long time since the discovery of statins; that a new avenue should come about to substantially reduce cholesterol levels. The realization that a complete LOF *PCSK9* mutation and/or inhibition of plasma PCSK9 results in very low cholesterol levels, suggests that PCSK9 inhibitors could be the next blockbuster drug to combat hypercholesterolemia, which would be a harbinger of things to come (Hall 2013). The outcomes of the multiple ongoing phase III clinical trials using PCSK9 mAbs are expected to be made public in 2016-2018, and are surely going to be revealing in terms of the wider use and safety of plasma PCSK9 inhibition (Sheridan *et al.* 2013).

4. Familial Hypercholesterolemia and Calcified Atherosclerosis

Given national guidelines mandating strict targets for LDL-C, the treatment of lipoprotein disorders has been one of the most successful therapeutic measures in the prevention and treatment of CVD (Anderson *et al.* 2013, Stone *et al.* 2013). The analysis of large-scale clinical trials using statins has led to an impressive decrease in cardiovascular events, cardiovascular mortality and total mortality, with no substantial side effects. The decrease in CVD appears to be related to dose and duration of treatment (Baigent *et al.* 2005). However in FH subjects this reduction in mortality has not necessarily translated into disease-free life expectancy. Indeed, even with combined lipid regimens and drastic LDL-C reduction using extracorporeal LDL removal (LDL-apheresis), patients develop premature and severe aortic valve and aortic wall calcification (Awan *et al.* 2008) (**Figure. 2**).

Generally, there are inhibitory and stimulatory factors that influence calcification in vessels and these are summarized in Chapter 5. These factors are not directly targeted by lipid lowering drugs, and thus they need to be addressed separately. In brief, vascular calcification is the net result of loss of inhibitory factors and the addition of stimulatory ones. The co-morbidities and mechanisms associated with this condition are further discussed in Chapter 5.

Currently there is no effective therapy targeting vascular calcification that does not also negatively impact the skeletal system (Fantus *et al.* 2013). Raising HDL-C has long been associated with CVD protection. However, using Mendelian randomization, the causal relationship between HDL-C and CVD has recently been called into question (Frikke *et al.* 2010; Voight *et al.* 2012). Thus, HDL-C may be considered more as a marker of cardiovascular health despite its strong epidemiological association with MI (Di Angelantonio 2009), and the biological plausibility linking HDL-C to cardiovascular health. Furthermore, there is little evidence that raising HDL-C pharmacologically with fibrates (Jun *et al.* 2010), niacin (Boden 2011, HPS2-THRIVE 2013) or the two inhibitors of cholesteryl ester transfer protein (CETP), torcetrapib and dalcetrapib, leads to an improvement in cardiovascular health and vascular calcification.

As previously discussed, a new class of lipid lowering agents, the PCSK9 inhibitors, has the potential to further lower LDL-C. A significant reduction in LDL-C levels has been reported with mAbs in phase 1 and 2 studies in healthy volunteers and

in subjects with FH or non-FH (Stein *et al.* 2012). Large scale phase 3 clinical trials are also underway to test the hypothesis that further LDL-C lowering with PCSK9 inhibitors will improve cardiovascular health. The agents being studied include evolocumab (FOURIER trial), alirocumab (ODYSSEY trial) and bevacizumab. However, their effect on vascular calcification is still unknown.

In spite of the impressive lowering of MI events attained with lipid lowering agents, there remains significant “residual risk” in the prevention and treatment of cardiometabolic disease. Meta-analysis of statin trials showed a 21% reduction in risk of CVD per mmol of LDL-C (Baigent *et al.* 2010). Furthermore, a search for other modifiable factors has led to novel targets in the atherosclerosis process. Indeed, a systematic review of available evidence determined that chronic inflammatory diseases result in a greater risk of developing CVD. However, further research of prospective cohort studies are needed to better quantify this risk (Roifman *et al.* 2011).

Nevertheless, inflammation is critical to the development of atherosclerosis. While conventional cardiovascular risk factors, such as elevated cholesterol, are causal in atherosclerosis, the mechanism by which cholesterol induces calcification is mediated to a great degree by the immune system. Elegant work by Duwell *et al.* has shown that excess cholesterol is taken up by macrophages that then elicits an inflammatory reaction, mediated by the release of interleukin-1 (IL-1β) (Duewell *et al.* 2010), and leading to an amplification of the immune response cascade (Maritnon *et al.* 2002). IL-1β and IL-6, together with other systemic inflammatory mediators, such as tumor necrosis-alpha (TNFα), are released into circulation, leading to hepatic production of C-reactive protein (CRP), a marker of systemic inflammation (Harris *et al.* 1999). Furthermore, work by our group has shown that the osteogenic Wnt pathway is implicated in calcified lesions in *Ldlr*(KO) mice (Awan *et al.* 2011); ox-LDL and inflammatory cytokines are hypothesized to be upstream to this pathway.

The strong association between CRP as a marker of systemic inflammation and CVD has been tested in multiple epidemiological studies and meta-analysis. These studies show that high sensitivity CRP is a graded and independent biomarker of cardiovascular risk. However, the causal link between CRP and atherosclerosis has been called into question by animal studies failing to support a pro-atherosclerotic role for CRP. Likewise, Mendelian randomization experiments have cast doubt on the causal role of CRP in atherosclerosis. Thus, the potential role of CRP in CVD is still a matter of

debate (Koening *et al.* 2013). Nevertheless, the use of CRP to identify patients at increased cardiovascular risk of recurrent cardiovascular events is the basis in launching the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER trial) (Ridker *et al.* 2008).

In JUPITER, 17,800 men (over 50 years of age) and women (over 60 years of age), with elevated CRP levels, but levels of LDL-C that did not warrant lipid-lowering therapy according to the standard of care at the time, were given either 20 mg of rosuvastatin (a potent statin) or a placebo. Two years after trial commencement, the Data and Safety Monitoring Board recommended that the study be halted due to the overwhelming benefit of rosuvastatin in patients. Patients in the 20 mg rosuvastatin treatment group had a 50% decrease in LDL-C and a 37% reduction in CRP, leading to a 44% reduction in CVD primary end-point, with no heterogeneity of treatment benefit in any sub-group analyzed (Ridker *et al.* 2008). This overwhelmingly positive result was reflected in updates of several national guidelines recommending more aggressive lipid lowering regimens (Anderson *et al.* 2013, Stone *et al.* 2013). However, the results of JUPITER questioned whether the benefits of rosuvastatin were due solely to the reduction in LDL-C or due the reduction in CRP. The trial was not designed to answer this apparently simple question, but offered a novel hypothesis that could be tested in large-scale, appropriate clinical trial (discussed later). In Chapter 4, we study blood samples and DNA from a subset of this population to draw associations between LDL-C, PCSK9 and many cardiometabolic markers including the inflammatory marker CRP.

4.1. Atherosclerosis and inflammation

The pathogenesis of atherosclerosis and inflammation is complex and many excellent reviews have been published on this subject (Libby *et al.* 2013). Furthermore, chronic inflammation and many of the cytokine mediators are considered inducers of vascular calcification (Chapter 5). This is why atherosclerotic soft plaques may stabilize, shrink and become less susceptible to rupture in response to statins, yet calcified plaques do not regress and the course of calcification is intimately linked to inflammation (Sia *et al.* 2009) (**Figure. 14**).

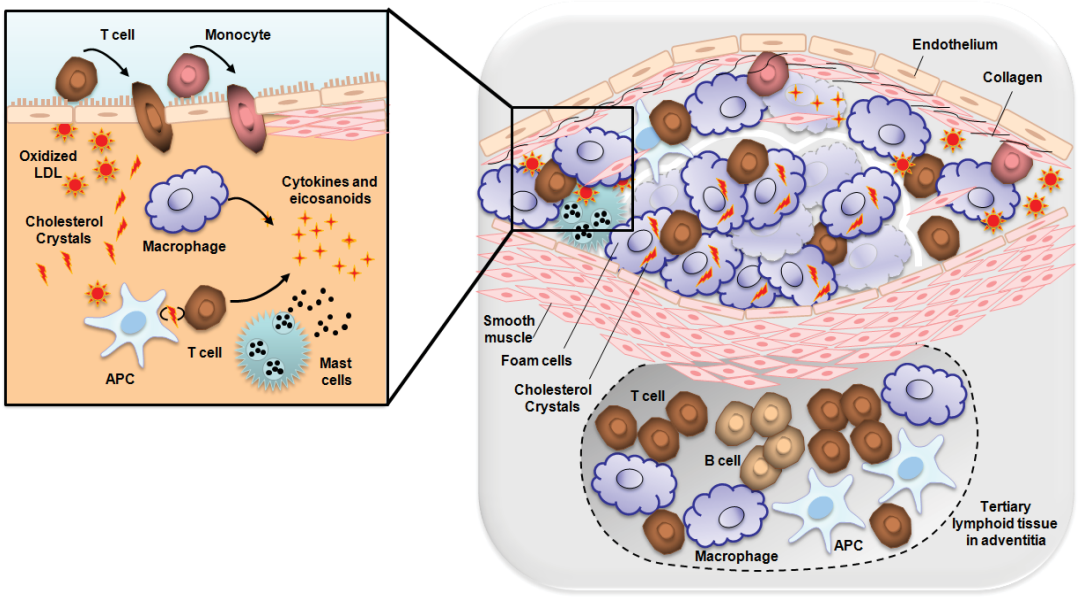

Figure 14: The immune cells populating the atherosclerotic plaque. The atheroma has a core of lipids, including cholesterol-crystals, apoptotic and living cells and a fibrous cap with collagen and smooth muscle cells. Excess lipoproteins accumulate in the subendothelial space. Several types of cells of the immune response are present throughout the atheroma including (1) T cells, (2) monocyte/macrophages, (4) APC and DCs and (3) mast cells. The plaque builds up in the intima, while cells of the immune system also accumulate outside advanced atheroma and may develop into tertiary lymphoid tissue with germinal centers. APC: antigen-presenting cell, DC: dendritic cells (adopted from Hansson and Klareskog 2011).

Arterial inflammation in response to stressors (cholesterol-crystals, cigarette-derived vascular toxins, protein modification by diabetes, and shear stress due to elevated blood pressure) leads to endothelial dysfunction. The hallmark of endothelial dysfunction is the expression of vascular cell adhesion molecules (VCAM) and monocyte chemotactic protein-1 (MCP-1). These proteins will attract antigen-presenting cells (APC), monocytes and dendritic cells (DC) to the arterial intima. In turn, these cells will differentiate into macrophages under the influence of macrophage colony stimulating factors and lead to the formation of foam cells together with more inflammation, cytokine release and T-cell recruitment. Unstable vulnerable plaques may rupture and cause platelet activation and thrombosis, leading to vessel obstruction in vital organs, such as the heart (MI), peripheral blood vessels (limb gangrene) and the brain (strokes). Interestingly, even in healthy aortas of children, the intima is continuously under surveillance by T cells and macrophages that have the capacity to express IL-1, IL-6 and TNF when they are induced (Kishikawa *et al.* 1993). Some studies have documented

endothelial activation, inflammation and premature atherosclerosis in children with FH phenotype (Guardamagna *et al.* 2009) (**Figure. 15**).

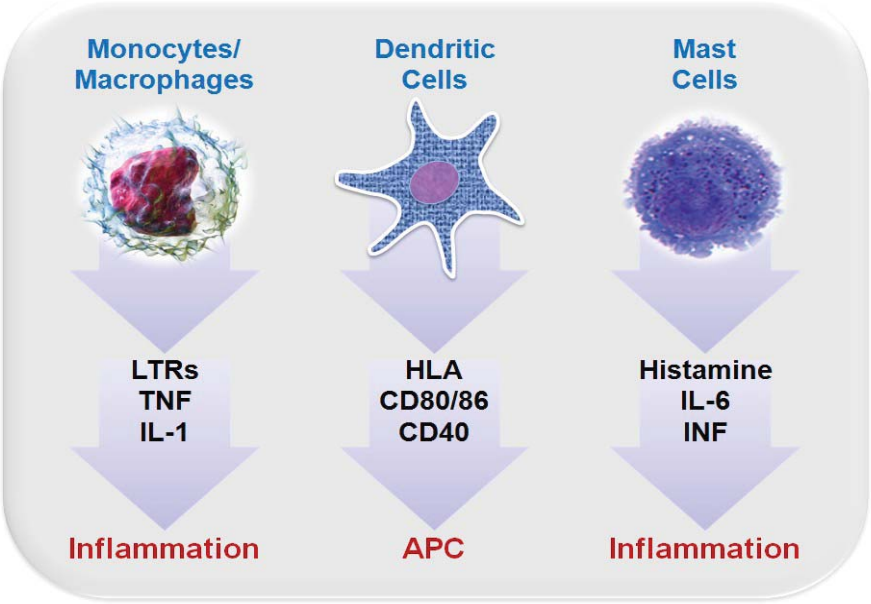

Figure 15: The functions of cells participating in atherosclerosis. The figure outlines the functions attributed to various cellular components in atherosclerosis. Mononuclear phagocytes represent the first line of the innate immune defenses. They give rise to macrophages which form foam cells, the hallmark of the arterial fatty streak. Inflammatory cells express high levels of Toll-like receptors (TLR), and release high levels of the cytokines TNF and IL-1. Dendritic cells present antigens to T cells, linking innate to adaptive immunity. Mast cells release many mediators as shown. Recent data support a causal role for mast cells in mouse atherosclerosis (adopted from Libby *et al.* 2009).

The oxidized LDL particles that intrude into the subendothelial space as a result of endothelial dysfunction are sensed and engulfed by macrophages and may trigger the expression of immune mediators. Under control of innate immunity, mononuclear phagocytes, mast cells, platelets, granulocytes, and the complement cascade contribute to amplifying the immune response and promoting the growth of atherosclerotic plaques (Libby *et al.* 2013) (**Figure. 15**). In the adaptive cellular immune response, T- and B-cells, antigen-presenting DCs, along with humoral immunity, exert modulatory effects on atherosclerosis. The interplay between human genetic susceptibility and response to environmental stressors additionally modulates adaptive and innate immunity. One of the best studied stressors is cholesterol elevation.

Current data implicates cholesterol crystals as being an initiating factor in the release of IL-1β through the activation of the NOD-Like Receptor family Pyrin domain containing 3 (or NLRP3) inflammasome (Duewell *et al.* 2010; Rajamäki *et al.* 2010). In innate immunity, NLRP3 inflammasomes are large, cytosolic multimeric protein complexes that connect metabolic stress sensors with the downstream proteolytic cleavage of prointerleukin-1β into bioactive IL-1β. Inflammasomes are thus responsible for the activation of inflammatory processes, and have been shown to induce cell pyroptosis (Church *et al.* 2008) (**Figure. 16**):

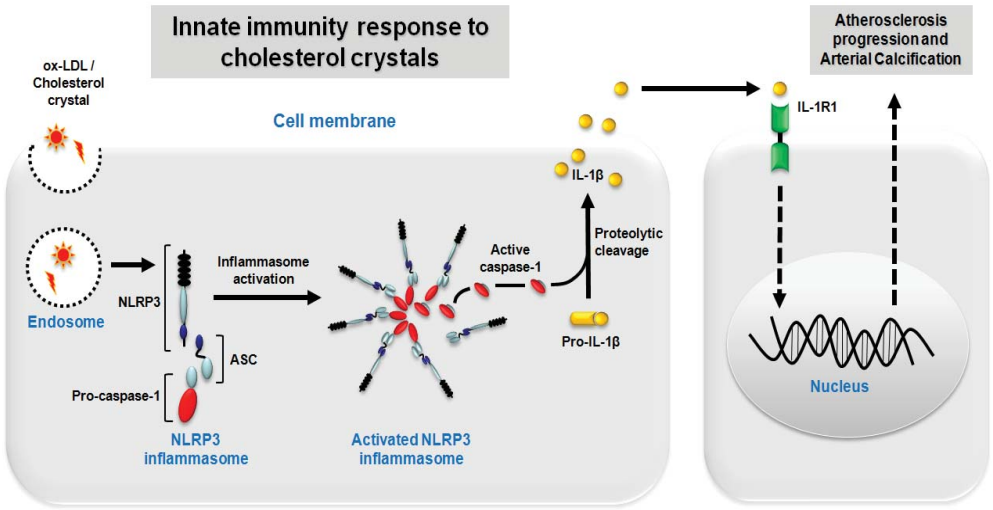

Figure 16: Innate immune response to cholesterol crystals. Cholesterol crystals represent an endogenous danger signal, are engulfed by macrophages in the subendothelial layer of the arterial wall and activate the NLRP3 inflammasome pathway, leading to the cleavage-activation of pro-IL-1β and its release in plasma. IL-1β acts as a proximal amplification to evoke more inflammation, atherosclerosis progression and promote arterial calcification. Subsequent steps leading to dysregulation of local mineralization are postulated, which includes activation of the Wnt/LRP5/β-catenin pathway of osteoblast differentiation. This establishes a link between inflammation, atherosclerosis initiation and progression of vascular calcification. Anti-IL-1β mAb selectively targets this critical step (adopted from Rader 2012).

The identification of individuals with autosomal dominant gain-of-function (GOF) mutations in the *NLRP3* gene associated with autoinflammatory process has supported the effort to develop orphan drugs inhibiting IL-1β (Walsh *et al.* 2009). The targeted inactivation of IL-1β decreases atherosclerosis in a mouse model of atherosclerosis; a double *Apoe*(KO) / *IL-1β*(KO) fed a high cholesterol diet (Kirii *et al.* 2003). In addition,

lack of IL-1β diminishes the burden of atherosclerosis in *Apoe*(KO) mice while prolonged treatment with IL-1β promotes arterial intimal thickening in the porcine coronary arteries (Shimokawa *et al.* 1996).

Furthermore, IL-1 has been implicated in atherothrombosis and plaque rupture in several pre-clinical and clinical studies (Alexander *et al.* 2012). Interleukin-1β in particular plays an essential role in triggering an inflammatory reaction to injury and mediating smooth muscle differentiation *via* the actions of TNFα on the Wnt canonical pathway (Church *et al.* 2008). In addition, genetic strategies may help identify unknown key players in inflammation in humans. GWAS studies and newer deep sequencing data have led to the identification of genetic variants in immune mediators and inflammatory responses that help to determine the severity of the atherosclerosis. The most recent CARDIoGRAMplusC4D Consortium GWAS offered a network analysis that implicated inflammation as one of the pathways most strongly associated with human CVD (Deloukas *et al.* 2013). Furthermore, recent genetic studies provide robust data linking the IL-6 receptor to coronary heart disease (Sarwar *et al.* 2012). These data provide a strong rational for targeting inflammatory pathways to narrow the gap in the residual risk associated with CVD beyond lipid lowering.

4.2. Atherosclerosis prevention using immunomodulation

Herein, I will discuss the advantages and disadvantages associated with the use of inflammation modulation (anti-inflammation) from the literature as well as ongoing major clinical trials that will likely change the nature of atherosclerosis prevention. From many immunomodulation methods, I will rationalize the selection of one that I will test in a drug-placebo animal experiment to test the hypothesis that immunomodulation can lower the degree of calcification seen in FH (more details in Chapter 6).

Evidence for Corticosteroids - In asthmatic patients, corticosteroids (CS) inhibit VCAM-1 induced by TNFα during the exacerbation of their disease. This suppression may contribute to their anti-inflammatory effect in bronchial epithelial cells in humans; however, this effect could not be extended to the vascular endothelium (Atsuta *et al.* 1999). Moreover, the long term beneficial effect of inhaled CS was only validated in asthmatic patients and not in cardiovascular patients (Camargo *et al.* 2008). However, in

mice high-dose CS for post MI use was found to exert cardiovascular protection through a novel mechanism involving the rapid, non-transcriptional activation of endothelial nitric oxide synthase (eNOS). Binding of CS to the glucocorticoid receptor, induces nitric oxide dependent vasorelaxation via eNOS activation and decreases vascular inflammation and myocardial infarct size (Hafezi-Moghadam *et al.* 2002).

Despite these interesting results, earlier reports suggested that CS may in fact promote atherosclerosis. In the early 1970s, Kalbak predicted the long-term outcome of CS on vascular calcification of the tibial artery as seen on X-ray films (Kalbak *et al.* 1972). Furthermore, the incidence of arteriosclerosis in patients with rheumatoid arthritis (RA) receiving long-term CS was elevated and arterial calcification was common and resembles Mönckeberg's sclerosis with little intimal occlusion. Many years later, long term treatment of coronary artery disease with high doses of CS was clearly associated with adverse effects on the cardiovascular system in the form of delayed myocardial scar formation post MI (Sholter *et al.* 2000). Moreover, in a systemic inflammatory disease like Systemic Lupus Erythematosus (SLE), aortic valve calcification was associated with CS use (P = 0.049) and the use of methotrexate (P = 0.0174) (Kiani *et al.* 2006). Furthermore, it was found that rheumatoid factor (RF)-positive but not RF-negative patients are at increased risk of CVD events following exposure to CS. These findings suggest that CS interacts with RF status to modulate the occurrence of an event in patients with RA (Davis *et al.* 2007). In a nested case-control analysis of MI in a cohort of 17,700 patients with RA and 3,001 patients with noninflammatory rheumatic showed that CS increased the risk of diabetes and hypertension as well as increasing the overall risk of MI (Wolfe *et al.* 2008). Finally, a study showing a dose-dependent increase in CVD risk with the chronic use of CS in RA patients, confirmed the futility of CS in cardiovascular protection (Greenberg *et al*. 2011). Therefore, there are no active clinical trials examining the role of CS in CVD.

Evidence for Methotrexate - Methotrexate (MTX) competitively and reversibly binds to dihydrofolate reductase preventing the conversion of tetrahydrofolate precursor to the co-factor necessary for DNA and RNA synthesis, arresting the cell cycle in S phase. MTX has been used for decades as an anti-cancer agent, as an immunomodulator in rheumatological and autoimmune skin diseases and as an agent to

terminate ectopic pregnancies. A narrow therapeutic index makes MTX notorious for its side effects. A systematic review of the effects of MTX on CVD in patients with RA concluded that its use was associated with a reduced CVD, suggesting that MTX not only improves disease-specific outcomes but concomitant atherosclerosis (Westlake *et al.* 2010). Importantly, in a large study of 10,156 RA patients treated with a variety of TNF antagonists, MTX and Disease-Modifying Antirheumatic Drug (DMARD), only TNF inhibitors were associated with a reduction in cardiovascular risk events. Interestingly, MTX was not associated with a reduced risk and CS use was associated with a dose-dependent increased risk as mentioned above (Greenberg *et al.* 2011). However, a systematic review and meta-analysis of evidence of relations of MTX with CVD occurrence were performed in patients with chronic inflammation (Micha *et al.* 2011). MTX was correlated with a 21% lower risk for CVD and the authors suggested that a direct treatment of inflammation may reduce CVD risk. Given its profound effect as a broad immunosuppressant, the National Heart, Lung, and Blood Institute (NHLBI) launched the cardiovascular inflammation reduction trial (CIRT) (http://clinicaltrials.gov - NCT01594333). This is a randomized clinical trial investigating whether taking low-dose MTX reduces heart attacks, strokes, or death in people with either type 2 diabetes or metabolic syndrome (known to be associated with vascular calcification) that have had a heart attack. The estimated study completion date is December 2018 (**Table 7**).

Table 7: Immunomodulation approaches to prevent residual CVD risk .

Approach	Trials name (Agent used)	Patients type (Ph; trial phase)	Effect on lipids and heart (Possible side effect)
Inhibit DNA synthesis	CIRT/NIH (Methotrexate)	2nd prevention (Ph 3 ends in 2018)	↔ **LDL-C** (Bone marrow suppression, Neutropenia, Infections)
Receptor antagonist	Kineret (IL-1R antagonist)	2nd prevention (Ph 3)	↑ **LDL-C, Heart failure** Injection site reactions, Infections, Malignancy
mAb antibody	Escape/Amgen (mAb TNFα)	1ry prevention (Ph 3 ends in 2015)	↑ **LDL-C, Heart failure** (Injection site reactions, Infusion reactions, Heart failure, Neutropenia, Infections, Demyelinating disease, Cutaneous reactions and Malignancy)
	Roche (mAb IL-6)	Post MI (Ph 3 ends in 2013)	↑ **LDL-C** (Injection site reactions, Infusion reactions, Infections, Liver function, Neutropenia and Diarrhea)
	CANTOS/Novartis * (mAb IL-1β)	previous MI (Ph 3 ends in 2016)	↔ **LDL-C** (Injection site reactions, Infections)

* CANTOS uses canakinumab, an FDA approved drug for the treatment of cryopyrin-associated periodic syndromes (source FDA reports and http://www.ehealthme.com accessed December 2013).

Evidence for Tumor necrosis factor alpha - TNFα or cachectin is a cytokine that induces cytolysis of certain tumors and acts as a potent pyrogen causing fever directly or via IL-1β stimulation. TNFα is synthesised as a pro-TNFα with a long and atypical signal peptide, which is absent from the mature and active cytokine (Sherry *et al.* 1990). Both the mature protein and a partially cleaved one are secreted into the circulation (Cseh *et al.* 1989). Many animal models, as well as some initial clinical trials, have suggested that downregulation of TNFα may improve cardiac performance. Soluble TNF receptor lessens myocardial inflammation, but myocardial thickening was not reversed in this model (Kubota *et al.* 2000). Subsequent to coronary artery ligation, etanercept (mAb TNFα) or intravenous immunoglobulin attenuate expression of genes involved in post MI remodeling in rats. This suggests that early immunomodulation post MI may prevent later development of heart failure (Gurantz *et al.* 2005). In addition, the previous study with 10,156 RA patients treated with a variety of TNF inhibitors, CS and MTX; demonstrated that only TNF antagonist were associated with a reduction in CVD (Greenberg *et al.* 2011).

On the other hand, preliminary results from a placebo-controlled study using TNFα inhibitors suggest no effect, or even an adverse effect of TNFα inhibitor therapy on mortality and hospitalization. In a three group study of RA patients that were treated with TNFα inhibitor, MTX and placebo, TNFα inhibition failed to completely restrain the inflammatory process and may promote atherogenesis (Cuchacovich *et al.* 2009). In a cross-sectional study of 23 patients with RA taking TNFα inhibitor therapy, inflammatory markers were non-better than classical risk markers, suggesting TNFα inhibitors could not be used alone in CVD prevention (Sandoo *et al.* 2012). Post MI, etanercept was shown to reduces systemic inflammation, but to increase platelet activation. This suggests that a TNFα inhibitor is unlikely to be a beneficial therapeutic strategy in patients with acute MI (Padfield *et al.* 2013).

Though a bit disappointing, these adverse effects do not necessarily dismiss the immunomodulation hypothesis. These results only underline the difficulties in selecting approaches that can modulate the cytokine network in beneficial ways to combat CVD. The route of TNF inhibition may be indicative of successfulness depending on how fast they reach the vascular bed. A significant decreased risk in MI in psoriasis subjects using etanercept subcutaneously compared to treatment with topical agents (Wu *et al.* 2013).

Currently, there is a model K/BxA (g7) mouse that recapitulates the human phenotype seen in RA, namely inflammatory arthritis followed by atherosclerosis. This new animal model may help identify the inflammatory component that is involved in the development of atherosclerosis without hindering the immune system (Rose *et al.* 2013).

Importantly, targeted use of TNFα inhibitors may be associated with the development or exacerbation of heart failure (HF). TNFα inhibitors that were tested in patients with moderate to severe heart failure did not demonstrate any clinical benefits and suggested that etanercept may adversely affect the course of the disease (Anker *et al.* 2004, Mann *et al.* 2011). Concern about this possible adverse effect stems from randomized clinical trials testing TNFα inhibitors as a potential therapy for HF and from postmarketing surveillance data gathered by the FDA (Ding *et al.* 2010). Given the evidence to date, patients with symptomatic HF should be treated with strategies other than TNFα inhibitors. In a patient who develops HF while on a TNFα inhibitor, a drug-induced cause should be suspected, and the use of the medication should be suspended (**Table 7**). Importantly, TNFα inhibitors have been associated with either the development or the exacerbation of neurologic disorders associated with demyelination, such as multiple sclerosis. However the true nature of this association has not been established (Pasumarthy *et al.* 2009). TNFα inhibitors should be discontinued immediately in patients suspected to have demyelination. At present, a study is being conducted in psoriasis patients using etanercept to determine whether less cardiovascular inflammation is attributed with the use of the drug (http://clinicaltrials.gov - NCT01522742).

Evidence for Interleukin 6 – IL-6 is a multifunction cytokine responsible for stimulating acute phase protein synthesis, as well as the production of neutrophils in the bone marrow. It is antagonistic to regulatory T-cells while it supports the growth of B-cells. Counter intuitively, IL-6 can also attenuate inflammation through its inhibitory effects on TNFα and IL-1, as well as activation of the IL-1 receptor antagonist (IL-1Ra) and IL-10 (Steensberg *et al.* 2003). IL-6 is secreted by macrophages involved in the atherosclerotic plaque in response to specific antigens, referred to as pathogen-associated molecular patterns (PAMPs) such as sodium monourate crystals and cholesterol crystals. PAMPs binds to a group of receptors in the innate immune system

named pattern recognition receptors or Toll-like receptors (TLRs). TLRs are present on the cell surface and induce cytokine production. In addition, smooth muscle cells in the tunica media of many blood vessels also produce IL-6 as well as adipocytes, and this may explain the reason why obese individuals have higher circulating CRP induced by IL-6 (Bastard *et al.* 1999).

In a carotid atherosclerotic model, IL-6 was found to destabilize plaques by downregulating a key intracellular enzyme required for synthesis of collagen (Zhang *et al.* 2012). Tocilizumab is a humanized anti-human IL-6 receptor antibody that competes for both the membrane-bound and the soluble forms of the human IL-6 receptor, thereby inhibiting the binding of the native cytokine to its receptor and interfering with its effects. Tocilizumab was shown to be effective in improving endothelial dysfunction and reducing arterial stiffness in RA patients (Protogerou *et al.* 2011, Kume *et al.* 2011). It is therapeutically effective in patients with RA, but adverse events, such as liver function disorders, neutropenia, diarrhea, infection and hyperlipidemia, were observed in clinical trials (**Table 7**) (Kawashiri *et al.* 2011). Drug-induced hyperlipidemia is generally tolerated, however as clinical trials are being conducted in CVD prevention, the current TNFα and IL-6 inhibitors increase LDL-C, and thus the issue of cardiovascular safety needs to be addressed. Recently, it was reported that tocilizumab is being considered in the prevention of CVD (Hingorani *et al.* 2012) and may be useful for the treatment of acute MI (http://clinicaltrials.gov - NCT01491074). The effect of tocilizumab in post MI will be evaluated by CRP, the myocardial necrosis marker troponin T and pro-BNP.

Evidence for Interleukin 1 - Stimulation of the IL-1 receptor type I (IL-1R1) on vascular endothelial cells leads to upregulation of iNOS, endothelin-1, chemokines/cytokines, and adhesion molecules, endothelial and smooth muscle proliferation and macrophage activation. These processes all contribute to endothelial dysfunction and progression of atherosclerosis (Fearon *et al.* 2008). Both IL-1α and IL-1β activate IL-1R1 to evoke a proinflammatory response, whereas IL-1Ra naturally inhibits their binding. IL-1α remains associated with the plasma membrane, whereas IL-1β is fully secreted and can act on IL-1R1 in cells distant from the cell of origin. Thus IL-1β has been thought to be of greater relevance to the human inflammation system.

IL-1Ra: is a naturally occurring glycoprotein inhibitor that can neutralize the IL-1 mediated inflammatory effects. A synthetic IL-1 receptor antagonist (Anakinra) can be used as a biologic DMARD. However, it is administered daily due to its short half life via a subcutaneous (SC) injection and acts through competitive binding with IL-1 for its receptor, but with no receptor signaling activation. It exhibits modest clinical efficacy and a dose-dependent increase in the risk of serious infection in patients with RA compared with placebo. The IL-1Ra:IL-1 ratio is critical in vascular inflammation. In obese patients, IL-1Ra levels are increased (Meier *et al.* 2002), partly secreted by adipose tissue. In this population, IL-1Ra promotes leptin and insulin resistance and weight gain (Meier *et al.* 2002). It was reported that anakinra may improve vascular and left ventricular function in patients with RA, but without coronary disease (Ikonomidis *et al.* 2008). This improvement was related to a concomitant reduction in nitrooxidative stress and endothelin-1 levels. A current study is evaluating whether or not anakinra also improves vascular and left ventricular function in patients with coronary diseases and RA (http://clinicaltrials.gov – NCT 01566201).

IL-1β: is a potent pyrogen that plays a role in the regulation of immune and inflammatory responses to infections or sterile insults. It is synthesised as a pro-IL-1β that needs Caspase-1 for its cleavage/activation. In clinical studies, IL-1β concentrations were found in high levels in atherosclerotic human coronary arteries (Galea *et al.* 1996). More significantly, IL-1β inhibition modulates a proinflammatory mediator that is neither essential for host defense nor critical to innate immunity. Thus, targeted inactivation of IL-1β decreased atherosclerosis in a mouse model of atherosclerosis fed a high cholesterol diet (Kirii *et al.* 2003). As alluded to earlier, a relationship between the earliest deposition of cholesterol crystals and the initiation of inflammation, is likely through the NLRP3 inflammasome. Even though the biological effects of IL-1β are mediated through its own receptor (i.e., IL-1R1), its activation leads to proinflammatory gene expressions similar to that of TNFα activation (Kida *et al*. 2005). In fact, some reports have shown that IL-1β also regulates the TNF receptor (Winzen *et al.* 1993, Hultner *et al.* 2000). In addition, IL-1β may play a role in the expression and shedding of the TNF receptor. One study demonstrated that IL-1β modified TNFα signaling through alterations in TNF receptor mRNA expression (Holtmann *et al.* 1987).

The development of an IL-1β inhibitor was promoted as an orphan drug (Walsh *et al.* 2009) by the European Medicines Agency (Doc. Ref.: EMEA/503722/2009) and thus granted the use of canakinumab (mAb IL-1β) in Cryopyrin-Associated Periodic Syndromes (CAPS), which is a genetic variance of *NLRP3* gene causing autoinflammatory disorders. An extensive program of toxicology studies was performed in marmoset monkeys and conducted with the murine form mAb IL-1β in mice. Using this murine mAb, it was further shown that neutralization of IL-1β does not inhibit the development of a T cell-dependent antibody response induced by immunization in the presence of aluminium hydroxide. Concerning the immune system, there were no differences in counts of lymphocytes and red blood cells, spleen and thymus in mice treated with a mAb IL-1β. Treatment of littermates with mAb IL-1β once a week for 2 month resulted in minimal inflammatory reactions at the sites of injection. These were reversible over a 4 week recovery period. In addition, the pharmacokinetics of mAb IL-1β was determined in mice after single and repeated injections. A dose-dependent increase was observed with a peak approximately 24 h after dosing and the elimination half-life ranged between 1 and 17 days. Male and female fertility was not affected by treatment. This initial safety profile led the way to launch the CANTOS trial (http://clinicaltrials.gov – NCT NCT01327846).

The primary outcome in CANTOS is the first occurrence of a major adverse cardiovascular event (a composite of CV death, non-fatal MI, and stroke). Changes from baseline in carotid plaque burden in the bifurcation region of the carotid artery and changes in insulin secretion rate (ISR) across the yearly visits will be evaluated. This will allow for the detection of new onset type 2 diabetes among patients with pre-diabetes within the 5 year follow-up period (Estimated primary completion date: July 2016). Given the above and the potential beneficial effect of IL-1β inhibition on atherosclerosis, we selected a mAb against IL-1β to test the hypothesis that immunomodulation may prevent aortic calcification. The choice of a mAb against IL-1β was based on the lack of evidence that it induces cardiovascular necrosis and its neutral effect on lipids levels. This we believe will be a direct answer to the question if inflammation is a residual risk in CVD. In Chapter 6, we test the hypothesis that inhibiting the inflammatory process induced by IL-1β will lead to a measurable reduction in vascular calcifications in mice.

4.3. Atherosclerosis immunization:

The future of treatment in atherosclerosis will be based on lipid lowering drugs in combination with anti-inflammatory therapies, as large expensive clinical trials are trying to prove. A more cost-effective alternative approach is training the immune system to self-recognise culprit proteins in the pathogenesis of early inflammation that is involved directly in the atherosclerosis formation (De Jager *et al.* 2011). Historically, the concept of active immunization against atherosclerosis was proposed by Gero *et al* more that 50 years ago using beta-lipoproteins (Gero *et al.* 1959), yet only in the last 10 years has it received attention in the field. Assisting in this interest is the enhanced understanding of the underlying role of immune responses in atherosclerosis. Additionally, a few labs have reported promising results with active immunization using antigenic epitopes found within the apoB-100 sequence to reduce atherosclerosis in an *Apoe*(KO) mouse model (Chyu *et al.* 2005). These proof-of-concept studies have raised the possibility that such an approach might be possible in humans. However, unlike the immunization against a definite pathogen, immunization against ubiquitous antigens in the vascular inflammation process is much harder. This is in addition to the technical challenges with approaches used: (i) active immunization, (ii) passive immunization or (iii) acquired tolerance to an antigen by suppression of cellular and humoral immune response to the introduced antigen formation (Chyu *et al.* 2013).

Additional antigens that have been tested in animal models to reduce atherosclerosis include; modified LDL, homogenates of plaques, functional group on oxidized phospholipid, heat shock protein 60 and cholesterol ester transfer protein (CETP) (Chyu *et al.* 2013). Interestingly, none of these are cytokine-derived antigens. However, the concept of cholesterol crystals initiating the pathogenesis of atherosclerosis may evoke the development of new immunization regiments that provides lifelong CVD protection and may possibly prevent vascular calcification.

Regardless of the antigen of choice, the documented side effects are so far cardiovascular in nature, counter intuitively, driving biomedical researchers away from this field (De Jager *et al.* 2011). As disappointing as this may be, vaccination against atherosclerosis remains a theoretical goal. The development of such vaccination would have a high impact on the future of atherosclerosis management especially as primary prevention.

In summary, the idea that inflammation is central to the development of atherosclerosis has been a fundamental principle of vascular biology, atherosclerosis and preventive medicine. However, therapeutic modulation of inflammation for the prevention and treatment of arteriosclerosis remains largely unproven, even more in vascular calcification. Indeed the use of steroids has been associated with unwanted side effects and the modulation of the IL-1β and IL-6 pathways has proven safe from the cardiovascular standpoint, but with an increased risk of infections. A sign of harm was noted in patients with heart failure given the TNFα blocker etanercept.

Basic and clinical research has led to quantum leaps forward in the identification of the role of both innate and adaptive immune system in arteriosclerosis. The tight balance and interplay of inhibitory and stimulatory pathways of the immune system regulate their participation in atherosclerosis progression and vascular calcifications. The redundancy in the immune system represents an opportunity and challenge to identify mediators in the host-defense mechanism susceptible to inhibition in a way that could diminish atherosclerosis without leading to immunosuppression, hindered tumor surveillance and increased susceptibility to opportunistic infections. The collective knowledge gained by studying the role of inflammation in atherosclerosis and the translational application of these advances in basic science will shape future clinical practice.

To date, two large-scale clinical trials of immunomodulation to prevent arteriosclerosis and its many complications are underway. In the Coronary Inflammation Reduction Trial (CIRT), low-dose MTX is being used as a broad-term upstream anti-inflammatory and in the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) the selective IL-1β mAb canakinumab will provide a much more focused approach to modulating the immune system. Importantly, neither approach appears to have much impact on the conventional cardiovascular risk factors (plasma levels of lipids, blood pressure and glucose) which will make them complementary to current treatments.

5. Hypothesis and Objectives of Thesis

Familial hypercholesterolemia registries will advance the genetic bases of the disease and thus the care of this population.

Through record keeping, history, physical examination, biochemical analysis, genetic analysis, cascade screening, next-generation sequencing and Sanger sequencing, new candidates genes in FH will be discovered and thus open the door for interesting biology. My objectives thus are:

- Improve FH diagnosis using available molecular testing
- Identify new loci associated with FH spectrum
- Implement a prevention strategy to identify asymptomatic yet at risk individuals

New biology of PCSK9 will be uncovered when young and old populations are examined as well as and when PCSK9 LOF mutations and APOE LOF mutations are subanalyzed.

Through measuring circulating PCSK9 levels by an ELISA assay and determining common physical parameters, biochemical parameters, apoE isoforms and *PCSK9* LOF variants, association and relations can be determined. My objectives thus are:

- Evaluate PCSK9 as a biomarker for response to treatment with statins
- Examine relevant cardiometabolic correlates
- Predict possible risk of PCSK9 inhibitors by studying natural LOF individuals

Animal models will advance our understanding of aortic calcification and may validate treatments options using immunomodulation therapy.

Using relevant animal models, atherogenic diet, method development to invoke vascular calcification *ex vivo* and pharmacological intervention, much can be learned. My objectives thus are:

- Confirm the utility of *Ldlr*-deficient animal models in FH-induce aortic calcification
- Validate the sensitivity of a detection method using micro-CT
- Investigate the pathogenesis of aortic calcification and identify key players
- Immunomodulation intervention using IL-1β inhibitor to reduce the burden of aortic calcification

CHAPTER 2

"FH and APOE"

ARTICLE (A)

CHAPTER 2: FH AND APOE

ARTICLE (A): *APOE p.Leu167del mutation in familial hypercholesterolemia*

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2. Foreword:

Familial Hypercholesterolemia (FH) is a monogenic disorder classically caused by mutations in the *LDLR* gene or mutation in its ligand encoded by *APOB* gene. Since the discovery of the third locus implicated in FH, the disease was renamed Autosomal recessive hypercholesterolemia (ADH) to capture the phenotypic diversity. Discovering more genes involved in ADH will increase our understanding of the disease.

In this article we report a relatively novel mutation within the *APOE* gene causing pure FH or ADH. Through a combination of physical examination, biochemical analysis, candidate gene approach, next-generation exome sequencing and Sanger sequencing, we uncovered the genetic basis of an ADH phenotype in a proband of an Italian origin. The collection of patients with familial dyslipidemia as in our FH registry was advantageous at multiple levels.

The first advantage was for the patient who is directed to a specialized care unit with experienced staff to lessen his disease burden. The second advantage was to the family who benefit from cascade screening and early follow up. The third advantage was to the scientific community who will gain significant insight in to the biology of many dyslipidemia diseases including FH. Initiation of nationwide registries thus will promote cardiovascular prevention in more than half of the FH population that are vulnerable to premature CVD.

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APOE p.Leu167del mutation in Familial Hypercholesterolemia

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Keywords: Autosomal dominant hypercholesterolemia, Familial hypercholesterolemia and *APOE* gene.

2.1 Abstract:

BACKGROUND: Autosomal dominant hypercholesterolemia (ADH) is caused by mutations in the low density lipoprotein receptor (*LDLR*), its ligand apoB (*APOB*) or proprotein convertase subtilisin/kexin type 9 (*PCSK9*) genes. Yet DNA sequencing does not identify mutations in these genes in a significant number of cases, suggesting that ADH has multiple genetic etiologies. METHODS: Through a combination of clinical examination, biochemical analysis, candidate gene approach and next-generation exome sequencing we investigated the genetic basis of an ADH phenotype in a proband of an Italian origin. RESULTS: The proband presented with an acute myocardial infarction at age 43. He had tendinous xanthomas, xanthelasmas and elevated levels of total and LDL cholesterol, at 11.2 and 9.69 mmol/L, respectively, with normal levels of HDL cholesterol and triglycerides at 1.62 and 1.13 mmol/L, respectively. HPLC lipoprotein profile showed selective increase in LDL-C. DNA sequencing did not identify any mutation in the *LDLR*, *PCSK9*, *LDLRAP1*and *APOB* gene. We then performed exome sequencing on three individuals from the family. The strongest evidence of association was found for the previously identified apolipoprotein E mutation (*APOE,* chromosome 19:45412053-55) known as *APOE* Leu167del, an in-frame three base-pair deletion. Computational biology confirmed the deleterious nature of this mutation. The Leu167del mutation is predicted to alter the protein structure of apoE near the α-helix within the receptor binding domain. CONCLUSIONS: This report confirms a previous report that ADH can be caused by mutations within the *APOE* gene and represents the 4th loci causing ADH. Standard screening for ADH should include *APOE* gene.

2.2 Introduction:

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by cutaneous xanthomas and xanthelasmas, a marked increase in low density lipoprotein (LDL)-cholesterol (LDL-C) and premature coronary artery disease. The genetic basis of FH is primarily from mutations within the *LDLR* gene, coding for the LDL receptor (LDLR) **[1,2]**. At least two other genes other than the *LDLR* have been found to cause a phenotype clinically similar to FH. These include the *APOB* **[3]** and *PCSK9* **[4,5]** genes. The term Autosomal Dominant Hypercholesterolemia (ADH) thus captures the genetic diversity of FH. Mutations in the *LDLRAP1* **[6]**, coding for the LDLR adaptor protein 1 cause the rare autosomal recessive hypercholesterolemia disorder. In population-based studies, ~20% of patients with the FH phenotype do not have identifiable mutations in these genes **[7].** Genome-wide association studies (GWAS) had broadened the search for genes contributing to LDL-C levels; this technique has allowed the identification of several genes that contribute to small variation in LDL-C levels **[8]**. Remarkably, mutations in several of the genes identified by GWAS have been previously identified in patients with FH. Contemporary techniques in high-throughput sequencing allow the sequencing of all exons expressed in the human genome. Using exome sequencing has allowed the identification of the genetic basis of rare diseases, usually of an extreme phenotype. Here, we present a mutation of the *APOE* gene in a kindred with a classical ADH phenotype.

ApoE is a multi-functional glycosylated protein (34 KDa) secreted from a variety of tissues, including the liver, brain, kidney, adrenals, adipocytes, macrophages and immune cells. It is a key component of all lipoproteins, but especially of triglyceride-rich lipoproteins (chylomicrons and chylomicron remnants), VLDL (very low density lipoproteins) and IDL (intermediate density lipoproteins), and participates in their catabolism through interacting with receptors belonging to the LDLR superfamily. ApoE is recognized by most receptors of this class, but especially the LDLR (also known as the apoB/E receptor), LDL-receptor related peptide-1 (LRP1) and LRP8 (apoE receptor 2) **[9]**. Three common polymorphisms at the *APOE* gene, apoE3, E4 and E2 have been recognized. ApoE2 binds with much less affinity to the LDLR. Homozygosity for the E2 allele is seen in approximately 0.5% of the population and a small percentage of these subjects will develop type III dyslipidemia, characterized by accumulation of remnant lipoprotein particles in plasma and premature vascular disease. A “second hit” is

postulated to contribute to the expression of the apoE2/E2 genotype into a clinical dyslipidemia phenotype **[10]**. Rare mutations of *APOE* also lead to a rare form of lipid glomerulopathy **[11]**. Genome-wide association studies have shown that apoE is strongly associated with LDL-C levels **[8]**. Marduel *et al* [**12**] were the first to report the strong genetic link between *APOE* p.Leu167del and the ADH phenotype in a large family.

2.3 Material and Methods

Patients with an FH phenotype were selected from the McGill University Health Centre (MUHC). In all patients, a cascade screening of first- and second-degree relatives is offered. Clinical FH were suspected when total cholesterol is above 7 mmol/L, LDL-cholesterol is above 5 mmol/L and triglycerides are below 3 mmol/L. The protocol for blood sampling, family studies and DNA analysis has been approved by the Research Ethics Board of the research institute of the MUHC. Follow-up data for a period of 3 years was retrieved. Proper informed consent was obtained prior to clinical photography. A lipid profile is obtained in the fasted state, preferably without lipid-lowering drugs. Such medications are not withheld in subjects at high cardiovascular risk. In all cases, medical records are sought to determine previous lipid profiles.

Plasma samples (150 µl) from subjects were separated into lipoprotein fractions using high performance liquid chromatography (HPLC) with a Superose 6 10/300 GL column (GE Healthcare) attached to a Beckman Coulter System Gold™ apparatus. A 150 mM NaCl mobile phase with a flow rate of 0.4 ml/min was used for the separation of the sample into 72x 400 µl-HPLC fractions that were collected in a 96-well plate using the ProteomeLab™ automated fraction collector (Beckman Coulter). Total cholesterol and triglyceride concentrations were subsequently analyzed on each fraction using the Infinity™ Cholesterol and Triglyceride Liquid Stable Reagents (Thermo Electron Corporation) following the manufacturer's instructions.

Using a candidate gene approach, we sequenced all exons of the *LDLR*, *PCSK9* and *LDLRAP1* genes as well as part of exon 26 of the *APOB* gene, associated with the apoB -LDLR interaction. In addition, a search for common copy number variants (CNV) at the 5' region of the LDLR was performed. We then performed whole exon sequencing

as previously described **[12,13].** All identified SNPs in non-coding regions or not expected to impart functional defects on PolyPhen-2 **[14]** and SIFT softwares **[15]** were excluded. We used the following filters: we removed variants unlikely to be causal in the LDL-C genome-wide association studies, variants with a minor allele frequency >5%, synonymous or intronic variants and variants that did not fit a dominant model of transmission.

Exome sequencing of individuals from the proband family are performed to confirm association. Suspected gene are then sequenced in 39 independent individuals with a clinical diagnosis of ADH (primarily diagnosed based on the Simon Broome criteria) from a tertiary lipid referral clinic in Southwestern Ontario; mutations in known ADH genes, namely *LDLR*, *APOB* and *PCSK9* were excluded in these patients.

Finally we used computer prediction and modeling software to identify and illustrate the impact of a mutation on a protein function. Protein Variation Effect Analyzer (PROVEAN) was used to determine if a given mutation is deleterious to the protein structure in multiple nucleotide involvement **[16].** Template-based protein structure modeling using the I-TASSER server was accessed and then illustrated with the PyMol viewer to show structural impact of the mutation **[17, 18].**

2.4 Results

Our proband is a 43 year-old man of Italian origin. He presented initially with an acute coronary syndrome; a coronary angiogram revealed severe three-vessel coronary artery disease. He underwent coronary artery bypass surgery. Because of the presence of tendinous xanthomas, xanthelasmas, Achilles tendons xanthomas, elevated total cholesterol and LDL-C levels (**Fig.1 A, B and C**), he was selected for further evaluation. The initial lipid and lipoprotein lipid profile revealed total cholesterol of 11.2 mmol/L (457 mg/dL), LDL-C of 9.69 mmol/L (374 mg/dL), HDL-C of 1.62 mmol/L (63 mg/dL) and triglyceride level of 1.13 mmol/L (100 mg/dL). He did not have any cause of secondary hypercholesterolemia nor was there any sign of a hematological disease and his abdominal ultrasound was unremarkable –specifically the spleen was not enlarged.

Compliance issues, including the development of myalgias on atorvastatin 80 mg/day led to unsatisfactory results during the initial year of treatment. On a combination of rosuvastatin 10 mg/day and ezetimibe 10 mg/day, the lipoprotein profile dropped to a total cholesterol of 3.87 mmol/L (150 mg/dL), and LDL-C of 2.3 mmol/L (90 mg/dL), a respective 67% and 76% reduction from baseline (**Fig. 1D**). While a target level of an LDL-C < 2.0 mmol/L (~80 mg/dL) was not achieved, these results are well within the current recommended Canadian Cholesterol guidelines **[19]**.

We then conducted a family study and performed DNA sequencing for the *LDLR*, *PCSK9*, *LDLRAP1* and exon 26 of the *APOB* genes; in addition, to common CNV in *LDLR* in the proband. No known mutation was identified and the several polymorphisms found were either in non-coding regions or were not expected to impart functional defects on PolyPhen **[14]** and SIFT software **[15].** Multiplex ligation-dependent probe amplification was not performed for the *LDLR* gene. The *APOE* gene was not considered as a candidate for the initial screening strategy. Until the report by Marduel *et al* [**12**], there was little biological plausibility to include *APOE* as causal in the ADH phenotype.

We then proceeded with exome sequencing on three individuals from the same family (proband #301 and both sisters #303 and #304). Approximately 54,000 variants now were identified. We used the previously mentioned filters in the method section and ended with 49 missense mutations, 1 frameshift and 2 in-frame deletions in 52 genes. The *APOE* gene Leu167del mutation was the most likely candidate gene since it was

recently discovered in a large family with FH **[12]**. The proband was found to be heterozygous for Leu167del as well as his sister (# 303). The other sister (#304) was found not to carry the *APOE* mutation (**Fig. 1E**). The *APOE* gene was then Sanger sequenced to confirm results obtained from the next-generation sequencing strategy. *APOE* genotyping showed that the proband and both sisters (**Fig. 1D**) were homozygous for the apoE3 allele.

HPLC profile on the subject's affected sister (#303) was performed who was not on lipid-lowering medication at the time. Because the apoE2/E2 phenotype is not associated with an isolated LDL elevation but rather a "broad beta" fraction, we sought to contrast the lipoprotein profile –as determined by HPLC separation- between a normal relative (#304), an *APOE* mutation subject (#303) and a subject with known type III hyperlipoproteinemia (apoE2/E2). Therefore the major particle abnormality in the affected subject (#303) resides in the LDL fraction and hence the selective increase in LDL-sized lipoproteins in the proband's sister is compatible with FH (type IIa hyperlipoproteinemia) (**Fig. 1F**).

The *APOE* gene mutation consists of a TCC deletion (c.500_502delTCC; p.Leu167del) coding for a leucine at position 167 in exon 4 of the *APOE* gene (chromosome 19:45412053-55) (**Fig. 2A and B**). The *APOE* p.Leu167del mutation was absent from 39 individuals with a clinical diagnosis of ADH ascertained in Ontario, Canada who had no mutation in any of the known ADH genes; indeed no rare mutations in *APOE* were found in this cohort. Using the protein structure algorithm online PROVEAN software to predict the effect of an amino acid loss on the protein functionality, *APOE* Leu167del scored -7.41 (default threshold -2.5) marking it deleterious to the protein structure **[16]**. Using established template-based protein structure modeling (I-TASSER server) **[17]** followed by 3-D structure alignment of both reference and Leu167del protein using PyMol viewer we could identify a leucine zipper disruption (**Fig. 2C**).

2.5 Discussion

Recently *APOE* p.Leu167del was reported in a kindred from France with ADH **[12]**. The *APOE* mutation was shown to segregate in a large family with elevated LDL-C. Based on kinetic studies, it was postulated that this mutation may cause a decrease in LDL clearance **[12]**. Furthermore, apoE is not only a ligand for the LDLR but also for many other LRPs in the LDLR family that may assist in LDL clearance and may be more affected by the mutation seen in our patient **[9]**. Interestingly recent data have shown that LDLR-mediated LDL uptake is regulated by LRP6 (another LDLR family member) that can potentially bind apoE and thus the Leu167del mutation may interfere with LDL internalisation **[20]**. This mutation has also been previously reported in a patient with sea-blue histiocytosis **[21]** and was known as *APOE* Leu149del, due to different numbering based on exclusion of the pro-peptide sequence. Thus, *APOE* p.Leu167del is associated with a range of clinical phenotypes, including classical ADH, hypertriglyceridemia with splenomegaly and sea-blue histiocytosis and familial combined hyperlipidemia **[12, 21-24].** The reasons behind expression of such a diverse range of phenotypes in carriers of this mutation, but not seen in our proband is unclear, but mechanism may be related to differences in genetic background of different carriers, gene-gene interactions, gene-environment interactions, or perhaps epigenetic and other non-mendelian interacting effects **[22-24].** Thus our report confirms the observation of Marduel *et al* **[12]**, and confirms that *APOE* is the 4th locus identified in ADH.

The structure of apoE has been extensively studied. The amino terminal portion has 4 α-helical structures that contain the receptor-binding region. The major lipid binding region is located near the carboxyl terminus **[25]**. The deletion of Leu167 is part of this highly conserved motif within the forth helix of the apoE protein. This region besides containing the receptor binding site (residues 136-150), it contain 112 and 158 residues, sites of common polymorphisms leading to apo E2, E3 and E4 isoforms and different binding affinity. We used computer prediction and modeling softwares to predict and illustrate the impact of this deletion on the 3D-structure of apoE **[16-18].** It is apparent that *APOE* p.Leu167del destabilizes a leucine zipper motif in a critical region of the protein that, in turn, would weaken the lipoprotein particle binding to the LDLR (**Fig. 2C**). However the proband was heterozygous therefore it was feasible to overcome this phenotype by statin therapy that is known to induce LDLR in the liver, therefore the pharmacological approach was remarkable. From a clinical point of view it would be

interesting to examine the effect of upcoming class of PCSK9 inhibitors that enhances LDLR availability on the liver surface and thus lowers LDL-C, with fewer myalgic effects than those experienced by our proband while on statin **[5]**.

In conclusion the p.Leu167del mutation was associated with ADH in a kindred of an Italian ancestry and was identified by next-generation exome sequencing; the candidate gene approach did not identify mutations in genes known to be associated with ADH or autosomal recessive hypercholesterolemia. ADH individuals with a confirmed molecular diagnosis **[26]** are known to have premature atherosclerotic vascular disease, especially coronary artery disease, which if left untreated will lead to clinical symptoms in the third to fourth decade of life in men, and approximately ten years later in women **[27-29]**. Accordingly, the proband was 43 years of age at the time of presentation and had classical and biochemical features of FH, but not recognized for an acute coronary syndrome prior to his hospitalization. This report may have an impact on large registries of FH patients and access to specialized medical care. With the unravelling of additional genes associated with FH, future generations will benefit from cascade gene screening in high risk families to prevent vascular disease.

2.6 ACKNOWLEDGEMENTS

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2.7 References

1. Talmud PJ, Shah S, Whittall R, et al. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. Lancet. 2013 Apr 13;381(9874):1293-301.

2. Rader DJ, Cohen J, Hobbs HH. Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. J Clin Invest. 2003 Jun;111(12):1795-803

3. Vega GL, Grundy SM. In vivo evidence for reduced binding of low density lipoproteins to receptors as a cause of primary moderate hypercholesterolemia. J Clin Invest. 1986 Nov;78(5):1410-4

4. Abifadel M, Varret M, Rabès JP, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet. 2003 Jun;34(2):154-6

5. Seidah NG. Proprotein Convertase Subtilisin Kexin 9 (PCSK9) inhibitors in the treatment of hypercholesterolemia and other pathologies. Curr Pharm Des. 2013 Jan

6. Filigheddu F, Quagliarini F, Campagna F, et al. Prevalence and clinical features of heterozygous carriers of autosomal recessive hypercholesterolemia in Sardinia. Atherosclerosis. 2009 Nov;207(1):162-7.

7. Marduel M, Carrié A, Sassolas A, et al. Molecular spectrum of autosomal dominant hypercholesterolemia in France. Hum Mutat. 2010 Nov;31(11):E1811-24.

8. Asselbergs FW, Guo Y, van Iperen EP, et al. Large-Scale Gene-Centric Meta-analysis across 32 Studies Identifies Multiple Lipid Loci. Am J Hum Genet. 2012 Nov 2;91(5):823-38.

9. Herz J, Chen Y. Reelin, lipoprotein receptors and synaptic plasticity. Nat Rev Neurosci. 2006;7:850-9.

10. Johansen CT, Kathiresan S, Hegele RA. Genetic determinants of plasma triglycerides. J Lipid Res. 2011;52:189-20

11. Tsimihodimos V, Elisaf M. Lipoprotein glomerulopathy. Curr Opin Lipidol. 2011;22(4):262-9

12. Marduel M, Ouguerram K, Serre V, et al. Description of a Large Family with Autosomal Dominant Hypercholesterolemia Associated with the APOE p.Leu167del Mutation. Hum Mutat. 2013 Jan;34(1):83-7

13. Reddy MV, Iatan I, Weissglas-Volkov D, et al. Exome sequencing identifies 2 rare variants for low high-density lipoprotein cholesterol in an extended family. Circ Cardiovasc Genet. 2012 Oct 1;5(5):538-46

14. PolyPhen-2 prediction of functional effects of human nsSNPs. Web site. http://genetics.bwh.harvard.edu/pph2. Accessed January 2013.

15. The J. Craig Venter Institute. The SIFT web site. http://www.jcvi.org. Accessed January 2013.

16. The J. Craig Venter Institute. Protein Variation Effect Ananlyzer (PROVEAN) web site. http://www.provean.jcvean.org. Accessed January 2013.

17. Y Zhang. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics, vol 9, 40 (2008)

18. The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. Web site. http://www.pymol.org. Accessed January 2013

19. Anderson TJ, Grégoire J, Hegele RA, et al. Society Guidelines 2012 Update of the Canadian Cardiovascular Society guidelines for the diagnosis and treatment of dyslipidemia and prevention of cardiovascular disease. 2012 Recommendations. Can J Cardiol 2013; 29(2):151-67

20. Ye ZJ, Go GW, Singh R, et al. LRP6 protein regulates low density lipoprotein (LDL) receptor-mediated LDL uptake. J Biol Chem 2012; 287(2):1335e44.

21. Nguyen T, Kruckeberg E, O'Brien F, et al. Familial splenomegaly: macrophage hypercatabolism of lipoproteins associated with apolipoprotein E mutation [apolipoprotein E (delta149 leu)]. J. Clin. Endocr. Metab. 85: 4354-4358, 2000.

22. Faivre L, Saugier-Veber P, Pais de Barros JP, et al. Variable expressivity of the clinical and biochemical phenotype associated with the apolipoprotein E p.Leu149del mutation. Eur J Hum Genet. 2005 Nov;13(11):1186-91

23. Solanas-Barca M, de Castro-Orós I, Mateo-Gallego R, et al. Apolipoprotein E gene mutations in subjects with mixed hyperlipidemia and a clinical diagnosis of familial combined hyperlipidemia. Atherosclerosis. 2012 Jun;222(2):449-55.

24. Brouwers C, van Greevenbroek M, Stehouwer D, et al. The genetics of familial combined hyperlipidaemia. Nat Rev Endocrinol. 2012 Feb 14;8(6):352-6

25. Hatters M, Peters-Libeu A, Weisgraber H. Apolipoprotein E structure: insights into function. Trends Biochem Sci. 2006 Aug;31(8):445-54.

26. Wang J, Ban MR, Hegele RA. Multiplex ligation-dependent probe amplification of LDLR enhances molecular diagnosis of familial hypercholesterolemia. J Lipid Res. 2005 Feb;46(2):366-72.

27. Mortality in treated heterozygous familial hypercholesterolaemia: implications for clinical management. Scientific Steering Committee on behalf of the Simon Broome Register Group. Atherosclerosis. 1999 Jan;142(1):105-12

28. Hansen PS, Defesche JC, Kastelein JJ, et al. Phenotypic variation in patients heterozygous for familial defective apolipoprotein B (FDB) in three European countries. Arterioscler Thromb Vasc Biol. 1997 Apr;17(4):741-7

29. Jansen AC, van Aalst-Cohen ES, Tanck MW, et al. Genetic determinants of cardiovascular disease risk in familial hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2005 Jul;25(7):1475-81

2.8 Legends and Figures:

Figure 1.

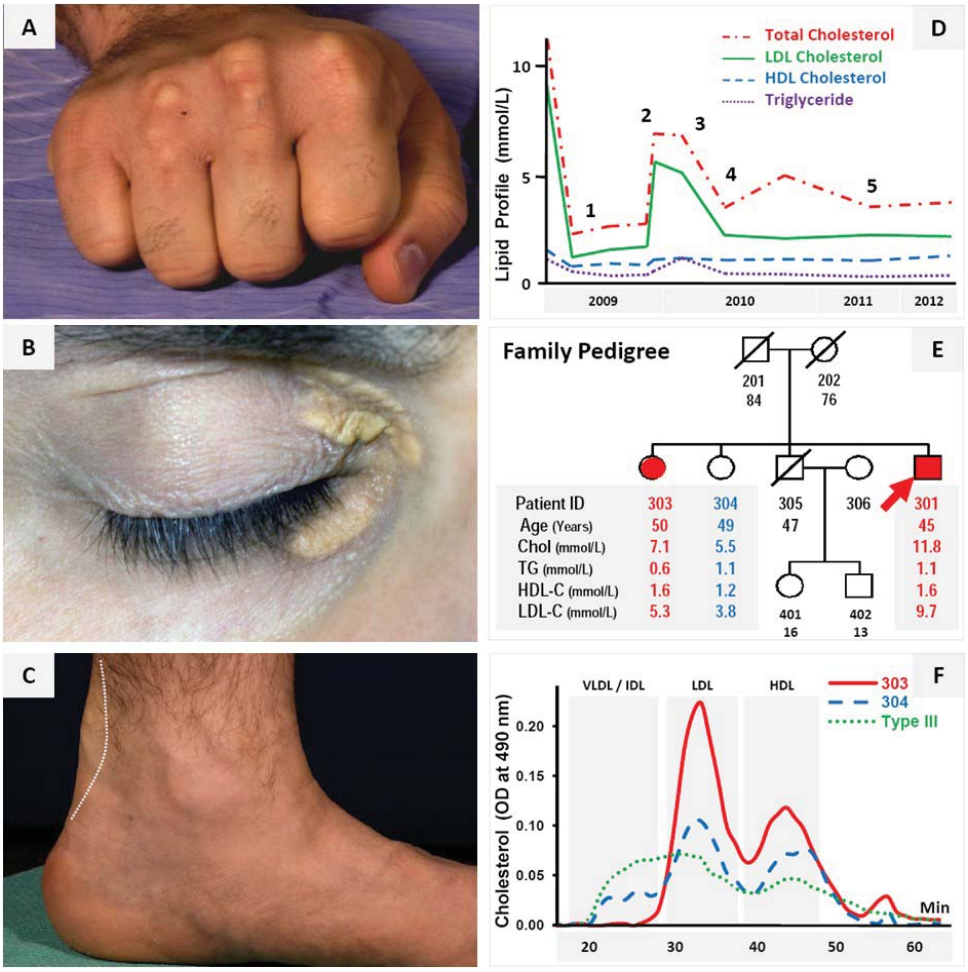

Figure 1. **A.** Tendinous xanthomas in the proband, at the time of initial diagnosis. **B.** Xanthelasmas around both eyes in the proband. **C.** Achilles tendon xanthomas in the proband are demarcated with the dash line. **D.** Lipid and lipoprotein lipids over a three-year period in response to change of medication. The proband developed myalgias to high-dose atorvastatin and finally tolerated rosuvastatin (Medication history indicated by numbers: 1-Atorvastatin 80mg and Ezetimibe 10mg, 2-No medication, 3-Atorvastatin 20mg, 4-Atorvastatin 20mg and Ezetimibe 10mg, 5-Rosuvastatin 10mg and Ezetimibe 10mg). **E.** Family tree of the kindred with *APOE* Leu167del. The proband (#301) is indicated by an arrow. The heterozygotes were subjects in red (#301 and #303) while the sister in blue (#304) was normal. **F.** HPLC profiles of the proband's affected sister subjects #303, unaffected sister r#304 and a type III hyperlipidemias associated with apoE2/E2.

Figure 2.

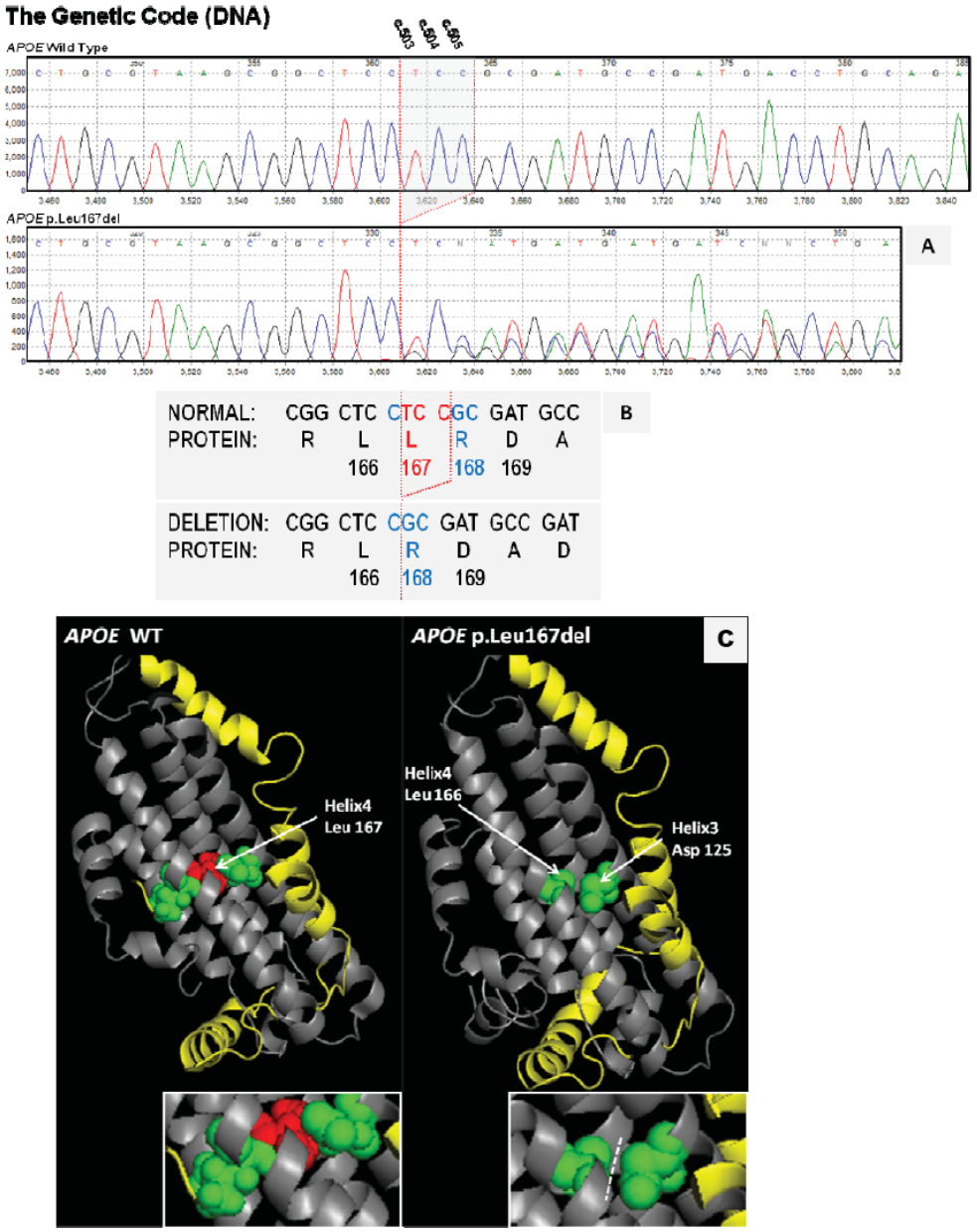

Figure 2. **A.** Shows chromosome 19 sequence data of the affected subject using next-generation sequencing. **B.** Part of *APOE* coding gene. The deleted leucine residue is shown in red creating an in-frame deletion (codon TCC). **C.** The predicted crystal structure of apoE protein with Leu167 deletion in compare to the wild type. Notice proximity of Leu167 residue (red) from LDL receptor binding site spanning between 136 to150 residues. The N-terminal portion (gray ribbon) contains the 4th α-helical region in which the leucine zipper motif is destabilized (dash line) by the Leu167del. The C-terminal portion of the protein is indicated by a yellow ribbon.

CHAPTER 3

"APOE and PCSK9"

ARTICLE (B)

CHAPTER 3: APOE AND PCSK9

ARTICLE (B): *Regional distribution and metabolic effect of PCSK9 insLEU and R46L gene mutations and apoE genotype*
AUTHORS: Zuhier Awan, Delvin E, Levy E, Genest J, Davignon J, Seidah NG, Baass A.

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3. Foreword:

APOE isoforms are one of the most frequent variants that determine the cholesterol levels in human due to its different affinity to the LDLR, and PCSK9 is a natural negative regulator of the LDLR. Therefore, studying the interaction of both genes may lead to better insight into the outcome of using PCSK9 inhibitors in future.

In the following paper, gene sequencing and screening of a large cohort of a pediatric population for loss-of-function (LOF) mutations in PCSK9 were undertaken and the gene-gene interactions with *APOE* isoforms were studied. Multiple parameters were captured from the study and were tested for association. The advantage of testing a young population is that the disease complexity should be relatively less advanced than adults and thus an association should be more accurate. In *PCSK9* p.R46L carriers, contribution of the apoE genotype better explains the cholesterol phenotype than the *PCSK9* variants alone. Patients, with a combination of p.R46L and apoE2 genotype showed a tendency toward insulin resistance as indicated by a two-fold increase in insulin, a homeostasis model assessment of insulin resistance and leptin concentrations, compared to other apoE isoforms.

This data shows the prevalence of the p.R46L SNP and the common in-frame insertion of leucine 10 (insLEU, L10) in the signal peptide of the PCSK9 protein, within 17 geographical regions of the province of Québec, Canada. The data show possible enrichment of the LOF mutations within specific regions of Québec and a possible protective founder effect to counter the FH genotype prevalence.

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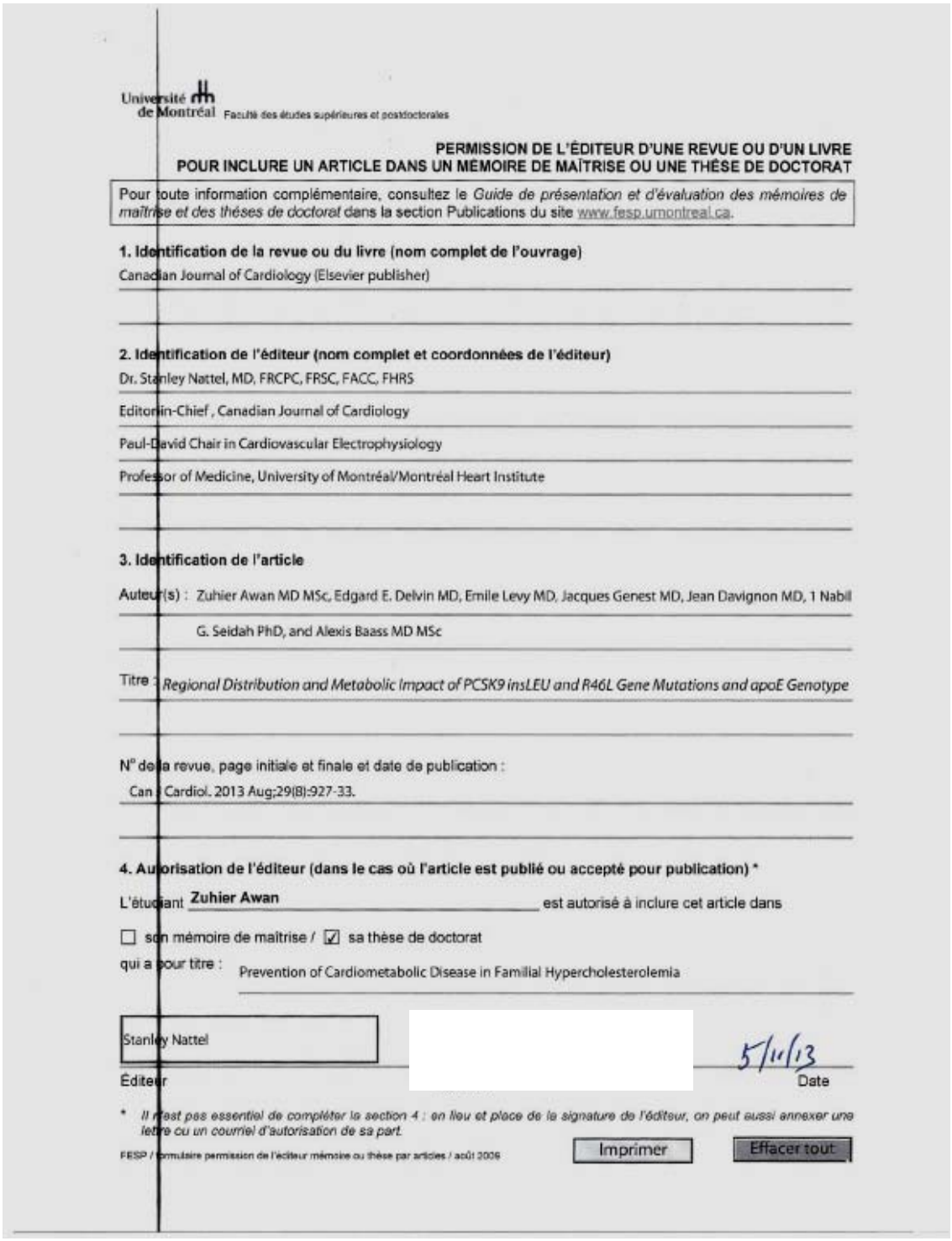

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Regional Distribution and Metabolic Effect of *PCSK9* insLEU and R46L Gene Mutations and apoE Genotype

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CANADA

3.1 Abstract:

BACKGROUND: Natural loss-of-function mutations in the proprotein convertase subtilisin/kexin type-9 (*PCSK9*) gene are associated with lower cholesterol and cardiovascular risk. Because a founder effect exists in French Canadians for many lipid-related genes, we sought to investigate *PCSK9* mutations and associated variables in this population. We also investigated the combined effect of PCSK9 mutations and apoE polymorphism on metabolic variables.

METHODS: Gene sequencing and screening was carried out in 1745 healthy individuals ages 9, 13 and 16 years from a provincially representative population sample. In parallel, we measured related metabolic markers and used appropriate statistical methods.

RESULTS: We report herein that the carrier rates of the R46L single nucleotide polymorphism were higher in the French Canadian population (4.8 %) than previously seen in Caucasians individuals (2.4 %). This is second to the most common variant insLEU at a carrier rate of 24%, making it the most common *PCSK9* loss-of-function mutation in French Canadians individuals. In R46L carriers the contribution of apoE genotype better explains the cholesterol phenotype than R46L mutation alone. Patients, with both R46L and apoE3/E2 genotype also showed a tendency toward insulin resistance as indicated by a twofold increase in insulin, homeostasis model assessment of insulin resistance and leptin concentrations, compared with those without apoE3/E2.

CONCLUSIONS: R46L and insertion of leucine mutations were more frequent in French Canadians and showed a specific geographical distribution. This might represent a gene selection to overcome clustering genes harboring familial hypercholesterolemia and might suggest a founder effect. Subjects with apoE3/E2 genotype and R46L have increased plasma insulin, homeostasis model assessment of insulin resistance and Leptin, an intriguing finding that warrants further investigation.

3.2 Introduction:

Proprotein convertase subtilisin kexin type-9 (PCSK9) is an autocatalytic enzyme with no known substrate other than itself, mainly excreted by the liver, intestine and kidney (**1**). PCSK9 is synthesized as a 72 kDa, 692–amino acid serine protease encoded by the *PCSK9* gene located on the short arm of chromosome one. In human it is the ninth member of a family of proprotein convertases with homologous sequences found across many other animal species (**2**). PCSK9 share similar structure with other proprotein convertase subtilisin/kexin family members; a signal peptide, prodomain, catalytic domain and C-terminal domain. The signal peptide of the PCSK9 has 9 residues of leucine arranged in short tandem repeats. PCSK9 acts as a cholesterol regulator that binds the low-density lipoprotein (LDL) receptor (LDLR) through its epidermal growth factor-like repeat-A and enhances LDLR degradation (**3, 4**) through an endosomal/lysosomal pathway (**5**), thereby prevent LDLR from capturing LDL particle from the circulation.

Mutations in the *PCSK9* gene are associated with either gain-of-function (leading to hypercholesterolemia) (**6**) or loss-of-function (LOF; leading to hypocholesterolemia) (**7-9**). Autosomal dominant hypercholesterolemia or familial hypercholesterolemia (FH) secondary to gain-of-function mutations in *PCSK9* accounts for approximately 2% of cases of FH (**2**). In 1 study, carriers of *PCSK9* LOF mutation R46L (3.6% of Caucasians individuals) and nonsense mutations Y142X and C679X (2.6% of African Americans individuals) were significantly protected from cardiovascular disease (10). The latter 2 nonsense mutations translate into a reduction of cardiovascular risk reaching 90%. The R46L single-nucleotide polymorphism (SNP) is associated with reduce risk of myocardial infarction with a significant 0.40 odds ratio in Caucasians individuals across multiple sites with overall minor allele frequency of 2.4% in a meta-analysis (11). Other LOF mutations have been described and are summarized in a recent article (**12**).

In addition to SNPs and stop codons leading to LOF in PCSK9 protein, an extra insertion of leucine (insLEU) in the leucine stretch of the PCSK9 signal peptide region is also mysteriously associated with lower populational levels of LDL cholesterol (LDL-C) and apolipoprotein (apo) B that can reach a carrier rate of 18% **(13, 14)**, but with few clinical outcome data (**13, 15, 16**). Insertion of insLEU was referred to as c.43_44insCTG

in the literature (**13**) and later to c.61_63dupCTG (L10) for an additional one insertion of leucine residue and c.61_63triCTG (L11) for two insertions of leucine (**17**).The in-frame insertion of an extra leucine (L10) beyond the existing 9 residuals (L9) in the signal peptide region of PCSK9 is constantly associated with lower LDL-C **(14, 18)**, unlike the controversial insertions of two extra leucine residues (L11) (**17**) which have been associated with familial combined hyperlipidemia in a single study.

At present many novel pharmacological compounds have been developed to target PCSK9 inhibition mimicking the many normal occurring LOF mutations. A more recent clinical study was published and showed the usefulness of a monoclonal antibody targeting PCSK9 as an efficient way to lower PCSK9 and LDL-C (**19**).With the development of such new medications, it becomes important to definitely understand the biological effects and metabolic consequences of different *PCSK9* gene variants to define those that require intervention.

The aim of this study was to identify the *PCSK9* mutations that determine the plasma PCSK9 concentration in a French Canadian population-based sample and to examine the combined impact of LOF mutations in *PCSK9* and other available data that influences cholesterol levels. Intriguingly apoE polymorphism was examined for a synergic effect with *PCSK9* variance on lipid and metabolic variables.

3.3 Methods:

Population Studied

A population sample of 1745 apparently healthy individuals aged 9, 13 and 16 years attending Québec schools who participated in the Québec Child and Adolescent Health and Social Survey (QCAHSS), conducted between January and May of 1999 was investigated. Information on the study design and methods has been published previously (**20**). In brief, the QCAHSS used a cluster sampling design to pull 3 independent, representative samples from the province of Québec. Only French Canadians individuals were selected for this study, which encompass roughly 80% of the available cohort. None of the participants were on glucose- or lipid-lowering agents. DNA samples were available for 48 % of eligible candidates (1745 out of 3613). There were no sex or weight differences between youth for whom data were included or not included for analysis. Written informed agreement and consent were acquired from participants and their legal guardians, respectively. The Ethics Review Board of the Centre Hospitalier Universitaire (CHU) Sainte-Justine approved the study.

Sample Collection and Measurements

Samples were taken from 1745 French Canadian participants. Blood was collected in the morning into EDTA-Vacutainer™ tubes after an overnight fast and kept on ice until separation. Plasma and peripheral leukocytes were obtained by centrifugation at 850 x g for 15 min at 4°C. After separation the plasma was frozen on dry ice, and sent to the laboratory within 24 h and stored at -80 °C until analysis.

Height and weight were recorded following a standardized protocol (**20**) and body mass index was computed as weight in kilograms divided by height in meters squared. Waist and hip circumferences were not measured (**20**). Furthermore, because of the anonymous design of the study, information concerning family history, such as history of cardiovascular disease or diabetes was not available. Total cholesterol, triglycerides (TG), high-density lipoprotein cholesterol, glucose and fasting plasma insulin concentrations were measured as previously described (**20, 21**). LDL-C was calculated using the Friedewald equation (**22**). ApoB and apoA-I concentrations were determined using nephelometry (Beckman Array Protein System). Plasma adiponectin was

measured using radioimmunoassay (Linco Research) (**23**), leptin was measured using a human leptin immunoassay kit (SPI-Bio, Montigny le Bretonneux, France) and apoE genotype was determined using amplification refractory mutation system (**24**) or restriction fragment length polymorphism (**25**). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to an earlier version described by Matthews *et al.* (**26**). PCSK9 measurement was performed using an in-house enzyme- linked immunosorbent assay (**27- 29**).

Genetic Approach and Sequencing

DNA was extracted from leukocytes using QIAmp Blood Maxi kit (Qiagen, Missisauga, ON) according to the manufacturer's instructions. The *PCSK9* gene was fully sequenced in study subjects in whom the plasma PCSK9 concentration was either under the 5th or over the 95th percentile. The primer sequences used for amplifying the 12 exons were obtained from NCBI at http://www.ncbi.nlm.nih.gov/genome/probe/ using the resequencing amplicons for *PCSK9*. Amplicon was treated with ExoSAP-It (USB corp), followed by sequencing with Bigdye V3.1. The extension products were then separated using capillary electrophoresis on a 3130 XL Genetic analyzer from Applied Biosystems. Sequences were then assembled with Sequencher software (Sequencher 5.1; Gene Codes Corp)

We then screened the whole cohort for the in-frame insLEU and the R46L SNP identified in Exon 1-*PCSK9*. The insLEU screening was done by fragment analysis detection: an amplicon in the region of leucine was generated with two specific primers, the forward carrying a fluorescent dye label (forward primer 6FAM GCCAGGACAGCAACCTCTC, reverse primer TAGCACCAGCTCCTCGTAGTC), so the fluorescent-labeled product could be separated by size on 3130xl Genetic analyzer and then interpreted using the geneMapper sofware (GeneMapper 3.7). Additional insertion of leucine differed only by the size of fragment obtained: from wild type (9L=159 base pairs [bp]), mutant (10L=162bp) or (11L=165bp). For R46L mutation, screening was performed using a TaqMan SNP genotyping assay, designed by lifetechnologies (assay ID: C_2018188_10); context sequence:

TACGAGGAGCTGGTGCTAGCCTTGC[G/T]TTCCGAGGAGGACGGCCTGGCCGAA.

Statistical Analysis

Samples were stratified according to their *PCSK9* genotype. Because of small numbers, the homozygotes were pooled with heterozygotes for the R46L mutation. A trend analysis was performed using GraphPad InStat and Prism4 software (GraphPad Software, La Jolla, CA). Mean, standard error of the mean and a *t*-test (two sided) was used to compare lipid and metabolic parameters between different groups with various levels of statistical significance: $P < 0.01$, $P < 0.001$, and $P < 0.0001$. Fisher's exact or X^2 tests were used to analyze contingency categorical variables, as appropriate. The additional stratification with apoE genotype was performed on groups with ≥12 unrelated individuals and groups <12 individuals were excluded from the analysis.

3.4 Results:

No gain-of-function mutations were identified at the 95th percentile end of the PCSK9 distribution, and 2 LOF mutations were detected at the 5th percentile end; that is the insLEU polymorphisms (L9/L10, L9/L11, L10/L10) and the missense mutation R46L (rs11591147). A full screen of these variants was therefore carried out on the available DNA sample to determine the frequency distribution of insLEU and R46L in each administrative region of the province of Québec (Supplemental Table S1 and Supplemental Fig. S1). *PCSK9* insLEU was the most frequent *PCSK9* LOF mutation in French Canadians individuals at a carrier rate of 24% followed by the R46L mutation with a carrier rate of 4.8%. Polymorphism with insLEU was found in 458 out of 1745 individuals (411 heterozygotes and 47 homozygote), with an allele frequency of 505/1745 chromosomes or 0.29, higher than the frequency previously reported in other population-based studies reported between 0.12 – 0.18 (**13- 15, 18**). The R46L polymorphism was found in 73 out of 1745 individuals (72 heterozygotes and one homozygote), therefore the allele frequency was 74/1745 chromosomes or 0.04, higher than any previously frequency reported in white subjects (**10**). In addition we observed geographical differences in the frequency of these *PCSK9* mutations in the province of Québec. The insLEU was somewhat homogeneously distributed with a frequency rate greater than 19% in all the regions, and the R46L was more frequent in certain regions such as the Saguenay-Lac-Saint-Jean (12.09%), Québec city (9.09%) and Outaouais (7.94%) (Supplementary Fig. S1).

Characteristics of the 1745 screened participating in this study, stratified by *PCSK9* genotype is shown in **Supplementary Table S2.** *PCSK9* genotyping led to the following distribution: wild type (*n*=1202), insLEU heterozygote (*n*= 411), insLEU homozygote (*n*=47), R46L heterozygote (*n*=72), R46L homozygote (*n*=1) and finally a compound homozygote for both insLEU and R46L (*n*=12). There were no significant differences in terms of age, sex, weight and body mass index in all groups.

We observed that PCSK9 concentrations were significantly lower by -3.7%, -8.6%, -19.3% and -34.7% in insLEU heterozygotes, insLEU homozygotes, R46L heterozygotes and compound heterozygotes respectively, with *P*-value of 0.024, 0.043, <0.0001 and <0.0001; (**Fig 1, A and B** and **Table 1**). Interestingly the phenotype resulting from compound heterozygous exhibited a synergistic effect when both mutations were present. An expected percentage drop in LDL-C was parallel with the PCSK9 drop, reaching significance in all genotypes group except insLEU homozygotes (**Fig 1, D and C; Table 1)**. However, effects on high-density lipoprotein cholesterol, apoA-I, TGs and free fatty acids (FFAs) were not statistically significant and were inconsistent for fasting glucose and insulin, HOMA-IR, adiponectin, leptin and high-sensitivity C-reactive protein across the different *PCSK9* mutations, except in the group of R46L heterozygotes in whom we observe near significant increment in fasting insulin (p=0.06) and HOMA-IR (p=0.05) in compared to wild type. Therefore, we tested the hypothesis that stratifying each *PCSK9* mutation according to apoE genotype would reveal some metabolic relationships. As expected, subgroups of individuals with the R46L allele and apoE3/E2, E3/E3 or E3/E4 alleles exhibited a statistically significant (p<0.05) additional drop in LDL-C (18%, 21% and 22%, respectively) in comparison with wild type, thereby revealing an additive effect between the *PCSK9* R46L mutation and apoE genotype on lipid variables (**Fig. 2A**). Also, the subgroup of individuals with a *PCSK9* insLEU mutation and apoE3/E3 genotype (n=269) had a significant lower TG concentration 0.79±0.02; (p=0.01) compared with both the apoE3/E2 and apoE3/E4 carriers (**Fig. 2B**). More interestingly, the subgroup of individuals with the R46L allele and apoE3/E2 genotype (n=12) now presented a significant increase in fasting insulin 87.9±30; (p=0.00003), HOMA-IR 3±1.1; (p=0. 00002) and leptin 15.8±4.6; (p=0. 006) compared with the E3/E3 group (**Fig 2, C-E**).

3.5 Discussions:

The present Québec French Canadian population mainly originates from 8,000 founder individuals that settled this region approximately 4 centuries ago and remain in genetic isolation for decades later (**30**). Genetic pools provide power to study genes contributing to complex conditions such as dyslipidemia and metabolic heart disease. Consequently a large number of individuals in the province carry LOF mutations in the *LDLR* gene leading to elevated LDL-C concentrations and thus prone to premature coronary heart disease. The founder effect for familial hypercholesterolemia (FH) in French Canadians has been extensively studied over many years given its high frequency that varies between 1:270 to 1:75 compared to 1:500 globally (**31, 32**). Thus we propose that a gene selection process has led to increase frequency of the *PCSK9* LOF mutations in this population as a survival process to lessen cardiovascular mortality. This is in alignment with other observations by Mayne *et al* (**33**) that indicated a prevalence of LOF mutations in the same population. Furthermore, a recent study by Huijgen *et al*, (**34**) showing that FH patients with the same LDLR activity may have very different lipid profiles depending on their PCSK9 concentrations, which is genetically determined, ranging from the upper range of normal cholesterol to 8 times higher than the general population. We have also established that carrier rates of insLEU are roughly homogeneous across the province (24 %), and carrier rates of R46L increase in certain regions such as the Saguenay-Lac-Saint-Jean and Québec City regions reaching frequencies between 1:12 to 1:8. It is interesting to note that the region where the R46L frequency is the highest (Saguenay-Lac-Saint-Jean) corresponds to the region with the highest frequency of FH in the Province of Québec (**32**). This is highly suggestive of a founder effect for R46L mutation and warrants further co-segregation analysis in family members, however because of the nature of the study the patient identity was unattainable and thus family studies were not possible.

Perceptive modulation of the LDLR by PCSK9 was a breakthrough in the understanding of the metabolism of this receptor. Natural LOF mutations in *PCSK9* were first identified in 2006 (**10, 11, 13, 35**), resulting in apparently healthy lifelong lower cholesterol concentrations (**3, 36**), thus opening the door to a new class of lipid lowering agents. Furthermore, a recent report advocates that the carrier status of *PCSK9* insLEU

(L10) is at an advantage of increase response to statin in FH patients (**37**). We have clearly shown that *PCSK9* insLEU negatively affected LDL-C by -3.4%, -7.5% and -28.9% in insLEU heterozygous (L9/L10), insLEU homozygous (L10/L10) and compound heterozygous (L9/L10 and R46L) subjects, with a *P* value of 0.027, 0.068 and 0.011, respectively (**Fig.1, C and D; Table 1**). This lowering effect does not stand true with the insertion of 2 leucine (L9/L11) (**Data not shown, *n*=18**), which behave more like wild type *PCSK9* and is in sharp contrast in our study with previous observations by Abifadel *et al* (**17**). In this study, a limited number of subjects (*n*=3) with the insLEU (L11) had familial combined hyperlipidemia that was attributed to this polymorphism without consideration of the apoE genotype. Because the insLEU appears as a prevalent variant in the French Canadian population, we propose that the previously observed association was a random relationship.

ApoE phenotype is known to influence lipid and lipoprotein profile in the general population. There is a well-known relation between apoE genotypes and LDL-C; apoE4 is associated with higher cholesterol concentrations, and apoE2 is associated with lower cholesterol concentrations compared to apoE3 carriers (**38- 41**). Such a relationship is observed in our cohort study, which also reveals an additive effect of the *PCSK9* mutation, most noticeably in R46L group, but also with insLEU group on LDL-C levels (**Fig. 2A**). It is important to note that subjects with both the R46L and apoE3/E2 genotype represented 0.73 % of our study population and was associated with LDL-C that was lower (-17.68 %) than the population average. This new association between the R46L and apoE3/E2 genotype might therefore be a relatively frequent cause of hypobetalipoproteinemia in the French Canadian population. It is noteworthy that a novel APOE p.Leu167del mutation can lead to FH (**42)** hence screening patients for single point mutation in *APOE* gene is informative.

Regarding the effects of apoE genotype on other lipid parameters, there are some inconsistencies in the literature. A meta-analysis (**43**) demonstrated that TG concentrations were significantly higher in apoE2/E2, E3/E2, E4/E3 and E4/E2 than in E3/E3 subsets. Further, this trend was found in samples of normolipidemic adults and children, in diabetic and obese individuals, and in hyperlipidemic subjects. These

observations indicate ubiquitous and consistent relationship. In our study the TGs concentration was also higher in apoE3/E2 and apoE3/E4 subset regardless of *PCSK9* mutation, and decreased significantly in apoE3/E3 with R46L mutation subgroup (**Fig. 2B**); with invariable plasma (FFA) concentrations (**Data not shown**). We also report for the first time an intriguing phenotype associated with the combination of apoE3/E2 genotype and R46L mutation characterized by higher HOMA-IR (2.17 fold, $p<0.0001$), insulin (2.05 fold $p<0.0001$) and leptin (1.99 fold, $p=0.006$), an alarming finding that warrants further investigation. To explain these observations additional *in vitro* studies are necessary, however we shown that fasting glucose, insulin and HOMA-IR were trending upward in total R46L and compound heterozygote groups (**Table 1**). Recent data confirmed that even modest elevations in FFA and glucose could cause glucolipotoxicity *in vivo,* which may represent an important mechanism leading to failure of β-cell mass expansion in pre-diabetes (**44**). The authors of the study speculated that the anti-proliferative effect of FFAs might represent the connection between increased expression of inhibitor of INK4 (cyclin dependent kinase 4) family cell cycle inhibitors p16 and p18 and genetic risk of diabetes. It will be interesting to investigate if this anti-proliferative effect is observed in patients carrying the R46L mutation and apoE3/E2 genotype.

In summary, we have identified 2 frequent LOF mutations in the French Canadian population and shown their specific geographic frequency distribution. We have also shown that the insLEU and R46L mutations in French Canadians are associated with a lowering effect on total cholesterol, LDL-C and apoB. Interestingly, TG concentration was negatively associated in subjects with simultaneous apoE3/E3 genotype and insLEU or R46L mutation, representing a favorable lipid phenotype. The discovery that subjects with apoE3/E2 genotype and R46L are associated with higher insulin, HOMA-IR and Leptin, is intriguing and warrants further investigation. Therefore it would be important to investigate the potential deleterious effects of the R46L mutation and apoE3/E2 genotype on insulin resistance in other populations. Attention should be taken when confirming the findings of this study in older cohorts to adjust for waist and hip circumferences between groups because they correlate better with central obesity. With further validation, the inhibition of PCSK9 might encounter some health hazard in a subset of patients with cardiometabolic risk tendency. ‘

3.6 Acknowledgment:

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3.7 References:

1. Seidah NG, Benjannet S, Wickham L, et al. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. Proc Natl Acad Sci USA 2003;100:928-33.
2. Seidah NG, Prat A. The proprotein convertases are potential targets in thetreatment of dyslipidemia. J Mol Med 2007;85:685-96.
3. Zhang DW, Lagace TA, Garuti R, et al. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. J Biol Chem 2007;282:18602-12.
4. Kwon HJ, Lagace TA, McNutt MC, et al. Molecular basis for LDL receptor recognition by PCSK9. Proc Natl Acad Sci USA 2008;105:1820-5.
5. Nassoury N, Blasiole DA, Tebon Oler A, et al. The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDLR. Traffic 2007;8:718-32.
6. Abifadel M, Varret M, Rabès JP, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 2003;34:154-6.
7. Cohen J, Pertsemlidis A, Kotowski IK, et al. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. Nat Genet 2005;37:161-5.
8. Zhao Z, Tuakli-Wosornu Y, Lagace TA, et al. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. Am J Hum Genet 2006;79:514-23.
9. Mayne J, Raymond A, Chaplin A, et al. Plasma PCSK9 levels correlate with cholesterol in men but not in women. Biochem Biophys Res Commun 2007;21;361:451-6.
10. Cohen JC, Boerwinkle E, Mosley TH Jr, et al. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 2006;354:1264-72.
11. Kathiresan S. A PCSK9 missense variant associated with a reduced risk of early-onset myocardial infarction. N Engl J Med 2008;358:2299-300.

12. Davignon J, Dubuc G, Seidah NG. The influence of PCSK9 polymorphisms on serum low-density lipoprotein cholesterol and risk of atherosclerosis. Curr Atheroscler Rep 2010;12:308-15.
13. Yue P, Averna M, Lin X, et al. The c.43_44insCTG variation in PCSK9 is associated with low plasma LDL-cholesterol in a Caucasian population. Hum Mutat 2006;27:460-6.
14. Fasano T, Cefalù AB, Di Leo E, et al. A novel loss of function mutation of PCSK9 gene in white subjects with low-plasma low-density lipoprotein cholesterol. Arterioscler Thromb Vasc Biol 2007;27:677-81.
15. Chen SN, Ballantyne CM, Gotto AM Jr, et al. A common PCSK9 haplotype, encompassing the E670G coding single nucleotide polymorphism, is a novel genetic marker for plasma low-density lipoprotein cholesterol levels and severity of coronary atherosclerosis. J Am Coll Cardiol 2005;45:1611-9.
16. Abifadel M, Rabès JP, Jambart S, et al. The molecular basis of familial hypercholesterolemia in Lebanon: spectrum of LDLR mutations and role of PCSK9 as a modifier gene. Hum Mutat 2009;30:682-91.
17. Abifadel M, Bernier L, Dubuc G, et al. A PCSK9 variant and familial combined hyperlipidaemia. J Med Genet 2008; 45:780-6.
18. Miyake Y, Kimura R, Kokubo Y, et al. Genetic variants in PCSK9 in the Japanese population: rare genetic variants in PCSK9 might collectively contribute to plasma LDL cholesterol levels in the general population. Atherosclerosis 2008;196:29-36.
19. Stein EA, Gipe D, Bergeron J, et al. Effect of a monoclonal antibody to PCSK9, REGN727/SAR236553, to reduce low-density lipoprotein cholesterol in patients with heterozygous familial hypercholesterolaemia on stable statin dose with or without ezetimibe therapy: a phase 2 randomised controlled trial. Lancet 2012;380:29-36.
20. Paradis G, Lambert M, O'Loughlin J, et al. The Québec Child and Adolescent Health and Social Survey: design and methods of a cardiovascular risk factor survey for youth. Can J Cardiol 2003;19:523-31.
21. Allard P, Delvin EE, Paradis G, et al. Distribution of fasting plasma insulin, free fatty acids, and glucose concentrations and of homeostasis model assessment of insulin resistance in a representative sample of Quebec children and adolescents. Clin Chem 2003;49:644-9.

22. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.
23. Punthakee Z, Delvin EE, O'loughlin J, et al. Adiponectin, adiposity, and insulin resistance in children and adolescents. J Clin Endocrinol Metab 2006;91:2119-25.
24. Main BF, Jones PJ, MacGillivray RT, et al. Apolipoprotein E genotyping using the polymerase chain reaction and allele-specific oligonucleotide primers. J Lipid Res 1991;32:183-7.
25. Zivelin A, Rosenberg N, Peretz H, et al. Improved method for genotyping apolipoprotein E polymorphisms by a PCR-based assay simultaneously utilizing two distinct restriction enzymes. Clin Chem 1997;43:1657-9.
26. Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-9.
27. Baass A, Dubuc G, Tremblay M, et al. Plasma PCSK9 is associated with age, sex, and multiple metabolic markers in a population-based sample of children and adolescents. Clin Chem 2009;55:1637-45.
28. Dubuc G, Tremblay M, Paré G, et al. A new method for measurement of total plasma PCSK9: clinical applications. J Lipid Res 2010;51:140-9.
29. Awan Z, Seidah NG, MacFadyen JG, et al. Rosuvastatin, proprotein convertase subtilisin/kexin type 9 concentrations, and LDL cholesterol response: the JUPITER trial. Clin Chem 2012;58:183-9.
30. Laberge AM, Michaud J, Richter A, et al. Population history and its impact on medical genetics in Quebec. Clin Genet 2005;68:287-301.
31. Bétard C, Kessling AM, Roy M, et al. Molecular genetic evidence for a founder effect in familial hypercholesterolemia among French Canadians. Hum Genet 1992;88:529-36.
32. Vohl MC, Moorjani S, Roy M, et al. Geographic distribution of French-Canadian low-density lipoprotein receptor gene mutations in the Province of Quebec. Clin Genet 1997;52:1-6.
33. Mayne J, Dewpura T, Raymond A, et al. Novel loss-of-function PCSK9 variant is associated with low plasma LDL cholesterol in a French-Canadian family and with impaired processing and secretion in cell culture. Clin Chem. 2011;57:1415-23

34. Huijgen R, Fouchier SW, Denoun M, et al. Plasma levels of PCSK9 and phenotypic variability in familial hypercholesterolemia. J Lipid 2012;53:979-83.
35. Scartezini M, Hubbart C, Whittall RA, et al. ThePCSK9 gene R46L variant is associated with lower plasma lipid levels andcardiovascular risk in healthy U.K. men. Clin Sci 2007;113:435-41.
36. Humphries SE, Neely RD, Whittall RA, et al. Healthy individuals carrying the PCSK9 p.R46L variant and familial hypercholesterolemia patients carrying PCSK9 p.D374Y exhibit lower plasma concentrations of PCSK9. Clin Chem 2009;55:2153-61.
37. Pisciotta, Sallo R, Rabacchi C, et al. Leucine 10 allelic variant in signal peptide of PCSK9 increases the LDL cholesterol-lowering effect of statins in patients with familial hypercholesterolaemia. Nutr Metab Cardiovasc Dis 2012;22:831-5.
38. Sing CF, Davignon J. Role of the apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. Am J Hum Genet 1985;37:268-85.
39. Eto M, Watanabe K, Ishii K. Reciprocal effects of apolipoprotein E alleles (epsilon 2 and epsilon 4) on plasma lipid levels in normolipidemic subjects. Clin Genet 1986;29:477-84.
40. Wilson PW. Relation of high-density lipoprotein subfractions and apolipoprotein E isoforms to coronary disease. Clin Chem 1995;41:165-9.
41. Xhignesse M, Lussier-Cacan S, Sing CF, et al. Influences of common variants of apolipoprotein E on measures of lipid metabolism in a sample selected for health. Arterioscler Thromb 1991;11:1100-10.
42. Marduel M, Ouguerram K, Serre V, et al. Description of a Large Family with Autosomal Dominant Hypercholesterolemia Associated with the APOE p.Leu167del Mutation. Hum Mutat. 2013;34:83-87
43. Dallongeville J, Lussier-Cacan S, Davignon J. Modulation of plasma triglyceride levels by apoE phenotype: a meta-analysis. J Lipid Res 1992;33:447-54.
44. Pascoe J, Hollern D, Stamateris R, et al. Free fatty acids block glucose-induced β-cell proliferation in mice by inducing cell cycle inhibitors p16 and p18. Diabetes 2012;61:632-41.

3.8 Legends, figures and tables:

Figure 1.

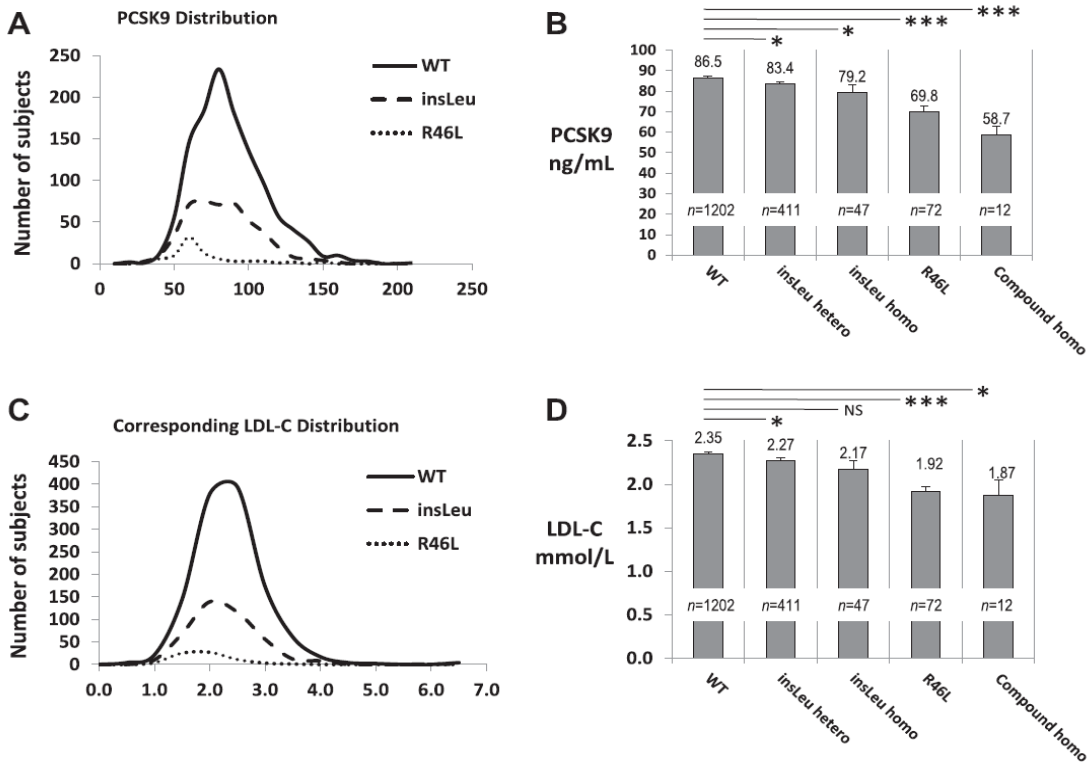


Figure 1. (A) Proprotein convertase subtilisin/kexin type-9 (PCSK9) concentration distribution for PCSK9 wild type (WT), insertion of leucine (insLEU), and R46L. **(B)** Average PCSK9 concentration of the WT, insLEU, and R46L carriers. **(C)** Low-density lipoprotein cholesterol (LDL-C) concentration distribution in PCSK9 WT allele, insLEU, and R46L. **(D)** Average LDL-C concentration of the WT, insLEU, and R46L carriers. * P < 0.01, ** P < 0.001, and *** P < 0.0001. hetero, heterozygotes; homo, homozygotes; NS, not significant.

Figure 2.

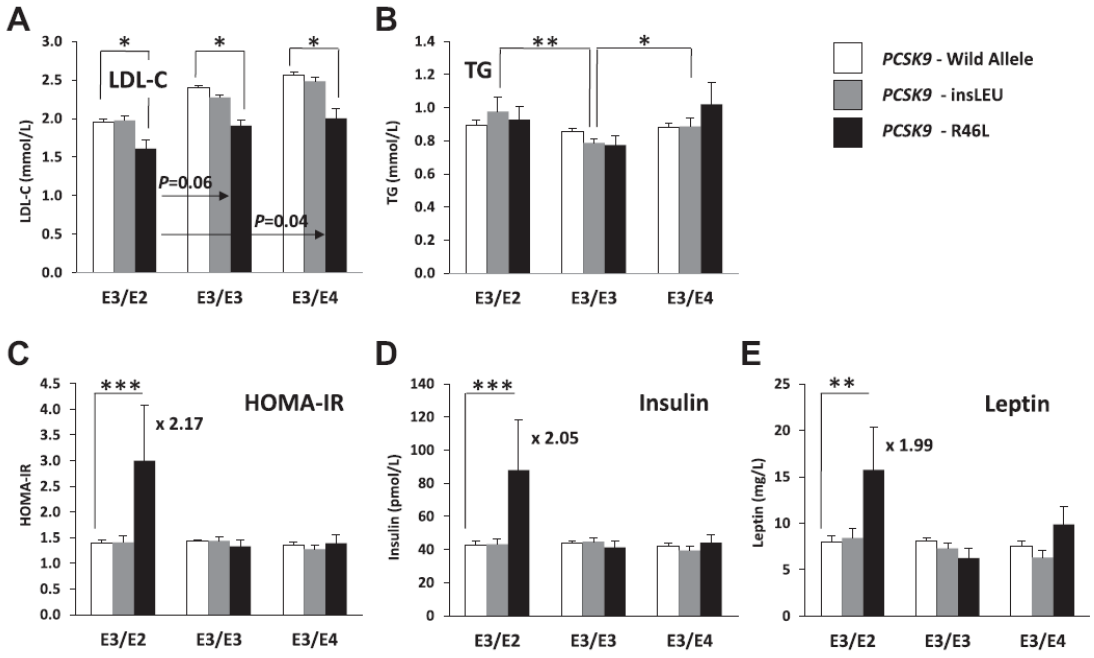


Figure 2. Subgroup analysis of proprotein convertase subtilisin/kexin type-9 (PCSK9) mutations depending on apolipoprotein (apo) E genotype. **(A)** Additive effect of R46L mutation and apoE genotype on low-density lipoprotein cholesterol (LDL-C). **(B)** Effect of the insertion of leucine (insLEU) mutation and apoE3/E3 genotype on triglycerides (TG). Carrying the R46L PCSK9 mutation and the apoE3/E2 genotype (n ¼ 12) encompass a significantly increased **(C)** homeostasis model assessment of insulin resistance (HOMA-IR; 3±1.1; *P* =0.00002); **(D)** fasting insulin (87.9±30; *P* =0.00003), and **(E)** leptin (15.8±4.6; *P* = 0.006). * *P* < 0.01, ** *P* < 0.001, and *** *P* < 0.0001.

Table 1.

Table 1. Lipid and metabolic effect of different PCSK9 mutations compared with wild type

	insLEU heterozygote (n = 411)		insLEU homozygote (n = 47)		R46L heterozygote (n = 72)		Compound heterozygote (n = 12)	
	Δ%	*P*	Δ%	*P*	Δ%	*P*	Δ%	*P*
Weight, kg	−0.28	0.89	−9.24	0.08	2.05	0.63	−7.9	0.81
BMI	0.00	0.59	−3.66	0.25	1.65	0.53	−14.6	0.66
PCSK9, ng/mL	−3.66	0.02	−8.58	0.04	−19.32	< 0.0001	−34.7	< 0.0001
Total cholesterol, mmol/L	−2.27	0.03	−5.09	0.07	−11.11	0.0001	−20.2	0.02
LDL-C, mmol/L	−3.44	0.03	−7.48	0.07	−18.67	0.00	−28.9	0.01
Apo B, g/L	−2.92	0.05	−8.39	0.03	−15.25	0.00	−43.2	0.01
HDL-C, mmol/L	0.00	0.91	0.92	0.75	0.18	0.94	−4.0	0.79
Apo A-I, g/L	0.00	0.63	1.74	0.42	−0.76	0.67	−16.0	0.84
TG, mmol/L	−3.17	0.25	−10.54	0.13	−3.38	0.54	−19.2	0.22
Free fatty acid, mmol/L	1.91	0.49	7.70	0.29	−0.35	0.95	−8.1	0.44
Fasting glucose, mmol/L	0.00	0.44	−0.33	0.76	0.30	0.74	8.2	0.82
Fasting insulin, pmol/L	−0.01	1.00	−13.34	0.14	14.78	0.06	13.9	0.77
HOMA-IR	−0.31	0.94	−13.86	0.15	16.29	0.05	23.3	0.76
Leptin, mg/L	0.00	0.18	−13.76	0.44	11.54	0.43	−34.9	0.84
Adiponectin, mg/L	0.00	0.40	6.85	0.23	−2.99	0.53	−3.8	0.45
CRP, mg/L	10.63	0.61	−39.25	0.44	45.83	0.31	−75.7	0.97

Apo, apolipoprotein; BMI, body mass index (calculated as weight in kg divided by height in m^2); CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; insLEU, insertion of leucine; LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type-9; TG, triglycerides.

Table 1. Lipid and metabolic effect of different PCSK9 mutations on individuals in compared to wild type (Hetero: heterozygote, Homo: homozygote, Comp: compound)

3.9 Supplemental Material:

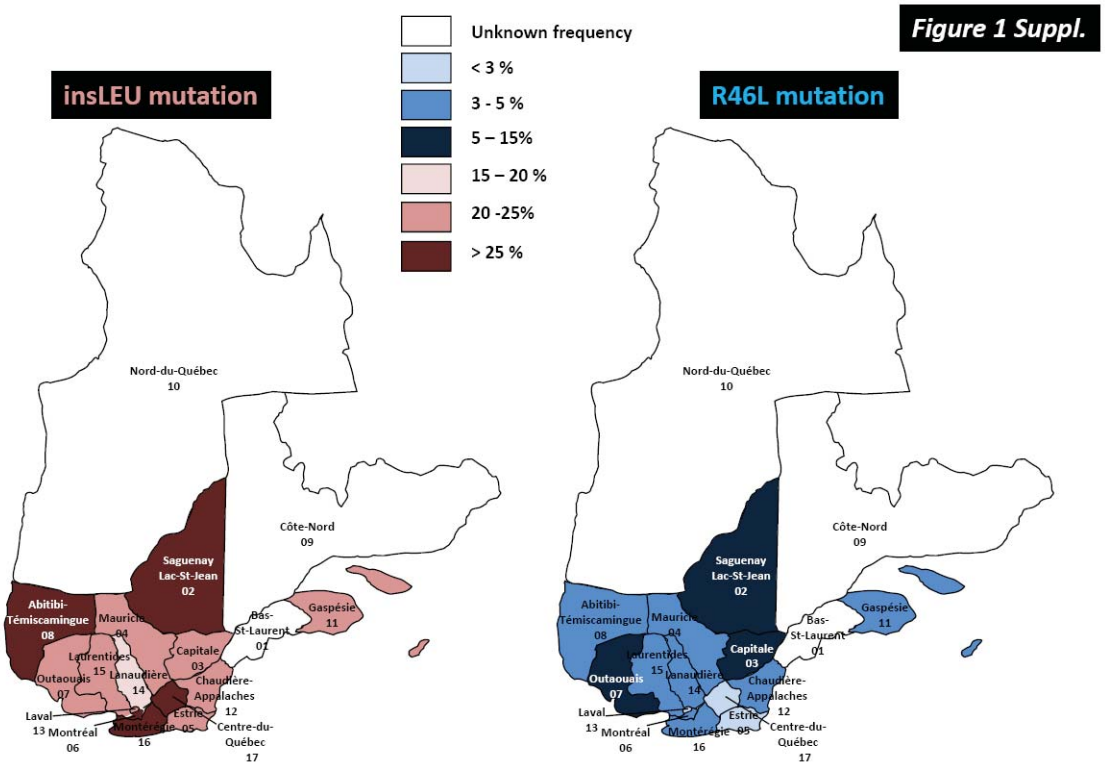
Suppl. Table 1: Frequency distribution of the insLEU and R46L PCSK9 mutations in each administrative region of the Quebec province

		insLEU			R46L		
ERAQC	Province	Ratio	%	(*P*=value)	Ratio	%	(*P*=value)
1	Bas-Saint-Laurent	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
2	Saguenay-Lac-Saint-Jean	26/91	28.57	(0.39)	11/91	12.09	(0.01)
3	Québec city	45/187	24.06	(0.94)	17/187	9.09	(0.02)
4	Mauricie	22/91	24.18	(0.93)	3/91	3.30	(0.80)
5	Estrie	17/69	24.64	(0.98)	2/68	2.94	(0.77)
6	Montréal	59/211	27.96	(0.24)	9/211	4.27	(0.88)
7	Outaouais	13/63	20.63	(0.58)	5/63	7.94	(0.22)
8	Abitibi-Témiscaminque	20/80	25.00	(0.93)	3/80	3.75	(1.00)
9	Cote-Nord	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
10	Nord-Du-Québec	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
11	Gaspésie-Iles-De-La-Madeleine	20/99	20.20	(0.35)	3/99	3.03	(0.62)
12	Chaudière-Appalaches	25/121	20.66	(0.43)	4/121	3.31	(0.65)
13	Laval	13/58	22.41	(0.84)	1/58	1.72	(0.52)
14	Lanaudière	22/116	18.97	(0.24)	4/116	3.45	(0.65)
15	Laurentides	30/137	21.90	(0.62)	5/137	3.65	(0.68)
16	Montérégie	85/337	25.22	(0.70)	14/337	4.15	(0.73)
17	Centre-Du-Québec	25/84	29.76	(0.25)	2/84	2.38	(0.43)
	Mean of all regions		24.2			4.8	

Suppl. Table 1. Frequency distribution of the insLEU and R46L PCSK9 mutations in each administrative region of the Quebec province (N.A: not available)

Suppl. Table 2: Characteristics, PCSK9 concentrations, lipids and metabolic markers (mean ± SEM) of *n*=1745 participant stratified by PCSK9 genotype.								
	Wild type		insLEU		R46L		Comp.	
n=	1202		458		73		12	
Age, years	13	±0.1	13	±0.1	13	±0.3	14	±0.7
Weight, kg	50	±0.5	50	±0.8	51	±2.1	52	±3.5
BMI, kg/m^2	20	±0.1	20	±0.2	21	±0.5	20	±0.9
PCSK9, ng/ml	87	±0.7	83	±1.1	70	±2.9	59	±4.2
Total Cholesterol, mmol/L	4.0	±0.02	3.9	±0.04	3.6	±0.08	3.5	±0.20
LDL-C, mmol/L	2.4	±0.02	2.3	±0.03	1.9	±0.06	1.9	±0.17
Apo B, g/L	0.7	±0.01	0.6	±0.01	0.6	±0.02	0.5	±0.03
HDL-C, mmol/L	1.3	±0.01	1.3	±0.01	1.3	±0.03	1.3	±0.08
Apo A-I, g/L	1.2	±0.01	1.2	±0.01	1.2	±0.02	1.2	±0.06
TG, mmol/L	0.9	±0.01	0.8	±0.02	0.9	±0.05	0.7	±0.05
Free fatty acid, mmol/L	0.4	±0.01	0.4	±0.01	0.4	±0.02	0.4	±0.04
Fasting glucose, mmol/L	5.2	±0.01	5.1	±0.02	5.2	±0.05	5.2	±0.09
Fasting insulin, pmol/L	43.5	±0.77	42.9	±1.68	49.8	±5.65	41.2	±3.43
HOMA-IR	1.4	±0.03	1.4	±0.05	1.6	±0.20	1.3	±0.12
Leptin, mg/L	7.9	±0.27	7.2	±0.42	8.8	±1.09	8.5	±2.68
Adiponectin, mg/L	10.2	±0.11	10.5	±0.19	9.9	±0.50	11.1	±1.35
CRP, mg/L	1.2	±0.12	1.2	±0.20	1.7	±0.91	1.2	±0.49

Suppl. Table 2. Characteristics, PCSK9 concentrations, lipids and metabolic markers (mean ± SEM) of *n*=1745 participant stratified by PCSK9 genotype; wild type (*n*=1202), insLEU (*n*=458), R46L (*n*=73) and compound (Comp) heterozygote (*n*=12)



Suppl. Figure 1. Geographical frequency distribution of PCSK9 mutations in each region of the province of Quebec

CHAPTER 4

"PCSK9 and LDL"

ARTICLE (C)

CHAPTER 4: PCSK9 AND LDL

ARTICLE (C): *Rosuvastatin, proprotein convertase subtilisin/kexin type 9 concentrations, and LDL cholesterol response: the JUPITER trial*

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4. Foreword:

While *PCSK9* and *LDLR* are under the regulation of SREBP, statin therapy is known to increase concentrations of LDLR and PCSK9. PCSK9 induction by statin will lead to more LDLR down regulation at the cell surface and less LDL cholesterol (LDL-C) cleared from blood. The magnitude of these relationships is unknown.

This study was undertaken to examine the relationship between PCSK9 and LDL-C reduction. The selected population was normolipidemic elderly individuals with no fluctuation in sex hormones. This is to eliminate all possible confounding factors from the study and to get a homogeneous result. PCSK9 was never evaluated in the same individual after one year or in the same individual before and after taking statin (rosuvastatin) in a relatively large sample size.

Our data indicate that among those taking rosuvastatin in the JUPITER trial, greater reductions in LDL-C were associated with greater increases in PCSK9 on both absolute and relative scales. However the LDL-C response to rosuvastatin could not be inferred by PCSK9 levels. While carriers of the p.R46L mutation (rs111591147) were found to have lower PCSK9 and LDL-C levels at study entry, the gene status did not alter the magnitude of LDL-C associated with rosuvastatin use. Therefore, future PCSK9 inhibitors should lower PCSK9 level beyond that seen in this population to see a significant drop in LDL-C.

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Rosuvastatin, Proprotein Convertase Subtilisin/Kexin Type 9 Concentrations, and LDL Cholesterol Response: the JUPITER Trial

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Key Words: Proprotein convertase subtilisin/kexin type 9, Low-density lipoprotein, Rosuvastatin, Statin, The JUPITER trial.

4.1 Abstract:

BACKROUND: Although statin therapy is known to increase concentrations of (PCSK9), whether this effect is related to the magnitude of LDL reduction is uncertain. This study was undertaken to understand the extent of this effect and examine the relationship between PCSK9 and LDL-C reduction.

METHODS: We measured plasma PCSK9 concentrations by ELISA at baseline and at one year in 500 men and 500 women participating in the *Justification for Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin* (JUPITER) trial that randomly allocated participant to rosuvastatin 20 mg daily or placebo was also evaluated rs11591147, a single nucleotide polymorphism known to have an impact on plasma PCSK9 concentrations.

RESULTS: At baseline, median (interquartile range) PCSK9 concentrations were higher in women 73(62-90) ng/mL than in men 69(57-81) ng/mL (P<0.005). During one year, there was no change in PCSK9 concentrations in the placebo arm, suggesting stability in time. In contrast, the rosuvastatin increased PCSK9 by 35% in women 101(82-117) ng/mL and 28% in men 89(71-109) ng/mL (P<0.0001). Among those allocated to rosuvastatin, greater reductions in LDL-C were associated with greater increases in PCSK9 on both absolute and relative scales (r=-0.15, P<0.0005). Furthermore PCSK9 (rs11591147) did not alter the magnitude of LDL-C reduction associated with rosuvastatin use.

CONCLUSIONS: In this randomized trial, rosuvastatin increased plasma concentration of PCSK9 in proportion to the magnitude of LDL-C reduction; the LDL-C response to statin could not be inferred by PCSK9 concentrations.

4.2 Introduction:

The major mechanism of action of statins is mediated through up-regulation of the LDL receptor (LDLR) found predominantly on hepatocytes (1). Recently, a critical role for the PCSK9 protein was found in the cellular processing of the LDLR (2,3) and it has subsequently been reported that mutations in the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene associate with wide variation in LDL cholesterol (LDL-C) concentrations. Specifically, gain-of-function mutations in PCSK9 are associated with marked increases in LDL-C similar to that seen in familial hypercholesterolemia due to defects in the LDLR protein (4), whereas loss of function mutations are associated with lower PCSK9 concentrations, low life-long LDL-C concentrations, and reduced cardiovascular risk (5,6). Given these inter-relationships, there has been considerable interest in understanding the effect of statins on PCSK9 concentrations, particularly since agents designed to inhibit PCSK9 are likely to be used as adjuncts to statin therapy. To date, it has been established that several statins increase plasma PCSK9 concentrations, but whether this effect is related to the magnitude of LDL-C reduction associated with statin treatment or is modified by genetic status at rs11591147 remains uncertain.

We addressed these issues among 500 men and 500 women participating in the Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial (7) these individuals underwent genotyping at rs11591147 and had baseline and 1-year blood samples evaluated for both PCSK9 and LDL-C concentrations.

4.3 Methods:

The patient population was derived from participants in the JUPITER trial, a primary prevention trial comparing rosuvastatin 20 mg daily to placebo in 17,802 apparently healthy men and women with LDL-C < 3.4 mmol/L (130 mg/dL) and high-sensitivity C-reactive protein (hsCRP) > 2.0 mg/L. For the purposes of the current analysis, 500 men and 500 women allocated to either rosuvastatin or placebo were selected using a stratified randomization method from compliant trial participants whom provided consent for genetic and plasma biomarker studies as approved by the Review Ethics Board.

Concentrations of LDL-C were measured centrally as part of the overall JUPITER trial protocol. Genotyping was performed using the Omni 1M Quad platform (Illumina) as part of an ongoing genome wide association study being conducted within the JUPITER trial. Using these data, genetic information on rs11591147, a previously described polymorphism at the PCSK9 gene locus that leads to an arginine for leucine substitution at position 46 (R46L), was available in 955 out of the 1000 participants. We evaluated plasma PCSK9 concentrations were evaluated using a sandwich ELISA as previously described (8). This assay measures total PCSK9, i.e., both mature and furin-cleaved forms (9, 10).

On the bases of our previous reports using PCSK9 ELISA in a healthy population, we assumed a mean value of 89 μg/L at baseline and a standard deviation of 32(8). With a likely effect of 12.5%, an α value of 0.01 and a 90% power, we expected that a minimum of 200 participants per group would be required. The estimated cohort size effect is considered a minimum and is likely to be larger. Because each subject serves as his or her own control, we anticipated achieving sufficient statistical robustness for firm conclusions. Therefore we analyzed 250 men and 250 women randomized to placebo and rosuvastatin and determined PCSK9 concentrations at baseline and at 1 year. In a sex-specific analysis, we compared baseline clinical characteristics between the placebo and rosuvastatin groups using t-tests for continuous variables and the X^2 statistic for categorical variables. The difference in the change in PCSK9 and LDL-C over time in the all groups was tested using Spearman rank correlation coefficients. We uesed ANOVA and Tukey-Kramer multiple comparison to determine differences in PCSK9 by quintile of LDL-C, adjusted for age, sex, body mass

index, blood pressure, serum glucose, serum concentrations of lipids and lipoprotein lipids (total cholesterol, triglycerides, LDL-C, HDL-C), apolipoproteins AI and B and hsCRP. Correlations between PCSK9 and these variables were analyzed by Spearman rank correlation coefficients.

4.4 Results:

Baseline characteristics of the 500 men and 500 women participating in this study, stratified by rosuvastatin or placebo use, are shown in **Table 1**. As would be anticipated in a randomized trial, there were no significant differences at study entry within each sex-specific group according to treatment allocation.

Baseline and 1-year concentrations of measured lipid parameters, hsCRP, and PCSK9 are shown in **Table 2** and **Fig. 1**. Median (interquartile range) baseline concentrations of PCSK9 were higher in women [73(62-90) ng/mL (*n*=499)] than in men [69(57-81) ng/mL (*n*=500)] (*P*<0.005). Participants randomized to placebo had no significant change in any measured variable, including PCSK9, suggesting considerable stability of PCSK9 concentrations over time. In contrast, participants randomized to rosuvastatin had significant reductions in total cholesterol, LDL-C, triglycerides, and hsCRP as well a modest increase in HDL-C. These effects were accompanied by a 35% increase in median concentrations of PCSK9 in women from 75(66-85) ng/mL to 101(82-117) ng/mL, *P*<0.0001 and a 28% increase in men from 69(57-81) ng/mL to 89(71-109) ng/mL, *P*<0.0001 (**Fig. 1**). At baseline, there was a significant correlation (*n*=999, *r*=0.15, *P*<0.0001) between concentrations of PCSK9 and LDL-C; however this relationship was no longer observed on rosuvastatin treatment (*n*=498, *r*=-0.01, *P*=0.84) (**see Supplemental. Fig. 1, A and B**).

Furthermore, the individual LDL-C percent change from baseline in response to rosuvastatin treatment was plotted in a declining manner as shown in **Fig. 2A** and corresponding individual percent change from baseline in PCSK9 is shown in **Fig. 2B.** Although, individual variation among those allocated to rosuvastatin was wide, a significant relationship was observed between the magnitude of LDL-C reduction and the increase in PCSK9 concentrations; this was seen in both an analysis of quintiles of LDL-C reduction (**Fig. 2C**) and when the LDL-C change was treated as a continuous variable (**Fig. 2D**). A similar response was observed for apo B and non-HDL-C changes and PCSK9 changes (data not shown).

Polymorphism at rs11591147 PCSK9 (R46L) was found in 46 of 955 individuals (24 male heterozygotes, 21 female heterozygotes and 1 male homozygote). The allele frequency was thus 47 of 1910 chromosomes or 0.0246, close to that previously reported in population-based studies (6). The sole homozygous subject was excluded

from further analysis. Serum PCSK9 at baseline was lower in carriers of the R46L SNP by -19% (P<0.005) (**see online Supplemental. Fig.2A**). And as seen in previous studies (6) the baseline LDL-C concentrations in R46L carriers were lower by -9% in men (n=24, mean 94 mg/dL *vs* n=451, mean 103 mg/dL) and by -7% in women (n=21, mean 100 mg/dL *vs* n=458, mean 107 mg/dL). In the rosuvastatin arm with available genetic status (n=478), no significant differences in LDL-C reduction were observed as influenced by the genetic status and thus no increased response to statin therapy in the R46L carriers. (**see online Supplemental. Figure 2B**).

4.5 Discussion:

We examined 1,000 patients in the JUPITER trial. Half (*n*=500) were randomized to placebo and half, to rosuvastatin 20 mg/day. We established that plasma PCSK9 concentrations are stable as a biomarker over time among those allocated to placebo, but increase by approximately 30% among those allocated to rosuvastatin 20 mg. Although, individual responses were variable, we observed a significant relationship between the magnitude of LDL-C reduction and the increase in PCSK9 concentrations on both an absolute and relative scale. Specifically, across the full study cohort, greater LDL-C reductions were associated with greater increases in plasma PCSK9 concentrations, an effect present in both sexes despite higher baseline PCSK9 concentrations in women compared to men.

PCSK9 gain-of-function mutations were identified as 1 of 4 molecular causes of familial hypercholesterolemia (4). PCSK9 is the ninth member of the mammalian proprotein convertase family of serine endoproteases (11). PCSK9 is recognized as a key regulator of serum LDL-C concentrations. The gene for PCSK9 is located on chromosome 1p34 and encodes a 692-amino acid protein that is mostly expressed in the liver and intestine (11). The protein comprises a 30 amino acid signal, peptide (SP), a pro-domain (amino acids 31-152), a catalytic domain (amino acids 153-454) and a cysteine- and histidine-rich C-terminal domain (amino acids 455-692) (12,13). The only known substrate for PCSK9 is itself. PCSK9 catalytic domain contains the main binding structure for the epidermal growth factor-like repeat A (EGF-A) domain on the LDLR (14), whereas the C-terminal domain binds cell surface proteins, including annexin A2 (15). The major function of PCSK9 is to mediate the degradation of the LDLR protein and evidence exists for both an intracellular and extracellular site of interaction between the LDLR EGF motif and PCSK9 (16-19). However the predominant source of circulating PCSK9 in blood originates from the extracellular pathway in the liver and this correlates with the concentration of plasma cholesterol (18).

PCSK9 is regulated at the transcriptional level by sterol regulatory element binding protein (SREBP)-2 (20, 21), possibly by SREBP-1c (22) and especially by hepatocyte nuclear factor-1 alpha (HNF-1 alpha) (23). PCSK9 expression is downregulated by cholesterol *via* SREBP-2 (24) and upregulated by statins *via* SREBP-2 (25). The Liver specific Receptor, LXR and insulin also regulate PCSK9 (26, 27).

Therefore, mRNA levels of LDLR and PCSK9 are regulated by SREBP-2 and statins, inducing an upregulation of both LDLR and PCSK9 (28). The results obtained in the present study are consistent with this observation. The concept that the statin-mediated increase in PCSK9 may limit the efficacy of statins in human (13, 29), however, is not supported by the present study.

The effect of statins on PCSK9 has been inferred or documented in previous studies (11, 30-32). Here we show that rosuvastatin at a daily dose of 20 mg reduces LDL-C by approximately 50% on average and increases PCSK9 by 28% in men and 34% in women. Baseline PCSK9 concentrations correlate with LDL-C (n=999; r=0.15 P<0.0001). Although a statistically significant correlation between serum concentrations of PCSK9 and LDL-C is present at baseline (r=0.15), only ~2.25% of the variance in LDL-C concentrations is explained by PCSK9 concentrations. On rosuvastatin, this correlation is no longer observed (n=498; r=-0.01; p=0.84) (**see online Supplemental Fig. 1, A and B**). Similar findings with the drug atorvastatin have been reported (31). The observation that the increase in PCSK9 concentrations with rosuvastatin is not associated with a blunted LDL-C response is counter-intuitive, based on the postulated mechanisms of action of PCSK9. In fact, the data presented here show a significant negative correlation between the percentage change in PCSK9 concentration on rosuvastatin and the percentage change of LDL-C (n=498; r=-0.15 P<0.0005) (**Fig. 2D**). This observation warrants further mechanistic explanation. Based on these data (**Fig. 2, A and B**), However, plasma PCSK9 concentrations cannot be used to predict individual response to statin therapy. It remains to be determined whether the measurement of PCSK9 concentration in blood aids in diagnosing patients who are refractory to statins. The development of PCSK9 inhibitors may require such measurement if it can be demonstrated that measuring PCSK9 concentrations influences the choice and success of treatment.

The R46L allele frequency in our study was 2.5%, similar to that of the general population, leading to approximately 8% lower LDL-C than non-carriers (6, 33). Given the ethnic heterogeneity of our population, we cannot, however, perform meaningful statistical comparisons without a much larger sample size. Nonetheless, the presence or absence of this single nucleotide polymorphism was not associated with a difference in the response to rosuvastatin (see online Supplemental. Fig. 2B); therefore, knowing the R46L variant would not likely guide therapy in our population.

We confirm the observation that statins increase PCSK9 (23, 25, 29). While individual LDL-C response to rosuvastatin can not be predicted by PCSK9 concentrations (**Fig. 2, A and B**); there is a significant association between the magnitude of change in LDL-C and changes in PCSK9 concentrations (**see online Supplemental Fig. 1C)**. A possible explanation is that the transcriptional regulatory protein SREBP-2 mediates the coordinate expression of the LDLR and PCSK9 in response to cellular cholesterol deprivation (24).

As a biomarker and a therapeutic target, PCSK9 is appealing (12), but because of stoichiometric interactions between the EGF-A region of the LDLR and PCSK9, it has, to date, not been amenable to be targeted by small molecules. Several approaches have thus been examined to decrease PCSK9 concentrations and are at an advanced stage. One such approach is the use of antisense mRNA that modulates the expression of PCSK9 mRNA leading to reduced PCSK9 production (34-36). Another approach is the inhibition of PCSK9 binding to the LDLR by using antibodies against PCSK9 leading to inhibition of LDLR degradation mediated by PCSK9 (19, 37). These approaches require the manufacture of biological substrates (antisense oligonucleotides or antibodies) and are likely to entail production costs that will restrict their application to patients in whom statins at maximally tolerated doses still do not allow target levels to be reached as proposed in national guidelines (38,39). Given this interest, several monoclonal antibodies to PCSK9 are already in phase two clinical trials (see www.clinicaltrials.gov) giving clinicians a potentially effective add-on treatment alternative. A strategy based on the measurement of LDL-C response and PCSK9 concentrations may help identify those statin-resistant subjects who may benefit from PCSK9 modulation for therapeutic benefits and those smaller proportions of subjects unable to tolerate statins (40).

4.6 Acknowledgments:

The data is the possession of the Investigators and Astra-Zeneca played no part in this manuscript. Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article. Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest: Employment or Leadership: None declared. Consultant or Advisory Role: P.M Ridker, AstraZeneca. Stock Ownership: None declared. Honoraria: J. Genest, speaker for JUPITER study AstraZeneca. Research Funding: The JUPITER study was sponsored by AstraZeneca. Zuhier Awan, King AbdulAziz University Funded Scholar; N.G. Seidah, Canadian Institutes of Health research grants (CTP 82946, MOP 102741); D.I. Chasman, funding for genomewide genotyping in JUPITER population from AstraZeneca; P.M Ridker, AstraZeneca. Expert Testimony: None declared. Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

4.7 References:

1. Hobbs HH, Russell DW, Brown MS, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. Annu Rev Genet 1990;24:133-70.

2. Maxwell KN, Breslow JL. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. Proc Natl Acad Sci U S A 2004;101:7100-5.

3. Benjannet S, Rhainds D, Essalmani R, Mayne J, Wickham L, Jin W, et al. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. J Biol Chem 2004;279:48865-75.

4. Abifadel M, Varret M, Rabès JP, Allard D, Ouguerram K, Devillers M, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 2003;34:154-6.

5. Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK, Hobbs HH. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. Nature Genetics 2005;37:161-5.

6. Benn M, Nordestgaard BG, Grande P, Schnohr P, Tybjaerg-Hansen A. PCSK9 R46L, low-density lipoprotein cholesterol levels, and risk of ischemic heart disease. 3 independent studies and meta-analyses. J Am Coll Cardiol 2010;55:2833-42.

7. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ,et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 2008;359:2195-207.

8. Dubuc G, Tremblay M, Paré G, Jacques H, Hamelin J, Benjannet S, et al. A new method for measurement of total plasma PCSK9: clinical applications. J Lipid Res 2010;51:140-9.

9. Benjannet S, Rhainds D, Hamelin J, Nassoury N, Seidah NG. The proprotein convertase (PC) PCSK9 is inactivated by furin and/or PC5/6A: functional consequences of natural mutations and post-translational modifications. J Biol Chem 2006;281:30561-72.

10. Essalmani R, Susan-Resiga D, Chamberland A, Abifadel M, Creemers JW, Boileau C, et al. In vivo evidence that furin from hepatocytes inactivates PCSK9. J Biol Chem 2011;286:4257-63.

11. Seidah NG, Benjannet S, Wickham L, Marcinkiewicz J, Jasmin SB, Stifani S, et al. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. Proc Natl Acad Sci U S A 2003;100:928-33.

12. Costet P, Krempf M, Cariou B. PCSK9 and LDL cholesterol: unravelling the target to design the bullet. Trends Biochem Sci 2008;33:426-34.

13. Seidah NG. PCSK9 as a therapeutic target of dyslipidemia. Expert Opin Ther targets 2009;13:19-28.

14. Kwon HJ, Lagace TA, McNutt MC, Horton JD, Deisenhofer J. Molecular basis for LDL receptor recognition by PCSK9. Proc Natl Acad Sci U S A 2008;105:1820-5.

15. Mayer G, Poirier S, Seidah NG. Annexin A2 is a C-terminal PCSK9-binding protein that regulates endogenous low density lipoprotein receptor levels. J Biol Chem 2008;283:31791-801.

16. Poirier S, Mayer G, Poupon V, McPherson PS, Desjardins R, Ly K, et al. Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation: evidence for an intracellular route. J Biol Chem 2009;284:28856-64.

17. Palmer-Smith H, Basak A. Regulatory effects of peptides from the pro and catalytic domains of proprotein convertase subtilisin/kexin 9 (PCSK9) on low density lipoprotein receptor (LDLR). Curr Med Chem 2010;17:2168-82.

18. Roubtsova A, Munkonda MN, Awan Z, Marcinkiewicz J, Chamberland A, Lazure C, et al. Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue. Arterioscler Thromb Vasc Biol 2011;31:785-91.

19. Ni YG, Di Marco S, Condra JH, Peterson LB, Wang W, Wang F, et al. A PCSK9-binding antibody that structurally mimics the EGF(A) domain of LDL-receptor reduces LDL cholesterol in vivo. J Lipid Res 2011;52:78-86.

20. Jeong HJ, Lee HS, Kim KS, Kim YK, Yoon D, Park SW. Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol regulatory element binding protein-2. J Lipid Res 2008;49:399-409.

21. Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc Natl Acad Sci U S A 2003;100:12027-32.

22. Costet P, Cariou B, Lambert G, Lalanne F, Lardeux B, Jarnoux AL, et al. Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. J Biol Chem 2006;281:6211-8.

23. Dong B, Wu M, Li H, Kraemer FB, Adeli K, Seidah NG, et al. Strong induction of PCSK9 gene expression through HNF1alpha and SREBP2: mechanism for the resistance to LDL-cholesterol lowering effect of statins in dyslipidemic hamsters. J Lipid Res 2010;51:1486-95.

24. Maxwell KN, Soccio RE, Duncan EM, Sehayek E, Breslow JL. Novel putative SREBP and LXR target genes identified by microarray analysis in liver of cholesterol-fed mice. J Lipid Res 2003;44:2109-1.

25. Dubuc G, Chamberland A, Wassef H, Davignon J, Seidah NG, Bernier L, Prat A. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. Arterioscler Thromb Vasc Biol 2004;24:1454-9.

26. Kourimate S, Le May C, Langhi C, Jarnoux AL, Ouguerram K, Zaïr Y, et al. Dual mechanisms for the fibrate-mediated repression of proprotein convertase subtilisin/kexin type 9. J Biol Chem 2008;283:9666-73.

27. Langhi C, Le May C, Kourimate S, Caron S, Staels B, Krempf M, et al. Activation of the farnesoid X receptor represses PCSK9 expression in human hepatocytes. FEBS Lett 2008;582:949-55.

28. Attie AD, Seidah NG. Dual regulation of the LDL receptor--some clarity and new questions. Cell Metab 2005;1:290-2.

29. Berge KE, Ose L, Leren TP. Missense mutations in the PCSK9 gene are associated with hypocholesterolemia and possibly increased response to statin therapy. Arterioscler Thromb Vasc Biol 2006 May;26:1094-100.

30. Lakoski SG, Lagace TA, Cohen JC, Horton JD, Hobbs HH. Genetic and metabolic determinants of plasma PCSK9 levels. J Clin Endocrinol Metab 2009;94:2537-43.

31. Welder G, Zineh I, Pacanowski MA, Troutt JS, Cao G, Konrad RJ. High-dose atorvastatin causes a rapid sustained increase in human serum PCSK9 and disrupts its correlation with LDL cholesterol. J Lipid Res 2010 Sep;51:2714-21.

32. Costet P, Hoffmann MM, Cariou B, Guyomarc'h Delasalle B, Konrad T, Winkler K. Plasma PCSK9 is increased by fenofibrate and atorvastatin in a non-additive fashion in diabetic patients. Atherosclerosis 2010;212:246-51.

33. Kathiresan S, Melander O, Anevski D, Guiducci C, Burtt NP, Roos C, et al. Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl J Med 2008;358:1240-9.

34. Frank-Kamenetsky M, Grefhorst A, Anderson NN, Racie TS, Bramlage B, Akinc A, et al. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. Proc Natl Acad Sci 2008;105:11915-20.

35. Graham MJ, Lemonidis KM, Whipple CP, Subramaniam A, Monia BP, Crooke ST, Crooke RM. Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice. J Lipid Res 2007;48:763-7.

36. Gupta N, Fisker N, Asselin MC, Lindholm M, Rosenbohm C, Ørum H, et al. A locked nucleic acid antisense oligonucleotide (LNA) silences PCSK9 and enhances LDLR expression in vitro and in vivo. PLoS One 2010;5:e10682.

37. Chan JC, Piper DE, Cao Q, Liu D, King C, Wang W, et al. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. Proc Natl Acad Sci 2009;106;9820-5.

38. Graham I, Atar D, Borch-Johnsen K, Boysen G, Burell G, Cifkova R, et al. European guidelines on cardiovascular disease prevention in clinical practice. Eur Heart J 2007;28:2375-414.

39. Genest J, McPherson R, Frohlich J, Anderson T, Campbell N, Carpentier A, et al. 2009 Canadian Cardiovascular Society/Canadian guidelines for the diagnosis and treatment of dyslipidemia and prevention of cardiovascular disease in the adult - 2009 recommendations. Can J Cardiol. 2009;25:567-79.

40. Bruckert E, Hayem G, Dejager S, Yau C, Bégaud B. Mild to moderate muscular symptoms with high-dosage statin therapy in hyperlipidemic patients--the PRIMO study. Cardiovasc Drugs Ther 2005;19:403-14.

4.8 Legends and figures:

Figure 1.

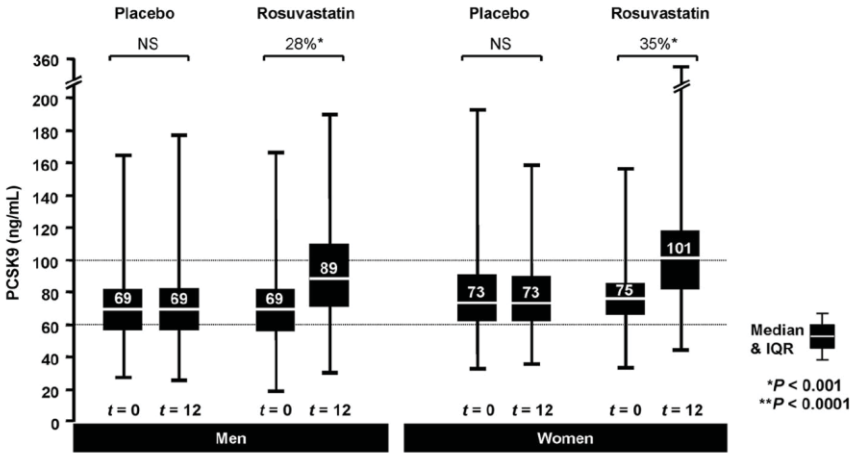


Fig. 1. Stability of PCSK9 concentrations over 1 year in the JUPITER placebo arm vs the rosuvastatin arm. PCSK9 concentrations in men and women at baseline and over 1 year.

Vertical bars, minimum and maximum values; box, interquartile range (IQR); horizontal bar, median; NS, nonsignificant; $t = 0$, baseline values; $t = 12$, 1-year values. $^*P < 0.0001$.

Figure 2.

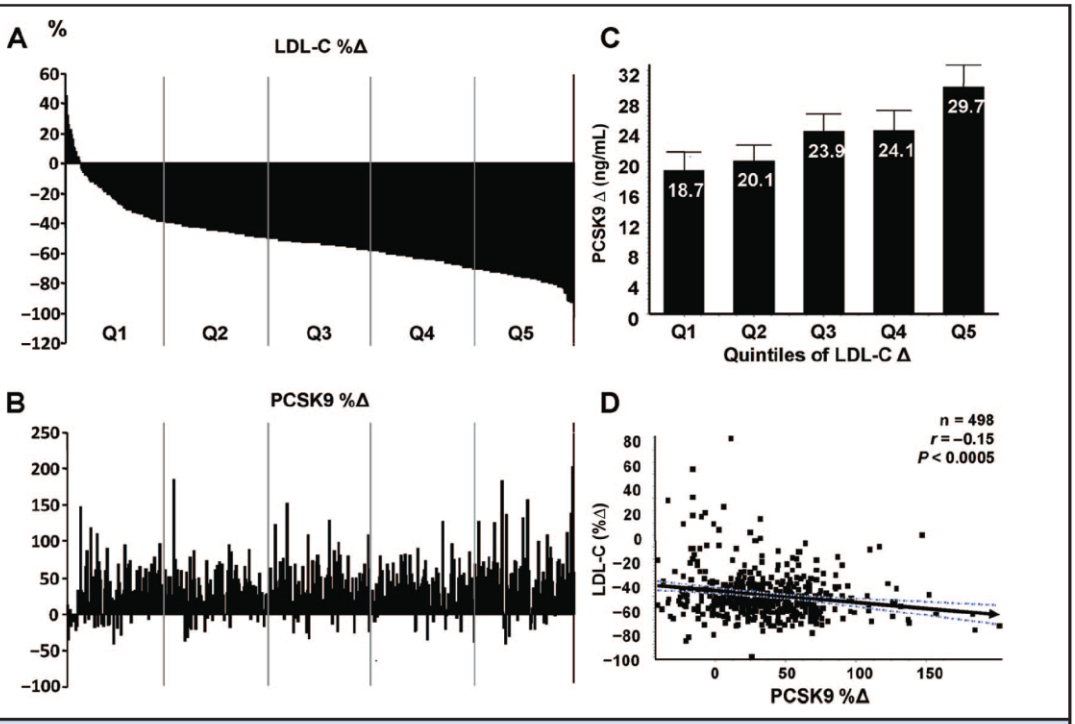


Fig. 2. Rosuvastatin increased plasma concentrations of PCSK9 in proportion to the magnitude of LDL-C reduction.

(A), Individual percentage LDL-C change in response to rosuvastatin 20 mg ranked by magnitude of effect. (B), Corresponding change in PCSK9 concentrations for each study subject ranked by the magnitude of LDL-C change. (C), Changes in PCSK9 ranked by quintiles of LDL-C change on rosuvastatin ($P = 0.0231$ by ANOVA). (D), LDL-C percentage change [(LDL-C at 12 months − LDL-C baseline)/LDL-C baseline × 100] on rosuvastatin correlates significantly with PCSK9 percentage change [(PCSK9 at 12 months − PCSK9 baseline)/(PCSK9 baseline × 100)].

4.9 Tables:

Table 1.

Table 1. Patient demographics stratified by sex and intervention group.[a]

	Men		Women	
	Placebo	Rosuvastatin	Placebo	Rosuvastatin
Age, years	64 (59–70)	62 (57–69)	70 (67–75)	70 (66–74)
Glucose				
mmol/L	5.3 (5.0–5.6)	5.3 (5.0–5.7)	5.2 (4.8–5.5)	5.2 (4.8–5.4)
mg/dL	95 (90–101)	96 (90–102)	93 (87–99)	93 (87–98)
Hb A_{1c}, %	5.6 (5.3–5.8)	5.6 (5.3–5.8)	5.7 (5.5–5.9)	5.6 (5.4–5.8)
Body mass index, kg/m^2	29 (26–32)	29 (26–32)	29 (25–33)	29 (25–33)
Systolic blood pressure	130 (122–140)	130 (120–140)	132 (120–142)	130 (122–140)
Diastolic blood pressure	80 (72–82)	80 (72–84)	78 (70–81)	77 (70–81)
Metabolic syndrome[b]	95 (38)	97 (39)	99 (40)	92 (37)
Smoking	41 (16)	38 (15)	13 (5)	18 (7)

[a] Data are median (25th–75th percentile) or n (%). No statistical difference exists between the 2 groups in either sex (n = 250 in each group).
[b] As defined by the consensus criteria of the American Heart Association.

Table 2.

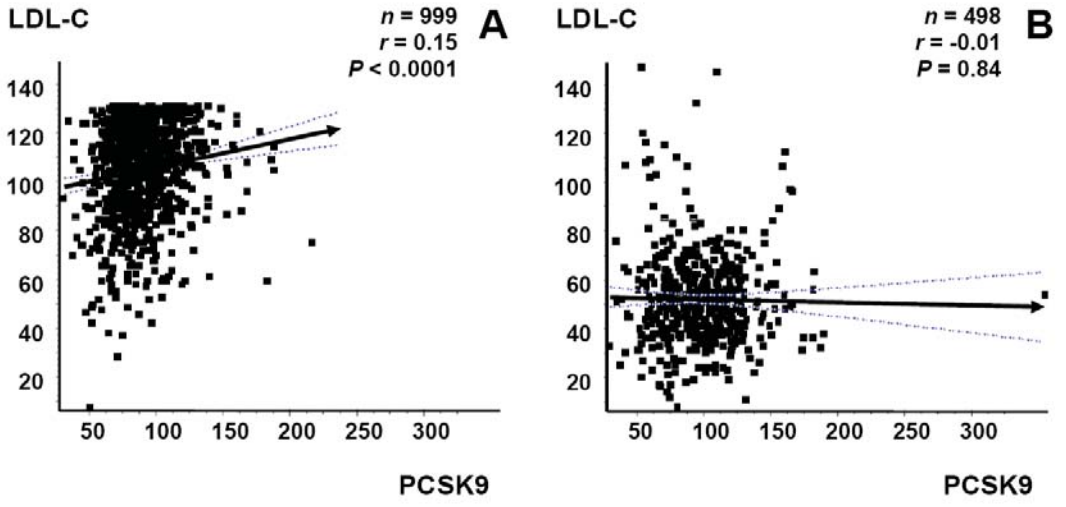
Table 2. Baseline and 12-month lipids, apolipoproteins A and B, and hsCRP.[a]

	Baseline		12 months	
	Placebo	Rosuvastatin	Placebo	Rosuvastatin
Men				
Cholesterol, mg/dL	184 (171–194)	180 (165–194)	182 (168–199)	125 (109–139)[b]
Triglycerides, mg/dL	123 (82–174)	125 (85–190)	121 (88–167)	99 (72–151)[b]
LDL-C, mg/dL	108 (95–118)	106 (88–117)	107 (94–120)	50 (40–61)[b]
HDL-C, mg/dL	46 (38–55)	44 (37–54)	46 (40–55)	49 (41–59)[b]
Apo B, mg/dL	107 (95–119)	106 (92–119)	108 (96–120)	64 (55–74)[b]
Apo A, mg/dL	153 (137–172)	152 (136–167)	157 (139–176)	159 (142–180)[b]
hsCRP, mg/L	3.8 (2.5–6.4)	3.5 (2.4–6.5)	3.2 (1.8–5.6)	2.2 (1.2–4.3)
Women				
Cholesterol, mg/dL	194 (179–206)	192 (179–207)	196 (181–214)[b]	134 (121–149)[b]
Triglycerides, mg/dL	116 (86–158)	117 (93–159)	116 (88–167)	98 (74–125)[b]
LDL-C, mg/dL	110 (95–121)	108 (96–120)	112 (97–124)[b]	49 (41–62)[b]
HDL-C, mg/dL	57 (48–70)	58 (49–68)	58 (49–67)	62 (53–73)[b]
Apo B, mg/dL	107 (94–119)	105 (93–118)	108 (94–120)	61 (54–70)[b]
Apo A, mg/dL	175 (156–201)	178 (158–198)	182 (161–203)	188 (168–204)[b]
hsCRP, mg/L	4.3 (3.0–6.9)	4.7 (3.1–7.6)	4.1 (2.5–6.7)	2.3 (1.3–4.1)[b]

[a] Data are median (25th–75th percentile).
[b] $P < 0.005$ baseline vs 12 months (n = 250 in each group unless stated otherwise in the text).

4.10 Supplemental Material:

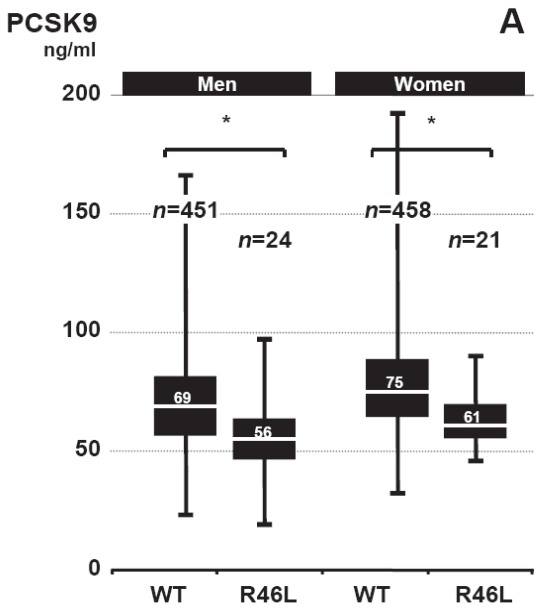
Supplemental Figure 1.



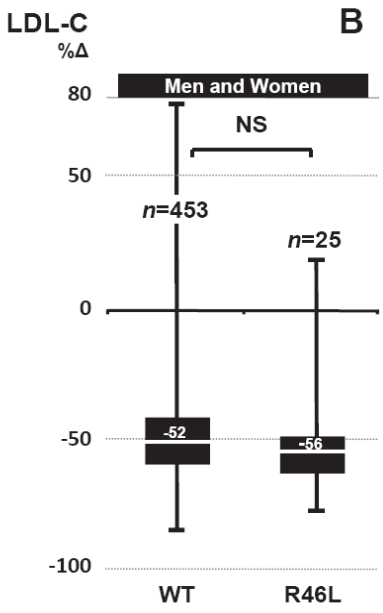
A. Baseline values in the placebo and rosuvastatin arms

B. One year in the rosuvastatin arm (Disruption of correlation)

Supplemental Figure 2.



A. Baseline values in the placebo and rosuvastatin arms (*n*=954)



B. One year in the rosuvastatin arm (*n*=478)

CHAPTER 5

"LDLR and Atherosclerosis"

ARTICLE (D)

CHAPTER 5: LDLR AND ATHEROSCLEROSIS

ARTICLE (D): *Aortic calcification: Novel insights from familial hypercholesterolemia and potential role for the low-density lipoprotein receptor*
AUTHORS: Fantus D, Zuhier Awan**,** Seidah NG, Genest J.

FIRST AUTHORS: Fantus D and Zuhier Awan (equal contribution)
CONTRIBUTION: Conception and design (70%), analysis and interpretation (50%) and writing and critical revising (40%)
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5. Foreword:

The LDLR deficient mouse as a model for aortic calcification was proposed by our group, and thus a novel method to objectively quantify calcium by micro-computed tomography (micro CT) was established. The mechanism of aortic wall calcification is poorly understood unlike the mechanism in aortic valve calcification, given the unique population of cells in each tissue. Once thought to be a passive process following atherosclerosis, now it is considered a tightly regulated process mediated by osteoblast like cells differentiation under the dysregulated interplay of shear stress, metabolites and inflammatory cytokines.

By studying the progression of atherosclerotic calcification in aorta and modifying the disease progression there is hope for FH patients to not only live beyond CVD but to surpass the premature aortic calcification process that contributes to lifelong morbidity.

Thus, using relevant animal models, an atherogenic diet, methods designed to objectively promote calcification *ex vivo* and pharmacologically intervention, much can be applied to humans in a translational manner. The timing of this project cannot be overemphasized since large clinical trials are being conducted to prevent secondary CVD from recurring in the context of secondary prevention. Conducting animal studies and applying new biology will steer the investigators on how to better utilize this huge effort effectively to look for relevant biomarkers of vascular health.

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Aortic Calcifications: Novel Insights from Familial Hypercholesterolemia and Potential Role for the Low-Density Lipoprotein Receptor

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CANADA

5.1 Abstract:

Once thought to be a passive process of calcium accrual in arterial vascular beds, vascular calcification is considered to be a tightly regulated process mediated by osteoblast-like cells under the dysregulated interplay of shear stress, metabolites, cytokines and transcription factors. Unfortunately, without effective medical interventions to prevent or regress vascular calcification, this process directly contributes to cardiovascular morbidity and mortality. We have previously shown that patients with familial hypercholesterolemia (FH) have severe, premature aortic calcifications and calcific aortic stenosis. We showed an age-related gene-dosage effect of deletion of the low-density lipoprotein receptor (LDL-R) gene on aortic calcifications in human subjects with FH. The LDL-R deficient mouse and transgenic mice overxpressing the LDL-R degrading protein PCSK9 also exhibit aortic calcifications, not fully explained by increased LDL cholesterol levels. Taken together, these data suggests a novel role for the LDL-R in the inhibition of vascular calcification. Understanding the molecular role of the LDL-R and its signaling partners in vascular calcification will be instrumental in identifying novel therapies for a common age-related process associated with a large burden of disease.

5.2 Abbreviations:

ANK: ankyrin family
AoCS: aortic calcium scores
BMP: bone morphogenetic proteins
Cbfa1: core binding factor alpha1
DKK1: Dikkopf protein
ESRD: end-stage renal disease
FH: familial hypercholesterolemia
GSK3B: glycogen synthase kinase 3 B
HSP: heat shock protein
LDL-R: low-density lipoprotein receptor protein
Ldlr$^{-/-}$: low-density lipoprotein receptor gene knockout in mice
LRP: low-density lipoprotein receptor-related protein
MGP: matrix gla protein
NPP I: ectonucleotide pyrophosphatase/phosphodiesterase I
OPG: osteoprotegerin
PEBP2: polyoma enhancer binding protein 2
PPARγ: peroxisome proliferator-activated receptor gamma
Pi: inorganic phosphate
PPi: inorganic pyrophosphate
RANKL: receptor activator of nuclear factor kappa-B ligand
Runx2: runt-related transcription factor 2
TGF: Transforming growth factor
TNFα: Tumor necrosis factor-alpha
VSMC: vascular smooth muscle cells
Wnt: a hybrid of Wg (wingless) and Int1 (integration1) genes in Drosophila
Hox8: Homeobox protein 8, now known as Msx2
Msx2: MSH homeobox 2, formerly Hox-8
Sox9: SRY (sex determining region Y) box 9
Osterix: Zinc Finger-Containing Transcription Factor
CCAAT: enhancer-binding proteins

It is estimated that one third of individuals over age 60 years have a progressive increase in calcium deposits in major arteries (1). As a consequence of calcium build-up in the vasculature, aortic and arterial elasticity is reduced and cardiovascular hemodynamics becomes compromised. The clinical impact of vascular calcification is widespread and contributes to arterial hypertension, aortic valve stenosis, limb ischemia, myocardial infarction and congestive heart failure (2). Calcific aortic stenosis is the leading cause of aortic valve replacement in Europe and North America and the third leading cause of cardiovascular disease (3-6). In the western world, 3% of adults over 75 years of age are affected by calcific aortic stenosis (7). Histologically, vascular calcification can occur in distinct layers of the blood vessel. Intimal calcification is associated with atherosclerosis whereas medial calcification (Monckeberg's sclerosis) is associated with age, diabetes and end-stage renal disease (ESRD). In ESRD, calcium and phosphate disorders are typically present (8). Both intimal and medial calcifications are markers of increased cardiovascular morbidity and mortality. Calcification of coronary arteries is an independent biomarker of atherosclerosis and myocardial infarction (9). Diffuse medial calcification can lead to decreased arterial compliance and increase cardiac after load contributing to the development of left ventricular hypertrophy and decreased cardiac perfusion (8). Arterial calcification in ESRD is considered a predictor of cardiovascular mortality (10). In dialysis patients, calciphylaxis represents a third form of vascular calcification; a systemic vascular calcification associated with calcific skin necrosis and global mortality (11).

The potential role of the low-density lipoprotein receptor (LDL-R) in the pathogenesis of intimal calcification was highlighted in a careful study of subjects with homozygous familial hypercholesterolemia due to mutations at the LDL-R gene locus (12, 13). Atherosclerotic intimal calcification appears to result from an interaction between inflammatory factors produced within an atherosclerotic plaque and a subpopulation of vascular cells that undergo osteogenic differentiation. Many of the conventional risk factors for coronary artery disease, namely hypercholesterolemia, hypertension, diabetes and tobacco use, have been linked to intimal calcification. It is thought that these pro-inflammatory agents induce oxidative stress in the milieu of the vascular endothelium that leads to atherosclerosis and calcification. On the basis of recent observations, this view is challenged.

5.3 Origin of cells involved in vascular calcification

The key regulatory mechanisms involved in vascular calcification have been described and many excellent reviews have already been written on this topic (5,14,15). **Fig.1** illustrates the proposed signaling pathways involved in vascular calcification. In the current model, vascular calcification is a process of active bone formation by osteoblast-like cells. Two types of lipid vesicles have been identified that nucleate vascular calcification; the apoptotic bodies of dead or dying cells and mineralizing matrix vesicles. The latter process appears to predominate in aortic calcification.

The origin of the cells that calcify the underlying matrix is a matter of continuous debate. Transdifferentiation of vascular smooth muscle cells (VSMC) in the vascular wall by reprogramming mature VSMCs to osteochondrocytic cells or calcifying vascular cells has been postulated as a process occurring in response to cytokines and oxidative stress stimuli (16). A second hypothesis, based on observation, suggests that nascent pericytes are involved in the process of calcification. Like calcifying vascular cells, pericytes form calcified nodules in culture (16). Pericytes play a role in skull wound repair, a process dependent on bone morphogenetic proteins (BMPs) (17). A third possibility, based on the finding of osteogenic cells in peripheral blood (18), is that the osteoblast-like cells responsible for vascular calcification arise from bone marrow-derived mesenchymal stem cell precursors. These stem cells have the ability to differentiate into osteoblasts, chondrocytes, myocytes and even adipocytes (19) (**Fig. 2**).

5.4 Regulation of vascular calcification

The signaling molecules that mediate vascular bone formation have been gradually elucidated. These include a variety of transcription factors, inflammatory stimuli and metabolites. The role of bone morphogenetic protein-2 (BMP2) in vascular calcification was elucidated at the time of its identification in human atherosclerotic plaques (20). To date, over twenty bone morphogenetic proteins have been discovered and belong to the Transforming growth factor-β (TGF β) superfamily of proteins. Since then, endothelial cells, foam cells and smooth muscle cells have all been found to express BMPs (16, 20). Mechanistically BMP2 acts through the transcription factor runt-related 2 (Runx2), which induces expression of type I collagen and alkaline phosphatase (**Fig 1**).

The other key genomic target of BMP signaling is the Wnt canonical pathway. This pathway involves signaling via the Frizzled (Fz) receptor and LRP 5/6 coreceptors. In the presence of Wnts, a ternary complex is formed at the cell surface that prevents glycogen synthase kinase 3 (GSK3B) from phosphorylating the transcription factor β-catenin. As a result, β-catenin can translocate to the nucleus. Once in the nucleus, β-catenin binds to proteins of the T-cell factor/lymphoid-enhancer factor-1 family and modulates the expression of several target genes including cyclin D, Cbfa 1 and Sox9. In the absence of Wnt, β-catenin is phosphorylated by GSK3B. Phosphorylated β-catenin undergoes ubiquitination and proteasomal degradation (19, 21, 22). Thus, *via* the canonical Wnt pathway, BMP2 promotes osteoblast differentiation and bone formation.

In addition to the canonical pathway, several Wnt proteins activate osteoblastogenesis through non-canonical pathways. Of the 19 Wnt family members, Wnt10b, Wnt7b and Wnt3a in particular have been shown to be involved in bone homeostasis in mice (19). Wnt10b promotes osteoblast differentiation through induction of the osteoblastogenic transcription factors Runx2 and Osterix and suppression of the adipogenic transcription factors CCAAT/enhancer binding protein alpha and peroxisome proliferator-activated receptor gamma (PPAR-γ) (19). Runx2 (also known as Cbfa1 or polyoma enhancer binding protein 2 (PEBP2), is thought to initiate osteoblast differentiation through activation of Osterix.

A protein upstream to Wnt signaling called Msx2 (Hox8), is a homeodomain transcription factor involved in craniofacial bone mineralization and trabecular and cortical bone formation. In murine models of vascular calcification, Msx2 expressing cells in the adventitia were found to control osteogenic differentiation of neighboring progenitors in the media *via* the vasa vasorum. Further work identified that Msx2 upregulates aortic expression of Wnt3a and Wnt7a (23). We have recently shown that Wnt5a and its co-receptor LRP5 are highly expressed in calcifying vessels in the absence of LDL-R in a mouse model of aortic calcification (24).

Two key players in bone biology that have great relevance in vascular calcification are the receptor activator of nuclear factor κB ligand (RANKL) and its inhibitor osteoprotegerin (OPG) (**Fig.1**). RANKL, a member of the TNF superfamily, signals *via* its receptor on monocyte/macrophage progenitors to promote the formation of bone-resorbing osteoclasts (14). OPG serves as a decoy receptor in osteoblasts and antagonizes RANKL. In the setting of vascular inflammation, RANKL is expressed by T cells, macrophages and endothelial cells near atherosclerotic lesions, promotes mineralization of VSMCs and aortic valve interstitial cells *in vitro* and upregulates BMP4 expression *via* its receptor. The role of RANKL's in vascular biology was further supported by the findings of severe medial and intimal vascular calcification in OPG-deficient mice (25). Interestingly, many of the same stimuli that induce cardiovascular disease in our aging population, namely inflammation (TNFα), reactive oxygen species and mechanical damage also upregulate these signaling pathways. Hydrogen peroxide, for example, promotes osteochondrocytic differentiation of VSMCs by upregulating Runx2 (15).

Interleukin-1b has been implicated in the stimulation of alkaline phosphatase (26) and may be a critical factor in the transdifferentiation of smooth muscle cells into an inflammatory phenotype (27, 28).

5.5 Inhibition of vascular calcification

Several circulating proteins have been discovered that normally inhibit vascular calcification (**Fig. 1**). Osteoprotegerin (OPG), for example, competes with RANKL for its receptor. Inorganic pyrophosphate directly inhibits hydroxyapatite formation and calcium deposition. While it serves a substrate for alkaline phosphatase, its activity is maintained by ectonucleotide pyrophosphatase/phosphodiesterase I (NPPI) and the cellular pyrophosphate exporter ankyrin (ANK) (13). The actions of BMP are inhibited by several proteins; including noggin, chordin and matrix gla protein (MGP). MGP functions as a noggin-like protein by inhibiting BMP-BMPR2 interactions as well as by binding and inhibiting BMP2 directly. It is the quantitative ratio of BMP2 to MGP that modulates BMP2 activity and osteogenic calcification (16). Interestingly, MGP is activated by

vitamin K-dependent gamma carboxylation. Therefore, non-gamma carboxylated MGP is associated with vascular calcification. The importance of MGP in vascular calcification has been confirmed *in vivo* by gene deletion studies in mice. In the absence of MGP, mice develop extensive vascular calcifications without atherosclerosis (26). The interplay between the BMP and MGP pathways of vascular calcification was elegantly illustrated in experiments showing that heat shock protein (HSP) 70, an endogenous MGP binding protein and antagonist of MGP function found in atherosclerotic plaques is upregulated by IL-6, an inflammatory cytokine important in diabetic vascular disease (14,30).

Fetuin (also known as alpha Heremans-Schmid glycoprotein) is a protein synthesized by the liver. It belongs to the cystatin superfamily of cysteine protease inhibitors (5). Its mechanism of action appears to be twofold. First, fetuin directly inhibits calcium phosphate crystal formation by binding amorphous calcium phosphate. Second, fetuin, along with MGP, have been shown to mediate VSMC-mediated uptake and clearance of matrix vesicles (29). As a result, the scaffold for matrix mineralization is removed and mineralization cannot take place. The clinical relevance of fetuin's protective role is supported by studies showing an inverse relationship between fetuin levels and mortality in end stage renal disease (31).

5.6 Familial hypercholesterolemia and aortic calcifications

Patients with familial hypercholesterolemia (FH) exhibit an age- and gene-dosage dependent increase in aortic calcification (**Fig. 3**). FH is a relatively common monogenic co-dominant Mendelian disorder (the allele frequency is often quoted as 1 in 500 in the general population) and is caused predominantly by mutations in the LDL-R gene (12, 13, 32, 33). In populations with a founder effect, such as the French Canadians, the prevalence of LDL-R mutations is much higher and reaches 1 in 80 in certain parts of the province of Quebec (34). The LDL-R is a cell surface glycoprotein that regulates plasma cholesterol *via* endocytosis of LDL particles (32, 33). Three other genes have been found to cause a phenotype similar to FH include the apolipoprotein (apo) B gene (APOB), the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*) and the adapter protein autosomal recessive hypercholesterolemia gene (ARH, encoded

by the *Ldlrap1* gene).FH is phenotypically characterized by markedly elevated levels of cholesterol, cutaneous xanthomas and xanthelasmas, corneal arcus, premature atherosclerosis and cardiovascular disease (12, 34-36). Before the advent of lipid lowering therapy, mortality was common in homozygous subjects before the second or third decade of life and cardiovascular disease developed in the third to fourth decade of life in heterozygotes. The development of selective LDL extracorporeal removal techniques (LDL apheresis) and lipid-lowering medication over the past few decades has resulted in prolonged survival and improved quality of life (12, 37-39).

Patients with homozygous FH develop premature aortic calcifications as determined by computed tomographic (CT) scan (12). A strong correlation was found between calcification scores and age but not with total cholesterol. Aortic calcifications in homozygotes and severe heterozygotes often lead to the development of a “porcelain” ascending aorta. In those patients with concomitant calcific aortic stenosis, resection of the entire ascending aorta under deep hypothermic circulatory arrest is often necessary. Thus coronary and aortic valve surgery presents unique challenges (40-42). Complications from such extensive surgery include calcific embolization, patient-valve mismatch and early coronary graft failure from proximal anastomotic occlusion and increased surgical mortality. Aortic calcifications were also observed in patients with heterozygous FH, though they tended to be less extensive and showed up 2 decades later than their homozygous counterparts (13) (**Fig. 3**). It is interesting to note that aortic calcifications developed despite appropriate medical therapy, including the combination of LDL apheresis, statins and ezetimibe, resulting in marked sustained reductions in LDL levels. In contrast, the progression of coronary artery disease was markedly reduced and tissue cholesterol deposits, including xanthomas and xanthelasmas, often regressed with lipid-lowering therapy in FH patients (12, 13).

*These clinical observations suggest that the calcification process may proceed independently of cholesterol levels, once sub-endothelial damage has occurred***.** Based on these clinical observations, we proposed a two hit hypothesis of vascular calcification in FH: first, intimal injury due to the initiation of an inflammatory reaction and early atherosclerosis. Second the deposition of calcium on matrix proteins in the intimal wall of

genetically susceptible individuals. This hypothesis establishes a timeline wherein athereosclerosis progresses to accelerated vascular calcification. This clinical course is also consistent with the results of several trials in humans that have failed to show a benefit of statins in the progression of calcific aortic valve stenosis (43). By the time vascular calcification is detected, lipid lowering therapy to prevent or reverse calcification appears to be of limited clinical benefit. While statins may considerably slow down or even regress the process of atherosclerosis, they do not stop the calcification process.

Similar observations were found in experimental animals. Aged *Ldlr*$^{-/-}$ mice expressing $ApoB_{100}$ developed extensive oxidative stress with aortic valve calcification and functional valvular disease (44). In addition, older rhesus monkeys and *Maccaca mulatta* fed high cholesterol diets developed atherosclerosis and vascular calcifications. When plasma cholesterol decreased on a regression diet, atherosclerosis but not calcification diminished (45).

5.7 Low-density lipoprotein receptor superfamily and osteogenesis

While statins and other LDL-lowering therapies markedly diminish the progression of atherosclerosis in patients with FH, aortic valve and aorta calcifications are minimally affected (13). This apparent dissociation between the process of atherosclerosis and intimal calcification is further emphasized in murine models of aortic calcification. Using the chow fed C57Bl/6 *Ldlr*$^{-/-}$ mouse model, Awan *et al*. have shown that extensive aortic calcifications occur in a distribution similar to that found in humans (24). Conversely, wild-type mice fed a high-cholesterol, high-fat diet have serum levels of cholesterol similar to the chow-fed *Ldlr*$^{-/-}$ mouse but do not develop calcifications (**Fig. 4**). These observations strongly support the concept that the LDL-R modulates vascular calcification independent of serum LDL. Our hypothesis is that osteogenic signaling pathways are modulated by an intact LDL-R protein. As a result, mutation at the *ldlr* gene locus results in dysregulated calcification signaling pathways in osteoblasts-like cells (**Fig. 2**).

The roles of LRP1, LRP5 and LRP6, proteins belonging to the LDL-R superfamily of proteins, are becoming increasingly important in vascular calcification. LRP1 expression levels are higher in mature chondrocytic cell lines suggesting a specific role of LRP1 in cartilage formation (46). Interestingly, *Lrp1*$^{-/-}$ mice were found to develop chondrogenesis of the vascular wall emphasizing the regulatory role of this LDL-R family member in cell differentiation (47-49). Both LRP5 and LRP6 are large single-pass transmembrane receptors also part of the LDL-R superfamily. Their role in the canonical Wnt signaling pathway has been extensively investigated in Drosophila, Xenopus and mice and is briefly described above. Although LRP5 has an established role in bone development, murine knockouts on a western diet showed both osteoporosis and dyslipidemia thanks to its putative roles in binding apolipoprotein E-containing lipoproteins and chylomicron clearance (51, 52). Human mutations in LRP6 have been identified in the context of severe osteoporosis and premature cardiovascular disease (49). Finally, LRP6 can bind and regulate the function of the LDL-R (50). This raises the possibility of molecular cross-talk between the LDL-R and LRP6. Given the role in LRP6 in the mineralization process, the possibility that the LDL-R modulates its function must be considered.

Ldlr$^{-/-}$ mouse aortic tissue exhibits abundant LRP5 and Wnt5a expression (24) (**Fig. 5**). This may be partly explained by the requirement of LRP pairing for LDL-R-clearance. LDL-R internalization is severely diminished when LRP6 is knocked down (50). Therefore in the absence of LRP6 and possibly LRP5 (48, 49), LDL-R cannot leave the membrane surface and intracellular signaling becomes disrupted.

The identification of oxidized lipoprotein particles in tissues such as bone has led to the concept that lipid oxidation products directly inhibit osteoblast differentiation and induce osteoclast differentiation further supports this concept (15, 52, 53). Whether vascular calcification develops as a consequence of increased bone resorption and serum calcium remains unclear. A study by Awan *et al* addressed this question by studying biomarkers of bone turnover in 19 patients with heterozygous FH (54). Mean bone mineral density (BMD) at the femoral neck in these patients was not found to differ from age-, and sex-matched mean BMD. However, negative correlations (r=-0.64,

p=0.0034) were seen between the aortic calcium score and serum concentrations of osteocalcin, a marker of bone formation. Despite the absence of significant bone loss in these patients, diminished levels of serum osteocalcin compared to controls suggest that bone formation in FH may be subtly altered, although the clinical significance of this observation remains uncertain. Lack of an LDL-R might modulate osteoblast function either locally in the vasculature, in bone or in both tissues simultaneously. This would provide a molecular explanation for the marked vascular calcifications seen in patients with nonfunctional LDL-R.

5.8 Conclusions:

The finding of accelerated vascular calcifications in patients with familial hypercholesterolemia that occurs independently of blood cholesterol levels coupled with data from mouse models of aortic calcifications supports a role for the LDL-R protein in the process of arterial mineralization. While statins, drugs that are known to increase LDL-R density and function at the cell surface, have markedly decreased the process of atherosclerosis, arterial calcifications remain unperturbed by this approach. Other avenues aimed at modulating the LDL-R signaling pathway may help identify novel therapeutic approaches to prevent vascular calcification.

5.9 Discussion:

The evolution of vascular calcification appears to be, at least in part, independent of LDL-cholesterol levels (24) and once osteoblast-like cells differentiate; the process may become only poorly reversible. Conventional treatment modalities may lower cholesterols level and prevent coronary artery disease but do not necessarily stop vascular calcification. If FH patients survive coronary artery disease, they encounter morbidities associated with premature calcification of the aorta, such as hypertension or embolizing calcified lesions. The intriguing finding that diet-induced hypercholesterolemia in mice to levels that of homozygous FH (*LDL-R*$^{-/-}$) alone does not lead to vascular calcification, may change established paradigms in the concept of arterial calcification (**Fig. 4**). Therefore we consider that vascular calcification requires two factors; one for initiation in the form of endothelial insult (oxidized LDL, angiotensin II, cytokine release and mainly intimal inflammation) and other factors for maintenance in the form of genetic predisposition (lacking cell surface receptors, lacking inhibitors of calcification). In turn, these factors are expected to trigger BMP2 activation of Msx2 and the Wnt/Lrp5/β-catenin signaling pathways, as well as the Runx2/Cbfa1 pathway and mediate activation of osteoblast transformation (**Fig. 2**).

LDL-R-deficient mice developed calcium deposition in the intimal space of the aorta similar to humans with FH (12). This implies that early sub-endothelial damage caused by foam cell formation during the atherosclerosis process results in accumulation of structural matrix (collagen). Therefore early screen and detection with a sensitive method like CT scan in human is essential for disease understanding, monitoring treatment response and planning for future surgical interventions.

Vascular intimal calcification stands in sharp contrast with the disturbances in calcium and phosphate metabolism in ESRD. Even when extracorporeal LDL-cholesterol filtration techniques (LDL aphaeresis) are used to normalize plasma LDL-cholesterol, patients do not suffer calcium levels alterations (54). Despite apparently normal levels of calcium, phosphate and bone mineral density, a negative correlation between osteocalcin levels and calcification merit further investigation. Therefore it would be of interest to examine bone metabolism in established animal models.

Unfortunately, at the present time, we have little in the way of medical therapy to halt or reverse vascular calcification. Anti-inflammatory agents, pyrophosphate inhibitors, chelating therapy, vitamin K, selective estrogen receptor modulators and calcium channel blockers all hold promise as potential therapies (55). The ideal agent would modulate the pathological calcification process –occurring at sites of tissue injury or inflammation, as seen in atherosclerosis, while leaving normal bone formation intact. Modulating inflammation is fraught with potential adverse effects; choosing an agent that leaves the immune defenses intact and decreasing inflammation would represent a potential therapeutic avenue. Interestingly, a randomized phase III (56) clinical trial is studying the role of subcutaneous canakinumab (an IL1β inhibitor) in stable post myocardial infarction patients. The purpose of this trial is to test the hypothesis that canakinumab treatment in patients with cardiovascular disease on lipid lowering drugs will prevent cardiovascular recurrence. It will be interesting to follow these patients for the development of calcific aortic stenosis and aortic calcification. This will help confirm the two hit hypothesis that is required to develop aortic calcification beyond cholesterol lowering.

It is interesting to note that the relationship between age and cholesterol levels has previously been observed in patients with homozygous FH [57]. This simple measure was seen as an index of “cholesterol burden” during lifetime. In our studies in patients with homozygous FH in whom premature aortic calcifications was determined by computed tomographic (CT) scan, a strong correlationwas found between aortic calcification score and age ($r = 0.73$, $p = 0.0001$), but not with total cholesterol ($r = -0.36$, $p = 0.0715$) [12, 13]. This suggests that age remains the most important factor in vascular calcification.

Based on the clinical trial evidence of statins on the progression of calcific aortic stenosis [43], and of trails showing that statins do not reduce the rate of progression of coronary calcifications despite a beneficial effect on the progression of atherosclerosis [58], it appears important not to solely reduce cholesterol markedly, but to do so early in order to prevent the calcification process. As statins increase the LDL-R on the cell surface, this should modulate osteocyte activity. Yet, this does not appear to be the case in the clinic. The molecular basis for this observation remains to be explained.

5.10 References:

1. Messika-Zeitoun D, Bielak LF, Peyser PA, et al. Aortic valve calcification: determinants and progression in the population. Arterioscler Thromb Vasc Biol. 2007; 27(3):642-8.

2. Kaplan NM. 2002. Clinical Hypertension. 8th Edition. Philadelphia, Lippincott, Williams and Wilkins. 4.

3. Butany J., Collins MJ, Demellawy DE et al. Morphological and clinical findings in 247 surgically excised native aortic valves. Can. J. Cardiol. 2005; 21: 747-55.

4. Nkomo VT., Gardin JM, Skelton TN et al. Burden of valvular heart diseases: a population-based study. Lancet. 2006; 368: 1005-11.

5. Akat K., Borggrefe M and J.J. Kaden. Aortic valve calcification: basic science to clinical practice. Heart. 2009; 95: 616-23.

6. Stewart B.F., Siscovick D, Lind BK et al. Clinical factors associated with calcific aortic valve disease: cardiovascular health study. J Am. Coll. Cardiol. 1997; 29: 630-4.

7. Cowell S.J., Newby DE, Prescott RJ et al. A randomized trial of intensive lipid-lowering therapy in calcific aortic stenosis. N. Engl. J. Med. 2005; 352: 2389-97.

8. Giachelli C.M. Vascular Calcification Mechanisms JASN 2004. 2959-2964.

9. Ferket BS, Genders TS, Colkesen EB et al., Systematic review of guidelines on imaging of asymptomatic coronary artery disease. J Am Coll Cardiol. 2011; 57(15):1591-600. Review.

10. Mizobuchi M., Towler D and Slatopolsky E. Vascular Calcification: The killer of patients with chronic kidney disease JASN 2009. 1453-64.

11. Coates T, Kirkland GS, Dymock RB et al. Cutaneous necrosis from calcific uremic arteriolopathy. Am J Kidney Dis. 1998; 32: 384–391.

12. Awan, Z., Alrasadi K, Francis GA et al.Vascular Calcifications in Homozygote Familial Hypercholesterolemia. Arterioscler. Thromb. Vasc. Biol. 2008; 28: 777-785.

13. Alrasadi K., Alwaili K, Awan Z, Valenti D, Couture P and J. Genest. Aortic calcification in familial hypercholesterolemia: Potential role of the low-density lipoprotein receptor gene. Heart Journal. 2009; 157: 171-176.

14. Shao J.S., Cheng SL, Sadhu J and D.A. Towler. Inflammation and the Osteogenic Regulation of Vascular Calcification: A review and perspective. Hypertension. 2010; 55: 579-592.

15. Sage A.P., Tintut Y and LL Demer. Regulatory mechanisms in vascular calcification. Nature Reviews Cardiology. 2010; 7: 528-536.

16. Johnson R.C., Leopold JA and J. Loscalzo. Vascular Calcification: Pathobiological Mechanisms and Clinical Implications. Circ. Res. 2006; 99: 1044-1059.

17. Sato K and Urist MR. Induced regeneration of calvaria by bone morphogenetic protein (BMP) in dogs. Clin Orthop Relat Res. 1985; 197: 301-311.

18. Otsuru S, Tamai K, Yamazaki T, Yoshikawa H and Y Kaneda. Bone marrow-derived osteoblast progenitor cells in circulating blood contribute to ectopic bone formation in mice. Biochemical and Biophysical Research Communications. 2007; 354: 453-458.

19. Kubota, T., Michigami T and K. Ozono. Wnt signaling in bone metabolism. J Bone Miner Metab. 2009; 27: 265-71.

20. Bostrom K., Watson KE, Horn S, Wortham C, Herman IM and L.L. Demer. Bone Morphogenetic Protein Expression in Human Atherosclerotic lesions. J. Clin. Invest. 1993; 91: 1800-1809.

21. Luo J., Chen J, Deng ZL et al. Wnt signaling and human diseases: what are the therapeutic implications? Lab. Invest. 2007; 87:97-103.

22. Rajamannan, N.M. Mechanisms of aortic valve calcification: the LDL density radius theory: a translation from cell signaling to physiology. Am J Physiol Heart Circ Physiol. 2010; 298: 5-15.

23. Shao, J.S., Cheng SL, Pingsterhaus JM, Charlton-Kachigian N, Loewy AP and D.A. Towler. Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals. J. Clin. Invest. 2005; 115: 1210-1220.

24. Awan, Z., Denis M. Bailey D. et al. The LDLR deficient mouse as a model for aortic calcification and quantification by micro-computed tomography. Atherosclerosis. 2011; 219: 455-462.

25. Bucay N, Sarosi I, Dunstan CR, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 1998;12:1260–8.

26. Lencel P, Delplace S, Pilet P et al. Cell-specific effects of TNFα and IL-1β on alkaline phosphatase: implication for syndesmophyte formation and vascular calcification.Lab Invest. 2011;91:1434-42.

27. Keuylian Z, De Baaij JH, Glorian M et al. The Notch pathway attenuates interleukin 1β (IL1β)-mediated induction of Adenylyl Cyclase 8 (AC8) expression during vascular smooth muscle cell (VSMC) trans-differentiation. J Biol Chem 2012; May 21.

28. Alexander MR, Murgai M, Moehle CW and GK Owens. Interleukin-1β modulates smooth muscle cell phenotype to a distinct inflammatory state relative to PDGF-DD via NF-κB-dependent mechanisms.Physiol Genomics. 2012;44:417-29.

29. Luo G, Ducy P, McKee MD et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature. 1997; 386: 78-81.

30. Yao Y, Watson AD, Ji S and KI Bostrom. Heat shock protein 70 enhances vascular bone morphogenetic protein-4 signaling by binding matrix GIa protein. Circ Res. 2009; 105: 575-84.

31. Ketteler M, Bongartz P, Westenfeld R et al. Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross sectional study. Lancet. 2003;361: 827-33.

32. Brown MS and Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science.1986. 232:34-47.

33. Hobbs H.H., Russell DW, Brown MS and J.L. Goldstein. The LDL receptor locus in familial hypercholesterolemia. Annu. Rev. Genet. 1990; 24: 133-70.

34. Scriver CR. Human genetics: lessons from Quebec populations. Annu Rev Genomics Hum Genet.2001; 2: 69-101.

35. Humphries SE, Whittall RA, Hubbart CS et al. Genetic causes of familial hypercholesterolaemia in patients in the UK: relation to plasma lipid levels and coronary heart disease risk. J Med Genet. 2006;43: 943-949.

36. Tosi I, Toledo-Leiva P, Neuwirth C, Naoumova RP and AK Soutar. Genetic defects causing familial hypercholesterolaemia: Identification of deletions and duplications in the LDL-receptor gene and summary of all mutations found in patients attending the Hammersmith Hospital Lipid Clinic.Atherosclerosis. 2007;194:102–111.

37. Thompson GR, Miller JP and JL Breslow. Improved survival of patients with homozygous familial hypercholesterolaemia treated with plasma exchange. BMJ (Clin Res Ed). 1985;291:1671–1673.

38. Naoumova RP, Thompson GR and AK Soutar. Current management of severe homozygous hypercholesterolaemias. Curr Opin Lipidol. 2004;15: 413–422. Review.

39. Thompsen J andPD Thompson. A systematic review of LDL apheresis in the treatment of cardiovascular disease. Atherosclerosis. 2006;189:31–38.

40. Grenon SM, Lachapelle K, Marcil M, Omeroglu A, Genest J and B de Varennes. Surgical strategies for severe calcifications of the aorta (porcelain aorta) in two patients with homozygous familial hypercholesterolemia. Can J Cardiol. 2007; 23: 1159-1161.

41. Saito S, Usui A, Akita T and Y Ueda. Surgery for calcific aortic root stenosis in homozygous familial hypercholesterolemia. Eur J Cardiothorac Surg. 2006. 29: 114-116.

42. Yasuda T, Kawasuji M, Sakakibara N and Y Watanabe. Aortic valve replacement for the calcified ascending aorta in homozygous familial hypercholesterolemia. Eur J Cardiothorac Surg. 2000. 18:249-250.

43. Teo KK, Corsi DJ, Tam JW, Dumesnil JG and KL Chan. Lipid Lowering on Progression of Mild to Moderate Aortic Stenosis: Meta-analysis of the Randomized Placebo-Controlled Clinical Trials on 2344 Patients. Can J Cardiol. 2011;27(6):800-8.

44. Weiss RM, Ohashi M, Miller JD, Young SG and DD Heistad. Calcific aortic valve stenosis in old hypercholesterolemic mice. Circulation. 2006; 114: 2065-2069.

45. Clarkson TB, Bond MG, Bullock BC and CA Marzetta. A study of atherosclerosis regression in Macaca mulatta. Exp Mol Pathol. 1984; 34: 345-368.

46. Kawata K, Eguchi T, Kubota S et al. Possible role of LRP1, a CCN2 receptor, in chondrocytes.Biochem Biophys Res Commun. 2006; 345(2):552-9.

47. Kim DH, Inagaki Y, Suzuki T et al. A new low-density lipoprotein receptor related protein, LRP5, is expressed in hepatocytes and adrenal cortex, and recognizes Apolipoprotein E. Journal of Biochem. 1998; 124: 1072-1076.

48. Fujinoa, TH, Asabab, MJ, Kang, Y et al. Low-density lipoprotein receptor-related protein 5 (LRP5) is essential for normal cholesterol metabolism and glucose-induced insulin secretion. PNAS. 2003; 100:229-34.

49. Mani A, Radhakrishnan J, Wang H et al. LRP6 mutation in a family with early coronary disease and metabolic risk factors. Science. 2007;315:1278-82.

50. Ye ZJ, Go GW, Singh R, Liu W, Keramati AR and A Mani. LRP6 protein regulates low density lipoprotein (LDL) receptor-mediated LDL uptake. J Biol Chem. 2012; 287(2):1335-44.

51. Niemeier A, Niedzielska D, Secer R et al. Uptake of postprandial lipoproteins into bone in vivo:Impact on osteoblast function. Bone. 2008; 43: 230-237.

52. Tintut Y, Morony S and LL Demer. Hyperlipidemia promotes osteoclastic potential of bone marrow cells ex vivo. Arter. Thromb. and vasc. biol. 2004; 24: e6-10.

53. Parhami F, Morrow AD, Balucan J et al. Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. Arter. Thromb. and vasc. biol. 1997; 17: 680-87.

54. Awan Z, Alwaili K, Alshahrani A, Langsetmo L, Goltzman D and J Genest. Calcium homeostasis and skeletal integrity in individuals with familial hypercholesterolemia and aortic calcification. Clin Chem. 2010; 56: 1599-1607.

55. Wallin R, Wajih N, Greenwood GT and DC Sane. Arterial calcification: a review of mechanisms, animal models, and the prospects for therapy. Med Res Rev. 2001; 21(4):274-301.

56. Ridker PM, Thuren T, Zalewski A and P Libby. Interleukin-1β inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). Am Heart J. 2011; 162(4):597-605.

57. Schmidt HH, Hill S, Makariou EV, Feuerstein IM, Dugi KA, Hoeg JM. Relation of cholesterol-year score to severity of calcific atherosclerosis and tissue deposition in homozygous familial hypercholesterolemia. Am J Cardiol 1996;77: 575e80.

58. Gill Jr EA. Does statin therapy affect the progression of atherosclerosis measured by a coronary calcium score? Curr Atheroscler Rep 2010;12(2):83e7.

5.11 Legends and figures:

Figure 1.

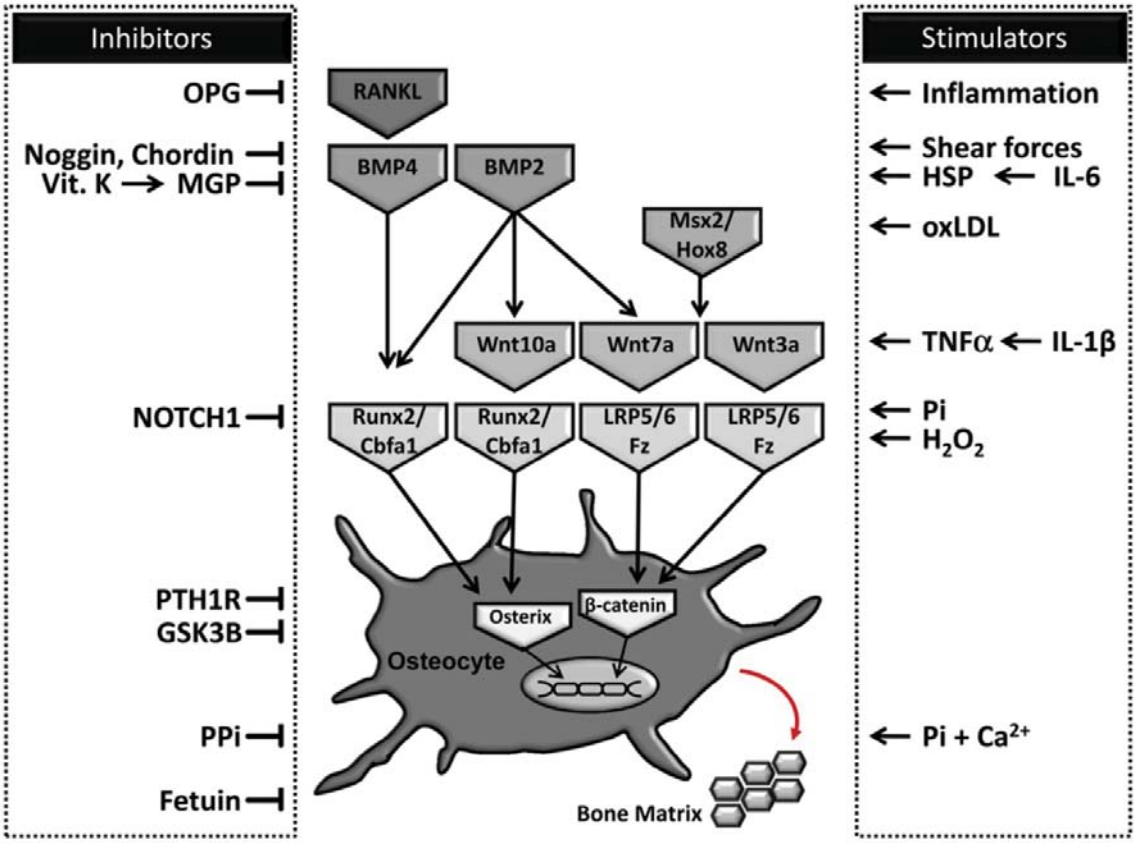

Figure 1. A schematic representation of the signaling pathways in vascular calcification in relation to known stimulatory and inhibitory factors

Figure 2.

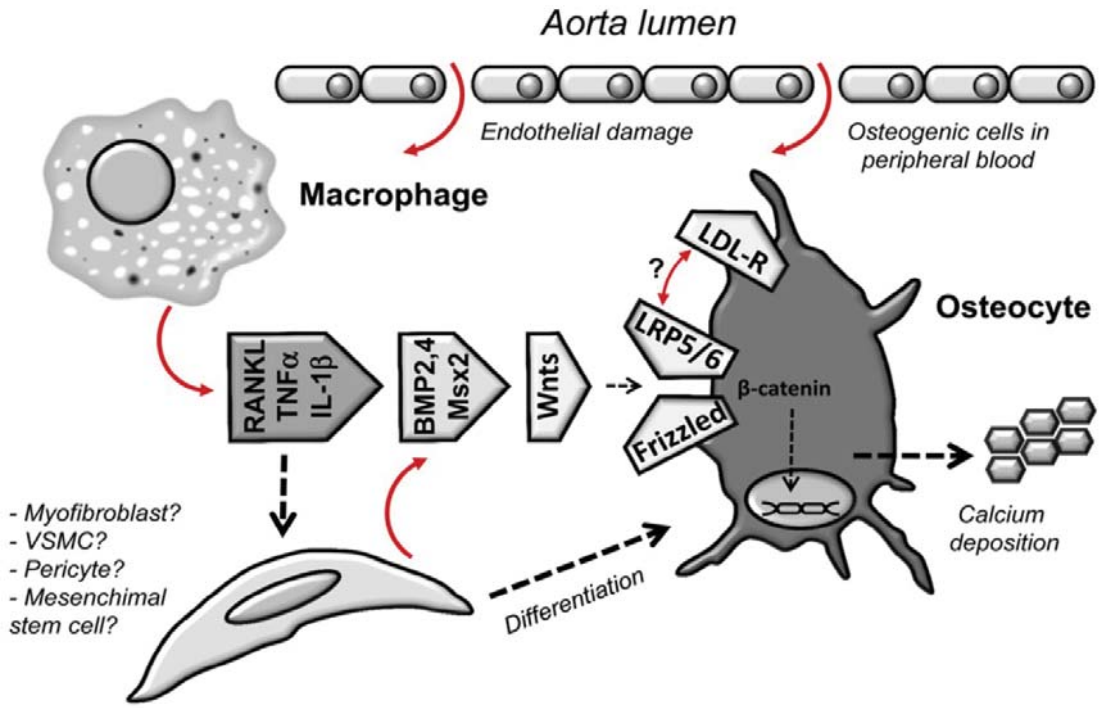

Figure 2. A proposed model linking the LDL-R and the Wnt canonical pathway. In this scheme, the inflammatory environment created by atherosclerosis lesions in the FH mouse model leads to differentiation of osteoblast precursors cells to osteocytes. The absence (complete or partial) of the LDL-R contributes to translocation of the β-catenin to the nucleus.

Figure 3.

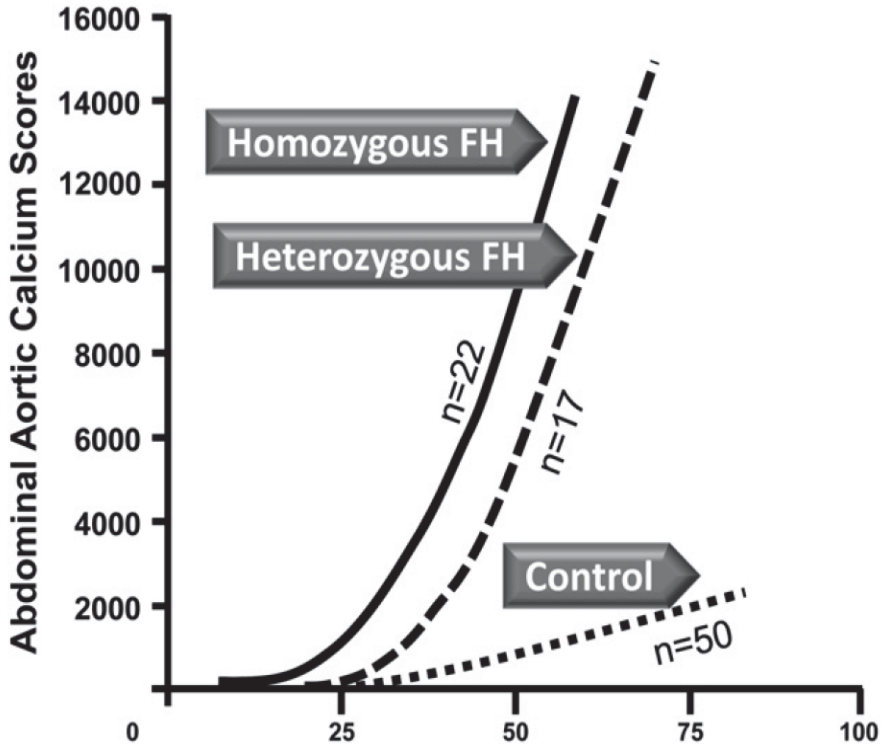

Figure 3. Age- and gene dosage-dependent increase in aortic calcification obtained by computed tomography in patients with homozygous (n = 22) heterozygous (n = 17) patients with known mutations at the *Ldlr* gene. Controls (n = 50) were patients undergoing virtual computer-tomography assisted colonoscopy.

Figure 4.

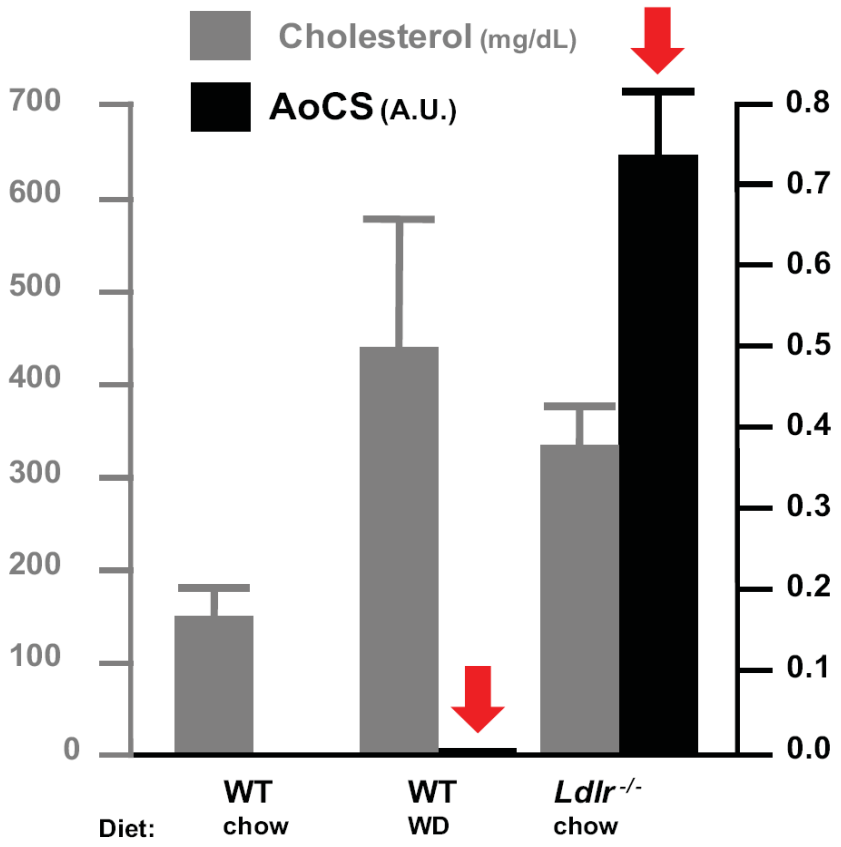

Figure 4. The effect of diet on serum cholesterol in wild type (WT) C57BL/6 background mice on both normal diet (chow) and high cholesterol, high fat diet (Western diet WD) in comparison to *Ldlr*$^{-/-}$ mice on a chow diet. Despite nearly comparable serum cholesterol levels, the degree of calcification was over 70 fold more in the *Ldlr*$^{-/-}$ mice fed a chow diet than in the WT fed a WD.

Figure 5.

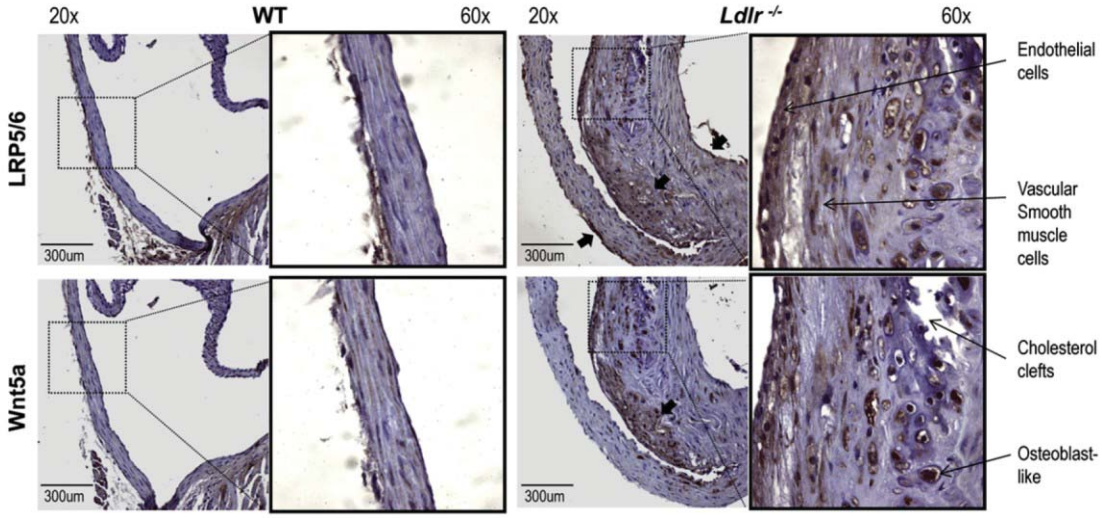

Figure 5. The immunohistochemical expression of LRP5/6 and Wnt5a in the aortic root of WT and *Ldlr*$^{-/-}$ mice. This sections indicating the involvement of the Wnt canonical pathway in the aortic calcification of *Ldlr*$^{-/-}$ mice *via* LRP5/6 signaling.

CHAPTER 6

"Atherosclerosis and Inflammation"

ARTICLE (E)

CHAPTER 6: ATHEROSCLEROSIS AND INFLAMMATION

ARTICLE (E): *Inhibition of Interleukin-1 β by a Monoclonal Antibody Therapy Reduces Vascular Calcification in LDLR Knockout Mice*
AUTHORS: Zuhier Awan, Maxime Denis, Anna Roubtsova, Amani Awan, Hermann Gram, Nabil G. Seidah and Jacques Genest

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6. Foreword:

Inflammation predates atherosclerosis and aortic calcification. In this last article, we examine the effects of interleukin-1β inhibition on the process of arterial calcification in two animal models. There is a strong translational aspect of this work to prevent the severe and premature aortic calcification in familial hypercholesterolemia and other chronic diseases.

Familial hypercholesterolemia is predicted to be affecting at least 70,000 individuals in Canada. Confirmed FH subjects are at a higher risk of premature CVD and thus at risk for premature aortic calcification. Despite intensive medical therapy, arterial calcification is currently not amenable to medical therapy.

As a proof-of-concept, it is experimentally possible now to modulate vascular calcification in mice by inhibiting interleukin-1β, a key inflammatory mediator involved in atherosclerosis and vascular calcification. Moreover, we showed that LDL-receptor-deficient (*Ldlr*$^{-/-}$) mice have a twofold higher level of interleukin-1β than LDL-receptor-attenuated *Pcsk9(Tg)* mice and this may contribute significantly to the difference in the degree of calcification seen in both models.

Inhibition of Interleukin-1β by a Monoclonal Antibody Therapy Reduces Vascular Calcification in *LdIr*$^{-/-}$ Mice

Brief title: *Inhibition of Calcification by Anti-IL-1β mAb*

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6.1 Abstract:

Objective: Given the link between cholesterol and activation of inflammation via interleukin-1β, we tested the effects of IL-1β inhibition on atherosclerotic calcification in mice.

Background: Patients with familial hypercholesterolemia (FH) develop extensive aortic calcification and calcific aortic stenosis. Although statins delay this process, LDL-cholesterol lowering alone is not enough to avert it. Data suggests that vascular inflammation initiated by hypercholesterolemia is followed by unchecked mineralization at sites of atherosclerotic plaques. LDL-receptor-deficient (*Ldlr*$^{-/-}$) and LDL-receptor-attenuated *Pcsk9(Tg)* mice are available animal models for pharmacological testing.

Methods and Results: A mouse monoclonal antibody (mAB) against IL-1β or placebo was administered subcutaneously to Ldlr$^{-/-}$ and *Pcsk9(Tg)* models fed a Western diet. Drug level, anthropometric, lipid and glucose profiles were determined. PCSK9, SAA1 and cytokine expressions were measured by ELISA. Aortic calcification was determined by micro-CT and X-Ray densitometry and aortic flow velocity was assessed by ultrasound. Circulating levels of IL-1β in *Ldlr*$^{-/-}$ mice were significantly greater (2-fold) than observed in *Pcsk9(Tg)* mice. MAb- and placebo-treated mice did not differ in their growth, lipid, glucose profiles and other cytokines while plasma SAA1 levels were lower in mAb-treated mice. Calcifications were significantly diminished in mAb-treatment *Ldlr*$^{-/-}$ mice (a reduction of 75% by X-ray and 96% by micro-CT) and reduced insignificantly in mAb-treatment *Pcsk9(Tg)* mice, whereas aortic flow velocity was unchanged in both models.

Conclusions: Herein, we demonstrate that aortic calcifications can be inhibited by an IL-1β mAb in LDL-receptor deficient mice. These results have a translational component to prevent vascular calcification in human and represent new evidence to rationalize targeting inflammation in cardiovascular disease.

Key Words: Familial Hypercholesterolemia, LDLR, PCSK9, IL-1β, Aortic Calcification.

6.2 Abbreviations:

AoCS = aortic calcium scores
FH = familial hypercholesterolemia
hsCRP =high-sensitivity C-reactive protein
IFNγ = interferon gamma
IL-1β = interleukin-1beta
iNOS = inducible nitric oxide synthase
Ldlr = low-density lipoprotein receptor gene in mice
Ldlr$^{-/-}$ = Ldlr knockout mice
Micro-CT = micro-computerized tomography
NLRP3 = NOD-like receptor family pyrin domain containing 3
PCSK9 = proprotein convertase subtilisin/kexin type 9
SAA1 = serum amyloid A1
Pcsk9 = proprotein convertase subtilisin/kexin type 9 gene in mice
Pcsk9(Tg) = *Pcsk9* transgenic mice
TNFα = tumor necrosis factor-alpha
WD = western-diet
Wnt = a hybrid of Wg (wingless) and Int1 (integration1) genes in Drosophila
WT = wild-type

6.3 Introduction:

Patients with familial hypercholesterolemia (FH) as a result of mutations in the low-density lipoprotein receptor (LDLR) gene experience severe and extensive vascular calcification in an age dependent fashion.[1] Using computerized tomography (CT) scans of the thoraco-abdominal aorta, revealed that this phenotype involves the entire aorta by the second decade of life in homozygous FH.[1] In heterozygous individuals, a similar degree of vascular calcification is seen 20 years later.[2] This stands in marked contrast with non-FH subjects in whom vascular calcification is not seen until the 7th decade of life. Interestingly, all relevant clinical and laboratory parameters in relation to bone and calcium homeostasis are within reference values.[3] We recently recapitulated this phenotype in two mouse models and validated a micro-computed tomography (micro-CT) technique.[4] Using this method it was established that *Ldlr*$^{-/-}$ mice develop aortic calcification in a pattern similar to that seen in human. Furthermore, similar findings were obtained in *Tg(Pcsk9),* mice that transgenic for proprotein convertase subtilisin/kexin type 9 (PCSK9)[5], a protein known to promote the LDLR degradation.[6] Intriguingly at a comparable levels of serum cholesterol, an adult *Ldlr*$^{-/-}$ mouse fed a normal Chow-diet develops 70-fold greater calcification in comparison to a wild-type (WT) mouse fed a Western-diet (WD). This led us to postulate a model in which vascular inflammation due to early hypercholesterolemia is followed by dysregulated mineralization of subendothelial tissues.[7]

The importance of vascular calcification in man is increasingly recognized in an aging population and in patients with FH. It is estimated that 30% of individuals over 60 years of age have increased calcium deposits in major arteries.[8] The hemodynamic consequences of calcium buildup in the vasculature include a reduction in aortic and arterial elastance, and cardiovascular hemodynamic response becomes compromised. Clinically, the impact of vascular calcification contributes to arterial hypertension, aortic valve stenosis, limb ischemia, myocardial infarction and congestive heart failure.[9] Furthermore, calcific aortic stenosis is the leading cause of aortic valve replacement in Western countries and the third leading cause of cardiovascular disease.[10-13] It is estimated that in the Western world approximately 3% of adults over 75 years of age are affected by calcific aortic stenosis.[14] Unfortunately, at the present time no efficient

therapy exists to stop or reverse vascular calcification.[15] Amongst the many potential targets to prevent vascular calcifications, several interfere with bone formation, an undesirable side effect in an aging population. Anti-inflammatory agents, pyrophosphate inhibitors, chelating therapy, vitamin K, selective estrogen receptor modulators and calcium channel blockers are a short list of potential drugs.[15-16]

In the present study, we investigated whether inhibition of the inflammatory cytokine interleukin-1β (IL-1β) prevents vascular calcification. Interleukin-1β plays an essential role in triggering an inflammatory response to injury and mediating the calcification process via the actions of IL-1β on Wnt signaling.[17] Current data implicates cholesterol micro-crystals as being the initiating factor in the release of IL-1β through the activation of the NLRP3 inflammasome. Inflammasomes are large, multimeric protein complexes that connect the metabolic stress sensing with proteolytic cleavage of prointerleukin-1β into bioactive IL-1β. Inflammasomes are thus responsible for activation of inflammatory processes, and have been shown to induce cell pyroptosis.[17] Stimulation of the IL-1 receptor on vascular endothelial cells leads to upregulation of iNOS, endothelin-1, chemokines/cytokines, adhesion molecules, endothelial and smooth muscle proliferation and macrophage activation, processes that contribute to endothelial dysfunction and progression of atherosclerosis.[18] The targeted inactivation of IL-1β decreased plague size in a mouse model of atherosclerosis (*apoe*$^{-/-}$*/IL-1β*$^{-/-}$) fed a high cholesterol diet.[19] In addition, lack of IL-1β decreased the severity of atherosclerosis in *apoe*$^{-/-}$mice while prolonged treatment with IL-1β promotes arterial intimal thickening in the porcine coronary artery.[20] In addition, IL-1β has been implicated in atherothrombosis and plaque rupture in several pre-clinical and clinical studies.[21] Apparently, IL-1β inhibition modulates both a pro-inflammatory mediator that is not absolute for host defense and a signaling process that is not critical to innate immunity. Furthermore, IL-1β inhibition does not modify plasma level of lipids and lipoproteins, thus making it a perfect addition to more classical lipid lowering agents. Herein, we tested the hypothesis that inhibiting the early inflammatory process with a murine monoclonal antibody against IL-1β (01BSUR, Novartis), will lead to a marked reduction in vascular calcifications in mice.

6.4 Methods

Animal protocol. Mice were fed a WD (Harlan Teklad # TD 88137) from weaning until euthanasia. The colony of *Ldlr*$^{-/-}$ mice (C57BL/6J background; stock 002529) (*n*=31) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). *Pcsk9(Tg)* mice overexpressing the *PCSK9* gene (*n*=27) were backcrossed to C57BL/6J for 10 generations.[5] Mice were kept in a pathogen free facility under 12 hour light/dark cycles and fasted for 3 hours prior to euthanasia and a total of 58 adult mice were used in this study. All procedures were approved by the animal care committee of the Clinical Research Institute of Montreal. The protocol for genotyping and the sequence of used primers can be found in supplementary materials and (**Supplementary Table 1**). The murine anti-mouse IL-1β monoclonal antibody (IgG2a/κ mAb)[22] was administered subcutaneously for 6 months. The half life of the antibody in mice is approximately 14 days. The 1st and 2nd doses were 10 mg/kg and subsequent injections were 7.5 mg/kg every other week for the remainder of the study. The study design is shown in **Supplementary Figure 1**. Conceptually, genetically modified mice deficient in LDLR or transgenic for *Pcsk9* respectively simulate homozygote and heterozygote FH phenotypes observed in humans. A treatment with either 01BSUR (treatment, + mAb) or NaCl 0.9% (placebo) was administered every two weeks after weaning and after the start of the Western diet (WD). Thus, 4 groups were analyzed: **(1)** *Ldlr*$^{-/-}$ on placebo (*n*= 15), **(2)** *Ldlr*$^{-/-}$ on anti-IL-1β mAb (*n*= 16), **(3)** *Pcsk9(Tg)* on placebo (*n*= 13) and **(4)** *Pcsk9(Tg)* on anti-IL-1β mAb (*n*= 14).

Tissue sampling and plasma analysis. Mice went for primary (4 month) and final (6 month) assessments. Blood was collected 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 850x*g* and 4°C) and kept at –20°C until assayed. Total cholesterol and triglyceride were measured by commercial kits Infinity (Thermo Fisher Scientific Inc, MA) and Wako (Pure Chemical Industries Ltd. Osaka, Japan), respectively. Mouse inflammatory cytokines were measured in two independent pools in each group by an expression ELISA array (SABiosciences, QIAGEN Company). SAA1 (main inflammatory marker in mice) and

PCSK9 levels were measured by a quantitative ELISA kit (MyBioSource and Cayman, respectively) according to the manufacturer's recommendations. An *in-house* ELISA assay was developed by Novartis to measure the level of circulating IL-1β mAb (01BSUR) given that measuring IL-1β in injected mice with anti-IL-1β mAb would be misleading (interference with IL-1β assay). Thus circulation levels of IL-1β mAb were determined by a competitive ELISA using highly purified goat anti-idiotypic antibodies raised against the Fab fragment of the mouse anti-IL-1β mAb. The ELISA is based on capture of a biotinylated anti-IL-1βmAb by plate-bound anti-idiotypic antibody and competition of this interaction by anti-IL-1β mAb present in the mouse serum. Serum concentrations of the antibody in mice were derived from standard curves generated with graded concentrations of the mouse anti-IL-1β mAb in mouse serum.

Aorta isolation and analysis. Under general anesthesia, mice were perfused and aorta fixed *in-situ* as previously reported.[23] Fine dissection of the excised aorta was done under microscopic inspection and each intact aorta was embedded in a single paraffin block for evaluation. For more details on surgical technique and histology protocol, see online (**Supplemental Material & Methods**). Vascular calcification in humans can be detected on a standard X-Ray or quantified more precisely on a computed tomography (CT) scan.[2-4,24] Thus, we applied a miniaturized version (Skyscan-1072, TomoNT version 3N.5, Belgium) to detect and quantify aortic calcification in mice with a resolution of 5-20 μm. A three-dimensional (3D) reconstruction image and video were produced using commercial software to evaluate calcification. For details on X-Ray and micro-CT settings and the assessments of aortic valve size using ultrasound and flow velocity, see online **Supplemental Material & Methods, Supplementary Figure 2**.

6.5 Results:

Inflammatory cytokine expression in aortic tissue of *Ldlr*$^{-/-}$ mice. Advanced atherosclerotic lesions were prominent in the aorta of *Ldlr*$^{-/-}$ mice. Expression of IL-1β, TNFα, and Mac2 (a marker of macrophages) was examined by immunohistochemistry (IHC) in WT and *Ldlr*$^{-/-}$ mice both fed a WD for 6 months. As described in **Supplemental Material and Methods**, aortic tissues were stained with appropriate primary and secondary antibodies. No expression of IL-1β (**Figure 1A**), TNFα, Mac2 was seen in WT control mice (*data not shown*). Note the intense subendothelial staining for IL-1β, TNFα and Mac2 (**Figure 1B, C & D**), reflecting complex atherosclerotic lesions.

Anti-IL-1β mAb treatment did not alter growth, glucose and lipid profiles in comparison to placebo groups. Similar growth rates (*data not shown*) and organ profiles were seen after 6 months of WD in both arms. There were no significant differences between liver, kidneys, spleen sizes (expressed as % of body weight) or glucose levels between IL-1β mAb-treated and placebo-treated mice **(Table 1)**. Lipid parameters were also not significantly changed between the two groups, including circulating PCSK9 levels between the *Ldlr*$^{-/-}$ mice groups (3,287±378 *vs.* 3,793±251 ng/mL; mAb-treatment *vs*. placebo, respectively; p=0.28) and between the *Pcsk9(Tg)* mice groups (35,273±3,201 *vs.* 29,899±2,084 ng/mL; mAb-treatment *vs.* placebo, respectively; p=0.44). As expected, *Ldlr*$^{-/-}$ mice had higher total cholesterol and triglyceride levels than *Pcsk9(Tg)* mice. Circulating plasma cholesterol levels were ~2.5-fold higher in *Ldlr*$^{-/-}$ mice relative to *Pcsk9(Tg)* mice (**Table 1**), consistent with a previous report.[3]

Circulating levels of endogenous IL-1β were two-fold higher in *Ldlr*$^{-/-}$ mice than *Pcsk9(Tg)* mice. During the treatment phase, groups did not suffer major skin reactions or death related to repeated injections with anti-IL-1β mAb. To determine the efficacy of mAb-treatment, levels of the anti-IL-1β mAb (01BSUR) were determined by an ELISA at 6 weeks (prior to initiation of treatment), then at 4 and 6 months (during the trough window of treatment). As seen in **Figure 2A**, therapeutic levels of 01BSUR were maintained throughout the course of the treatment.

Previous reports have shown that endogenous IL-1β levels increase by two-fold in *Ldlr*$^{-/-}$ mice compared to WT, post stimulation.[25] Inflammatory cytokines therefore were measured by an expression ELISA array (SABiosciences, QIAGEN Company). Endogenous levels of IL-1β were determined at 6 months in placebo-treated *Ldlr*$^{-/-}$ and *Pcsk9(Tg)* mice maintained on WD diet. Intriguingly, endogenous levels of IL-1β in *Pcsk9(Tg)* mice, were half those observed in *Ldlr*$^{-/-}$ mice (p<0.010) (**Figure 2B**). Other than IL-1β, plasma levels of IL-1α, IL-2, IL-4, IL-6, IL-10, IFNγ or TNFα were not significantly different between *Ldlr*$^{-/-}$ and *Pcsk9(Tg)* mice on placebo nor between mAb-treated and placebo-treated on the same background (**Figure 3A**). Interestingly, the level of serum amyloid A1 (SAA1, the major systemic marker of inflammation in mice) between mAb-treated and placebo-treated groups was 30% lower in *Ldlr*$^{-/-}$ mice and 15% lower in *Pcsk9(Tg)* mice (but not reaching significance p=0.44 and p=0.59, respectively) (**Figure 3B**). Considering the plasma levels of cholesterol, triglycerides and IL-1β, the *Ldlr*$^{-/-}$ mice fed a WD show a more pro-atherogenic and pro-inflammatory profile than the *Pcsk9(Tg)* mice at 6 months of age.

Significant reduction in aortic calcification in IL-1β mAb treated *Ldlr*$^{-/-}$ mice. *Ldlr*$^{-/-}$ mice fed a WD developed extensive atherosclerotic lesions, as seen previously.[4] Compared with placebo-treated *Ldlr*$^{-/-}$ mice, IL-1β mAb-treated mice exhibited a marked decrease in aortic calcification, as measured by X-Ray densitometry and by micro-CT aortic calcium scores (AoCS) (**Figure 4**). As shown from X-Ray densitometry, *Ldlr*$^{-/-}$ mice on anti-IL-1β mAb showed a marked attenuation of vascular calcification by 4.3-fold (-75%) (0.17± 0.05 in mAb *vs.* 0.70±0.19 in placebo, P=0.007) and by 27-fold (96%) based on micro-CT scan quantification (0.12± 0.10 in mAb *vs.* 3.34±1.42 in placebo, P=0.043) **(Table 1)**.

As previously seen, the level of calcification in the *Pcsk9(Tg)* mice was lower and showed a later onset compared to *Ldlr*$^{-/-}$ mice.[4] In *Pcsk9(Tg)* mice, the effect of the IL-1β mAb treatment on parameters of aortic calcification was not significantly different between placebo- and mAb-treated groups at 6 months on WD (**Figure 5**). *Pcsk9(Tg)* mice on anti-IL-1β mAb showed a trend towards attenuation of vascular calcification by 55% as shown from X-Ray densitometry (0.07±0.03 in mAb *vs.* 0.16±0.04 in placebo, P=0.09) and micro-CT scan quantification (0.00±0.00 in mAb *vs.* 0.0015±0.001 in placebo, P=N.S). Interestingly, *Pcsk9(Tg)* mice had levels of plasma lipids and IL-1β markedly lower than that of the *Ldlr*$^{-/-}$ mice (**Table 1, Figure 2A**).

Flow velocities were unchanged between anti-IL-1β mAb and placebo. Because human subjects with familial hypercholesterolemia develop severe and premature aortic calcification, calcific aortic stenosis is often seen in FH patients, especially in homozygous FH. We thus examined aortic flow velocity in *Ldlr*$^{-/-}$ and *Pcsk9(Tg)* mice by Doppler ultrasound. At 6 months on a WD, IL-1β mAb-treatment had no effect on aortic valve size in either model (*data not shown*). The difference in flow velocity across the valve in *Ldlr*$^{-/-}$ mice on anti-IL-1β mAb showed no significant difference from placebo (1.44 ±0.34 in mAb *vs*. 1.22 ±0.30 in placebo, *P*=0.15) (**Supplementary Figure 1D**).

6.6 Discussion:

FH is a relatively common monogenic co-dominant Mendelian disorder and is caused predominantly by mutations in the *LDLR* gene,[26] coding for a cell surface glycoprotein that regulates plasma cholesterol *via* endocytosis of LDL particles.[27] A phenotype similar to FH can be caused by mutations in the LDLR ligand, apolipoprotein (apo) B gene (*ApoB*) and the regulatory protein produced by the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*).[28] At least in FH, the calcification process proceeds independently of cholesterol levels once subendothelial inflammation and atherosclerosis has occurred.[1] This supports the two-hit model of vascular calcification,[7] were the *first hit* consists of intimal injury caused by lifelong hypercholesterolemia leading to accumulation of inflammatory cells, including monocytes/macrophages. The uptake of oxidized LDL by macrophages of the innate immune system is a critical step in the initiation of the atherosclerotic plaque and subendothelial inflammation. Recent data from Duewel *et al*[29] and Rajamäki *et al*[30] shows that micro-crystals of cholesterol are taken up by macrophages in the subendothelial layer of the arterial wall and activate the NLRP3 inflammasome, leading to the cleavage-activation of pro-IL-1β and its release in plasma. The contemporary view of vascular calcification has been extensively reviewed.[7,8,15,31-34]

The *second hit* involves the calcification process by which calcium is deposited on matrix proteins in the intimal wall of genetically susceptible individuals. The involvement of inflammation in the calcification process has been shown previously.[18,30] Importantly, IL-1β acts as a proximal amplification mechanism, causing the release of TNFα and the activation of the Wnt pathway[35]. Furthermore, IL-1β has been implicated in the stimulation of alkaline phosphatase[36] and likely critical in the transdifferentiation of smooth muscle cells into an inflammatory phenotype.[21,37] Therefore subsequent events leading to dysregulated mineralization have been postulated to involve a multi-step pathway, which include subendothelial injury and release of IL-1β, activation of the Wnt canonical pathway of osteoblasts and the decreased cross-talk between the LRLR and LRP5 cell surface receptors on osteoblasts[7]. This hypothesis establishes a link between atherosclerosis initiation and progression, and accelerated vascular calcification. Furthermore this is also consistent with the results of several trials that have failed to show a benefit of statins in the progression of calcific aortic stenosis.[38] By the time vascular calcification is detected, lipid lowering therapy to prevent or reverse calcification

appears to be of limited clinical benefit. While statins may considerably slow down the process of atherosclerosis, they do not directly alter calcification. Conversely, elevated cholesterol seen in the WT mouse on a WD, does not lead to aortic calcifications per say, in sharp contrast to in the *Ldlr*$^{-/-}$ mouse model of aortic calcification.[4] Moreover, aged *Ldlr*$^{-/-}$ mice expressing ApoB^{100} developed extensive oxidative stress with aortic valve calcification and functional valvular disease.[39] Thus, our data indicated for the first time the potential role for IL-1β immune modulation in preventing vascular calcification. In addition, to the associate presence of IL-1β and TNFα in atherosclerotic aortic wall on *Ldlr*$^{-/-}$ mice **(Figure 1**), the plasma levels of IL-1β were shown to be markedly higher in the *Ldlr*$^{-/-}$ mice compared to *Pcsk9(Tg)* mice (**Figure 2**). The reasons for this observation have not yet been elucidated. This may explain why, *Ldlr*$^{-/-}$ mice develop extensive aortic calcifications at 6 months on WD, while *Pcsk9(Tg)* mice develop severe aortic calcifications only at 12 months of WD.[4] However, a milder calcification process was also observed in the current report at 6 months on WD (**Figure 5**).

Most importantly, subtle changes (non-significant and less than 15%) in plasma levels of IL-1α, IL-2, IL-4, IL-8, IL-10, Interferon-γ, or TNFα, in mice treated with IL-1β mAb and placebo, suggesting that selective IL-1β inhibition does not result in a major alteration of innate immunity signaling (**Figure 3**).However the trivial positive changes in cytokine profile seen in *Ldlr*$^{-/-}$ IL-1β treated mice was not anticipated and may reflect the IL-1β-mAb complex retained in the blood, that was not seen in *Pcsk9(Tg)* mice at 6 month (**Figure 2A**). Notably these changes were both in proinflammatory and anti-inflammatory suggesting a desirable balance and a neutral net effect. After 6 months of treatment, *Ldlr*$^{-/-}$ mice with anti-IL-1β mAb reported a marked reduction in aortic calcification determined by X-ray densitometry (4.3-fold reduction) or by AoCS determined by the precise micro-CT scan (27-fold reduction), compared with saline-treated mice (**Figure 4**). Although *Pcsk9(Tg)* showed a trend toward reduced calcification upon mAb treatment, those results did not reach statistical significance, perhaps due to lower basal calcification in those mice compared to *Ldlr*$^{-/-}$ mice (**Figure 5**). The *Pcsk9(Tg)* mouse model is characterized by lower levels of cholesterol and IL-1β than the *Ldlr*$^{-/-}$ mouse and serves as a model of heterozygous familial hypercholesterolemia.[4] This observation parallels that observed in human, where aortic calcifications in heterozygous familial hypercholesterolemia lag approximately 2 decades, compared with homozygous FH.[2]

In conclusion, as a proof-of-concept, the murine IL-1β monoclonal antibody (01BSUR) represents a potential therapy to prevent vascular calcifications. We demonstrate that aortic calcifications can be prevented by selective inhibition of the inflammatory cytokine IL-1β and to a lesser extent by the reduction of the systemic marker of inflammation, SAA1. Interestingly, a randomized phase III clinical trial is currently underway to investigate the effect of subcutaneous injections of a fully humanized antibody directed against IL1β, canakinumab,[40] in stable post myocardial infarction patients showing elevated levels of the inflammatory biomarker hsCRP. A translational component of this study therefore may apply to the overall treatment of homozygous and severe heterozygous FH patients by initiating immune modulating IL-1β agents to decrease aortic calcification. The incidence of FH is often quoted at 1:500 in the general population and it is much higher (1:80) in some communities with a founder effect, such as in the Lac St. Jean area of Quebec. These patients are at potential risk of premature aortic calcification and late aortic complications that are associated with impaired functional status and survival. Individuals with gain of function mutation in *PCSK9* gene that promotes the LDLR degradation can similarly benefit from this approach.

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6.8 References:

1. Awan Z, Alrasadi K, Francis GA, et al. Vascular Calcifications in Homozygote Familial Hypercholesterolemia. Atheroscler Thromb Vasc Biol 2008;28:777-785.
2. Alrasadi K, Alwaili K, Awan Z, Valenti D, Marcil M, Genest J. Aortic calcifications in familial hypercholesterolemia: potential role of the low-density lipoprotein receptor gene. Am Heart J 2009;157:170-6.
3. Awan Z, Alwaili K, Alashaharani A, Goltzman D, Genest J. Calcium Homeostasis and Cortical Bone Integrity in Aortic Calcification in Subjects with Familial Hypercholesterolemia. Clin Chem. 2010;56(10):1599-607.
4. Awan Z, Denis M, Bailey D, et al. The LDL-R Deficient Mouse as a Model for Aortic Calcification and Quantify by micro computer tomography. Atheroscler 2011;219:455-62.
5. Zaid A, Roubtsova A, Essalmani R, et al. Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. Hepatology. 2008 Aug;48(2):646-54.
6. Seidah NG, Prat A. The biology and therapeutic targeting of the proprotein convertases. Nat Rev Drug Discov 2012;11:367-83.
7. Fantus D. Awan Z, Seidah N, Genest J. Aortic Calcifications: Novel Insights from Familial Hypercholesterolemia and Potential Role for the Low-Density Lipoprotein Receptor. Atheroscler 2013 Jan;226(1):9-15.
8. Messika-Zeitoun D, Bielak LF, Peyser PA, et al. Aortic valve calcification: determinants and progression in the population. Arterioscler Thromb Vasc Biol.2007 Mar;27(3):642-8.
9. Kaplan NM, Lieberman E, Neal W. In Kaplan's Clinical Hypertension. 8th ed. Philadelphia, Pa: Lippincott Williams & Wilkins; 2002;28 –9. 12.
10. Butany J, Collins MJ, Demellawy DE, et al. Morphological and clinical findings in 247 surgically excised native aortic valves. Can J Cardiol. 2005 Jul;21(9):747-55.
11. Nkomo VT, Gardin JM, Skelton TN, Gottdiener JS, Scott CG, Enriquez-Sarano M. Burden of valvular heart diseases: a population-based study. Lancet. 2006 Sep 16;368(9540):1005-11.
12. Akat K., Borggrefe M, Kaden JJ. Aortic valve calcification: basic science to clinical practice. Heart. 2009; 95: 616-23.

13. Stewart BF, Siscovick D, Lind BK, et al. Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. J Am Coll Cardiol. 1997 Mar 1;29(3):630-4.
14. Cowell SJ, Newby DE, Prescott RJ, et al. A randomized trial of intensive lipid-lowering therapy in calcific aortic stenosis. N Engl J Med. 2005 Jun 9;352(23):2389-97.
15. Wallin R, Wajih N, Greenwood GT, Sane DC. Arterial calcification: a review of mechanisms, animal models, and the prospects for therapy. Med Res Rev. 2001; 21(4):274-301.
16. Baron R, Hesse E. Update on Bone Anabolics in Osteoporosis Treatment: Rationale, Current Status, and Perspectives. J Clin Endocrinol Metab 97: 311–325, 2012.
17. Church LD, Cook GP, McDermott MF. Primer: inflammasomes and interleukin 1beta in inflammatory disorders. Nat Clin Pract Rheumatol. 2008 Jan;4(1):34-42
18. Fearon WF, Fearon DT. Inflammation and cardiovascular disease: role of the interleukin-1 receptor antagonist. Circulation. 2008 May 20;117(20):2577-9.
19. Kirii H, Niwa T, Yamada Y, et al. Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-deficient mice. Arterioscler Thromb Vasc Biol. 2003 Apr 1;23(4):656-60.
20. Shimokawa H, Ito A, Fukumoto Y, et al. Chronic treatment with interleukin-1 beta induces coronary intimal lesions and vasospastic responses in pigs in vivo. The role of platelet-derived growth factor. J Clin Invest. 1996 Feb 1;97(3):769-76.
21. Alexander MR, Murgai M, Moehle CW, GK Owens. Interleukin-1β modulates smooth muscle cell phenotype to a distinct inflammatory state relative to PDGF-DD via NF-κB-dependent mechanisms. Physiol Genomics. 2012;44:417-29.
22. Osborn O, Brownell SE, Sanchez-Alavez M, Salomon D, Gram H, Bartfai T. Treatment with an Interleukin 1 beta antibody improves glycemic control in diet-induced obesity. Cytokine. 2008 Oct;44(1):141-8.
23. Baglione J, Smith JD. Quantitative assay for mouse atherosclerosis in the aortic root. Methods Mol Med. 2006;129:83-95.
24. Iijima K, Hashimoto H, Hashimoto M, et al. Aortic arch calcification detectable on chest X-ray is a strong independent predictor of cardiovascular events beyond traditional risk factors. Atherosclerosis. 2010 May;210(1):137-44.
25. Netea MG, Demacker PN, Kullberg BJ, et al. Increased interleukin-1alpha and interleukin-1beta production by macrophages of low-density lipoprotein receptor

knock-out mice stimulated with lipopolysaccharide is CD11c/CD18- receptor mediated. Immunology. 1998 Nov;95(3):466-72.

26. Tosi I, Toledo-Leiva P, Neuwirth C, Naoumova RP, Soutar AK. Genetic defects causing familial hypercholesterolaemia: Identification of deletions and duplications in the LDL-receptor gene and summary of all mutations found in patients attending the Hammersmith Hospital Lipid Clinic. Atherosclerosis. 2007;194:102–111.
27. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science. 1986. 232:34-47.
28. Abifadel M, Rabès JP, Devillers M, et al. Mutations and polymorphisms in the proprotein convertase subtilisin kexin 9 (PCSK9) gene in cholesterol metabolism and disease. Hum Mutat. 2009 Apr;30(4):520-9.
29. Duewell P, Kono H, Rayner KJ, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature. 2010 Apr 29;464(7293):1357-61.
30. Rajamäki K, Lappalainen J, Oörni K, et al. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. PLoS One. 2010 Jul 23;5(7):e11765.
31. Johnson RC, Leopold JA, Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. Circ Res. 2006 Nov 10;99(10):1044-59.
32. Miller JD, Weiss RM, Heistad DD. Calcific aortic valve stenosis: methods, models, and mechanisms. Circ Res. 2011 May 27;108(11):1392-412.
33. Sage AP, Tintut Y, Demer LL. Regulatory mechanisms in vascular calcification. Nat Rev Cardiol. 2010 Sep;7(9):528-36.
34. Johnson RC, Leopold JA, Loscalzo J. Vascular Calcification: Pathobiological Mechanisms and Clinical Implications. Circ. Res. 2006; 99: 1044-1059.
35. Shao J.S., Cheng SL, Sadhu J, Towler DA. Inflammation and the Osteogenic Regulation of Vascular Calcification: A review and perspective. Hypertension. 2010; 55: 579-592.
36. Lencel P, Delplace S, Pilet P, et al . Cell-specific effects of TNFα and IL-1β on alkaline phosphatase: implication for syndesmophyte formation and vascular calcification. Lab Invest. 2011 Oct;91(10):1434-42.
37. Keuylian Z, de Baaij JH, Gueguen M, et al. The Notch pathway attenuates interleukin 1β (IL1β)-mediated induction of adenylyl cyclase 8 (AC8) expression during vascular

smooth muscle cell (VSMC) trans-differentiation. J Biol Chem. 2012 Jul 20;287(30):24978-89.

38. Teo KK, Corsi DJ, Tam JW, Dumesnil JG, Chan KL. Lipid Lowering on Progression of Mild to Moderate Aortic Stenosis: Meta-analysis of the Randomized Placebo-Controlled Clinical Trials on 2344 Patients. Can J Cardiol. 2011;27(6):800-8.
39. Weiss RM, Ohashi M, Miller JD, Young SG, Heistad DD. Calcific aortic valve stenosis in old hypercholesterolemic mice. Circulation. 2006 Nov 7;114(19):2065-9.
40. Ridker PM, Thuren T, Zalewski A, Libby P. Interleukin-1β inhibition and the prevention of recurrent cardiovascular events: rationale and design of the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS). Am Heart J. 2011 Oct;162(4):597-60.

6.9 Legends, figures and tables:

Figure 1.

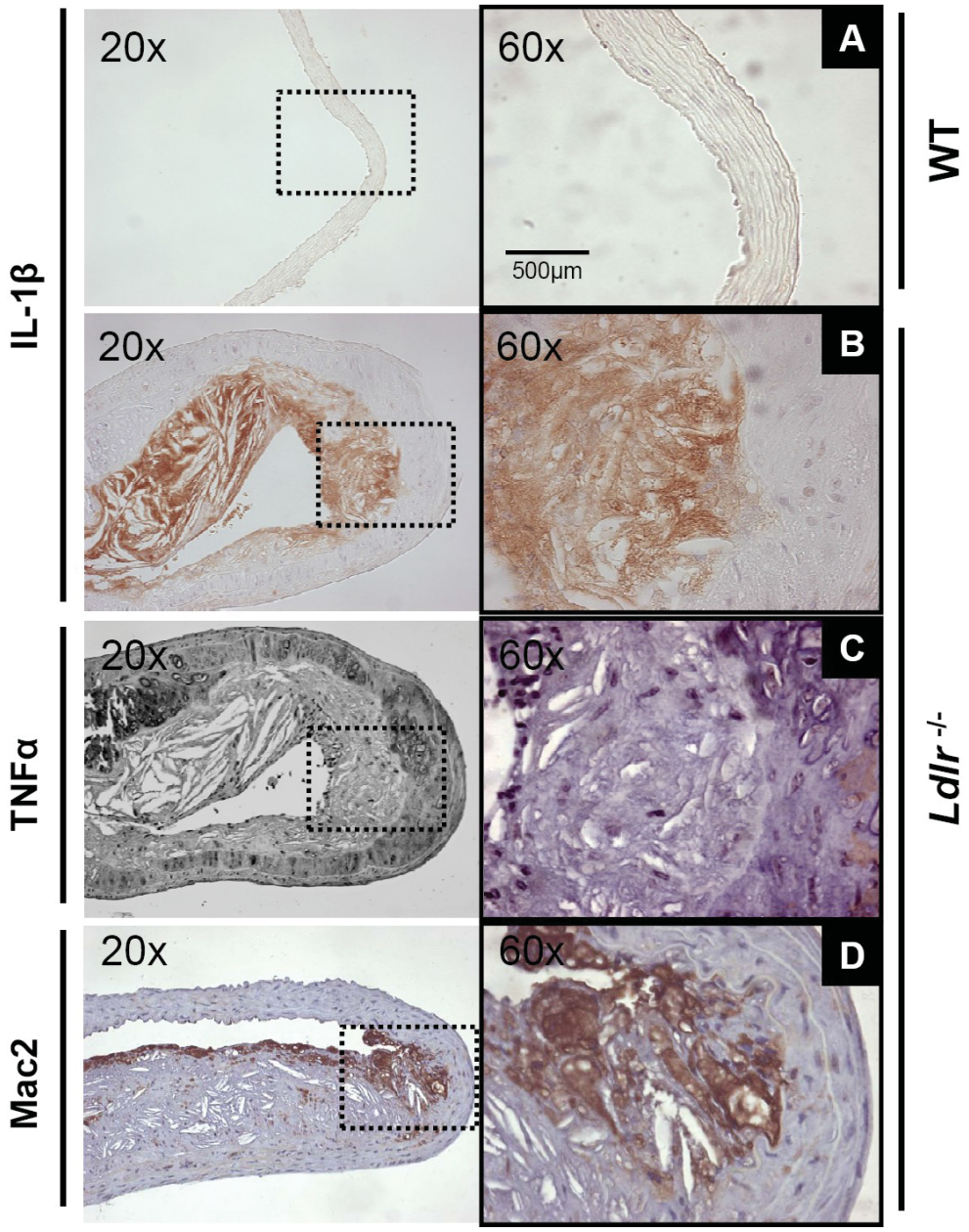


Figure 1. Tissue expression of inflammatory cytokines in the aortic tissue of *Ldlr*$^{-/-}$ mice. **(A&B**) Immunohistochemistry (IHC) expression of IL-1β in subendothelium area of ascending aorta in the WT mouse (above) and the *Ldlr*$^{-/-}$ mouse (below) both fed a WD for 6 months. **(C)** IHC expression of TNFα and **(D)** Mac2 in subendothelium area of ascending aorta in *Ldlr*$^{-/-}$ mouse fed a WD for 6 months.

Figure 2.

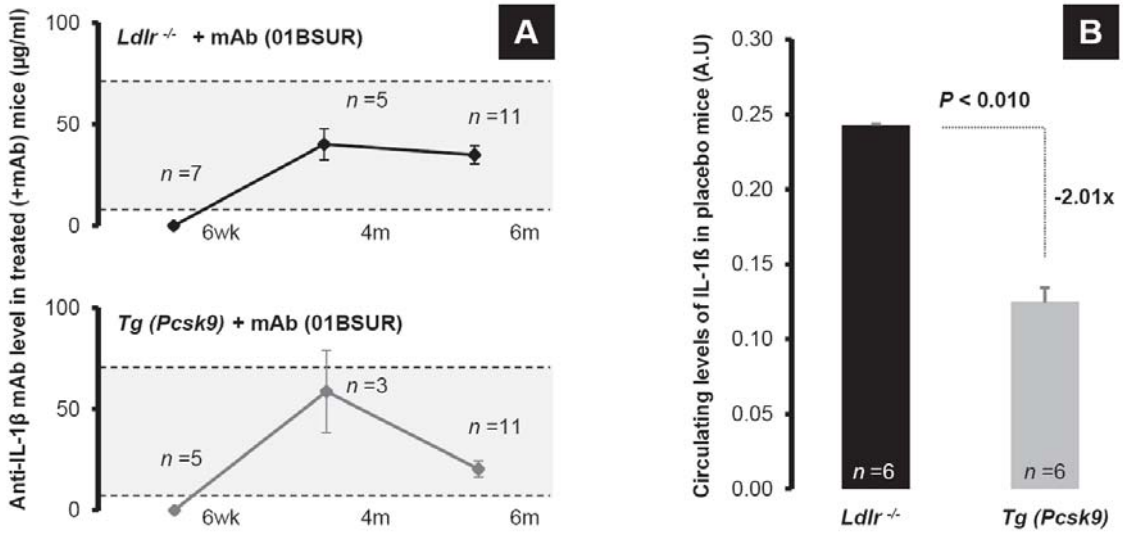


Figure 2. Drug levels and endogenous circulating levels of IL-1β. (A) Levels of anti-IL-1β mAb (01BSUR) at 6 weeks (before treatment), 4 and 6 months in treated animals. **(B)** Circulating IL-1β levels in *Ldlr*$^{-/-}$ and *Pcsk9(Tg)* on WD at 6 months.

Figure 3.

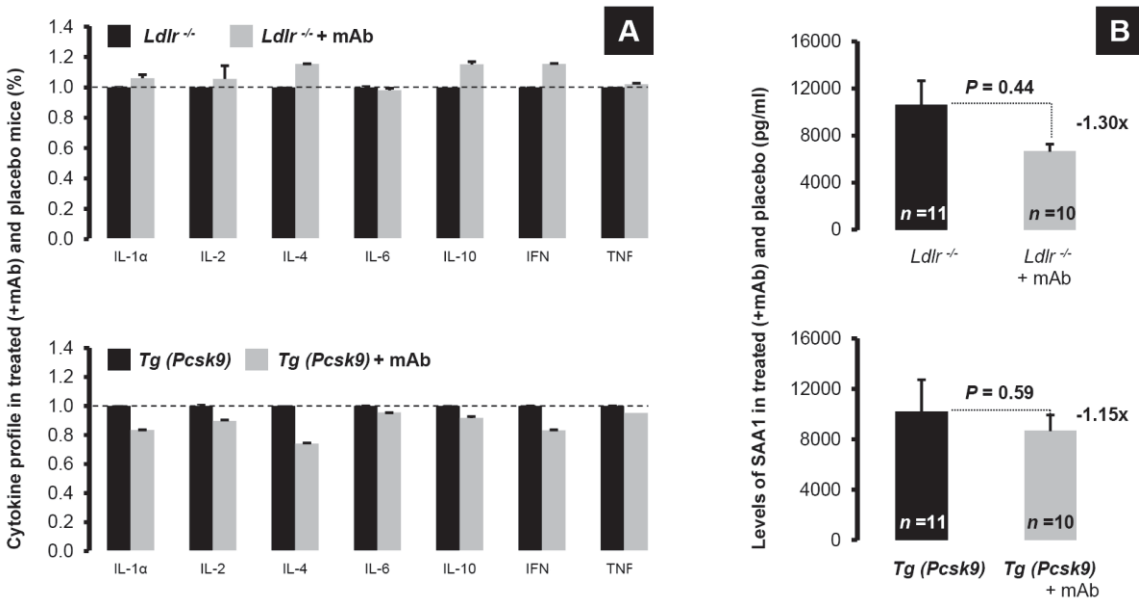


Figure 3. Cytokine levels and serum amyloid A1. (A) Placebo-treated and anti-IL-1β mAb-treated (+ mAb) plasma levels of IL-1α, IL-2, IL-4, IL-6, IL-10, IFNγ or TNFα in both *Ldlr*$^{-/-}$ (top panel) and in *Tg (PCSK9)* mice (bottom panel). **(B)** Plasma levels of SAA1 in both placebo-treated and anti-IL-1β mAb-treated (+ mAb) *Ldlr*$^{-/-}$ (top panel) and *Pcsk9(Tg)* mice (bottom panel).

Figure 4.

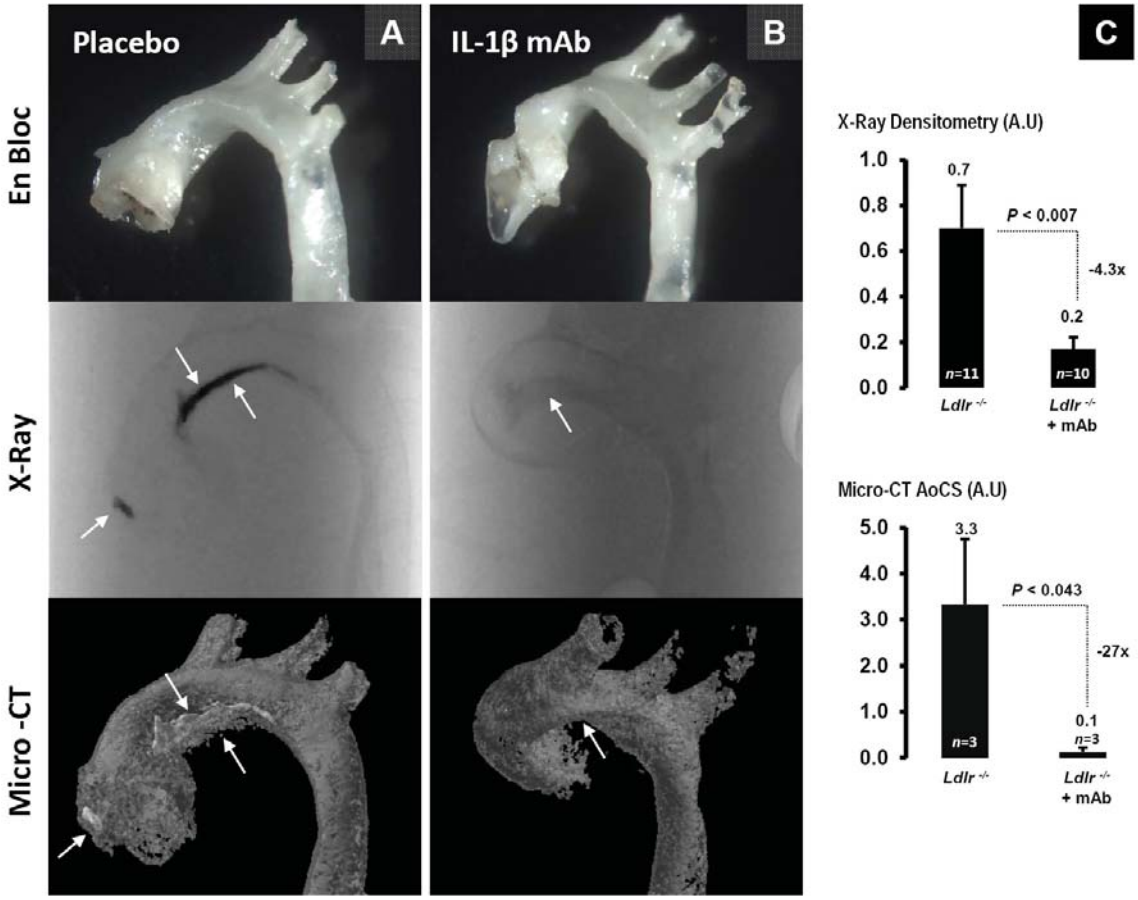


Figure 4. Significant reduction in aortic calcification in IL-1β mAb-treated *Ldlr*$^{-/-}$ mice. Aortas from placebo-treated **(panel A)** and anti-IL-1β mAb-treated **(panel B)** *Ldlr*$^{-/-}$ mice. The top panel shows the thoracic aorta, removed en bloc, the middle panel shows the X-ray of the aorta and the lower panel shows the 3 dimensional micro-CT reconstituted aortas; arrows points toward sites of calcification progression and resolution **(Panel C)** shows the X-Ray densitometry quantification (top) and aortic calcium score (AoCS) expressed in arbitrary unit (A.U).

Figure 5.

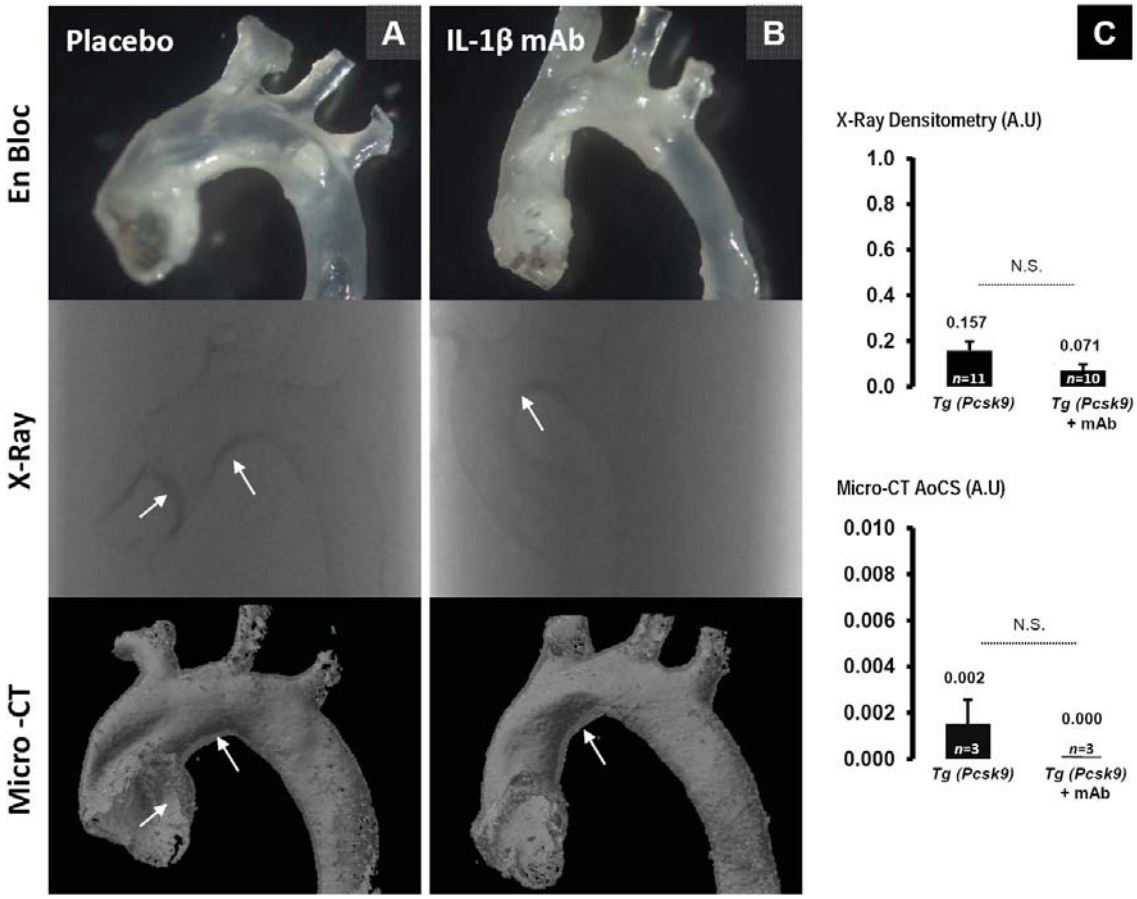


Figure 5. Attenuation of aortic calcification in IL-1β mAb-treated *Pcsk9(Tg)* mice. Aortas from placebo-treated mice **(panel A)** and anti-IL-1β mAb-treated **(panel B)** *Pcsk9(Tg)* mice. The top panel shows the thoracic aorta, removed en bloc, the middle panel shows the X-ray of the aorta and the lower panel shows the 3 dimensional micro-CT reconstituted aortas; arrows points toward sites of calcification progression and resolution **(Panel C)** shows the X-Ray densitometry quantification (top) and aortic calcium score (AoCS) expressed in arbitrary unit (A.U).

Table 1.

Genetic Background	*Ldlr* $^{-/-}$		*Tg(Pcsk9)*	
Treatment	Placebo	anti-IL-1β mAb	Placebo	anti-IL-1β mAb
Number	*n*=10	*n*=11	*n*=10	*n*=11
Liver (% BW)	5.6 %	5.2%	6.7%	4.3%
Kidney (% BW)	0.7%	0.6%	0.6%	0.6%
Spleen (% BW)	0.5%	0.5%	0.3%	0.3%
Sugar (mmol/L)	11.0±0.81	12.9±1.08 (N.S)	11.3±0.85	14.0±1.39 (N.S)
Cholesterol (mg/dL)	1679±190	1867±75 (N.S)	687±53	737±33 (N.S)
Triglycerides (mg/dL)	750±140	895±108 (N.S)	139±13	118±14 (N.S)
PCSK9 (ng/mL)	3793±251	3287±378 (N.S)	29899±2084	35273±3201 (N.S)
X-Ray Densitometry (A.U)	0.70±0.19	0.17± 0.05**	0.16±0.04	0.07±0.03 (N.S)
Number	*n*=3	*n*=3	*n*=3	*n*=3
Micro-CT AoCS (A.U)	3.34±1.42	0.12± 0.10*	0.0015±0.001	0.000±0.000 (N.S)

A.U (arbitrary unit), %BW (% body weight), data presented as (mean±SEM), * (p<0.05), ** (p<0.005), and N.S (Non-significant)

6.10 Supplemental Material:

Genotyping. The *Ldlr* and *Pcsk9* genotyping was performed by polymerase chain reaction amplification on genomic DNA isolated from mouse tails. Polymerase chain reaction products were then separated on a 3130Xl Genetic Analyzer (Applied Biosystems) and identified on the basis of fragment size and expected fluorescence, according to the manufacturer's instructions and as previously described (**1**). Primers used for the genotyping are in (**Supplementary Table 1**).

Aorta isolation and fixation. At term, under general anesthesia, mice were perfused fixed *in-situ* as previously reported (**2**). Isoflurane at rate of 2% in 1L of oxygen was administered and blood was evacuated through an intact diaphragm intracardiac puncture and the systemic circulation was flushed with 5mL phosphate buffered saline (PBS) through the left ventricle at a pump rate of 0.4mL per minute for 10 min, followed by *in-situ* 4% phosphate-buffered paraformaldehyde (PBP) fixation. Organs were isolated and weighted in each animal while the heart and aorta was extracted as a whole by opening the chest wall and gently lifting the heart and excising posterior to the thoracic and abdominal aorta until the renal artery, where it was cut. The apex of the extracted heart was excised and the aortic tissue was further submerged *ex-vivo* in 4% PBP at 4°C overnight. Finally a fine dissection under microscopic inspection was performed and each intact aorta was embedded in a single paraffin block for evaluation.

Histochemistry. Immunostaining for IL-1β (Abcam, Cambridge, MA), TNFα (Abcam, Cambridge, MA), and macrophage markers, Mac2 (Santa Cruz Biotechnology, Santa Cruz, CA) of WT and *Ldlr*$^{-/-}$ mice sections taken at the level of the ascending aorta were carried out according to the manufacturer's protocol. Deparaffinized sections were incubated overnight at 4°C with anti-mouse IL-1β, TNFα and macrophage Mac2 antibodies (1:20, 1:50 and 1:50, respectively). Sections were subsequently immunostained using an avidin-biotin-peroxidase kit (Vectastain,Vector Labs, CA). Nuclei were counterstained with hematoxylin (Sigma). After the end of the treatment

protocol, sections were performed at 6-month-old mice fed a WD and stained with Fast Green and 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 (*data not shown*) as previously published (**3**) Images were acquired using a Retiga EXi camera (Q-Imaging) mounted on a Leica DMRB microscope. Normalized total grey values from labeling monochromatic images (8 bits - 256 greyscale values) were obtained using Matlab R2010a (The MathWorks Inc.).

Calcification and lesion analysis. We applied X-Ray and micro computed tomography (micro-CT), a miniaturized version, to detect and quantify aortic calcification in mice with a resolution of 5-20 µm. After dissection, the entire aorta was exposed to digital imaging capture. Aortas were then embedded in paraffin blocks as previously published (**3**). The whole cohort underwent X-Ray densitometric evaluation of calcification while representative samples underwent detailed and precise micro-CT evaluation. The radiological images of the entire aorta and aortic valve were acquired by digital X-Ray densitometry and then calcified areas were quantified by imageJ software (http://imagej.nih.gov/ij/, 1997-2012). Three independent reading were done blindly on two axes, averaged and expressed as % of calcification to whole aorta in arbitrary unit (A.U). Micro-CT evaluation was done on the scanner (Skyscan-1072, TomoNT version 3N.5, Belgium) adopting the following parameters: 40 kV voltage; 248 µA current; 50 X zoom; 5.63µm 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 (mm^3) multiplied by 100 and expressed in arbitrary unit (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).

Ultrasound and flow velocity assessment. Animals were maintained under anesthesia with 2% isoflurane in oxygen during the whole procedure while monitoring the heart rate and body temperature as previously described (**4**). A commercial hair remover cream was used to clean the mouse chest, and echography transmission gel was applied before scanning (Aquasonic-100 from Parker Laboratories). Flow velocities were determined in the "Power-Doppler" mode ultrasonography with a Vevo 770 apparatus (Visual Sonics, Toronto, Canada) equipped with a 30-MHz probe, with a depth focus at 12.7 mm. Three-second cine- loop movies were recorded for further offline analysis. A left parasternal long-axis view was used to visualize the aortic valves, aortic root, and ascending aorta (**Supplementary Figure 2A&B**) in order to position the probe. All measurements were performed by an operator blinded for genotypes. Velocities were expressed as meters per second (m/s) and the differential velocity was calculated by subtracting upstream from downstream velocities (**Supplementary Figure 2**).

Statistical Analysis. Categorical variables are expressed as mean ± stander error of the mean (SEM). Commercially available software (GraphPad Prism software, La Jolla, CA) was used for statistical analysis and graphs. Two-way analysis of variance (between treatment status, IL-1β mAb or placebo), are examined at any given time by Student's *t* test for significance. A *P value* < 0.05 was considered statistically significant.

REFERENCES

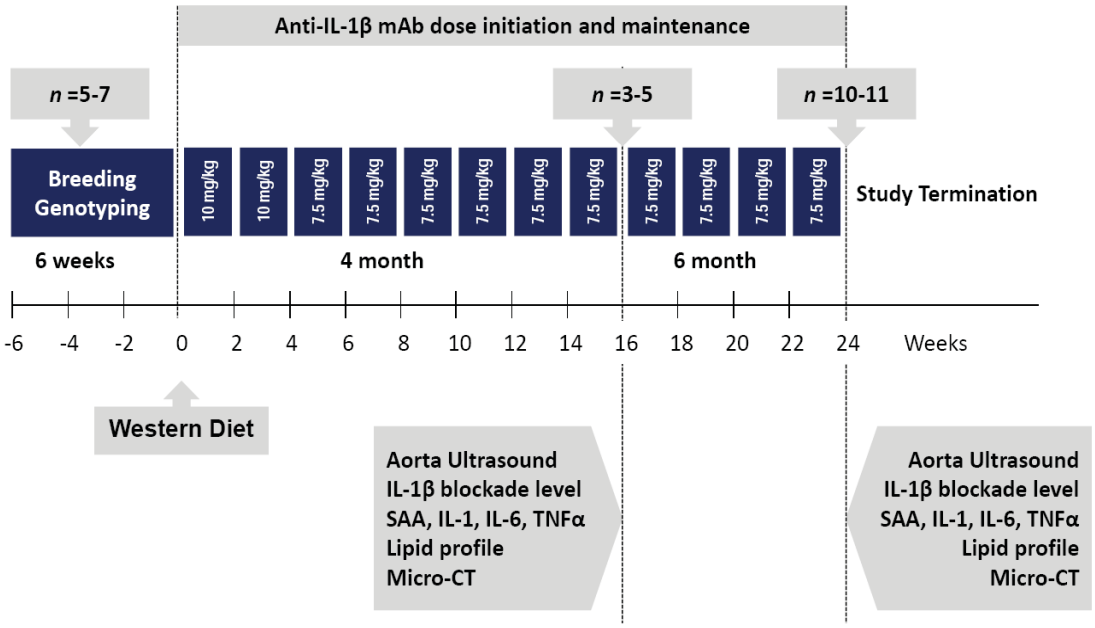
1. Zaid A, Roubtsova A, Essalmani R, et al. Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. Hepatology. 2008 Aug;48(2):646-54.
2. Baglione J, Smith JD. Quantitative assay for mouse atherosclerosis in the aortic root. Methods Mol Med. 2006;129:83-95.
3. Awan Z, Denis M, Bailey D, et al. The LDL-R Deficient Mouse as a Model for Aortic Calcification and Quantify by micro computer tomography. Atheroscler 2011;219:455-62.
4. Denis M, Marcinkiewicz J, Zaid A, et al. Gene inactivation of proprotein convertase subtilisin/kexin type 9 reduces atherosclerosis in mice. Circulation. 2012 Feb 21;125(7):894-901.

Supplementary Tables

Supplementary Table 1: Primers used for the genotyping of mice.

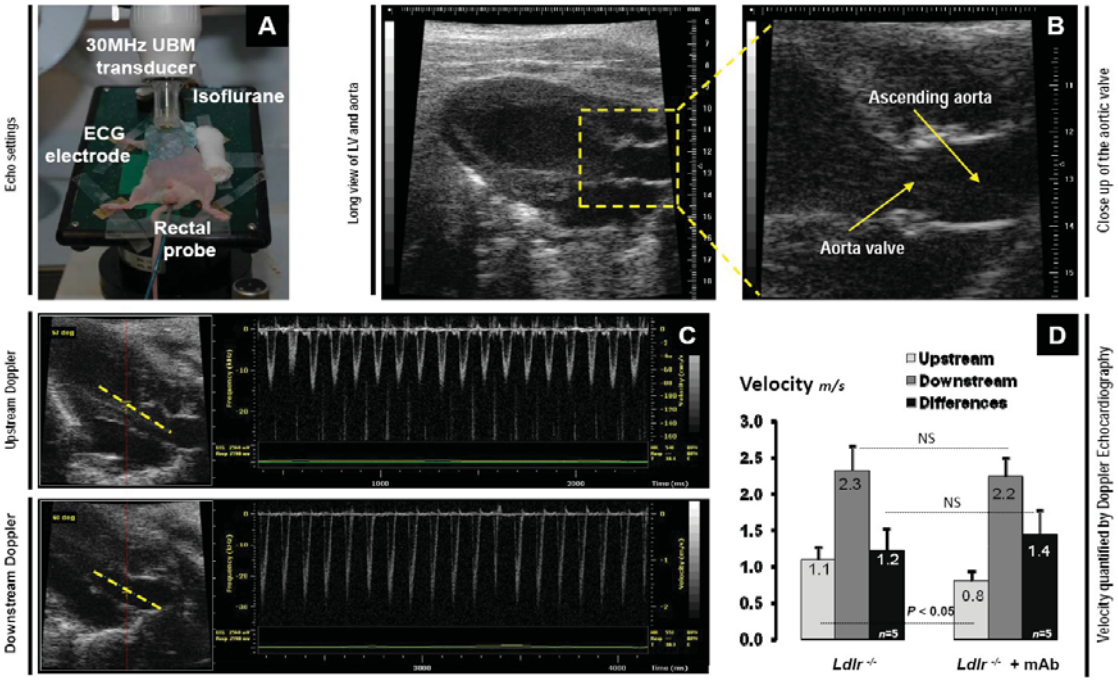
Gene	Sense	Primer sequence	Allele	Fragment size
Ldlr	Forward	VIC – CCATATGCATCCCCAGTCTT		
	Reverse	AATCCATCTTGTTCAATGGCCGATC	WT	167 bp
	Reverse	GCGATGGATACACTCACTGC	KO	350 bp
Pcsk9	Forward	FAM– ACTCCCCACAACTACGGACTGT		
	Reverse	ATCTTTCAGGATGCTGCATTT	WT	213 bp
	Reverse	CATCCCTTTAGGCACAGAGCTT	KO	338 bp

Supplementary Figure 1



Supplementary Figure 1: Protocol and study design. A total of 58 mice were used in the present study. Mice were weaned for 6 weeks. Baseline measurements were obtained with additional matching 12 mice. At the 4-month time-point, 3-5 mice were sacrificed to determine plasma levels of anti-IL-1β mAb. At 6 months, aortic ultrasonography and later plasma levels for lipid and glucose were collected in 10-11 mice before euthanasia.

Supplementary Figure 2



Supplementary Figure 2: Doppler ultrasound echocardiography of the aortic valve was performed in the aortic outflow track and downstream of the aortic valve. (A) Image showing the positioning of the Vevo 770 scanhead on a mouse. (B) Suprasternal view and close up along the long axis of the left ventricle and aortic root. (C) Pulsed-wave Doppler (PW Doppler) taken from upstream and downstream to the aortic valve. (D) Flow velocity across the aortic valve, the difference in flow velocity was calculated and represented in a histogram for IL-1β mAb-treated and placebo *Ldlr*$^{-/-}$ mice at 6 months. No significance in flow velocity across the aortic valve was seen.

CHAPTER 7

"*CLOSING REMARKS*"

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 7: CLOSING REMARKS

7. General discussion

Familial hypercholesterolemia (FH) is the most frequent genetic lipoprotein disorder associated with premature cardiovascular disease (CVD) and vascular calcification. As an autosomal dominant genetic disorder, the transmission of the affected allele is 50%. If left untreated, CVD develops in men in the third to fourth decade of life and in women ten years later (Simon *et al.* 1999, Hansen *et al.* 1997, Jansen *et al.* 2005). Unfortunately, the majority of identified new cases are made in patients with advanced atherosclerosis or sudden cardiac death. Treatment options include lifestyle modification and administration of a potent statin, often in combination with other lipid-lowering agents. In some individuals, statins alone are not sufficient to lower lipoproteins to the recommended levels or are associated with serious side effect(s). Furthermore, genetic background and life-long exposure to cholesterol can foster an unhealthy vasculature that is chronically inflamed and prone to ectopic calcification as inflammation induces the differentiation of vascular cells to an osteoblast-like phenotype. The hope for primary prevention in CVD lies in its earlier identification combined with the administration of new classes of anti-PCSK9 and anti-inflammatory drugs. A Canadian registry of FH patients would provide an invaluable tool to determine health outcomes, resource allocation and health economic issues.

7.1. A need for a FH registry (and earlier screening)

The purpose of creating a registry of subjects with FH is to capture clinical, biochemical and demographic information. Patient specimens (plasma/serum and DNA) are usually collected for bio-banking in order to perform specialized lipid and genetic testing. This registry would, on one hand, help clinicians collaborate in patient management and identify relatives for screening and treatment (cascade family screening). On the other hand, the registry would provide advice to general practitioners and support collaborative studies in biomedical, clinical and health economics research. Access to anonymized data from this registry would be available to researchers, clinicians, biopharmaceutical industries and government agencies through a peer-reviewed process.

Undoubtedly, using data from referral centers and small FH registries has already led to many advances. The identification of the *APOE* mutation associated with FH from Italian and French families represents one example of the potential benefits in terms of novel gene discovery (Awan *et al.* 2013, Marduel *et al.* 2013). Furthermore, the identified *APOE* mutation was discovered following next-generation exome sequencing (a second level of genetic screening) after a candidate gene approach failed to identify a mutation in genes known to be causal in FH or autosomal recessive hypercholesterolemia. The discovery of the *APOE* mutation emphasizes the role of novel techniques in exploring phenotype-associated genes, which are not possible using traditional methods. Confirming the molecular basis of FH may be required in many instances since FH individuals with a molecular diagnosis (Wang *et al.* 2005) are known to have premature CVD and develop aortic calcifications (as confirmed by CT scan) (Awan *et al.* 2008), making early and aggressive treatment mandatory. Indeed, FH patients with a confirmed DNA diagnosis (definite FH) on statin regimens have a higher survival rate as compared to FH patients without treatment (Versmissen *et al.* 2008) (Figure 1).

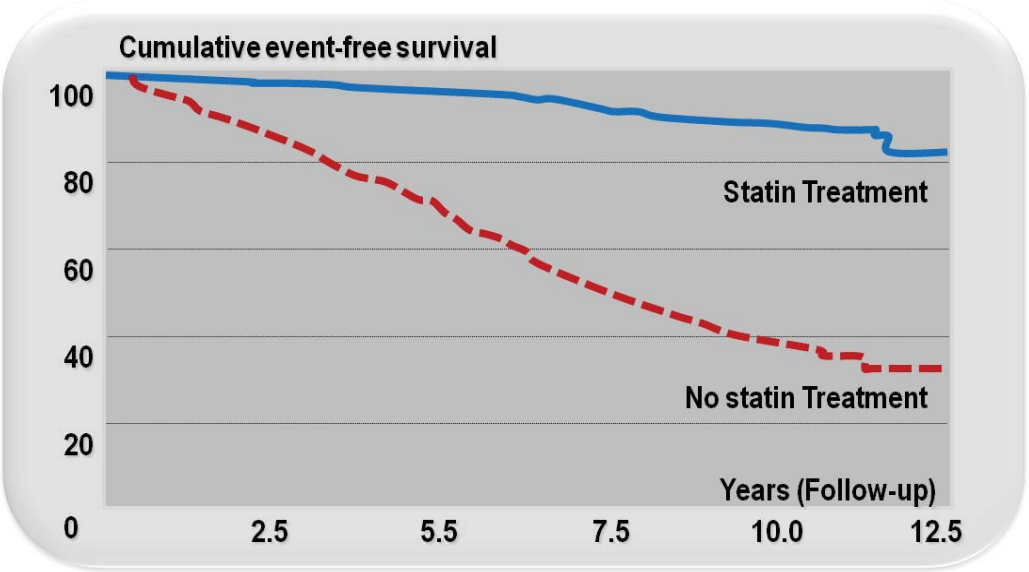


Figure 1: Kaplan-Meier survival curve for FH patients: FH patients on statin treatment have a better prognosis than FH patients without statin treatment. The survival rate is represented on the vertical axis and follow-up time is represented on the horizontal axis (adopted from Versmissen *et al.* 2008).

Accordingly, our reported proband with the *APOE* mutation was a 43 year old man at the time of presentation and had classical and biochemical features of FH. Sadly, he was not recognized for being at risk for CVD prior to his hospitalization, even though more than one individual in the family died from a CVD event (which argues strongly in favour of cascade screening). Nonetheless, the use of cascade screening of this proband family was successful in identifying one asymptomatic carrier (the proband's sister) who was also heterozygous for the same *APOE* mutation and would ultimately benefit from close follow up in a specialized lipid clinic. Thus, *APOE* mutations should be screened together with the classical *LDLR, APOB* and *PCSK9* genes in individuals with an FH phenotype. This will help disseminate the finding that the *APOE* gene is in fact the forth loci implemented in FH and possibly a causative gene in many of the FH negative-*LDLR/APOB/PCSK9* patients (Figure 2).

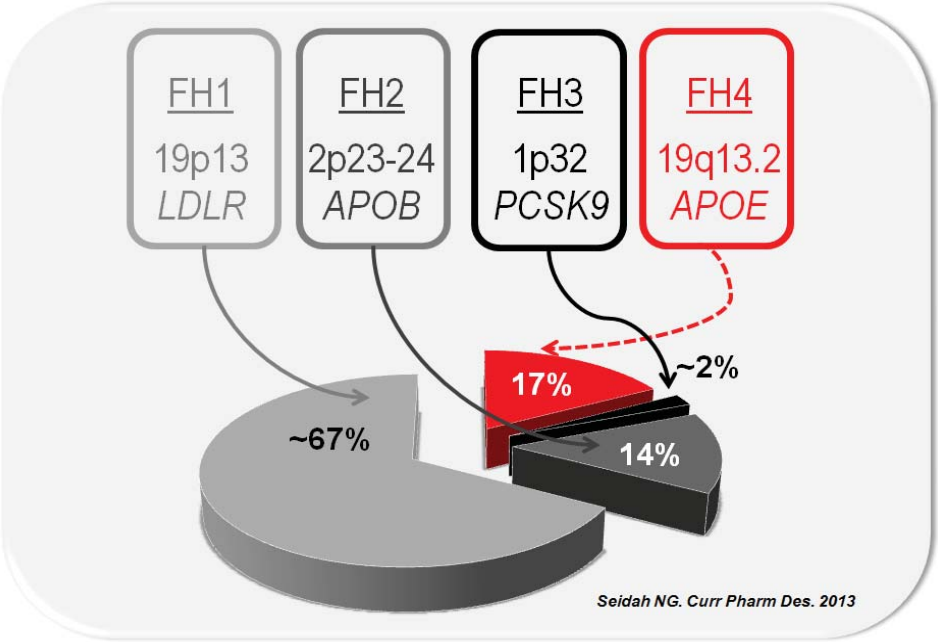


Figure 2: Contribution of known mutations in FH. The 3 genes implicated in FH are LDLR (FH1), APOB (FH2) and PCSK9 (FH3); the contribution of mutations in these three genes ranges from 67%, 14% and 2%, respectively. However, there are a considerable number (17%) of unknown mutations, which could be attributed to *APOE* mutations. Therefore, expanding the candidate gene approach to include screening for the *APOE* gene may help decrease the percentage of unknown mutations (adopted from Seidah 2013).

From a basic science point of view, the understanding of how a mutation in *APOE* actually causes an isolated increase in LDL-C (in spite of absence of *LDLR, APOB* and *PCSK9* mutations) is not completely understood. Initial work by Marduel *et al* has shown a decreased catabolism of LDL particle using a cell-based assay. These findings suggest a new role for apoE in enhancing LDLR binding. However, with the help of properly implemented FH registries and systematic screening for mutations in the *APOE* gene, it would be possible to identify a patient homozygous for an *APOE* mutation and eventually test *in vivo* the LDLR binding capacity. The latter test would determine if the poor binding of the ligand (LDL) to the LDLR is indeed the mechanism or if an alternative mechanism exists. By analogy to apoB (Sun *et al.* 2012, Kosenko *et al.* 2013), one could hypothesize that wild-type apoE may be required to prevent PCSK9 from targeting the LDLR to degradation and mimicking a FH phenotype.

We are anticipating that this report will have a positive impact on the creation of large registries for FH patients and ultimately allow access to specialized medical care for these patients. The diagnosis is critical to give patients access to novel and costly therapies that are given only to FH patients with a DNA diagnosis. Examples for such therapies are extracorporeal LDL filtration, anti-PCSK9 therapy, Lomitapide (an oral inhibitor of MTP), Mipomersen (anti-apoB antisense oligonucleotide), and, in the future, anti-inflammatory therapy. Furthermore, identifying additional genes associated with FH negative-*LDLR/APOB/PCSK9* patients will advance cascade family screening and thus allow for efficient identification of more individuals at risk, which ultimately translates into CVD prevention.

The close patient follow-up in specialized centers will ensure the identification of metabolic signs of harm, and trigger rapid action to prevent disease progression and provide optimal care. These complications will be entered into the FH registry and allow quality performance reviews comparing with national standards. Cardiometabolic aspects that modulate cardiovascular disease in FH will contribute to improve treatment modalities. Clinical and metabolic signs of harm include: elevated blood pressure related to aortic calcification or anemia related to chronic LDL-aphaeresis therapy or statin-related muscle toxicity (rhabdomyolysis), renal toxicity (elevated creatinine), liver toxicity (elevated alanine aminotransferase), and glucose intolerance related to statins or, conceivably, infection related to anti-inflammation modulation. Monitoring and

recognizing all these metabolic signs of harm require dedicated teams and proper facilities. In the end, patients, caregivers and society will benefit.

The establishment of national registries has proven to be a valuable asset in nations that have implemented them for many years (e.g.; Netherlands) (Al-Sarraf *et al.* 2013). The challenges of implementing a national FH registry includes convincing governmental institutions to provide the required funding while ensuring a return on investment in terms of reduced rates of mortality, morbidity and health care costs. Fortunately, a Canadian FH registry has recently been initiated and I was delighted to be invited in there first assembly at the time of this thesis (http://clinicaltrials.gov - NCT02009345). This database will hopefully encompass all the new findings and discoveries related to patient care and thus prove itself useful in the very near future. For example, DNA diagnosis confirmed FH should be evaluated by CT scan prior to aortic valve surgery since associated calcification may increase intra-operative mortality (Grenon *et al.* 2007). Furthermore, the number of surgical procedures in this group will decrease once alternative therapy is made available (Chapter 6).

Prospective from screening and following-up Familial Hypercholesterolemia (FH) patients and maintaining an FH registry:

- Encourage the wide spectrum of FH recognition and the multiple associated genes. We included one of two simultaneous reports that confirm that FH can be caused by mutations within the *APOE* gene and represents the 4th locus causing FH.

- Information from GWAS and next-generation exome sequencing should supplement our findings and therefore should be examined in selected FH individuals, since in many cases the current candidate gene approach will fail to identify a significant number (~20%) of FH patients.

- This genetic approach should encourage lipid specialist to strive for confirmation of a molecular diagnosis in an individual with a FH phenotype so that extended family members of the affected index may benefit from early detection. Genetically confirmed FH patients have a higher risk for premature CVD and thus should be managed closely.

- It would be interesting to screen FH negative-*LDLR/APOB/PCSK9* cohorts for *APOE* mutations and report the prevalence of FH due to *APOE* mutations in these cohorts.

- The criteria by which the entry into a FH registry needs to be simplified to capture the whole range of FH. It should include a lipid profile (isolated increases in LDL-C / apoB) and at least one of the following:

    1) Family history of premature CVD.

    2) Skin stigmata of dyslipidemia (Xanthoma / Xanthelasma).

    3) Molecular diagnosis / dominant mode of inheritance.

    4) Functional analysis of LDLR (skin fibroblast).

- Unrecognized phenotypes are easily captured in FH registries and specialized lipid centers. This was the case for the French-Canadian mutation that was ultimately associated with a severe form of aortic calcification that prompted us to study this phenomenon further (Awan *et al.* 2008, Alrasadi *et al.* 2009).

- Study of long-term data in FH indicates that despite marked LDL-C lowering by statins, aortic calcification persists in a gene- and age-dependent manner and contributes to significant lifelong morbidity. Hopefully, this will encourage more biomedical researcher to examine the underlying mechanism (Awan *et al.* 2011).

- It should be emphasized that vascular calcification was found to stand in sharp contrast with disturbances in bone metabolism and ectopic calcification as seen in end-stage renal disease. In fact, Bone Mineral Density (BMD) does not appear to be significantly disturbed in FH human subjects (Awan *et al.* 2010). The ectopic calcification on a background of normal bone calcification is intriguing and deserves more investigation since the bone is the main calcium reservoir.

- It should be emphasized that early screening and monitoring of calcification in FH using a sensitive method like either CT scan (in human) or micro-CT scan (in animals), will help optimize disease detection (Awan *et al.* 2011), evaluate treatment responses (chapter 6) and plan future surgical interventions (Grenon *et al.* 2007).

7.2. PCSK9 is a "cause" and "cure" for FH (as anti-PCSK9 therapy)

The *PCSK9* gene has been established as the 3rd locus for FH since 2003 and is thought to decrease LDLR activity at the cell surface. Soon after the discovery of PCSK9 and its critical role in regulating hepatic LDLR, it became a therapeutic target for LDL-C lowering and CVD prevention. Although PCSK9 is considered a member of the PC family, little is known about its potential substrate(s). Apart from cleaving itself and targeting the hepatic LDLR to the degradation pathway (and possibly other candidate receptors implicated in lipid and fat metabolism), there is very little known about its function in extrahepatic tissues. Therefore, before inhibiting PCSK9 on a large scale in man, we need to study situations that simulate an environment of low PCSK9 availability. Hence, the purpose of the two cohort studies (reported in chapters 3 and 4) was to address the yet undisclosed human biology of PCSK9. In addition, an increasing number of reports (chapters 1) are exploring the role of PCSK9 by examining the state of PCSK9 protein deficiency in animals or LOF mutations in humans under different conditions. This will simulate how a PCSK9 inhibitor would function *in vivo* and predict potential side effects. Until more is known about PCSK9 function(s) and its potential partner(s), health providers cannot recommend PCSK9 inhibitors as a first line of lipid lowering medication. In certain health circumstances (e.g.; liver disease or pre-diabetic state), medical doctors could even prohibit treatment with a lipid lowering medication that targets PCSK9. Large-scale clinical trials with PCSK9 inhibitors are currently underway and will help physicians determine the appropriate patient for this new class of medications.

The first population we studied was the homogeneous French-Canadians of Quebec. Four centuries ago 3,000-5,000 individual founders shaped the French-Canadian community. Thus, this small genetic pool is an ideal population to study genes contributing to both Mendelian monogenic traits and complex traits. One common lipid determinant is the apoE phenotype, known to influence lipoprotein profiles in the French-Canadian as well as the general population (Robitaille *et al.* 1996). There is a well-known relationship between apoE genotypes and LDL-C levels, namely apoE2 is associated with lower cholesterol levels compared to the prevalent apoE3 isoform (Sing *et al.* 1985, Eto *et al.* 1986, Wilson *et al.* 1995, Xhignesse *et al.* 1991). However, only 5% of the individuals possessing a homozygous apoE2/E2 genotype will develop a severe hyperlipidemia phenotype (type III dyslipidemia), which is characterized by

tuberous xanthoma, palmar creases and premature CVD (Johansen *et al.* 2011). In addition, *PCSK9* gene mutations contribute further to the wide variability seen in plasma lipids.

Prior to this report, there were no published reports of an interaction between the *ApoE* and *PCSK9* genes on the modulation of plasma lipids. We found that the carrier rates of the *PCSK9* natural LOF p.R46L variant is surprisingly enriched in certain regions, such as Saguenay-Lac-Saint-Jean and Québec City, reaching frequencies between 1:12 and 1:8. These two regions also have a high frequency of FH mutations (Vohl *et al.* 1997). The high prevalence of mutations causing low LDL-C (PCSK9 LOF) in areas enriched for LDLR mutations causing FH may seem counter-intuitive. One potential explanation may be that gene selection would favour the survival of protective genes to overcome deleterious ones. The likelihood of a founder effect for the p.R46L mutation will need to be addressed by co-segregation analysis in family members. However, due to the nature of the study, the identity of the patients was beyond our reach and thus family studies were not possible.

Interestingly, when we combined the analysis of *APOE* and *PCSK9* variants, we observed for the first time an intriguing phenotype associated with the combination of apoE2 genotype and p.R46L mutation that was characterized by higher HOMA-IR, insulin and leptin, which are known markers of insulin and leptin resistance. This is an alarming finding that warrants further investigation, and points out the importance of gene-gene interactions. The mechanism of insulin and leptin resistance with the combination of *APOE* and *PCSK9* LOF may be partially explained by the increased density of LDLR (mainly in the liver) and VLDLR (mainly in non fat tissue) as a result of lower circulating PCSK9 levels. Furthermore, the additional LOF in *APOE* gene is suggested to further concentrate the elevated cholesterol and TG rich particles in many tissues that express VLDLR, including adipose tissue and the pancreas, since the apoE2 isoform has a weaker affinity to LDLR. To explain further these observations, additional *in vivo* studies are necessary. In fact, one would anticipate that expressing both apoE2 and p.R46L variants in transgenic mice may elucidate the underlying mechanism. Current *in vivo* data confirms that even modest elevations in free fatty acids and glucose could cause lipotoxicity and glucotoxicity, respectively. This may represent an important mechanism leading to failure of β-cell mass adaptation in the pre-diabetic state (Pascoe *et al.* 2012). It will be interesting to investigate if this anti-adaptive effect is observed in

older patients carrying the apoE2 genotype and *PCSK9* p.R46L mutation (or PCSK9 inhibitor). However, this remains a cross-sectional observation with its limitation, thus confirmation in other cohorts is necessary. Caution should be exercised when confirming the findings of this study in older cohorts by adjusting for waist and hip circumferences between groups, as they correlate better with visceral adiposity. As seen in the *Pcsk9* (KO) animal model, there is a tendency to accumulate visceral fat without total weight gain (Roubtsova *et al.* 2011). This visceral adiposity can contribute to insulin resistance, glucose intolerance and pre-diabetes.

Diabetes mellitus was also associated with statin in many clinical trials, while statin-induce PCSK9 elevation is apparently contradictory to our finding with LOF in *PCSK9* gene leading to insulin resistance, the mechanism of diabetes in statin is unknown and is thought to be very different in each case. Indeed a small but significant increase in the rate of physician-reported diabetes was reported with rosuvastatin in the JUPITER study, as well as a small increase in the median value of glycated hemoglobin. Increases in glucose and glycated hemoglobin levels and the incidence of newly diagnosed diabetes have been also reported in many statin trials (Ridker *et al.* 2008). Nevertheless, the small increase in the rate of physician-reported diabetes in the JUPITER study (270 reports of diabetes, vs. 216 in the placebo group; P=0.01) could have been a random observation. Hence, further studies are needed before any causative effect can be established between statin or the related increase in PCSK9 levels and diabetes.

Collectively, overexpressing *Pcsk9* in mice leads to hypercholesterolemia and aortic calcification, while both animal and human studies confirm signs of insulin resistance in *Pcsk9* (KO) mice and in human *PCSK9* LOF mutations. Therefore we believe that the normal PCSK9 values have a bell-shaped curve distribution, where at the high end they lead to hypercholesterolemia and promote atherosclerosis and vascular calcification, while at the lower end they lead to hypocholesterolemia and may promote visceral obesity and insulin resistance in high risk individuals (Figure 3).

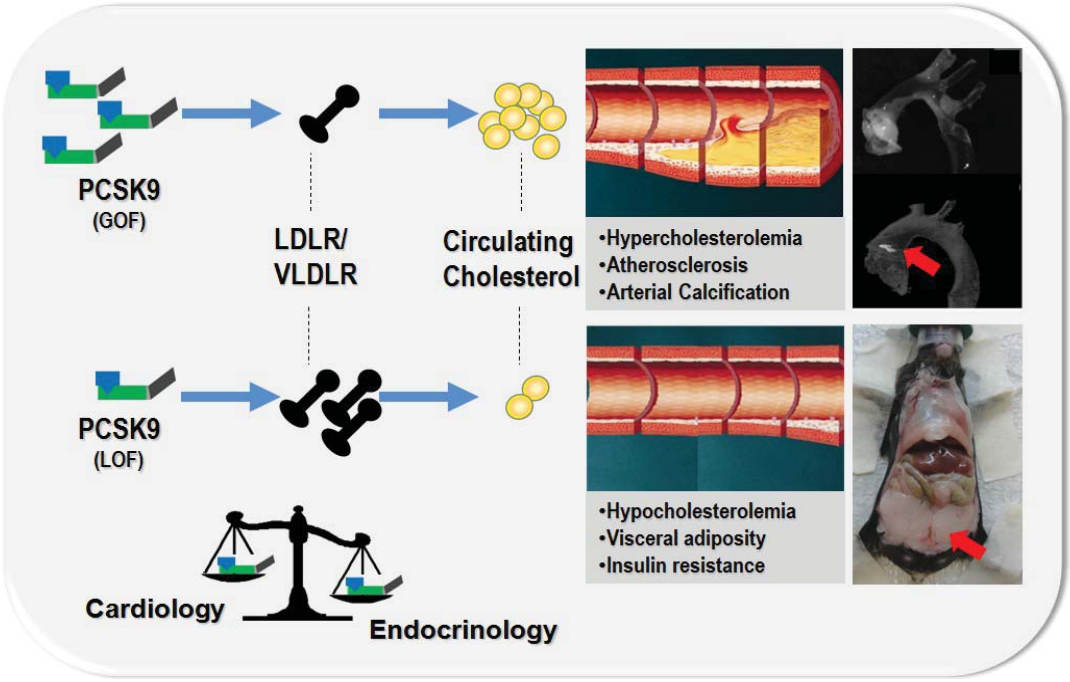


Figure 3: PCSK9 GOF and LOF and the risk of cardiometabolic disease. PCSK9 GOF mutations leads to less LDLR/VLDLR on the cell surface of tissues and this will contribute to hypercholesterolemia, atherosclerosis and arterial calcification. PCSK9 LOF mutations (or use of inhibitors) leads to higher LDLR/VLDLR accumulation on the cell surface and contribute to hypocholesterolemia, visceral adiposity and insulin resistance. Therefore, targeting PCSK9 should be a balance between benefit and harm (adapted from Awan *et al.* 2011, Roubtsova *et al.* 2011 and candidate private collection).

In an attempt to further understand PCSK9 biology, we turned to a second population with a completely different mixed genetic background. The p.R46L allele frequency in this adult population is 2.5%, which is half of that seen in the French-Canadian population and similar to that of the general population, leading to ~8% lower LDL-C than the non-p.R46L allele carriers (Benn *et al.* 2010, Kathiresan *et al.* 2008). Given the ethnic heterogeneity of this population, we cannot perform reliable subgroup statistical comparisons without a much larger sample size. Nevertheless, the presence or absence of the p.R46L variant was not associated with a difference in the response to statin, as one might expect, and therefore knowing whether the patient is a p.R46L carrier would not likely alter therapeutic decisions. The criteria to include patients based on low cholesterol levels (less than 3.4 mmol/L) could have been a confounding factor (selection bias) leading to a blunt response to rosuvastatin. This raises the question of whether or not p.R46L carriers could have showed a stronger response to rosuvastatin

in a hypercholesterolemia cohort. However, in the same study we examined 1,000 patients at two time points and established that plasma PCSK9 concentrations are stable as a biomarker over time among those allocated to placebo, but increase by approximately 30 % among those allocated to 20 mg rosuvastatin.

Not surprisingly, individual responses to statin were variable. However, we established a significant relationship between the magnitude of LDL-C reduction and the increase in PCSK9 concentrations on both the absolute and the relative scale. Across the entire group of patients, LDL-C reductions were associated with increases in plasma PCSK9 concentrations, an effect present across genders despite higher baseline PCSK9 concentrations in women as compared to men. The concept that a statin-related increase in PCSK9 level may limit statins efficacy (Seidah and Attie *et al.* 2005, Berge *et al.* 2006) was not supported by the present study. This can be explained by the presence of an intracellular pathway for PCSK9 (Poirier *et al.* 2009). The PCSK9 intracellular pathway might exhibit a wide variation among individuals in a way that may confound meaningful correlations. Nevertheless, an approach based on the measurement of LDL-C response and residual PCSK9 concentrations may identify subjects with statin resistance (who may benefit from PCSK9 inhibitors) and subjects unable to tolerate statins.

More common loss-of-function (LOF) mutations in *PCSK9* gene are associated with lower circulating PCSK9 and reduction in both LDL-C and CVD. PCSK9-inhibition approaches are at phase III clinical trials and are reported to be powerful enough to lower LDL-C to levels seen in LOF mutations in PCSK9. We sought to determine the effect of LOF mutations in this large registry hoping to be able to predict the LDL-C reduction by statin therapy from the initial PCSK9 levels. However, due to the effect of a statin on the transcription of both LDLR and PCSK9, a net result seams in favour of the LDLR elevation as suggested by the overall ~52% reduction in LDL-C (n=478). This is partially explained by the PCSK9 mode of action since it is mainly mediated by liver excretion and the protein-protein interaction in plasma, in comparison to the direct accumulation of LDLR on the surface of hepatocytes. In addition, we cannot exclude the co-expression of other PCSK9-inhibitors such as AnxA2 and apoB (Kosenko et al. 2013) or the co-regulation of other nuclear binding factors and receptors (HNF1α, HINFP, FXR, PPARs) by statin therapy. Nevertheless, in LOF R46L individuals after 20mg of rosuvastatin were associated with an overall ~56% reduction in LDL-C (n=25) and reflect

a non-significant additional 8% reduction in LDL-C compared to wild type PCSK9. This emphasizes that PCSK9-inhibition based therapy should exceed the 20% lower PCSK9 plasma levels seen in LOF R46L in order to further lower LDL-C significantly.

By observing the variation in the measured PCSK9 values in different populations, we conclude that PCSK9 exhibits diurnal rhythm and pubertal rhythm, but is stable over time as a biomarker, and has a skewed distribution toward higher values. PCSK9 is also higher in subjects with FH and subjects taking statin or fibrate drugs, females compared to males, and premenopausal and pregnant women compared to postmenopausal and non-pregnant women. PCSK9 is also higher postprandial and post systemic inflammation. PCSK9 is lower in a newborn compared to his mother, in women taking estrogen induction therapies for infertility, and in periods of overnight fasting. With more physiological and pathological conditions being reported, a better understanding of the biological functions PCSK9 may be obtained; this may influence the selection of candidates for this class of medications. We can also predict populations at potential risk when treated with PCSK9 inhibitors by combining human and animal studies. Those who may be at risk includes: patients with acute or chronic viral hepatitis, pregnant women, individuals with insulin intolerance or diabetes mellitus, individuals with any LOF variants in PCSK9, individuals with neurological and psychiatric conditions, patients recovering from surgical operations (including liver resection) and individuals with cancer, metastasis and inflammation. Current on-going clinical trials will determine if these potential risks are seen in treated patients.

Furthermore, the reason for the higher level of PCSK9 in premenopausal and pregnant women as opposed to postmenopausal and non-pregnant women is largely unknown. However, hormonal regulation is suggested to influence the level of PCSK9 through different transcription factors and nuclear receptors. Candidate hormones include; estrogen, progesterone, gonadotropin-releasing hormone (GnRH) and growth hormone as suggested by infertility hormonal induction medications (Persson et al. 2012). Furthermore, higher PCSK9 levels in premenopausal than postmenopausal women may reflect central regulation (Lakoski et al. 2009), while higher PCSK9 levels in pregnant than non-pregnant women may reflect higher cholesterol demand of the fetus (Peticca et al. 2013). Nevertheless, *in vivo* studies in animal models are required to further elucidate this relationship.

Prospective from studying populations with PCSK9 LOF mutations to predict the biological effect of a PCSK9 inhibitor:

- Cardiometabolic risk required close monitoring in patients taking PCSK9 inhibitors. Screening should include weight, blood pressure, glucose levels and insulin levels. As statins treatments may tend to increase the incidence of diabetes in subjects with the metabolic syndrome, obesity or insulin intolerance, PCSK9 inhibitors may have a similar effect.

- Testing for the apoE genotype is another consideration. The observation of insulin and leptin resistance associated with *PCSK9* LOF mutations and the apoE2 isoform provides an interesting hypothesis and a research question that needs to be addressed.

- Inhibition of PCSK9 may lead to visceral obesity, in *Pcsk9* (KO) animal models. The complete absence of PCSK9 in animal models (likely not achieved by any drugs in human), is associated with visceral obesity and glucose intolerance but does not affect inflammatory biomarkers (data not shown).

- LDL-C response to statin treatment could not be predicted by measuring circulating plasma PCSK9 levels, possibly due to the intracellular pathway of PCSK9 action. However, PCSK9 levels could probably still be used in predicting the best responder to PCSK9 inhibitors. Thus, PCSK9 levels should be measured before and after administration with a PCSK9 inhibitor and analyzed for an associated LDL-C response in large-scale clinical trials. This will determine the clinical usefulness of PCSK9–guided lipid-lowering therapies.

- Interestingly, PCSK9 LOF mutations are more common than GOF mutations. Depending on the population, GOF mutations may account for ~2% of mutations causing FH, while LOF mutations may reach ~24% in some isolated populations of Québec associated with FH (as a survival gene selection).

7.3. Immunomodulation in FH management (anti-inflammation therapy)

We have shown that FH patients with LOF mutations in LDLR (or GOF mutations in PCSK9 as predicted in mice) develop premature calcification of the aorta approximately 25-30 years earlier than subjects without FH. Conventional treatment modalities lower cholesterol levels and slow down the progression of atherosclerosis, but do not prevent the development of aortic calcification. Thus, there is a **residual risk** beyond lipid lowering and it is manifested as increased risk of vascular calcifications even when plasma levels of LDL-C have been normalized.

Accelerated vascular calcifications in patients with FH occur independently of blood cholesterol levels in adults. These findings, coupled with data from mouse models of aortic calcification, support a role for the LDLR and its downstream targets. In particular, the osteogenic Wnt canonical pathway is implicated in *Ldlr* (KO) mice (Awan *et al.* 2011). Targeting elements in this pathway might bring about unwanted side effects since the Wnt pathway is involved in a wide range of cellular functions including cell differentiation. An alternative approach is to modulate the local and upstream inflammatory stimulus, which is in line with recent data showing that micro-crystals of cholesterol are taken up by macrophages in the subendothelial layer of the arterial wall and activate the NLRP3 inflammasome (Duewell *et al.* 2010, Rajamäki *et al.* 2010), leading to the cleavage-activation of pro-IL-1β and its release. After its release, IL-1β acts as a proximal amplification hub causing the of the Wnt pathway implicated in calcification (Shao *et al.* 2010). Furthermore, IL-1β has been shown to be involved in downstream stimulation of alkaline phosphatase essential to calcification (Lencel *et al.* 2011) and was linked to a possible role in smooth muscle cells transdifferentiation in an inflammatory phenotype (Alexander *et al.* 2012, Keuylian *et al.* 2012).

In Figure 4, we summarize our current mechanism for aortic calcification. This mechanism includes the uptake of oxidized LDL and cholesterol crystals by macrophages, a key step in the initiation of the atherosclerotic plaque and vascular cell transdifferentiation to an osteoblast-like phenotype.

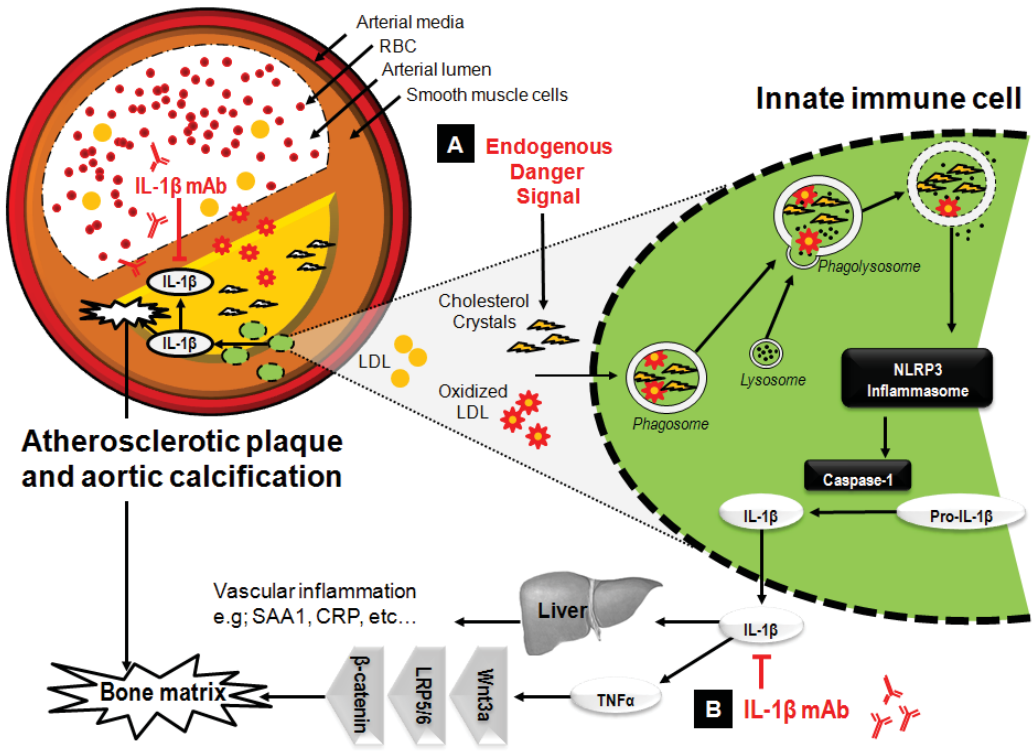


Figure 4: Targeting IL-1β to inhibit aortic calcification: Anti-inflammation drugs may enhance vascular health in FH. Abbreviations – RBC: red blood cells, NLRP3: NOD-Like Receptor family Pyrin domain containing 3, SAA1: serum amyloid A1, CRP: C-reactive protein (adopted from chapter 6).

In Figure 4, the aortic calcification process probably precedes atherosclerotic plaque accumulation by many years. In hypercholesterolemia, micro-crystals represent an endogenous danger-signal for the macrophages of the innate immune system in the subendothelial layer of the arterial wall (Figure 4A). Thus, micro-crystals promote the NLRP3 inflammasome assembly and the release of IL-1β in subendothelium and plasma. This results in the differentiation of aortic cells to an osteoblast-like cell and the release of inflammatory markers by the liver (Figure 4B). Therefore, micro-crystals and IL-1β serve to maintain the state of chronic subendothelial inflammation; leading to dysregulated mineralization, which is postulated to involve a multi-step pathway that includes activation of the Wnt canonical pathway of osteoblast differentiation (Figure 4B). Indeed, immunohistochemical analyses revealed an increased expression of the receptor LRP5 and Wnt ligand in aortas of *Ldlr* (KO) and *Pcsk9(Tg)* mice at 6 months of age as compared to wild type mice (Awan *et al.* 2011). This observation establishes a

link between inflammation, atherosclerosis initiation and progression of vascular calcification. Anti-IL-1β mAb selectively targets this critical step.

The model shown in Figure 4 supports the two-hit model of vascular calcification (Fantus *et al.* 2013), where the ***first hit*** consists of intimal injury caused by lifelong hypercholesterolemia (thus the rationale to start statin treatment as soon as the diagnosis of FH is confirmed) leading to accumulation of inflammatory cells, including monocytes/macrophages. The uptake of micro-crystals and oxidized LDL by macrophages of the innate immune system is a key element in the initiation of the atherosclerotic plaque and the maintenance of chronic subendothelial inflammation. The ***second hit*** involves the calcification process, where calcium is deposited on matrix proteins in the intimal wall of genetically susceptible individuals including individuals with a hyperactive immune response (thus the rational to combined statin and immunomodulation).

Activation of cytokine IL-1β will induce systemic inflammation, reflected by the hepatic secretion of CRP and SAA-1 (Figure 4B). Furthermore, transdifferentiation of aortic tissue occurs when cholesterol remains elevated and inflammation remains active, and with time leads to osteoblast-like differentiation and accelerated arterial calcification. Therefore, measuring both CRP and cholesterol may help in predicting the development of arterial calcification in comparison to cholesterol alone.

Primary prevention strategies in newly diagnosed FH without a CVD should include a potent statin together with lifestyle modification. The FH population is at high risk to develop atherosclerosis and aortic calcification (immunization against atherosclerosis may be available in future). **Secondary prevention** with a history of CVD in FH should include a high dose of a potent statin (PCSK9 inhibitor, if indicated), and likely immunomodulation therapy if clinical trials confirm long-term safety (Figure 5).

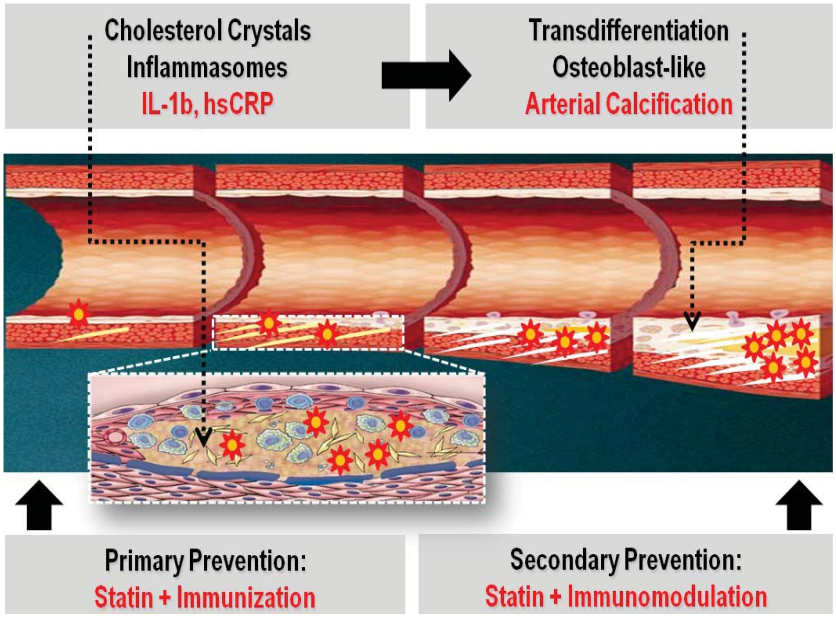


Figure 5: Management of cardiometabolic disease in FH. A statin (and in future immunization methods) is proposed for the CVD primary prevention in asymptomatic molecularly-confirmed FH individuals. High dose statin in combination with immunomodulation is proposed as a secondary prevention in FH individuals (adapted from Klingenberg *et al.* 2009).

An ideal agent that prevents vascular calcification should modulate the pathological calcification process occurring at the level of the subendothelium, as seen in atherosclerosis, while leaving normal bone formation intact. Moreover, modulating inflammation is fraught with potential adverse effects (increase risk of infection, cancer, etc.). Choosing a target that leaves the immune defenses intact and decreases inflammation would represent a potential beneficial therapeutic class. IL-1β targeting fits this category since it is not critical for innate immunity. Our data indicates for the first time the potential role for IL-1β immune modulation in the prevention of vascular calcification, as seen by lower X-ray intensity and lower micro-CT calcium scoring, and the prevention of vascular calcification in *Ldlr* (KO) mice taking anti-IL-1β mAb.

Conversely, *Pcsk9(Tg)* mice did not significantly benefit from IL-1β immune modulation, possibly due to the lower burden of calcification in this model and/or lack of drug efficacy, since the mAb levels declined over time. It would be interesting to examine the kidney pathology in this animal model since the mAb-PCSK9 complex may have altered the filtration capacity of the nephrons, which is a general but theoretical concern

in therapeutic approaches using mAbs. A limitation to our study was that the kidneys and bone were not evaluated and the immune system was not challenged with a pathogen.

However, the immune system in humans is more complex than in the mouse model. Nevertheless, these models are basic safety screens and the only guarantee of the success of these approaches is long-term outcomes in clinical trials. Screening must be meticulously done and it is important to pay attention to all of the data displayed in our animal models. Furthermore, immunomodulation approaches should be evaluated after the development of advanced plaques since this should simulate the real-life situation where the patient usually starts the medication after a CVD event has occurred or after a CT scan showing premature aortic calcification. Therefore, a mouse experiment where a Western diet is introduced first for a few months to accelerate atherosclerotic plaque formation, and then IL-1β mAb is administered in an escalating dose and frequency protocol, followed by micro-CT evaluation, would be very informative. Other anti-inflammatory approaches discussed earlier should also be evaluated in a similar protocol to demonstrate the superiority of one anti-inflammatory drug over the other (IL-1β mAb *vs.* low dose methotrexate, for example).

Prospective from studying human and animal models to advance the understanding of arterial calcification and prevent disease:

- By establishing mouse models with moderate to extensive vascular calcification that replicate the disease observed in humans, we can advance our understanding of the disease. These two animal models mimic absolute LDLR-deficiency and low LDLR-availability. The latter phenotype is mimicked by over expressing *Pcsk9*. The two models should facilitate the studies of both the pathophysiology of vascular calcification as well as other metabolic risk.

- Having validated micro-CT as a sensitive technique to quantify aortic calcification in murine models, we can now begin to test different therapeutic approaches. Micro-CT allows us to measure changes in calcification in a precise, quantifiable and informative manner and hence the efficacy of different treatment regimens can be compared.

- The mice used in our study demonstrate that aortic calcification is multifactorial and is associated with aging, LDLR-deficiency and is accelerated by Western diet. *Pcsk9(Tg)* confirmed the possible role of LDLR in calcium deposition. The role for the LDLR protein needs to be further examined.

- The mechanism of aortic calcification in human FH could not be elucidated from this work. However, macrophage markers, TNFα and IL-1β were highly expressed in calcified aorta. Furthermore, the canonical Wnt pathway was induced, at least in mice, suggesting an inflammatory proliferative and remodeling mechanism.

- We demonstrate that aortic calcification can be inhibited by IL-1β mAb in LDL-receptor deficient mice. These results may be applied to the prevention of vascular calcification in humans. Future therapeutic approaches may include a combination of a statin with an anti-inflammation agent. Further testing in aortic calcification is warranted using different drug doses and patterns of administration.

- Interestingly, a randomized phase III clinical trial is currently underway to investigate the effectiveness of subcutaneous injections of a fully humanized antibody directed against IL1β, canakinumab (Ridker *et al.* 2011) in stable post MI patients (secondary prevention) with elevated levels of the inflammatory biomarker CRP. It would be interesting to evaluate coronary calcification before and after canakinumab in a subset of these patients to confirm the findings we obtained in mice. However, failure to regress aortic calcification in these secondary prevention patients is expected, whereas the reasonable expectation should be attenuation of calcification.

7.4. Conclusions

The incidence of FH in the general population is approximately 1/500 and it is much higher in populations with a founder effect, such as in the Lac St Jean and Kamouraska regions of Quebec. Given the incidence of FH in the general population, it is predicted that FH affects at least 70,000 individuals in Canada. Without adequate screening and treatment strategies for FH, vulnerable individuals are missing a golden opportunity in preventive medicine. Cascade screening should be implemented and DNA analysis should now include the *APOE* gene. Long-term follow-up of FH subjects will help identify success and failure of new treatment modalities and will help provide patients access to novel treatments. While on therapy, some FH subjects may develop statin intolerance and many experience premature aortic calcification that is associated with impaired functional status and survival. Similarly, individuals with GOF mutations in the *PCSK9* gene that promotes degradation of the LDLR might be at risk (as seen in animal models).

Therefore, PCSK9 inhibitors are a very attractive class of medications that will become an add-on to statins or an alternative to statins in statin-intolerant patients due to its unique mode of action. Currently, several clinical trials using different approaches to inhibit PCSK9 have reached phase III and continue to report satisfactory responses. With PCSK9 inhibitors, used in assisted monotherapy or in combination with statins, safety and tolerability are a concern. Therefore, we and others are focusing on predicting the long-term effects of these drugs on cardiometabolic health. PCSK9 inhibition, as seen in natural LOF mutations, is successful in maintaining lower cholesterol; however, care should be taken in individuals with cardiometabolic risk, particularly insulin resistance. *APOE* genotyping may assist in identifying individuals at risk.

Aortic calcification is currently under-diagnosed and unresponsive to existing therapies. Subendothelial inflammation may represent the missing link between cholesterol and calcification. Administration of a murine IL-1β monoclonal antibody was successful in preventing vascular calcifications in mice. This study therefore indicates a potential means of treating homozygous and severe heterozygous FH patients to prevent aortic calcification.

REFERENCES:

Abidi P, Zhou Y, Jiang JD, Liu J. Extracellular signal-regulated kinase-dependent stabilization of hepatic low-density lipoprotein receptor mRNA by herbal medicine berberine. Arterioscler Thromb Vasc Biol. 2005 Oct;25(10):2170-6. Epub 2005 Aug 11. PubMed PMID: 16100034.

Abifadel M, Varret M, Rabès JP, Allard D, Ouguerram K, Devillers M, Cruaud C, Benjannet S, Wickham L, Erlich D, Derré A, Villéger L, Farnier M, Beucler I, Bruckert E, Chambaz J, Chanu B, Lecerf JM, Luc G, Moulin P, Weissenbach J, Prat A, Krempf M, Junien C, Seidah NG, Boileau C. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet. 2003 Jun;34(2):154-6. PubMed PMID: 12730697.

Alexander MR, Murgai M, Moehle CW, Owens GK. Interleukin-1β modulates smooth muscle cell phenotype to a distinct inflammatory state relative to PDGF-DD via NF-κB-dependent mechanisms. Physiol Genomics. 2012 Apr 2;44(7):417-29. doi:10.1152/physiolgenomics.00160.2011. Epub 2012 Feb 7. PubMed PMID: 22318995; PubMed Central PMCID: PMC3339851.

Allard D, Amsellem S, Abifadel M, Trillard M, Devillers M, Luc G, Krempf M, Reznik Y, Girardet JP, Fredenrich A, Junien C, Varret M, Boileau C, Benlian P, Rabès JP. Novel mutations of the PCSK9 gene cause variable phenotype of autosomal dominant hypercholesterolemia. Hum Mutat. 2005 Nov;26(5):497. Erratum in: Hum Mutat. 2005 Dec;26(6):592. PubMed PMID: 16211558.

Al-Mashhadi RH, Sørensen CB, Kragh PM, Christoffersen C, Mortensen MB, Tolbod LP, Thim T, Du Y, Li J, Liu Y, Moldt B, Schmidt M, Vajta G, Larsen T, Purup S, Bolund L, Nielsen LB, Callesen H, Falk E, Mikkelsen JG, Bentzon JF. Familial hypercholesterolemia and atherosclerosis in cloned minipigs created by DNA transposition of a human PCSK9 gain-of-function mutant. Sci Transl Med. 2013 Jan 2;5(166):166ra1. doi: 10.1126/scitranslmed.3004853. PubMed PMID: 23283366.

Alrasadi K, Alwaili K, Awan Z, Valenti D, Couture P, Genest J. Aortic calcifications in familial hypercholesterolemia: potential role of the low-density lipoprotein receptor

gene. Am Heart J. 2009 Jan;157(1):170-6. doi:10.1016/j.ahj.2008.08.021. PubMed PMID: 19081415.

Al-Sarraf A, Allard M, Martinka M, Frohlich J. Regional and national familial hypercholesterolemia registries: present international application, importance, and needs for Canada. Can J Cardiol. 2013 Jan;29(1):6-9. doi: 10.1016/j.cjca.2012.04.019. Epub 2012 Jun 19. Review. PubMed PMID: 22717249.

Abbate A, Kontos MC, Grizzard JD, Biondi-Zoccai GG, Van Tassell BW, Robati R, Roach LM, Arena RA, Roberts CS, Varma A, Gelwix CC, Salloum FN, Hastillo A, Dinarello CA, Vetrovec GW; VCU-ART Investigators. Interleukin-1 blockade with anakinra to prevent adverse cardiac remodeling after acute myocardial infarction (Virginia Commonwealth University Anakinra Remodeling Trial [VCU-ART] Pilot study). Am J Cardiol. 2010 May 15;105(10):1371-1377.e1. doi: 10.1016/j.amjcard.2009.12.059. Epub 2010 Apr 2. PubMed PMID: 20451681.

Anderson RG, Goldstein JL, Brown MS. A mutation that impairs the ability of lipoprotein receptors to localise in coated pits on the cell surface of human fibroblasts. Nature. 1977 Dec 22-29;270(5639):695-9. PubMed PMID: 201867.

Anderson TJ, Grégoire J, Hegele RA, Couture P, Mancini GB, McPherson R, Francis GA, Poirier P, Lau DC, Grover S, Genest J Jr, Carpentier AC, Dufour R, Gupta M, Ward R, Leiter LA, Lonn E, Ng DS, Pearson GJ, Yates GM, Stone JA, Ur E. 2012 update of the Canadian Cardiovascular Society guidelines for the diagnosis and treatment of dyslipidemia for the prevention of cardiovascular disease in the adult. Can J Cardiol. 2013 Feb;29(2):151-67. doi: 10.1016/j.cjca.2012.11.032. Review. PubMed PMID: 23351925.

André P, Perlemuter G, Budkowska A, Bréchot C, Lotteau V. Hepatitis C virus particles and lipoprotein metabolism. Semin Liver Dis. 2005 Feb;25(1):93-104. Review. PubMed PMID: 15732001.

Anker SD, von Haehling S. Inflammatory mediators in chronic heart failure: an overview. Heart. 2004 Apr;90(4):464-70. PubMed PMID: 15020532; PubMed Central PMCID: PMC1768165.

Arca M, Zuliani G, Wilund K, Campagna F, Fellin R, Bertolini S, Calandra S, Ricci G, Glorioso N, Maioli M, Pintus P, Carru C, Cossu F, Cohen J, Hobbs HH. Autosomal recessive hypercholesterolaemia in Sardinia, Italy, and mutations in ARH: a clinical and molecular genetic analysis. Lancet. 2002 Mar 9;359(9309):841-7. PubMed PMID: 11897284.

Asselbergs FW, Guo Y, van Iperen EP, Sivapalaratnam S, Tragante V, Lanktree MB, Lange LA, Almoguera B, Appelman YE, Barnard J, Baumert J, Beitelshees AL, Bhangale TR, Chen YD, Gaunt TR, Gong Y, Hopewell JC, Johnson T, Kleber ME, Langaee TY, Li M, Li YR, Liu K, McDonough CW, Meijs MF, Middelberg RP, Musunuru K, Nelson CP, O'Connell JR, Padmanabhan S, Pankow JS, Pankratz N, Rafelt S, Rajagopalan R, Romaine SP, Schork NJ, Shaffer J, Shen H, Smith EN, Tischfield SE, van der Most PJ, van Vliet-Ostaptchouk JV, Verweij N, Volcik KA, Zhang L, Bailey KR, Bailey KM, Bauer F, Boer JM, Braund PS, Burt A, Burton PR, Buxbaum SG, Chen W, Cooper-Dehoff RM, Cupples LA, deJong JS, Delles C, Duggan D, Fornage M, Furlong CE, Glazer N, Gums JG, Hastie C, Holmes MV, Illig T, Kirkland SA, Kivimaki M, Klein R, Klein BE, Kooperberg C, Kottke-Marchant K, Kumari M, LaCroix AZ, Mallela L, Murugesan G, Ordovas J, Ouwehand WH, Post WS, Saxena R, Scharnagl H, Schreiner PJ, Shah T, Shields DC, Shimbo D, Srinivasan SR, Stolk RP, Swerdlow DI, Taylor HA Jr, Topol EJ, Toskala E, van Pelt JL, van Setten J, Yusuf S, Whittaker JC, Zwinderman AH; LifeLines Cohort Study, Anand SS, Balmforth AJ, Berenson GS, Bezzina CR, Boehm BO, Boerwinkle E, Casas JP, Caulfield MJ, Clarke R, Connell JM, Cruickshanks KJ, Davidson KW, Day IN, de Bakker PI, Doevendans PA, Dominiczak AF, Hall AS, Hartman CA, Hengstenberg C, Hillege HL, Hofker MH, Humphries SE, Jarvik GP, Johnson JA, Kaess BM, Kathiresan S, Koenig W, Lawlor DA, März W, Melander O, Mitchell BD, Montgomery GW, Munroe PB, Murray SS, Newhouse SJ, Onland-Moret NC, Poulter N, Psaty B, Redline S, Rich SS, Rotter JI, Schunkert H, Sever P, Shuldiner AR, Silverstein RL, Stanton A, Thorand B, Trip MD, Tsai MY, van der Harst P, van der Schoot E, van der Schouw YT, Verschuren WM, Watkins H, Wilde AA, Wolffenbuttel BH, Whitfield JB, Hovingh GK, Ballantyne CM, Wijmenga C, Reilly MP, Martin NG, Wilson JG, Rader DJ, Samani NJ, Reiner AP, Hegele RA, Kastelein JJ, Hingorani AD, Talmud PJ, Hakonarson H, Elbers CC, Keating BJ, Drenos F. Large-scale gene-centric meta-analysis across 32 studies identifies multiple lipid loci. Am J Hum

Genet. 2012 Nov 2;91(5):823-38. doi: 10.1016/j.ajhg.2012.08.032. Epub 2012 Oct 11. PubMed PMID: 23063622; PubMed Central PMCID: PMC3487124.

Atsuta J, Plitt J, Bochner BS, Schleimer RP. Inhibition of VCAM-1 expression in human bronchial epithelial cells by glucocorticoids. Am J Respir Cell Mol Biol. 1999 Apr;20(4):643-50. PubMed PMID: 10100995.

Attie AD, Seidah NG. Dual regulation of the LDL receptor--some clarity and new questions. Cell Metab. 2005 May;1(5):290-2. Review. PubMed PMID: 16054075.

Awan Z, Alrasadi K, Francis GA, Hegele RA, McPherson R, Frohlich J, Valenti D, de Varennes B, Marcil M, Gagne C, Genest J, Couture P. Vascular calcifications in homozygote familial hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2008 Apr;28(4):777-85. doi: 10.1161/ATVBAHA.107.160408. Epub 2008 Jan 31. PubMed PMID: 18239150.

Awan Z, Alwaili K, Alshahrani A, Langsetmo L, Goltzman D, Genest J. Calcium homeostasis and skeletal integrity in individuals with familial hypercholesterolemia and aortic calcification. Clin Chem. 2010 Oct;56(10):1599-607. doi: 10.1373/clinchem.2010.147066. Epub 2010 Aug 11. PubMed PMID: 20702785.

Awan Z, Bailey D, Hafiane A, Genest J. Acquired severe hypercholesterolemia and hypoalphalipoproteinemia. J Clin Lipidol. 2009 Dec;3(6):393-7. doi: 0.1016/j.jacl.2009.10.001. Epub 2009 Oct 8. PubMed PMID: 21291840.

Awan Z, Delvin EE, Levy E, Genest J, Davignon J, Seidah NG, Baass A. Regional distribution and metabolic effect of PCSK9 insLEU and R46L gene mutations and apoE genotype. Can J Cardiol. 2013 Aug;29(8):927-33. doi: 10.1016/j.cjca.2013.03.004. Epub 2013 Jun 3. PubMed PMID: 23743349.

Awan Z, Denis M, Bailey D, Giaid A, Prat A, Goltzman D, Seidah NG, Genest J. The LDLR deficient mouse as a model for aortic calcification and quantification by micro-computed tomography. Atherosclerosis. 2011 Dec;219(2):455-62. doi:10.1016/j.atherosclerosis.2011.08.035. Epub 2011 Aug 26. PubMed PMID: 22051553.

Awan Z, Seidah NG, MacFadyen JG, Benjannet S, Chasman DI, Ridker PM, Genest J. Rosuvastatin, proprotein convertase subtilisin/kexin type 9 concentrations, and LDL

cholesterol response: the JUPITER trial. Clin Chem. 2012 Jan;58(1):183-9. doi: 10.1373/clinchem.2011.172932. Epub 2011 Nov 7. PubMed PMID: 22065156

Baass A, Dubuc G, Tremblay M, Delvin EE, O'Loughlin J, Levy E, Davignon J, Lambert M. Plasma PCSK9 is associated with age, sex, and multiple metabolic markers in a population-based sample of children and adolescents. Clin Chem. 2009 Sep;55(9):1637-45. doi: 10.1373/clinchem.2009.126987. Epub 2009 Jul 23. PubMed PMID: 19628659.

Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, Peto R, Barnes EH, Keech A, Simes J,Collins R Cholesterol; ; (Cholesterol Treatment Trialists' CTT) Collaborators. Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. Lancet. 2010 Nov 13;376(9753):1670-81. doi: 10.1016/S0140-6736(10)61350-5. Epub 2010 Nov 8. PubMed PMID: 21067804; PubMed Central PMCID: PMC2988224.

Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, Kirby A, Sourjina T, Peto R, Collins R, Simes R; (Cholesterol Treatment Trialists' CTT) Collaborators. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet. 2005 Oct 8;366(9493):1267-78. Epub 2005 Sep 27. Erratum in: Lancet. 2008 Jun 21;371(9630):2084. Lancet. 2005 Oct 15-21;366(9494):1358. PubMed PMID: 16214597.

Bailyes EM, Bennett DL, Hutton JC. Proprotein-processing endopeptidases of the insulin secretory granule. Enzyme. 1991;45(5-6):301-13. Review. PubMed PMID: 1843283.

Bassi DE, Mahloogi H, Klein-Szanto AJ. The proprotein convertases furin and PACE4 play a significant role in tumor progression. Mol Carcinog. 2000 Jun;28(2):63-9. Review. PubMed PMID: 10900462.

Bastard JP, Jardel C, Delattre J, Hainque B, Bruckert E, Oberlin F. Evidence for a link between adipose tissue interleukin-6 content and serum C-reactive protein concentrations in obese subjects. Circulation. 1999 Apr 27;99(16):2221-2. PubMed PMID: 10217702.

Benjannet S, Hamelin J, Chrétien M, Seidah NG. Loss- and gain-of-function PCSK9 variants: cleavage specificity, dominant negative effects, and low density lipoprotein receptor (LDLR) degradation. J Biol Chem. 2012 Sep 28;287(40):33745-55. Epub 2012 Aug 8. PubMed PMID: 22875854; PubMed Central PMCID: PMC3460471.

Benjannet S, Rhainds D, Essalmani R, Mayne J, Wickham L, Jin W, Asselin MC, Hamelin J, Varret M, Allard D, Trillard M, Abifadel M, Tebon A, Attie AD, Rader DJ, Boileau C, Brissette L, Chrétien M, Prat A, Seidah NG. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. J Biol Chem. 2004 Nov 19;279(47):48865-75. Epub 2004 Sep 9. PubMed PMID: 15358785.

Benjannet S, Rhainds D, Hamelin J, Nassoury N, Seidah NG. The proprotein convertase (PC) PCSK9 is inactivated by furin and/or PC5/6A: functional consequences of natural mutations and post-translational modifications. J Biol Chem. 2006 Oct 13;281(41):30561-72. Epub 2006 Aug 15. PubMed PMID: 16912035.

Benjannet S, Saavedra YG, Hamelin J, Asselin MC, Essalmani R, Pasquato A, Lemaire P, Duke G, Miao B, Duclos F, Parker R, Mayer G, Seidah NG. Effects of the prosegment and pH on the activity of PCSK9: evidence for additional processing events. J Biol Chem. 2010 Dec 24;285(52):40965-78. doi: 10.1074/jbc.M110.154815. Epub 2010 Oct 11. PubMed PMID: 20937814; PubMed Central PMCID: PMC3003396.

Benn M, Nordestgaard BG, Grande P, Schnohr P, Tybjaerg-Hansen A. PCSK9 R46L, low-density lipoprotein cholesterol levels, and risk of ischemic heart disease: 3 independent studies and meta-analyses. J Am Coll Cardiol. 2010 Jun 22;55(25):2833-42. doi: 10.1016/j.jacc.2010.02.044. Review. PubMed PMID: 20579540.

Berge KE, Ose L, Leren TP. Missense mutations in the PCSK9 gene are associated with hypocholesterolemia and possibly increased response to statin therapy. Arterioscler Thromb Vasc Biol. 2006 May;26(5):1094-100. Epub 2006 Jan 19. PubMed PMID: 16424354.

Bergeron F, Leduc R, Day R. Subtilase-like pro-protein convertases: from molecular specificity to therapeutic applications. J Mol Endocrinol. 2000 Feb;24(1):1-22. Review. PubMed PMID: 10656993.

Berthold HK, Seidah NG, Benjannet S, Gouni-Berthold I. Evidence from a randomized trial that simvastatin, but not ezetimibe, upregulates circulating PCSK9 levels. PLoS One. 2013;8(3):e60095. doi: 10.1371/journal.pone.0060095. Epub 2013 Mar 27. PubMed PMID: 23544125; PubMed Central PMCID: PMC3609731.

Bhat, Mamatha [Master's thesis 2010, McGill University. http://digitool.library.mcgill.ca]

Boden WE, Probstfield JL, Anderson T, Chaitman BR, Desvignes-Nickens P, Koprowicz K, McBride R, Teo K, Weintraub W. Niacin in patients with low HDL cholesterol levels receiving intensive statin therapy. N Engl J Med. 2011 Dec 15;365(24):2255-67. doi: 10.1056/NEJMoa1107579. Epub 2011 Nov 15. Erratum in: N Engl J Med. 2012 Jul 12;367(2):189. PubMed PMID: 22085343.

Brouwers MC, Konrad RJ, van Himbergen TM, Isaacs A, Otokozawa S, Troutt JS, Schaefer EJ, van Greevenbroek MM, Stalenhoef AF, de Graaf J. Plasma proprotein convertase subtilisin kexin type 9 levels are related to markers of cholesterol synthesis in familial combined hyperlipidemia. Nutr Metab Cardiovasc Dis. 2013 Jan 17. doi:pii: S0939-4753(12)00266-9. 10.1016/j.numecd.2012.11.008. [Epub ahead of print] PubMed PMID: 23333725.

Brown MS, Goldstein JL. Biomedicine. Lowering LDL--not only how low, but how long? Science. 2006 Mar 24;311(5768):1721-3. PubMed PMID: 16556829.

Brown MS, Goldstein JL. Expression of the familial hypercholesterolemia gene in heterozygotes: mechanism for a dominant disorder in man. Science. 1974 Jul 5;185(4145):61-3. PubMed PMID: 4366052.

Brown MS, Goldstein JL. Lipoprotein receptors in the liver. Control signals for plasma cholesterol traffic. J Clin Invest. 1983 Sep;72(3):743-7. Review. PubMed PMID: 6309907; PubMed Central PMCID: PMC1129238.

Brown MS, Herz J, Goldstein JL. LDL-receptor structure. Calcium cages, acid baths and recycling receptors. Nature. 1997 Aug 14;388(6643):629-30. PubMed PMID: 9262394.

Camargo CA Jr, Barr RG, Chen R, Speizer FE. Prospective study of inhaled corticosteroid use, cardiovascular mortality, and all-cause mortality in asthmatic women. Chest. 2008 Sep;134(3):546-51. doi: 10.1378/chest.07-3126. Epub 2008 Jul 18. PubMed PMID: 18641096; PubMed Central PMCID: PMC2643337.

Cameron J, Ranheim T, Kulseth MA, Leren TP, Berge KE. Berberine decreases PCSK9 expression in HepG2 cells. Atherosclerosis. 2008 Dec;201(2):266-73. doi: 10.1016/j.atherosclerosis.2008.02.004. Epub 2008 Feb 15. PubMed PMID: 18355829.

Canuel M, Sun X, Asselin MC, Paramithiotis E, Prat A, Seidah NG. Proprotein convertase subtilisin/kexin type 9 (PCSK9) can mediate degradation of the low density lipoprotein receptor-related protein 1 (LRP-1). PLoS One. 2013 May 13;8(5):e64145. doi: 10.1371/journal.pone.0064145. Print 2013. PubMed PMID: 23675525; PubMed Central PMCID: PMC3652815.

Cao A, Wu M, Li H, Liu J. Janus kinase activation by cytokine oncostatin M decreases PCSK9 expression in liver cells. J Lipid Res. 2011 Mar;52(3):518-30. doi: 10.1194/jlr.M010603. Epub 2010 Dec 31. PubMed PMID: 21196532; PubMed Central PMCID: PMC3035688.

Cariou B, Le May C, Costet P. Clinical aspects of PCSK9. Atherosclerosis. 2011 Jun;216(2):258-65. doi: 10.1016/j.atherosclerosis.2011.04.018. Epub 2011 Apr 22. Review. PubMed PMID: 21596380.

Centers for Disease Control and Prevention (CDC). Vital signs: prevalence, treatment, and control of high levels of low-density lipoprotein cholesterol--United States, 1999-2002 and 2005-200. MMWR Morb Mortal Wkly Rep. 2011 Feb 4;60(4):109-14. PubMed PMID: 21293326.

Chan JC, Piper DE, Cao Q, et al. A proprotein convertase subtilisin/kexin type 9 neutralizing antibody reduces serum cholesterol in mice and nonhuman primates. Proc Natl Acad Sci U S A. 2009 Jun 16;106(24):9820-5. doi: 10.1073/pnas.0903849106. Epub 2009 May 14. PubMed PMID: 19443683; PubMed Central PMCID: PMC2682542.

Chan KL, Teo K, Dumesnil JG, Ni A, Tam J; ASTRONOMER Investigators. Effect of Lipid lowering with rosuvastatin on progression of aortic stenosis: results of the aortic stenosis progression observation: measuring effects of rosuvastatin (ASTRONOMER) trial. Circulation. 2010 Jan 19;121(2):306-14. doi: 10.1161/CIRCULATIONAHA.109.900027. Epub 2010 Jan 4. PubMed PMID: 20048204.

Church LD, Cook GP, McDermott MF. Primer: inflammasomes and interleukin 1beta in inflammatory disorders. Nat Clin Pract Rheumatol. 2008 Jan;4(1):34-42. doi: 10.1038/ncprheum0681. Review. PubMed PMID: 18172447.

Chyu KY, Shah PK. Can We Vaccinate Against Atherosclerosis? J Cardiovasc Pharmacol Ther. 2013 Sep 13. [Epub ahead of print] PubMed PMID: 24038015.

Chyu KY, Zhao X, Reyes OS, Babbidge SM, Dimayuga PC, Yano J, Cercek B, Fredrikson GN, Nilsson J, Shah PK. Immunization using an Apo B-100 related epitope reduces atherosclerosis and plaque inflammation in hypercholesterolemic apo E (-/-) mice. Biochem Biophys Res Commun. 2005 Dec 30;338(4):1982-9. Epub 2005 Nov 2. PubMed PMID: 16288717.

Circulation. Coronary heart disease in seven countries. Summary. 1970 Apr;41(4 Suppl):I186-95. PubMed PMID: 5442782.

Civeira F, Ros E, Jarauta E, Plana N, Zambon D, Puzo J, Martinez de Esteban JP, Ferrando J, Zabala S, Almagro F, Gimeno JA, Masana L, Pocovi M. Comparison of genetic versus clinical diagnosis in familial hypercholesterolemia. Am J Cardiol. 2008 Nov 1;102(9):1187-93, 1193.e1. doi: 10.1016/j.amjcard.2008.06.056. Epub 2008 Aug 27. PubMed PMID: 18940289.

Civeira F. International Panel on Management of Familial Hypercholesterolemia. Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia. Atherosclerosis. 2004 Mar;173(1):55-68. Review. PubMed PMID: 15177124.

Cnop M, Hannaert JC, Grupping AY, Pipeleers DG. Low density lipoprotein can cause death of islet beta-cells by its cellular uptake and oxidative modification. Endocrinology. 2002 Sep;143(9):3449-53. PubMed PMID: 12193557.

Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK, Hobbs HH. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. Nat Genet. 2005 Feb;37(2):161-5. Epub 2005 Jan 16. Erratum in: Nat Genet. 2005 Mar;37(3):328. PubMed PMID: 15654334.

Cohen JC, Boerwinkle E, Mosley TH Jr, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med. 2006 Mar 23;354(12):1264-72. PubMed PMID: 16554528.

Costet P, Cariou B, Lambert G, Lalanne F, Lardeux B, Jarnoux AL, Grefhorst A, Staels B, Krempf M. Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. J Biol Chem. 2006 Mar 10;281(10):6211-8. Epub 2006 Jan 6. PubMed PMID: 16407292.

Cseh K, Beutler B. Alternative cleavage of the cachectin/tumor necrosis factor propeptide results in a larger, inactive form of secreted protein. J Biol Chem. 1989 Sep 25;264(27):16256-60. PubMed PMID: 2777790.

Cuchacovich R, Espinoza LR. Does TNF-alpha blockade play any role in cardiovascular risk among rheumatoid arthritis (RA) patients? Clin Rheumatol. 2009 Oct;28(10):1217-20. doi: 10.1007/s10067-009-1208-x. Epub 2009 Jun 11. PubMed PMID: 19517156.

Cuchel M, Bloedon LT, Szapary PO, Kolansky DM, Wolfe ML, Sarkis A, Millar JS, Ikewaki K, Siegelman ES, Gregg RE, Rader DJ. Inhibition of microsomal triglyceride transfer protein in familial hypercholesterolemia. N Engl J Med. 2007 Jan 11;356(2):148-56. PubMed PMID: 17215532.

Cunningham D, Danley DE, Geoghegan KF, Griffor MC, Hawkins JL, Subashi TA, Varghese AH, Ammirati MJ, Culp JS, Hoth LR, Mansour MN, McGrath KM, Seddon AP, Shenolikar S, Stutzman-Engwall KJ, Warren LC, Xia D, Qiu X. Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. Nat Struct Mol Biol. 2007 May;14(5):413-9. Epub 2007 Apr 15. PubMed PMID: 17435765.

Davignon J, Dubuc G, Seidah NG. The influence of PCSK9 polymorphisms on serum low-density lipoprotein cholesterol and risk of atherosclerosis. Curr Atheroscler Rep.

2010 Sep;12(5):308-15. doi: 10.1007/s11883-010-0123-6. Review. PubMed PMID: 20623344.

Davignon J. Beneficial cardiovascular pleiotropic effects of statins. Circulation. 2004 Jun 15;109(23 Suppl 1):III39-43. Review. PubMed PMID: 15198965.

Davis JM 3rd, Maradit Kremers H, Crowson CS, Nicola PJ, Ballman KV, Therneau TM, Roger VL, Gabriel SE. Glucocorticoids and cardiovascular events in rheumatoid arthritis: a population-based cohort study. Arthritis Rheum. 2007 Mar;56(3):820-30. PubMed PMID: 17330254.

de Jager SC, Kuiper J. Vaccination strategies in atherosclerosis. Thromb Haemost. 2011 Nov;106(5):796-803. doi: 10.1160/TH11-05-0369. Epub 2011 Oct 20. Review. PubMed PMID: 22012002.

de Lorenzo F, Feher M, Martin J, Collot-Teixeira S, Dotsenko O, McGregor JL. Statin therapy-evidence beyond lipid lowering contributing to plaque stability. Curr Med Chem. 2006;13(28):3385-93. Review. PubMed PMID: 17168712.

Defesche JC. Defining the challenges of FH screening for familial hypercholesterolemia. J Clin Lipidol. 2010 Sep-Oct;4(5):338-41. doi:10.1016/j.jacl.2010.08.022. PubMed PMID: 21122674.

Deloukas P, Kanoni S, Willenborg C, Farrall M,Assimes TL, Thompson JR, Ingelsson E, Saleheen D, Erdmann J, Goldstein BA, Stirrups K, König IR, Cazier JB, Johansson A, Hall AS, Lee JY, Willer CJ, Chambers JC, Esko T, Folkersen L, Goel A, Grundberg E, Havulinna AS, Ho WK, Hopewell JC, Eriksson N, Kleber ME, Kristiansson K, Lundmark P, Lyytikäinen LP, Rafelt S, Shungin D, Strawbridge RJ, Thorleifsson G, Tikkanen E, Van Zuydam N, Voight BF, Waite LL, Zhang W, Ziegler A, Absher D, Altshuler D, Balmforth AJ, Barroso I, Braund PS, Burgdorf C, Claudi-Boehm S, Cox D, Dimitriou M, Do R; DIAGRAM Consortium; CARDIOGENICS Consortium, Doney AS, El Mokhtari N, Eriksson P, Fischer K, Fontanillas P, Franco-Cereceda A, Gigante B, Groop L, Gustafsson S, Hager J, Hallmans G, Han BG, Hunt SE, Kang HM, Illig T, Kessler T, Knowles JW, Kolovou G, Kuusisto J, Langenberg C, Langford C, Leander K, Lokki ML, Lundmark A, McCarthy MI, Meisinger C, Melander O, Mihailov E, Maouche S, Morris AD, Müller-Nurasyid M; MuTHER Consortium, Nikus K, Peden JF, Rayner NW, Rasheed A, Rosinger S, Rubin D, Rumpf MP,

Schäfer A, Sivananthan M, Song C, Stewart AF, Tan ST, Thorgeirsson G, van der Schoot CE, Wagner PJ; Wellcome Trust Case Control Consortium, Wells GA, Wild PS, Yang TP, Amouyel P, Arveiler D, Basart H, Boehnke M, Boerwinkle E, Brambilla P, Cambien F, Cupples AL, de Faire U, Dehghan A, Diemert P, Epstein SE, Evans A, Ferrario MM, Ferrières J, Gauguier D, Go AS, Goodall AH, Gudnason V, Hazen SL, Holm H, Iribarren C, Jang Y, Kähönen M, Kee F, Kim HS, Klopp N, Koenig W, Kratzer W, Kuulasmaa K, Laakso M, Laaksonen R, Lee JY, Lind L, Ouwehand WH, Parish S, Park JE, Pedersen NL, Peters A, Quertermous T, Rader DJ, Salomaa V, Schadt E, Shah SH, Sinisalo J, Stark K, Stefansson K, Trégouët DA, Virtamo J, Wallentin L, Wareham N, Zimmermann ME, Nieminen MS, Hengstenberg C, Sandhu MS, Pastinen T, Syvänen AC, Hovingh GK, Dedoussis G, Franks PW, Lehtimäki T, Metspalu A, Zalloua PA, Siegbahn A, Schreiber S, Ripatti S, Blankenberg SS, Perola M, Clarke R, Boehm BO, O'Donnell C, Reilly MP, März W, Collins R, Kathiresan S, Hamsten A, Kooner JS, Thorsteinsdottir U, Danesh J, Palmer CN, Roberts R, Watkins H, Schunkert H, Samani NJ. Large-scale association analysis identifies new risk loci for coronary artery disease. Nat Genet. 2013 Jan;45(1):25-33. doi: 10.1038/ng.2480. Epub 2012 Dec 2. PubMed PMID: 23202125; PubMed Central PMCID: PMC3679547.

Denis M, Marcinkiewicz J, Zaid A, Gauthier D, Poirier S, Lazure C, Seidah NG, Prat A. Gene inactivation of proprotein convertase subtilisin/kexin type 9 reduces atherosclerosis in mice. Circulation. 2012 Feb 21;125(7):894-901. doi: 10.1161/CIRCULATIONAHA.111.057406. Epub 2012 Jan 18. PubMed PMID: 22261195.

Dewpura T, Raymond A, Hamelin J, Seidah NG, Mbikay M, Chrétien M, Mayne J. PCSK9 is phosphorylated by a Golgi casein kinase-like kinase ex vivo and circulates as a phosphoprotein in humans. FEBS J. 2008 Jul;275(13):3480-93. doi: 10.1111/j.1742-4658.2008.06495.x. Epub 2008 May 22. PubMed PMID: 18498363.

Emerging Risk Factors Collaboration, Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. JAMA. 2009 Nov 11;302(18):1993-2000. doi: 10.1001/jama.2009.1619. PubMed PMID: 19903920; PubMed Central PMCID: PMC3284229.

Ding T, Ledingham J, Luqmani R, Westlake S, Hyrich K, Lunt M, Kiely P, Bukhari M, Abernethy R, Bosworth A, Ostor A, Gadsby K, McKenna F, Finney D, Dixey J, Deighton C; Standards, Audit and Guidelines Working Group of BSR Clinical Affairs Committee; BHPR. BSR and BHPR rheumatoid arthritis guidelines on safety of anti-TNF therapies. Rheumatology (Oxford). 2010 Nov;49(11):2217-9. doi: 10.1093/rheumatology/keq249a. Epub 2010 Sep 12. PubMed PMID: 20837498.

Dubuc G, Chamberland A, Wassef H, Davignon J, Seidah NG, Bernier L, Prat A. Statins upregulate PCSK9, the gene encoding the proprotein convertase neural apoptosis-regulated convertase-1 implicated in familial hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2004 Aug;24(8):1454- 9. Epub 2004 Jun 3. PubMed PMID: 15178557

Dubuc G, Tremblay M, Paré G, Jacques H, Hamelin J, Benjannet S, Boulet L, Genest J, Bernier L, Seidah NG, Davignon J. A new method for measurement of total plasma PCSK9: clinical applications. J Lipid Res. 2010 Jan;51(1):140-9. doi: 10.1194/jlr.M900273-JLR200. Epub . PubMed PMID: 19571328; PubMed Central PMCID: PMC2789774.

Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nuñez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature. 2010 Apr 29;464(7293):1357-61. doi: 10.1038/nature08938. Erratum in: Nature. 2010 Jul 29;466(7306):652. PubMed PMID: 20428172; PubMed Central PMCID: PMC2946640.

Eberlé D, Hegarty B, Bossard P, Ferré P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie. 2004 Nov;86(11):839-48. Review. PubMed PMID: 15589694.

Essalmani R, Susan-Resiga D, Chamberland A, Abifadel M, Creemers JW, Boileau C, Seidah NG, Prat A. In vivo evidence that furin from hepatocytes inactivates PCSK9. J Biol Chem. 2011 Feb 11;286(6):4257-63. doi: 10.1074/jbc.M110.192104. Epub 2010 Dec 8. PubMed PMID: 21147780; PubMed Central PMCID: PMC3039354.

Eto M, Watanabe K, Ishii K. Reciprocal effects of apolipoprotein E alleles (epsilon 2 and epsilon 4) on plasma lipid levels in normolipidemic subjects. Clin Genet. 1986 Jun;29(6):477-84. PubMed PMID: 3461894.

Fantus D, Awan Z, Seidah NG, Genest J. Aortic calcification: Novel insights from familial hypercholesterolemia and potential role for the low-density lipoprotein receptor. Atherosclerosis. 2013 Jan;226(1):9-15. doi:10.1016/j.atherosclerosis.2012.08.026. Epub 2012 Sep 18. Review. PubMed PMID: 23040868.

Fearon WF, Fearon DT. Inflammation and cardiovascular disease: role of the interleukin-1 receptor antagonist. Circulation. 2008 May 20;117(20):2577-9. doi: 10.1161/CIRCULATIONAHA.108.772491. Review. PubMed PMID: 18490534.

Feingold KR, Moser AH, Shigenaga JK, Patzek SM, Grunfeld C. Inflammation stimulates the expression of PCSK9. Biochem Biophys Res Commun. 2008 Sep 19;374(2):341-4. doi: 10.1016/j.bbrc.2008.07.023. Epub 2008 Jul 16. PubMed PMID: 18638454; PubMed Central PMCID: PMC2571081.

Finkelshtein D, Werman A, Novick D, Barak S, Rubinstein M. LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. Proc Natl Acad Sci U S A. 2013 Apr 30;110(18):7306-11. doi:10.1073/pnas.1214441110. Epub 2013 Apr 15. PubMed PMID: 23589850; PubMed Central PMCID: PMC3645523.

Fisher TS, Lo Surdo P, Pandit S, Mattu M, Santoro JC, Wisniewski D, Cummings RT, Calzetta A, Cubbon RM, Fischer PA, Tarachandani A, De Francesco R, Wright SD, Sparrow CP, Carfi A, Sitlani A. Effects of pH and low density lipoprotein (LDL) on PCSK9-dependent LDL receptor regulation. J Biol Chem. 2007 Jul 13;282(28):20502-12. Epub 2007 May 10. PubMed PMID: 17493938.

Fitzgerald K, Frank-Kamenetsky M, Shulga-Morskaya S, Liebow A, Bettencourt BR, Sutherland JE, Hutabarat RM, Clausen VA, Karsten V, Cehelsky J, Nochur SV, Kotelianski V, Horton J, Mant T, Chiesa J, Ritter J, Munisamy M, Vaishnaw AK, Gollob JA, Simon A. Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. Lancet. 2013 Oct 1. doi:pii: S0140-6736(13)61914-5. 10.1016/S0140-6736(13)61914-5. [Epub ahead of print] PubMed PMID: 24094767.

Frank-Kamenetsky M, Grefhorst A, Anderson NN, Racie TS, Bramlage B, Akinc A, Butler D, Charisse K, Dorkin R, Fan Y, Gamba-Vitalo C, Hadwiger P, Jayaraman M, John M, Jayaprakash KN, Maier M, Nechev L, Rajeev KG, Read T, Röhl I, Soutschek J, Tan P, Wong J, Wang G, Zimmermann T, de Fougerolles A, Vornlocher HP, Langer R, Anderson DG, Manoharan M, Koteliansky V, Horton JD, Fitzgerald K. Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. Proc Natl Acad Sci U S A. 2008 Aug 19;105(33):11915-20. doi: 10.1073/pnas.0805434105. Epub 2008 Aug 11. PubMed PMID: 18695239; PubMed Central PMCID: PMC2575310.

Fredrickson DS, Lees RS. A System for Phenotyping Hyperlipoproteinemia. Circulation. 1965 Mar;31:321-7. PubMed PMID: 14262568.

Fredrickson DS. An international classification of hyperlipidemias and hyperlipoproteinemias. Ann Intern Med. 1971 Sep;75(3):471-2. PubMed PMID:5568160.

Frikke-Schmidt R. Genetic variation in the ABCA1 gene, HDL cholesterol, and risk of ischemic heart disease in the general population. Atherosclerosis. 2010 Feb;208(2):305-16. doi: 10.1016/j.atherosclerosis.2009.06.005. Epub 2009 Jun 11. Review. PubMed PMID: 19596329.

Fugère M, Limperis PC, Beaulieu-Audy V, Gagnon F, Lavigne P, Klarskov K, Leduc R, Day R. Inhibitory potency and specificity of subtilase-like pro-protein convertase (SPC) prodomains. J Biol Chem. 2002 Mar 8;277(10):7648-56. Epub 2001 Nov 26. PubMed PMID: 11723118.

Galea J, Armstrong J, Gadsdon P, Holden H, Francis SE, Holt CM. Interleukin-1 beta in coronary arteries of patients with ischemic heart disease. Arterioscler Thromb Vasc Biol. 1996 Aug;16(8):1000-6. PubMed PMID: 8696938.

Genest J, Libby P. Chapter 47: Lipoprotein Disorders and CAD. Braunwald's Heart Disease 9th Edition. Braunwald E, Libby P, Zipes D, Bonow R, Editors. 2011. Saunders, New York

Genest JJ Jr, Martin-Munley SS, McNamara JR, Ordovas JM, Jenner J, Myers RH, Silberman SR, Wilson PW, Salem DN, Schaefer EJ. Familial lipoprotein disorders in

patients with premature coronary artery disease. Circulation. 1992 Jun;85(6):2025-33. PubMed PMID: 1534286.

Gero S, Gergely J, Jakab L, Szekely J, Virag S, Farkas K, Czuppon A. Inhibition of cholesterol atherosclerosis by immunisation with beta-lipoprotein. Lancet. 1959 Jul 4;2(7088):6-7. PubMed PMID: 13673567.

Goldberg AC, Robinson JG, Cromwell WC, Ross JL, Ziajka PE. Future issues, public policy, and public awareness of familial hypercholesterolemias: recommendations from the National Lipid Association Expert Panel on Familial Hypercholesterolemia. J Clin Lipidol. 2011 Jun;5(3 Suppl):S46-51. doi: 10.1016/j.jacl.2011.04.002. Epub 2011 Apr 8. PubMed PMID: 21600529.

Goldstein JL, Brown MS, Anderson RG, Russell DW, Schneider WJ. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu Rev Cell Biol. 1985;1:1-39. Review. PubMed PMID: 2881559.

Goldstein JL, Brown MS. Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. J Biol Chem. 1974 Aug 25;249(16):5153-62. PubMed PMID: 4368448.

Goldstein JL, Brown MS. Regulation of low-density lipoprotein receptors: implications for pathogenesis and therapy of hypercholesterolemia and atherosclerosis. Circulation. 1987 Sep;76(3):504-7. PubMed PMID: 3621516.

Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. The metabolic and molecular bases of inherited disease. New York, NY: McGraw-Hill Companies, Inc, 2001:2863–914.

Gotto AM Jr. In memoriam Donald S. Fredrickson, MD: 1924-2002. Arterioscler Thromb Vasc Biol. 2002 Sep 1;22(9):1506-8. PubMed PMID: 12231574.

Greenberg JD, Kremer JM, Curtis JR, Hochberg MC, Reed G, Tsao P, Farkouh ME, Nasir A, Setoguchi S, Solomon DH; CORRONA Investigators. Tumour necrosis factor antagonist use and associated risk reduction of cardiovascular events among patients with rheumatoid arthritis. Ann Rheum Dis. 2011 Apr;70(4):576-82. doi: 10.1136/ard.2010.129916. Epub 2010 Nov 24. PubMed PMID: 21109516.

Gregory, Linda C. Lipids and Lipoproteins. Appleton & Lange's outline review clinical chemistry, McGraw-Hill, 2001

Grenon SM, Lachapelle K, Marcil M, Omeroglu A, Genest J, de Varennes B. Surgical strategies for severe calcification of the aorta (porcelain aorta) in two patients with homozygous familial hypercholesterolemia. Can J Cardiol. 2007 Dec;23(14):1159-61. PubMed PMID: 18060104; PubMed Central PMCID: PMC2652009.

Grundy SM, Mok HY, Zech L, Berman M. Influence of nicotinic acid on metabolism of cholesterol and triglycerides in man. J Lipid Res. 1981 Jan;22(1):24-36. PubMed PMID: 7217784.

Grundy, SM. (1990) Cholesterol and Atherosclerosis: Diagnosis and Treatment. New York, NY: Gower Medical Publishing.

Guardamagna O, Abello F, Saracco P, Baracco V, Rolfo E, Pirro M. Endothelial activation, inflammation and premature atherosclerosis in children with familial dyslipidemia. Atherosclerosis. 2009 Dec;207(2):471-5. doi:10.1016/j.atherosclerosis.2009.06.006. Epub 2009 Jun 11. PubMed PMID: 19560772.

Guardamagna O, Baracco V, Abello F, Bona G. Identification and management of dyslipidemic children. Minerva Pediatr. 2009 Aug;61(4):391-8. Review. PubMed PMID: 19752848

Guardamagna O, Restagno G, Rolfo E, Pederiva C, Martini S, Abello F, Baracco V, Pisciotta L, Pino E, Calandra S, Bertolini S. The type of LDLR gene mutation predicts cardiovascular risk in children with familial hypercholesterolemia. J Pediatr. 2009 Aug;155(2):199-204.e2. doi: 10.1016/j.jpeds.2009.02.022. Epub 2009 May 15. PubMed PMID: 19446849.

Gupta N, Fisker N, Asselin MC, Lindholm M, Rosenbohm C, Ørum H, Elmén J, Seidah NG, Straarup EM. A locked nucleic acid antisense oligonucleotide (LNA) silences PCSK9 and enhances LDLR expression in vitro and in vivo. PLoS One. 2010 May 17;5(5):e10682. doi: 10.1371/journal.pone.0010682. PubMed PMID: 20498851; PubMed Central PMCID: PMC2871785.

Gurantz D, Yndestad A, Halvorsen B, Lunde OV, Omens JH, Ueland T, Aukrust P, Moore CD, Kjekshus J, Greenberg BH. Etanercept or intravenous immunoglobulin attenuates expression of genes involved in post-myocardial infarction remodeling. Cardiovasc Res. 2005 Jul 1;67(1):106-15. Epub 2005 Mar 23. PubMed PMID: 15949474.

Hafezi-Moghadam A, Simoncini T, Yang Z, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K, Liao JK. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nat Med. 2002 May;8(5):473-9. PubMed PMID: 11984591; PubMed Central PMCID: PMC2668717.

Hall SS. Genetics: a gene of rare effect. Nature. 2013 Apr 11;496(7444):152-5. doi: 10.1038/496152a. PubMed PMID: 23579660.

Hansen PS, Defesche JC, Kastelein JJ, Gerdes LU, Fraza L, Gerdes C, Tato F, Jensen HK, Jensen LG, Klausen IC, Faergeman O, Schuster H. Phenotypic variation in patients heterozygous for familial defective apolipoprotein B (FDB) in three European countries. Arterioscler Thromb Vasc Biol. 1997 Apr;17(4):741-7. PubMed PMID: 9108789.

Hansson GK, Klareskog L. Pulling down the plug on atherosclerosis: cooling down the inflammasome. Nat Med. 2011 Jul 7;17(7):790-1. doi: 10.1038/nm0711-790.PubMed PMID: 21738158.

Harris TB, Ferrucci L, Tracy RP, Corti MC, Wacholder S, Ettinger WH Jr, Heimovitz H, Cohen HJ, Wallace R. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. Am J Med. 1999 May;106(5):506-12. PubMed PMID: 10335721.

Heath KE, Day IN, Humphries SE. Universal primer quantitative fluorescent multiplex (UPQFM) PCR: a method to detect major and minor rearrangements of the low density lipoprotein receptor gene. J Med Genet. 2000 Apr;37(4):272-80. PubMed PMID: 10745045; PubMed Central PMCID: PMC1734566.

Hedrick JA. Targeting PCSK9 for the treatment of hypercholesterolemia. Curr Opin Investig Drugs. 2009 Sep;10(9):938-46. PubMed PMID: 19705336.

Herz J, Chen Y. Reelin, lipoprotein receptors and synaptic plasticity. Nat Rev Neurosci. 2006 Nov;7(11):850-9. Review. PubMed PMID: 17053810.

Hingorani AD, Casas JP. The interleukin-6 receptor as a target for prevention of coronary heart disease: a mendelian randomisation analysis. Lancet. 2012 Mar 31;379(9822):1214-24. doi: 10.1016/S0140-6736(12)60110-X. Epub 2012 Mar 14. PubMed PMID: 22421340; PubMed Central PMCID: PMC3316968.

Hobbs HH, Russell DW, Brown MS, Goldstein JL. The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. Annu Rev Genet. 1990;24:133-70. Review. PubMed PMID: 2088165.

Holtmann H, Wallach D. Down regulation of the receptors for tumor necrosis factor by interleukin 1 and 4 beta-phorbol-12-myristate-13-acetate. J Immunol. 1987 Aug 15;139(4):1161-7. PubMed PMID: 3038998.

Hooper AJ, Marais AD, Tanyanyiwa DM, Burnett JR. The C679X mutation in PCSK9 is present and lowers blood cholesterol in a Southern African population. Atherosclerosis. 2007 Aug;193(2):445-8. Epub 2006 Sep 20. PubMed PMID: 16989838.

Horton JD, Goldstein JL, Brown MS. SREBPs: transcriptional mediators of lipid homeostasis. Cold Spring Harb Symp Quant Biol. 2002;67:491-8. Review. PubMed PMID: 12858575.

HPS2-THRIVE Collaborative Group. HPS2-THRIVE randomized placebo-controlled trial in 25 673 high-risk patients of ER niacin/laropiprant: trial design, pre-specified muscle and liver outcomes, and reasons for stopping study treatment. Eur Heart J. 2013 May;34(17):1279-91. doi: 10.1093/eurheartj/eht055. Epub 2013 Feb 26. PubMed PMID: 23444397; PubMed Central PMCID: PMC3640201.

Hültner L, Kölsch S, Stassen M, Kaspers U, Kremer JP, Mailhammer R, Moeller J, Broszeit H, Schmitt E. In activated mast cells, IL-1 up-regulates the production of several Th2-related cytokines including IL-9. J Immunol. 2000 Jun 1;164(11):5556-63. PubMed PMID: 10820229.

Huijgen R, Boekholdt SM, Arsenault BJ, Bao W, Davaine JM, Tabet F, Petrides F, Rye KA, DeMicco DA, Barter PJ, Kastelein JJ, Lambert G. Plasma PCSK9 levels and clinical outcomes in the TNT (Treating to New Targets) trial: a nested case-control study. J Am Coll Cardiol. 2012 May 15;59(20):1778-84. doi: 10.1016/j.jacc.2011.12.043. Retraction in: J Am Coll Cardiol. 2013 Apr 23;61(16):1751. PubMed PMID: 22575316.

Huijgen R, Fouchier SW, Denoun M, Hutten BA, Vissers MN, Lambert G, Kastelein JJ. Plasma levels of PCSK9 and phenotypic variability in familial hypercholesterolemia. J Lipid Res. 2012 May;53(5):979-83. doi:10.1194/jlr.P023994. Epub 2012 Feb 27. PubMed PMID: 22375030; PubMed Central PMCID: PMC3329397.

Ikonomidis I, Lekakis JP, Nikolaou M, Paraskevaidis I, Andreadou I, Kaplanoglou T, Katsimbri P, Skarantavos G, Soucacos PN, Kremastinos DT. Inhibition of interleukin-1 by anakinra improves vascular and left ventricular function in patients with rheumatoid arthritis. Circulation. 2008 May 20;117(20):2662-9. doi: 10.1161/CIRCULATIONAHA.107.731877. Epub 2008 May 12. PubMed PMID: 18474811.

Innerarity TL, Mahley RW, Weisgraber KH, Bersot TP, Krauss RM, Vega GL, Grundy SM, Friedl W, Davignon J, McCarthy BJ. Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia. J Lipid Res. 1990 Aug;31(8):1337-49. Review. PubMed PMID: 2280177.

Ishikawa M, Iwasaki Y, Yatoh S, Kato T, Kumadaki S, Inoue N, Yamamoto T, Matsuzaka T, Nakagawa Y, Yahagi N, Kobayashi K, Takahashi A, Yamada N, Shimano H. Cholesterol accumulation and diabetes in pancreatic beta-cell-specific SREBP-2 transgenic mice: a new model for lipotoxicity. J Lipid Res. 2008 Dec;49(12):2524-34. doi: 10.1194/jlr.M800238-JLR200. Epub 2008 Aug 5. PubMed PMID: 18682608.

Jacobsson LT, Turesson C, Gülfe A, Kapetanovic MC, Petersson IF, Saxne T, Geborek P. Treatment with tumor necrosis factor blockers is associated with a lower incidence of first cardiovascular events in patients with rheumatoid arthritis. J Rheumatol. 2005 Jul;32(7):1213-8. PubMed PMID: 15996054.

Jänis MT, Tarasov K, Ta HX, Suoniemi M, Ekroos K, Hurme R, Lehtimäki T, Päivä H, Kleber ME, März W, Prat A, Seidah NG, Laaksonen R. Beyond LDL-C lowering:

distinct molecular sphingolipids are good indicators of proprotein convertase subtilisin/kexin type 9 (PCSK9) deficiency. Atherosclerosis. 2013 Jun;228(2):380-5. doi: 10.1016/j.atherosclerosis.2013.03.029. Epub 2013 Apr 6. PubMed PMID: 23623011.

Jansen AC, van Aalst-Cohen ES, Tanck MW, Cheng S, Fontecha MR, Li J, Defesche JC, Kastelein JJ. Genetic determinants of cardiovascular disease risk in familial hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2005 Jul;25(7):1475-81. Epub 2005 May 5. PubMed PMID: 15879303.

Jensen MD. Fate of fatty acids at rest and during exercise: regulatory mechanisms. Acta Physiol Scand. 2003 Aug;178(4):385-90. Review. PubMed PMID: 12864743

Johansen CT, Kathiresan S, Hegele RA. Genetic determinants of plasma triglycerides. J Lipid Res. 2011 Feb;52(2):189-206. doi: 10.1194/jlr.R009720. Epub 2010 Nov 1. Review. PubMed PMID: 21041806; PubMed Central PMCID: PMC3023540.

Jun M, Foote C, Lv J, Neal B, Patel A, Nicholls SJ, Grobbee DE, Cass A, Chalmers J, Perkovic V. Effects of fibrates on cardiovascular outcomes: a systematic review and meta-analysis. Lancet. 2010 May 29;375(9729):1875-84. doi: 10.1016/S0140-6736(10)60656-3. Epub 2010 May 10. Review. PubMed PMID: 20462635.

Kalbak K. Incidence of arteriosclerosis in patients with rheumatoid arthritis receiving long-term corticosteroid therapy. Ann Rheum Dis. 1972 May;31(3):196-200. PubMed PMID: 5032452; PubMed Central PMCID: PMC1005898.

Kane JP, Havel RJ. Disorders of the biogenesis and secretion of lipoproteins containing the B apolipoproteins. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic and Molecular Bases of Inherited Disease. 8th ed. New York, NY: McGraw-Hill; 2001:2717-2752.

Kannel WB, Dawber TR, Kagan A, Revotskie N, Stokes J 3rd. Factors of risk in the development of coronary heart disease--six year follow-up experience. The Framingham Study. Ann Intern Med. 1961 Jul;55:33-50. PubMed PMID: 13751193.

Kathiresan S, Melander O, Anevski D, Guiducci C, Burtt NP, Roos C, Hirschhorn JN, Berglund G, Hedblad B, Groop L, Altshuler DM, Newton-Cheh C, Orho-Melander M. Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl

J Med. 2008 Mar 20;358(12):1240-9. doi: 10.1056/NEJMoa0706728. PubMed PMID: 18354102.

Kawashiri SY, Kawakami A, Yamasaki S, Imazato T, Iwamoto N, Fujikawa K, Aramaki T, Tamai M, Nakamura H, Ida H, Origuchi T, Ueki Y, Eguchi K. Effects of the anti-interleukin-6 receptor antibody, tocilizumab, on serum lipid levels in patients with rheumatoid arthritis. Rheumatol Int. 2011 Apr;31(4):451-6. doi: 10.1007/s00296-009-1303-y. Epub 2009 Dec 19. PubMed PMID: 20024554.

Keuylian Z, de Baaij JH, Gueguen M, Glorian M, Rouxel C, Merlet E, Lipskaia L, Blaise R, Mateo V, Limon I. The Notch pathway attenuates interleukin 1β (IL1β)-mediated induction of adenylyl cyclase 8 (AC8) expression during vascular smooth muscle cell (VSMC) trans-differentiation. J Biol Chem. 2012 Jul 20;287(30):24978-89. doi: 10.1074/jbc.M111.292516. Epub 2012 May 21. Erratum in: J Biol Chem. 2013 Jan 11;288(2):1278. Gueguen, Marie [added]. PubMed PMID: 22613711; PubMed Central PMCID: PMC3408176.

Keys A, Taylor HL, Blackburn H, Brozek J, Anderson JT, Simonsen E. Coronary heart disease among Minnesota business and professional men followed fifteen years. Circulation. 1963 Sep;28:381-95. PubMed PMID: 14059458.

Keys A. Seven countries: a multivariate analysis of death and coronary heart disease. London: Harvard University Press, 1980

Khachadurian AK. The inheritance of essential familial hypercholesterolemia. Am J Med. 1964 Sep;37:402-7. PubMed PMID: 14209286.

Kiani AN, Fishman EK, Petri M. Aortic valve calcification in systemic lupus erythematosus. Lupus. 2006;15(12):873-6. PubMed PMID: 17211993.

Kida Y, Kobayashi M, Suzuki T, Takeshita A, Okamatsu Y, Hanazawa S, Yasui T, Hasegawa K. Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. Cytokine. 2005 Feb 21;29(4):159-68. Epub 2004 Dec 2. PubMed PMID: 15652448.

Kirii H, Niwa T, Yamada Y, Wada H, Saito K, Iwakura Y, Asano M, Moriwaki H, Seishima M. Lack of interleukin-1beta decreases the severity of atherosclerosis in ApoE-

deficient mice. Arterioscler Thromb Vasc Biol. 2003 Apr 1;23(4):656-60. Epub 2003 Feb 27. PubMed PMID: 12615675.

Kishikawa H, Shimokama T, Watanabe T. Localization of T lymphocytes and macrophages expressing IL-1, IL-2 receptor, IL-6 and TNF in human aortic intima. Role of cell-mediated immunity in human atherogenesis. Virchows Arch A Pathol Anat Histopathol. 1993;423(6):433-42. PubMed PMID: 8291216.

Klingenberg R, Hansson GK. Treating inflammation in atherosclerotic cardiovascular disease: emerging therapies. Eur Heart J. 2009 Dec;30(23):2838-44. doi: 10.1093/eurheartj/ehp477. Epub 2009 Oct 30. Review. PubMed PMID: 19880848.

Knott TJ, Rall SC Jr, Innerarity TL, Jacobson SF, Urdea MS, Levy-Wilson B, Powell LM, Pease RJ, Eddy R, Nakai H, et al. Human apolipoprotein B: structure of carboxyl-terminal domains, sites of gene expression, and chromosomal localization. Science. 1985 Oct 4;230(4721):37-43. PubMed PMID: 2994225.

Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, Wang Y, Wang Z, Si S, Pan H, Wang S, Wu J, Wang Y, Li Z, Liu J, Jiang JD. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat Med. 2004 Dec;10(12):1344-51. Epub 2004 Nov 7. PubMed PMID: 15531889.

Kosenko T, Golder M, Leblond G, Weng W, Lagace TA. Low density lipoprotein binds to proprotein convertase subtilisin/kexin type-9 (PCSK9) in human plasma and inhibits PCSK9-mediated low density lipoprotein receptor degradation. J Biol Chem. 2013 Mar 22;288(12):8279-88. doi: 10.1074/jbc.M112.421370. Epub 2013 Feb 11. PubMed PMID: 23400816; PubMed Central PMCID: PMC3605646.

Kosenko T, Golder M, Leblond G, Weng W, Lagace TA. Low density lipoprotein binds to proprotein convertase subtilisin/kexin type-9 (PCSK9) in human plasma and inhibits PCSK9-mediated low density lipoprotein receptor degradation. J Biol Chem. 2013 Mar 22;288(12):8279-88. doi: 10.1074/jbc.M112.421370. Epub 2013 Feb 11. PubMed PMID: 23400816; PubMed Central PMCID: PMC3605646.

Kotowski IK, Pertsemlidis A, Luke A, Cooper RS, Vega GL, Cohen JC, Hobbs HH. A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein

cholesterol. Am J Hum Genet. 2006 Mar;78(3):410-22. Epub 2006 Jan 20. PubMed PMID: 16465619; PubMed Central PMCID: PMC1380285.

Kourimate S, Le May C, Langhi C, Jarnoux AL, Ouguerram K, Zaïr Y, Nguyen P, Krempf M, Cariou B, Costet P. Dual mechanisms for the fibrate-mediated repression of proprotein convertase subtilisin/kexin type 9. J Biol Chem. 2008 Apr 11;283(15):9666-73. doi: 10.1074/jbc.M705831200. Epub 2008 Feb 3. PubMed PMID: 18245819.

Kubota T, Bounoutas GS, Miyagishima M, Kadokami T, Sanders VJ, Bruton C, Robbins PD, McTiernan CF, Feldman AM. Soluble tumor necrosis factor receptor abrogates myocardial inflammation but not hypertrophy in cytokine-induced cardiomyopathy. Circulation. 2000 May 30;101(21):2518-25. PubMed PMID: 10831527.

Kume K, Amano K, Yamada S, Hatta K, Ohta H, Kuwaba N. Tocilizumab monotherapy reduces arterial stiffness as effectively as etanercept or adalimumab monotherapy in rheumatoid arthritis: an open-label randomized controlled trial. J Rheumatol. 2011 Oct;38(10):2169-71. doi: 10.3899/jrheum.110340. Epub 2011 Aug 1. PubMed PMID: 21807781.

Kwon HJ, Lagace TA, McNutt MC, Horton JD, Deisenhofer J. Molecular basis for LDL receptor recognition by PCSK9. Proc Natl Acad Sci U S A. 2008 Feb 12;105(6):1820-5. doi: 10.1073/pnas.0712064105. Epub 2008 Feb 4. PubMed PMID: 18250299; PubMed Central PMCID: PMC2538846.

Labonté P, Begley S, Guévin C, Asselin MC, Nassoury N, Mayer G, Prat A, Seidah NG. PCSK9 impedes hepatitis C virus infection in vitro and modulates liver CD81 expression. Hepatology. 2009 Jul;50(1):17-24. doi: 10.1002/hep.22911. PubMed PMID: 19489072.

Lagace TA, Curtis DE, Garuti R, McNutt MC, Park SW, Prather HB, Anderson NN, Ho YK, Hammer RE, Horton JD. Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. J Clin Invest. 2006 Nov;116(11):2995-3005. PubMed PMID: 17080197; PubMed Central PMCID: PMC1626117.

Lakoski SG, Lagace TA, Cohen JC, Horton JD, Hobbs HH. Genetic and metabolic determinants of plasma PCSK9 levels. J Clin Endocrinol Metab. 2009 Jul;94(7):2537-43. doi: 10.1210/jc.2009-0141. Epub 2009 Apr 7. PubMed PMID: 19351729; PubMed Central PMCID: PMC2708952.

Lakoski SG, Xu F, Vega GL, Grundy SM, Chandalia M, Lam C, Lowe RS, Stepanavage ME, Musliner TA, Cohen JC, Hobbs HH. Indices of cholesterol metabolism and relative responsiveness to ezetimibe and simvastatin. J Clin Endocrinol Metab. 2010 Feb;95(2):800-9. doi: 10.1210/jc.2009-1952. Epub 2009 Dec 4. Erratum in: J Clin Endocrinol Metab. 2011 May;96(5):1582. PubMed PMID: 19965915; PubMed Central PMCID: PMC3079219.

Lalanne F, Lambert G, Amar MJ, Chétiveaux M, Zaïr Y, Jarnoux AL, Ouguerram K, Friburg J, Seidah NG, Brewer HB Jr, Krempf M, Costet P. Wild-type PCSK9 inhibits LDL clearance but does not affect apoB-containing lipoprotein production in mouse and cultured cells. J Lipid Res. 2005 Jun;46(6):1312-9. Epub 2005 Mar 1. PubMed PMID: 15741654.

Lambert G, Ancellin N, Charlton F, Comas D, Pilot J, Keech A, Patel S, Sullivan DR, Cohn JS, Rye KA, Barter PJ. Plasma PCSK9 concentrations correlate with LDL and total cholesterol in diabetic patients and are decreased by fenofibrate treatment. Clin Chem. 2008 Jun;54(6):1038-45. doi: 10.1373/clinchem.2007.099747. Epub 2008 Apr 24. PubMed PMID: 18436719.

Lambert G, Jarnoux AL, Pineau T, Pape O, Chetiveaux M, Laboisse C, Krempf M, Costet P. Fasting induces hyperlipidemia in mice overexpressing proprotein convertase subtilisin kexin type 9: lack of modulation of very-low-density lipoprotein hepatic output by the low-density lipoprotein receptor. Endocrinology. 2006 Oct;147(10):4985-95. Epub 2006 Jun 22. PubMed PMID: 16794006.

Langhi C, Le May C, Gmyr V, Vandewalle B, Kerr-Conte J, Krempf M, Pattou F, Costet P, Cariou B. PCSK9 is expressed in pancreatic delta-cells and does not alter insulin secretion. Biochem Biophys Res Commun. 2009 Dec 25;390(4):1288-93. doi: 10.1016/j.bbrc.2009.10.138. Epub 2009 Oct 28. PubMed PMID: 19878649.

Laurie AD, George PM. Evaluation of high-resolution melting analysis for screening the LDL receptor gene. Clin Biochem. 2009 Apr;42(6):528-35. doi: 10.1016/j.clinbiochem.2008.11.015. Epub 2008 Dec 11. PubMed PMID: 19118540.

Law SW, Lackner KJ, Hospattankar AV, Anchors JM, Sakaguchi AY, Naylor SL, Brewer HB Jr. Human apolipoprotein B-100: cloning, analysis of liver mRNA, and assignment of the gene to chromosome 2. Proc Natl Acad Sci U S A. 1985 Dec;82(24):8340-4. PubMed PMID: 3001697; PubMed Central PMCID: PMC390911.

Le May C, Berger JM, Lespine A, Pillot B, Prieur X, Letessier E, Hussain MM, Collet X, Cariou B, Costet P. Transintestinal cholesterol excretion is an active metabolic process modulated by PCSK9 and statin involving ABCB1. Arterioscler Thromb Vasc Biol. 2013 Jul;33(7):1484-93. doi: 10.1161/ATVBAHA.112.300263. Epub 2013 Apr 4. PubMed PMID: 23559630.

Le May C, Kourimate S, Langhi C, Chétiveaux M, Jarry A, Comera C, Collet X, Kuipers F, Krempf M, Cariou B, Costet P. Proprotein convertase subtilisin kexin type 9 null mice are protected from postprandial triglyceridemia. Arterioscler Thromb Vasc Biol. 2009 May;29(5):684-90. doi: 10.1161/ATVBAHA.108.181586. Epub 2009 Mar 5. PubMed PMID: 19265033.

Lencel P, Delplace S, Pilet P, Leterme D, Miellot F, Sourice S, Caudrillier A, Hardouin P, Guicheux J, Magne D. Cell-specific effects of TNF-α and IL-1β on alkaline phosphatase: implication for syndesmophyte formation and vascular calcification. Lab Invest. 2011 Oct;91(10):1434-42. doi: 10.1038/labinvest.2011.83. Epub 2011 May 9. PubMed PMID: 21555997.

Levy RI, Fredrickson DS. Diagnosis and management of hyperlipoproteinemia. Am J Cardiol. 1968 Oct;22(4):576-83. Review. PubMed PMID: 4880101.

Li H, Dong B, Park SW, Lee HS, Chen W, Liu J. Hepatocyte nuclear factor 1alpha plays a critical role in PCSK9 gene transcription and regulation by the natural hypocholesterolemic compound berberine. J Biol Chem. 2009 Oct 16;284(42):28885-95. doi: 10.1074/jbc.M109.052407. Epub 2009 Aug 17. PubMed PMID: 19687008; PubMed Central PMCID: PMC2781434.

Libby P, Lichtman AH, Hansson GK. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. Immunity. 2013 Jun 27;38(6):1092-104. doi: 10.1016/j.immuni.2013.06.009. Review. Erratum in: Immunity. 2013 Aug 22;39(2):413. PubMed PMID: 23809160; PubMed Central PMCID: PMC3764500.

Libby P, Ridker PM, Hansson GK; Leducq Transatlantic Network on Atherothrombosis. Inflammation in atherosclerosis: from pathophysiology to practice. J Am Coll Cardiol. 2009 Dec 1;54(23):2129-38. doi: 10.1016/j.jacc.2009.09.009. Review. PubMed PMID: 19942084; PubMed Central PMCID: PMC2834169.

Libby P, Theroux P. Pathophysiology of coronary artery disease. Circulation. 2005 Jun 28;111(25):3481-8. Review. PubMed PMID: 15983262.

Lindgren V, Luskey KL, Russell DW, Francke U. Human genes involved in cholesterol metabolism: chromosomal mapping of the loci for the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl-coenzyme A reductase with cDNA probes. Proc Natl Acad Sci U S A. 1985 Dec;82(24):8567-71. PubMed PMID: 3866240; PubMed Central PMCID: PMC390958.

Lipari MT, Li W, Moran P, Kong-Beltran M, Sai T, Lai J, Lin SJ, Kolumam G, Zavala-Solorio J, Izrael-Tomasevic A, Arnott D, Wang J, Peterson AS, Kirchhofer D. Furin-cleaved proprotein convertase subtilisin/kexin type 9 (PCSK9) is active and modulates low density lipoprotein receptor and serum cholesterol levels. J Biol Chem. 2012 Dec 21;287(52):43482-91. doi: 10.1074/jbc.M112.380618. Epub 2012 Nov 7. PubMed PMID: 23135270; PubMed Central PMCID: PMC3527935.

Mann HF, Vencovský J. Clinical trials roundup in idiopathic inflammatory myopathies. Curr Opin Rheumatol. 2011 Nov;23(6):605-11. doi: 10.1097/BOR.0b013e32834ba787. Review. PubMed PMID: 21926626.

Marduel M, Ouguerram K, Serre V, Bonnefont-Rousselot D, Marques-Pinheiro A,Erik Berge K, Devillers M, Luc G, Lecerf JM, Tosolini L, Erlich D, Peloso GM,Stitziel N, Nitchké P, Jaïs JP; French Research Network on ADH, Abifadel M, Kathiresan S, Leren TP, Rabès JP, Boileau C, Varret M. Description of a large family with autosomal dominant hypercholesterolemia associated with the APOE p.Leu167del mutation. Hum Mutat. 2013 Jan;34(1):83-7. doi: 10.1002/humu.22215. Epub 2012 Oct 11. PubMed PMID: 22949395; PubMed Central PMCID: PMC3638718.

Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002 Aug;10(2):417-26. PubMed PMID: 12191486.

Maxwell KN, Breslow JL. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. Proc Natl Acad Sci U S A. 2004 May 4;101(18):7100-5. Epub 2004 Apr 26. PubMed PMID: 15118091; PubMed Central PMCID: PMC406472.

Mayer G, Poirier S, Seidah NG. Annexin A2 is a C-terminal PCSK9-binding protein that regulates endogenous low density lipoprotein receptor levels. J Biol Chem. 2008 Nov 14;283(46):31791-801. doi: 10.1074/jbc.M805971200. Epub 2008 Sep 17. PubMed PMID: 18799458.

Mayne J, Dewpura T, Raymond A, Bernier L, Cousins M, Ooi TC, Davignon J, Seidah NG, Mbikay M, Chrétien M. Novel loss-of-function PCSK9 variant is associated with low plasma LDL cholesterol in a French-Canadian family and with impaired processing and secretion in cell culture. Clin Chem. 2011 Oct;57(10):1415-23. doi: 10.1373/clinchem.2011.165191. Epub 2011 Aug 3. PubMed PMID: 21813713.

Mayne J, Dewpura T, Raymond A, Cousins M, Chaplin A, Lahey KA, Lahaye SA, Mbikay M, Ooi TC, Chrétien M. Plasma PCSK9 levels are significantly modified by statins and fibrates in humans. Lipids Health Dis. 2008 Jun 11;7:22. doi: 10.1186/1476-511X-7-22. PubMed PMID: 18547436; PubMed Central PMCID: PMC2432057.

Mayne J, Ooi TC, Raymond A, Cousins M, Bernier L, Dewpura T, Sirois F, Mbikay M, Davignon J, Chrétien M. Differential effects of PCSK9 loss of function variants on serum lipid and PCSK9 levels in Caucasian and African Canadian populations. Lipids Health Dis. 2013 May 10;12:70. doi: 10.1186/1476-511X-12-70. PubMed PMID: 23663650; PubMed Central PMCID: PMC3661383.

Mbikay M, Sirois F, Mayne J, Wang GS, Chen A, Dewpura T, Prat A, Seidah NG, Chretien M, Scott FW. PCSK9-deficient mice exhibit impaired glucose tolerance and pancreatic islet abnormalities. FEBS Lett. 2010 Feb 19;584(4):701-6. doi: 10.1016/j.febslet.2009.12.018. Epub 2009 Dec 16. PubMed PMID: 20026049.

McNutt MC, Kwon HJ, Chen C, Chen JR, Horton JD, Lagace TA. Antagonism of secreted PCSK9 increases low density lipoprotein receptor expression in HepG2 cells. J Biol Chem. 2009 Apr 17;284(16):10561-70. doi: 10.1074/jbc.M808802200. Epub 2009 Feb 17. PubMed PMID: 19224862; PubMed Central PMCID: PMC2667743.

Meier CA, Bobbioni E, Gabay C, Assimacopoulos-Jeannet F, Golay A, Dayer JM. IL-1 receptor antagonist serum levels are increased in human obesity: a possible link to the resistance to leptin? J Clin Endocrinol Metab. 2002 Mar;87(3):1184-8. PubMed PMID: 11889184.

Meredith LW, Wilson GK, Fletcher NF, McKeating JA. Hepatitis C virus entry: beyond receptors. Rev Med Virol. 2012 May;22(3):182-93. doi: 10.1002/rmv.723. Epub 2012 Mar 6. PubMed PMID: 22392805.

Micha R, Imamura F, Wyler von Ballmoos M, Solomon DH, Hernán MA, Ridker PM, Mozaffarian D. Systematic review and meta-analysis of methotrexate use and risk of cardiovascular disease. Am J Cardiol. 2011 Nov 1;108(9):1362-70. doi: 10.1016/j.amjcard.2011.06.054. Epub 2011 Aug 17. Review. PubMed PMID: 21855836; PubMed Central PMCID: PMC3196048.

Nassoury N, Blasiole DA, Tebon Oler A, Benjannet S, Hamelin J, Poupon V, McPherson PS, Attie AD, Prat A, Seidah NG. The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDLR. Traffic. 2007 Jun;8(6):718-32. Epub 2007 Apr 25. Erratum in: Traffic. 2007 Jul;8(7):950. PubMed PMID: 17461796.

Neary NM, Booker OJ, Abel BS, Matta JR, Muldoon N, Sinaii N, Pettigrew RI, Nieman LK, Gharib AM. Hypercortisolism is associated with increased coronary arterial atherosclerosis: analysis of noninvasive coronary angiography using multidetector computerized tomography. J Clin Endocrinol Metab. 2013 May;98(5):2045-52. doi: 10.1210/jc.2012-3754. Epub 2013 Apr 4. PubMed PMID: 23559084; PubMed Central PMCID: PMC3644598.

Niesen M, Bedi M, Lopez D. Diabetes alters LDL receptor and PCSK9 expression in rat liver. Arch Biochem Biophys. 2008 Feb 15;470(2):111-5. Epub 2007 Nov 22. PubMed PMID: 18054320.

Nordestgaard BG, Chapman MJ, Humphries SE, Ginsberg HN, Masana L, Descamps OS, Wiklund O, Hegele RA, Raal FJ, Defesche JC, Wiegman A, Santos RD, Watts GF, Parhofer KG, Hovingh GK, Kovanen PT, Boileau C, Averna M, Borén J, Bruckert E, Catapano AL, Kuivenhoven JA, Pajukanta P, Ray K, Stalenhoef AF, Stroes E, Taskinen MR, Tybjærg-Hansen A; European Atherosclerosis Society Consensus Panel. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. Eur Heart J. 2013 Dec;34(45):3478-90a. doi: 10.1093/eurheartj/eht273. Epub 2013 Aug 15. PubMed PMID: 23956253; PubMed Central PMCID: PMC3844152.

Olsson U, Camejo G, Hurt-Camejo E, Elfsber K, Wiklund O, Bondjers G. Possible functional interactions of apolipoprotein B-100 segments that associate with cell proteoglycans and the ApoB/E receptor. Arterioscler Thromb Vasc Biol. 1997 Jan;17(1):149-55. PubMed PMID: 9012650.

Ouguerram K, Chetiveaux M, Zair Y, Costet P, Abifadel M, Varret M, Boileau C, Magot T, Krempf M. Apolipoprotein B100 metabolism in autosomal-dominant hypercholesterolemia related to mutations in PCSK9. Arterioscler Thromb Vasc Biol. 2004 Aug;24(8):1448-53. Epub 2004 May 27. PubMed PMID: 15166014.

Padfield GJ, Din JN, Koushiappi E, Mills NL, Robinson SD, Cruden Nle M, Lucking AJ, Chia S, Harding SA, Newby DE. Cardiovascular effects of tumour necrosis factor α antagonism in patients with acute myocardial infarction: a first in human study. Heart. 2013 Sep;99(18):1330-5. doi: 10.1136/heartjnl-2013-303648. Epub 2013 Apr 10. PubMed PMID: 23574969; PubMed Central PMCID: PMC3756454.

Park SW, Moon YA, Horton JD. Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. J Biol Chem. 2004 Nov 26;279(48):50630-8. Epub 2004 Sep 22. PubMed PMID: 15385538.

Pascoe J, Hollern D, Stamateris R, Abbasi M, Romano LC, Zou B, O'Donnell CP, Garcia-Ocana A, Alonso LC. Free fatty acids block glucose-induced β-cell proliferation in mice by inducing cell cycle inhibitors p16 and p18. Diabetes.2012

Mar; 61(3):632-41. doi: 10.2337/db11-0991. Epub 2012 Feb 14. PubMed PMID:22338094; PubMed Central PMCID: PMC3282818.

Pasumarthy L, Srour J, Choudhary C. Common pitfalls in management of inflammatory bowel disease. Gastroenterology Research 2009;2(4):200-208.

Persson L, Cao G, Ståhle L, Sjöberg BG, Troutt JS, Konrad RJ, Gälman C, Wallén H, Eriksson M, Hafström I, Lind S, Dahlin M, Amark P, Angelin B, Rudling M. Circulating proprotein convertase subtilisin kexin type 9 has a diurnal rhythm synchronous with cholesterol synthesis and is reduced by fasting in humans. rterioscler Thromb Vasc Biol. 2010 Dec;30(12):2666-72. doi: 10.1161/ATVBAHA.110.214130. Epub 2010 Sep 30. PubMed PMID: 20884874.

Persson L, Henriksson P, Westerlund E, Hovatta O, Angelin B, Rudling M. Endogenous estrogens lower plasma PCSK9 and LDL cholesterol but not Lp(a) or bile acid synthesis in women. Arterioscler Thromb Vasc Biol. 2012 Mar;32(3):810-4. doi: 10.1161/ATVBAHA.111.242461. Epub 2011 Dec 29. PubMed PMID: 22207727.

Peterson AS, Fong LG, Young SG. PCSK9 function and physiology. J Lipid Res. 2008 Jul;49(7):1595-9. PubMed PMID: 18663786.

Peticca P, Raymond A, Gruslin A, Cousins M, Adetola E, Abujrad H, Mayne J, Ooi TC. Human Serum PCSK9 Is Elevated at Parturition in Comparison to Nonpregnant Subjects While Serum PCSK9 from Umbilical Cord Blood is Lower Compared to Maternal Blood. ISRN Endocrinol. 2013 Jun 5;2013:341632. doi: 10.1155/2013/341632. Print 2013. PubMed PMID: 23862071; PubMed Central PMCID: PMC3687493.

Piper DE, Jackson S, Liu Q, Romanow WG, Shetterly S, Thibault ST, Shan B, Walker NP. The crystal structure of PCSK9: a regulator of plasma LDL-cholesterol. Structure. 2007 May;15(5):545-52. PubMed PMID: 17502100.

Pisciotta L, Priore Oliva C, Cefalù AB, Noto D, Bellocchio A, Fresa R, Cantafora A, Patel D, Averna M, Tarugi P, Calandra S, Bertolini S. Additive effect of mutations in LDLR and PCSK9 genes on the phenotype of familial hypercholesterolemia. Atherosclerosis. 2006 Jun;186(2):433-40. Epub 2005 Sep 23. PubMed PMID: 16183066.

Poirier S, Mayer G, Benjannet S, Bergeron E, Marcinkiewicz J, Nassoury N, Mayer H, Nimpf J, Prat A, Seidah NG. The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. J Biol Chem. 2008 Jan 25;283(4):2363-72. Epub 2007 Nov 26. PubMed PMID: 18039658.

Poirier S, Mayer G, Poupon V, McPherson PS, Desjardins R, Ly K, Asselin MC, Day R, Duclos FJ, Witmer M, Parker R, Prat A, Seidah NG. Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation: evidence for an intracellular route. J Biol Chem. 2009 Oct 16;284(42):28856-64. doi: 10.1074/jbc.M109.037085. Epub 2009 Jul 27. PubMed PMID: 19635789; PubMed Central PMCID: PMC2781431.

Poirier S, Prat A, Marcinkiewicz E, Paquin J, Chitramuthu BP, Baranowski D, Cadieux B, Bennett HP, Seidah NG. Implication of the proprotein convertase NARC-1/PCSK9 in the development of the nervous system. J Neurochem. 2006 Aug;98(3):838-50. PubMed PMID: 16893422.

Protogerou AD, Zampeli E, Fragiadaki K, Stamatelopoulos K, Papamichael C, Sfikakis PP. A pilot study of endothelial dysfunction and aortic stiffness after interleukin-6 receptor inhibition in rheumatoid arthritis. Atherosclerosis. 2011 Dec;219(2):734-6. doi: 10.1016/j.atherosclerosis.2011.09.015. Epub 2011 Sep 16. PubMed PMID: 21968316.

Qian YW, Schmidt RJ, Zhang Y, Chu S, Lin A, Wang H, Wang X, Beyer TP, Bensch WR, Li W, Ehsani ME, Lu D, Konrad RJ, Eacho PI, Moller DE, Karathanasis SK, Cao G. Secreted PCSK9 downregulates low density lipoprotein receptor through receptor-mediated endocytosis. J Lipid Res. 2007 Jul;48(7):1488-98. Epub 2007 Apr 20. PubMed PMID: 17449864.

Rader DJ. IL-1 and atherosclerosis: a murine twist to an evolving human story. J Clin Invest. 2012 Jan 3;122(1):27-30. doi: 10.1172/JCI61163. Epub 2011 Dec 27. PubMed PMID: 22201674; PubMed Central PMCID: PMC3248309.

Rader DJ, Cohen J, Hobbs HH. Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. J Clin Invest. 2003 Jun;111(12):1795-803. Review. PubMed PMID: 12813012; PubMed Central PMCID: PMC161432.

Rajamäki K, Lappalainen J, Oörni K, Välimäki E, Matikainen S, Kovanen PT, Eklund KK. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. PLoS One. 2010 Jul 23;5(7):e11765. doi: 10.1371/journal.pone.0011765. PubMed PMID: 20668705; PubMed Central PMCID: PMC2909263.

Rashid S, Curtis DE, Garuti R, Anderson NN, Bashmakov Y, Ho YK, Hammer RE, Moon YA, Horton JD. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc Natl Acad Sci U S A. 2005 Apr 12;102(15):5374-9. Epub 2005 Apr 1. PubMed PMID: 15805190; PubMed Central PMCID: PMC556275.

Reddy MV, Iatan I, Weissglas-Volkov D, Nikkola E, Haas BE, Juvonen M, Ruel I, Sinsheimer JS, Genest J, Pajukanta P. Exome sequencing identifies 2 rare variants for low high-density lipoprotein cholesterol in an extended family. Circ Cardiovasc Genet. 2012 Oct 1;5(5):538-46. doi: 10.1161/CIRCGENETICS.112.963264. Epub 2012 Aug 25.PubMed PMID: 22923419; PubMed Central PMCID: PMC3515660.

Richard C, Couture P, Desroches S, Benjannet S, Seidah NG, Lichtenstein AH, Lamarche B. Effect of the Mediterranean diet with and without weight loss on surrogate markers of cholesterol homeostasis in men with the metabolic syndrome. Br J Nutr. 2012 Mar;107(5):705-11. doi: 10.1017/S0007114511003436. Epub 2011 Jul 26. PubMed PMID: 21787450.

Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Glynn RJ; JUPITER Study Group. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med. 2008 Nov 20;359(21):2195-207. doi: 10.1056/NEJMoa0807646. Epub 2008 Nov 9. PubMed PMID: 18997196.

Robitaille N, Cormier G, Couture R, Bouthillier D, Davignon J, Pérusse L. Apolipoprotein E polymorphism in a French Canadian population of northeastern Quebec: allele frequencies and effects on blood lipid and lipoprotein levels. Hum Biol. 1996 Jun;68(3):357-70. PubMed PMID: 8935317.

Roehrich ME, Mooser V, Lenain V, Herz J, Nimpf J, Azhar S, Bideau M, Capponi A, Nicod P, Haefliger JA, Waeber G. Insulin-secreting beta-cell dysfunction induced by

human lipoproteins. J Biol Chem. 2003 May 16;278(20):18368-75. Epub 2003 Feb 18. PubMed PMID: 12594227.

Roifman I, Beck PL, Anderson TJ, Eisenberg MJ, Genest J. Chronic inflammatory diseases and cardiovascular risk: a systematic review. Can J Cardiol. 2011 Mar-Apr;27(2):174-82. doi: 10.1016/j.cjca.2010.12.040. Review. PubMed PMID: 21459266.

Roncaglioni MC, Santoro L, D'Avanzo B, Negri E, Nobili A, Ledda A, Pietropaolo F, Franzosi MG, La Vecchia C, Feruglio GA, et al. Role of family history in patients with myocardial infarction. An Italian case-control study. GISSI-EFRIM Investigators. Circulation. 1992 Jun;85(6):2065-72. PubMed PMID: 1591825.

Rose S, Eren M, Murphy S, Zhang H, Thaxton CS, Chowaniec J, Waters EA, Meade TJ, Vaughan DE, Perlman H. A novel mouse model that develops spontaneous arthritis and is predisposed towards atherosclerosis. Ann Rheum Dis. 2013 Jan;72(1):89-95. doi: 10.1136/annrheumdis-2012-201431. Epub 2012 Jun 26. PubMed PMID: 22736097; PubMed Central PMCID: PMC3551222.

Roubtsova A, Munkonda MN, Awan Z, Marcinkiewicz J, Chamberland A, Lazure C, Cianflone K, Seidah NG, Prat A. Circulating proprotein convertase subtilisin/kexin 9 (PCSK9) regulates VLDLR protein and triglyceride accumulation in visceral adipose tissue. Arterioscler Thromb Vasc Biol. 2011 Apr;31(4):785-91. doi: 10.1161/ATVBAHA.110.220988. Epub 2011 Jan 27. PubMed PMID: 21273557.

Ruff CT, Braunwald E. The evolving epidemiology of acute coronary syndromes. Nat Rev Cardiol. 2011 Mar;8(3):140-7. doi: 10.1038/nrcardio.2010.199. Epub 2010 Dec 21. Review. PubMed PMID: 2117379.

Saavedra YG, Day R, Seidah NG. The M2 module of the Cys-His-rich domain (CHRD) of PCSK9 protein is needed for the extracellular low-density lipoprotein receptor (LDLR) degradation pathway. J Biol Chem. 2012 Dec 21;287(52):43492-501. doi: 10.1074/jbc.M112.394023. Epub 2012 Oct 26. PubMed PMID: 23105118; PubMed Central PMCID: PMC3527936.

Sachais BS, Katz J, Ross J, Rader DJ. Long-term effects of LDL apheresis in patients with severe hypercholesterolemia. J Clin Apher. 2005 Dec;20(4):252-5. PubMed PMID: 15880364.

Sandoo A, Kitas GD, Carroll D, Veldhuijzen van Zanten JJ. The role of inflammation and cardiovascular disease risk on microvascular and macrovascular endothelial function in patients with rheumatoid arthritis: a cross-sectional and longitudinal study. Arthritis Res Ther. 2012 May 17;14(3):R117. doi: 10.1186/ar3847. PubMed PMID: 22594788; PubMed Central PMCID: PMC3446498.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. 1977. Biotechnology. 1992;24:104-8. PubMed PMID: 1422003.

Sarwar N, Butterworth AS, Freitag DF, Interleukin-6 receptor pathways in coronary heart disease: a collaborative meta-analysis of 82 studies. Lancet. 2012 Mar 31;379(9822):1205-13. doi: 10.1016/S0140-6736(11)61931-4. Epub 2012 Mar 14. PubMed PMID: 22421339; PubMed Central PMCID: PMC3316940.

Saxena U, Klein MG, Goldberg IJ. Identification and characterization of the endothelial cell surface lipoprotein lipase receptor. J Biol Chem. 1991 Sep 15;266(26):17516-21. PubMed PMID: 1654330.

Scriver CR. Human genetics: lessons from Quebec populations. Annu Rev Genomics Hum Genet. 2001;2:69-101. Review. PubMed PMID: 11701644.

Seidah NG. PCSK9 as a therapeutic target of dyslipidemia. Expert Opin Ther Targets. 2009 Jan;13(1):19-28. doi: 10.1517/14728220802600715 . Review. PubMed PMID: 19063703.

Seidah NG. Proprotein convertase subtilisin kexin 9 (PCSK9) inhibitors in the treatment of hypercholesterolemia and other pathologies. Curr Pharm Des. 2013;19(17):3161-72. Review. PubMed PMID: 23317404.

Seidah NG, Benjannet S, Wickham L, Marcinkiewicz J, Jasmin SB, Stifani S, Basak A, Prat A, Chretien M. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. Proc Natl Acad Sci U S A. 2003 Feb 4;100(3):928-33. Epub 2003 Jan 27. PubMed PMID: 12552133; PubMed Central PMCID: PMC298703.

Seidah NG, Mayer G, Zaid A, Rousselet E, Nassoury N, Poirier S, Essalmani R, Prat A. The activation and physiological functions of the proprotein convertases. Int J Biochem Cell Biol. 2008;40(6-7):1111-25. doi: 10.1016/j.biocel.2008.01.030. Epub 2008 Feb 8. Review. PubMed PMID: 18343183.

Seidah NG, Mowla SJ, Hamelin J, Mamarbachi AM, Benjannet S, Touré BB, Basak A, Munzer JS, Marcinkiewicz J, Zhong M, Barale JC, Lazure C, Murphy RA, Chrétien M, Marcinkiewicz M. Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. Proc Natl Acad Sci U S A. 1999 Feb 16;96(4):1321-6. PubMed PMID: 9990022; PubMed Central PMCID: PMC15461.

Seidah NG, Poirier S, Denis M, Parker R, Miao B, Mapelli C, Prat A, Wassef H, Davignon J, Hajjar KA, Mayer G. Annexin A2 is a natural extrahepatic inhibitor of the PCSK9-induced LDL receptor degradation. PLoS One. 2012;7(7):e41865. doi: 10.1371/journal.pone.0041865. Epub 2012 Jul 27. PubMed PMID: 22848640; PubMed Central PMCID: PMC3407131.

Seidah NG, Prat A. The proprotein convertases are potential targets in the treatment of dyslipidemia. J Mol Med (Berl). 2007 Jul;85(7):685-96. Epub 2007 Mar 10. Review. PubMed PMID: 17351764.

Shan L, Pang L, Zhang R, Murgolo NJ, Lan H, Hedrick JA. PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide. Biochem Biophys Res Commun. 2008 Oct 10;375(1):69-73. doi: 10.1016/j.bbrc.2008.07.106. Epub 2008 Jul 31. PubMed PMID: 18675252.

Sheridan C. Phase 3 data for PCSK9 inhibitor wows. Nat Biotechnol. 2013 Dec;31(12):1057-8. doi: 10.1038/nbt1213-1057. PubMed PMID: 24316621.

Shao JS, Cheng SL, Sadhu J, Towler DA. Inflammation and the osteogenic regulation of vascular calcification: a review and perspective. Hypertension. 2010 Mar;55(3):579-92. doi: 10.1161/HYPERTENSIONAHA.109.134205. Epub 2010 Jan 25. Review. PubMed PMID: 20101002; PubMed Central PMCID: PMC2853014.

Sheridan C. Phase 3 data for PCSK9 inhibitor wows. Nat Biotechnol. 2013 Dec 6;31(12):1057-8. doi: 10.1038/nbt1213-1057. PubMed PMID: 24316621.

Sherry B, Jue DM, Zentella A, Cerami A. Characterization of high molecular weight glycosylated forms of murine tumor necrosis factor. Biochem Biophys Res Commun. 1990 Dec 31;173(3):1072-8. PubMed PMID: 2268312.

Shimokawa H, Ito A, Fukumoto Y, Kadokami T, Nakaike R, Sakata M, Takayanagi T, Egashira K, Takeshita A. Chronic treatment with interleukin-1 beta induces coronary intimal lesions and vasospastic responses in pigs in vivo. The role of platelet-derived growth factor. J Clin Invest. 1996 Feb 1;97(3):769-76. PubMed PMID: 8609234; PubMed Central PMCID: PMC507115.

Sholter DE, Armstrong PW. Adverse effects of corticosteroids on the cardiovascular system. Can J Cardiol. 2000 Apr;16(4):505-11. Review. PubMed PMID: 10787466.

Sia YT, Dulay D, Burwash IG, Beauchesne LM, Ascah K, Chan KL. Mobile ventricular thrombus arising from the mitral annulus in patients with dense mitral annular calcification. Eur J Echocardiogr. 2010 Mar;11(2):198-201. doi: 10.1093/ejechocard/jep181. Epub 2009 Nov 17. PubMed PMID: 19919957.

Simon Broome Register Group: Mortality in treated heterozygous familial hypercholesterolaemia: implications for clinical management. [No authors listed]. Scientific Steering Committee on behalf of the Simon Broome Register Group. Atherosclerosis. 1999 Jan;142(1):105-12. PubMed PMID: 9920511.

Simon Broome Register Group: Risk of fatal coronary heart disease in familial hypercholesterolaemia. Scientific Steering Committee on behalf of the Simon Broome Register Group. BMJ. 1991 Oct 12;303(6807):893-6. PubMed PMID: 1933004; PubMed Central PMCID: PMC1671226.

Sing CF, Davignon J. Role of the apolipoprotein E polymorphism in determining normal plasma lipid and lipoprotein variation. Am J Hum Genet. 1985 Mar;37(2):268-85. PubMed PMID: 3985008; PubMed Central PMCID: PMC1684560.

Steensberg A, Fischer CP, Keller C, Møller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. Am J Physiol Endocrinol Metab. 2003 Aug;285(2):E433-7. PubMed PMID: 12857678.

Stefanutti C, Vivenzio A, Colombo C, Di Giacomo S, Mazzarella B, Berni A, Nigri A, Koga N. Treatment of homozygous and double heterozygous familial

hypercholesterolemic children with LDL-apheresis. Int J Artif Organs. 1995 Feb;18(2):103-10. PubMed PMID: 7558394.

Stein EA, Gipe D, Bergeron J, Gaudet D, Weiss R, Dufour R, Wu R, Pordy R. Effect of a monoclonal antibody to PCSK9, REGN727/SAR236553, to reduce low-density lipoprotein cholesterol in patients with heterozygous familial hypercholesterolaemia on stable statin dose with or without ezetimibe therapy: a phase 2 randomised controlled trial. Lancet. 2012 Jul 7;380(9836):29-36. doi: 10.1016/S0140-6736(12)60771-5. Epub 2012 May 26. PubMed PMID: 22633824.

Stein EA, Honarpour N, Wasserman SM, Xu F, Scott R, Raal FJ. Effect of the proprotein convertase subtilisin/kexin 9 monoclonal antibody, AMG 145, in homozygous familial hypercholesterolemia. Circulation. 2013 Nov 5;128(19):2113-20. doi: 10.1161/CIRCULATIONAHA.113.004678. Epub 2013 Sep 6. PubMed PMID: 24014831.

Stein EA. Low-density lipoprotein cholesterol reduction by inhibition of PCSK9. Curr Opin Lipidol. 2013 Dec;24(6):510-7. PubMed PMID: 24184943.

Stone NJ, Robinson J, Lichtenstein AH, Merz CN, Blum CB, Eckel RH, Goldberg AC, Gordon D, Levy D, Lloyd-Jones DM, McBride P, Schwartz JS, Shero ST, Smith SC Jr, Watson K, Wilson PW. 2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. Circulation. 2013 Nov 12. [Epub ahead of print] PubMed PMID: 24222016.

Sun H, Samarghandi A, Zhang N, Yao Z, Xiong M, Teng BB. Proprotein convertase subtilisin/kexin type 9 interacts with apolipoprotein B and prevents its intracellular degradation, irrespective of the low-density lipoprotein receptor. Arterioscler Thromb Vasc Biol. 2012 Jul;32(7):1585-95. doi: 10.1161/ATVBAHA.112.250043. Epub 2012 May 10. PubMed PMID: 22580899.

Sun X, Essalmani R, Day R, Khatib AM, Seidah NG, Prat A. Proprotein convertase subtilisin/kexin type 9 deficiency reduces melanoma metastasis in liver. Neoplasia. 2012 Dec;14(12):1122-31. PubMed PMID: 23308045; PubMed Central PMCID: PMC3540939.

Talmud PJ, Shah S, Whittall R, Futema M, Howard P, Cooper JA, Harrison SC, Li K, Drenos F, Karpe F, Neil HA, Descamps OS, Langenberg C, Lench N, Kivimaki M, Whittaker J, Hingorani AD, Kumari M, Humphries SE. Use of low-density lipoprotein cholesterol gene score to distinguish patients with polygenic and monogenic familial hypercholesterolaemia: a case-control study. Lancet. 2013 Apr 13;381(9874):1293-301. doi: 10.1016/S0140-6736(12)62127-8. Epub 2013 Feb 22. PubMed PMID: 23433573.

Tang Z, Jiang L, Peng J, Ren Z, Wei D, Wu C, Pan L, Jiang Z, Liu L. PCSK9 siRNA suppresses the inflammatory response induced by oxLDL through inhibition of NF-κB activation in THP-1-derived macrophages. Int J Mol Med. 2012 Oct;30(4):931-8. doi: 10.3892/ijmm.2012.1072. Epub 2012 Jul 20. PubMed PMID: 22825241.

Tavori H, Fan D, Blakemore JL, Yancey PG, Ding L, Linton MF, Fazio S. Serum proprotein convertase subtilisin/kexin type 9 and cell surface low-density lipoprotein receptor: evidence for a reciprocal regulation. Circulation. 2013 Jun 18;127(24):2403-13. doi: 10.1161/CIRCULATIONAHA.113.001592. Epub 2013 May 20. PubMed PMID: 23690465; PubMed Central PMCID: PMC3806304.

Tavori H, Giunzioni I, Linton MF, Fazio S. Loss of Plasma Proprotein Convertase Subtilisin/Kexin 9 (PCSK9) After Lipoprotein Apheresis. Circ Res. 2013 Oct 11. [Epub ahead of print] PubMed PMID: 24122718.

Teslovich TM, Musunuru K, Smith AV, Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010 Aug 5;466(7307):707-13. doi: 10.1038/nature09270. PubMed PMID: 20686565; PubMed Central PMCID: PMC3039276.

The Lipid Research Clinics Coronary Primary Prevention Trial results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering. JAMA. 1984 Jan 20;251(3):365-74. PubMed PMID: 6361300.

Thorsson B, Sigurdsson G, Gudnason V. Systematic family screening for familial hypercholesterolemia in Iceland. Arterioscler Thromb Vasc Biol. 2003 Feb 1;23(2):335-8. PubMed PMID: 12588780.

Timms KM, Wagner S, Samuels ME, Forbey K, Goldfine H, Jammulapati S, Skolnick MH, Hopkins PN, Hunt SC, Shattuck DM. A mutation in PCSK9 causing autosomal-dominant hypercholesterolemia in a Utah pedigree. Hum Genet. 2004 Mar;114(4):349-53. Epub 2004 Jan 15. PubMed PMID: 14727179.

Toldo S, Mezzaroma E, Van Tassell BW, Farkas D, Marchetti C, Voelkel NF, Abbate A. Interleukin-1β blockade improves cardiac remodelling after myocardial infarction without interrupting the inflammasome in the mouse. Exp Physiol. 2013 Mar;98(3):734-45. doi: 10.1113/expphysiol.2012.069831. Epub 2012 Nov 23. PubMed PMID: 23180808.

Tsimihodimos V, Elisaf M. Lipoprotein glomerulopathy. Curr Opin Lipidol. 2011 Aug;22(4):262-9. doi: 10.1097/MOL.0b013e328345ebb0. Review. PubMed PMID: 21464714.

Urban D, Pöss J, Böhm M, Laufs U. Targeting the proprotein convertase subtilisin/kexin type 9 for the treatment of dyslipidemia and atherosclerosis. J Am Coll Cardiol. 2013 Oct 15;62(16):1401-8. doi: 10.1016/j.jacc.2013.07.056. Epub 2013 Aug 21. PubMed PMID: 23973703.

van Poelgeest EP, Swart RM, Betjes MG, Moerland M, Weening JJ, Tessier Y, Hodges MR, Levin AA, Burggraaf J. Acute Kidney Injury During Therapy With an Antisense Oligonucleotide Directed Against PCSK9. Am J Kidney Dis. 2013 Oct;62(4):796-800. doi: 10.1053/j.ajkd.2013.02.359. Epub 2013 Apr 3. PubMed PMID: 23561896.

Varas-Lorenzo C, Rodriguez LA, Maguire A, Castellsague J, Perez-Gutthann S. Use of oral corticosteroids and the risk of acute myocardial infarction. Atherosclerosis. 2007 Jun;192(2):376-83. Epub 2006 Jun 19. PubMed PMID: 16787647.

Varret M, Rabès JP, Saint-Jore B, Cenarro A, Marinoni JC, Civeira F, Devillers M, Krempf M, Coulon M, Thiart R, Kotze MJ, Schmidt H, Buzzi JC, Kostner GM, Bertolini S, Pocovi M, Rosa A, Farnier M, Martinez M, Junien C, Boileau C. A third major locus for autosomal dominant hypercholesterolemia maps to 1p34.1-p32. Am J Hum Genet. 1999 May;64(5):1378-87. PubMed PMID: 10205269; PubMed Central PMCID: PMC1377874.

Versmissen J, Oosterveer DM, Yazdanpanah M, Defesche JC, Basart DC, Liem AH, Heeringa J, Witteman JC, Lansberg PJ, Kastelein JJ, Sijbrands EJ. Efficacy of statins in familial hypercholesterolaemia: a long term cohort study. BMJ. 2008 Nov 11;337:a2423. doi: 10.1136/bmj.a2423. PubMed PMID: 19001495; PubMed Central PMCID: PMC2583391.

Villéger L, Abifadel M, Allard D, Rabès JP, Thiart R, Kotze MJ, Béroud C, Junien C, Boileau C, Varret M. The UMD-LDLR database: additions to the software and 490 new entries to the database. Hum Mutat. 2002 Aug;20(2):81-7. Review. PubMed PMID: 12124988.

Vohl MC, Moorjani S, Roy M, Gaudet D, Torres AL, Minnich A, Gagné C, Tremblay G, Lambert M, Bergeron J, Couture P, Perron P, Blaichman S, Brun LD, Davignon J, Lupien PJ, Després JP. Geographic distribution of French-Canadian low-density lipoprotein receptor gene mutations in the Province of Quebec. Clin Genet. 1997 Jul;52(1):1-6. PubMed PMID: 9272705.

Voight BF, Peloso GM, Orho-Melander M et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. Lancet. 2012 Aug 11;380(9841):572-80. doi: 10.1016/S0140-6736(12)60312-2. Epub 2012 May 17. Erratum in: Lancet. 2012 Aug 11;380(9841):564. PubMed PMID: 22607825; PubMed Central PMCID: PMC3419820.

von Eckardstein A, Nofer JR, Assmann G. High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. Arterioscler Thromb Vasc Biol. 2001 Jan;21(1):13-27. Review. PubMed PMID: 11145929.

Walsh GM. Canakinumab for the treatment of cryopyrin-associated periodic syndromes. Drugs Today (Barc). 2009 Oct;45(10):731-5. doi: 1396674/dot.2009.45.10.1416583. Review. PubMed PMID: 20069137.

Wang J, Ban MR, Hegele RA. Multiplex ligation-dependent probe amplification of LDLR enhances molecular diagnosis of familial hypercholesterolemia. J Lipid Res. 2005 Feb;46(2):366-72. Epub 2004 Dec 1. PubMed PMID: 15576851.

Watts GF, Lewis B, Sullivan DR. Familial hypercholesterolemia: a missed opportunity in preventive medicine. Nat Clin Pract Cardiovasc Med. 2007 Aug;4(8):404-5. Epub 2007 Jun 26. PubMed PMID: 17593912.

Wei L, MacDonald TM, Walker BR. Taking glucocorticoids by prescription is associated with subsequent cardiovascular disease. Ann Intern Med. 2004 Nov 16;141(10):764-70. PubMed PMID: 15545676.

Westlake SL, Colebatch AN, Baird J, Kiely P, Quinn M, Choy E, Ostor AJ, Edwards CJ. The effect of methotrexate on cardiovascular disease in patients with rheumatoid arthritis: a systematic literature review. Rheumatology (Oxford). 2010 Feb;49(2):295-307. doi: 10.1093/rheumatology/kep366. Epub 2009 Nov 27. Review. PubMed PMID: 19946022.

Williams RR, Hunt SC, Schumacher MC, Hegele RA, Leppert MF, Ludwig EH, Hopkins PN. Diagnosing heterozygous familial hypercholesterolemia using new practical criteria validated by molecular genetics. Am J Cardiol. 1993 Jul 15;72(2):171-6. PubMed PMID: 8328379.

Wilson PW. Relation of high-density lipoprotein subfractions and apolipoprotein E isoforms to coronary disease. Clin Chem. 1995 Jan;41(1):165-9. Review. PubMed PMID: 7813073.

Winzen R, Wallach D, Kemper O, Resch K, Holtmann H. Selective up-regulation of the 75-kDa tumor necrosis factor (TNF) receptor and its mRNA by TNF and IL-1. J Immunol. 1993 May 15;150(10):4346-53. PubMed PMID: 8387093.

Wolfe F, Michaud K. The risk of myocardial infarction and pharmacologic and nonpharmacologic myocardial infarction predictors in rheumatoid arthritis: a cohort and nested case-control analysis. Arthritis Rheum. 2008 Sep;58(9):2612-21. doi: 10.1002/art.23811. PubMed PMID: 18759273.

Wonderling D, Umans-Eckenhausen MA, Marks D, Defesche JC, Kastelein JJ, Thorogood M. Cost-effectiveness analysis of the genetic screening program for familial hypercholesterolemia in The Netherlands. Semin Vasc Med. 2004 Feb;4(1):97-104. PubMed PMID: 15199439.

Wu CY, Tang ZH, Jiang L, Li XF, Jiang ZS, Liu LS. PCSK9 siRNA inhibits HUVEC apoptosis induced by ox-LDL via Bcl/Bax-caspase9-caspase3 pathway. Mol Cell Biochem. 2012 Jan;359(1-2):347-58. doi: 10.1007/s11010-011-1028-6. Epub 2011 Aug 17. PubMed PMID: 21847580.

Wu JJ, Poon KY, Bebchuk JD. Association between the type and length of tumor necrosis factor inhibitor therapy and myocardial infarction risk in patients with psoriasis. J Drugs Dermatol. 2013 Aug;12(8):899-903. PubMed PMID: 23986163.

Xhignesse M, Lussier-Cacan S, Sing CF, Kessling AM, Davignon J. Influences of common variants of apolipoprotein E on measures of lipid metabolism in a sample selected for health. Arterioscler Thromb. 1991 Jul-Aug;11(4):1100-10. PubMed PMID: 2065031.

Xiao HB, Sun ZL, Zhang HB, Zhang DS. Berberine inhibits dyslipidemia in C57BL/6 mice with lipopolysaccharide induced inflammation. Pharmacol Rep. 2012 Jul;64(4):889-95. PubMed PMID: 23087140.

Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L; INTERHEART Study Investigators. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet. 2004 Sep 11-17;364(9438):937-52. PubMed PMID: 15364185.

Zaid A, Roubtsova A, Essalmani R, Marcinkiewicz J, Chamberland A, Hamelin J, Tremblay M, Jacques H, Jin W, Davignon J, Seidah NG, Prat A. Proprotein convertase subtilisin/kexin type 9 (PCSK9): hepatocyte-specific low-density lipoprotein receptor degradation and critical role in mouse liver regeneration. Hepatology. 2008 Aug;48(2):646-54. doi: 10.1002/hep.22354. PubMed PMID: 18666258.

Zhang DW, Garuti R, Tang WJ, Cohen JC, Hobbs HH. Structural requirements for PCSK9-mediated degradation of the l

ow-density lipoprotein receptor. Proc Natl Acad Sci U S A. 2008 Sep 2;105(35):13045-50. doi: 10.1073/pnas.0806312105. Epub 2008 Aug 27. PubMed PMID: 18753623; PubMed Central PMCID: PMC2526098.

Zhang DW, Lagace TA, Garuti R, Zhao Z, McDonald M, Horton JD, Cohen JC, Hobbs HH. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. J Biol Chem. 2007 Jun 22;282(25):18602-12. Epub 2007 Apr 23. PubMed PMID: 17452316.

Zhang K, Huang XZ, Li XN, Feng M, Li L, Cai XJ, Zhang C, Liu XL, Zhang MX, Zhang Y, Wang XL, Zhang M. Interleukin 6 destabilizes atherosclerotic plaques by downregulating prolyl-4-hydroxylase α1 via a mitogen-activated protein kinase andc-Jun pathway. Arch Biochem Biophys. 2012 Dec 15;528(2):127-33. doi: 10.1016/j.abb.2012.09.007. Epub 2012 Sep 27. PubMed PMID: 23022409.

Zhao Z, Tuakli-Wosornu Y, Lagace TA, Kinch L, Grishin NV, Horton JD, Cohen JC, Hobbs HH. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. Am J Hum Genet. 2006 Sep;79(3):514-23. Epub 2006 Jul 18. PubMed PMID: 16909389; PubMed Central PMCID: PMC1559532.

Zuliani G, Arca M, Signore A, Bader G, Fazio S, Chianelli M, Bellosta S, Campagna F, Montali A, Maioli M, Pacifico A, Ricci G, Fellin R. Characterization of a new form of inherited hypercholesterolemia: familial recessive hypercholesterolemia. Arterioscler Thromb Vasc Biol. 1999 Mar;19(3):802-9. PubMed PMID: 10073989.

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APPENDIX

APPENDIX: I

The Québec Child and Adolescent Health and Social Survey: design and methods of a cardiovascular risk factor survey for youth

PMID: 12717488

CJC Canadian Journal of Cardiology
Journal canadien de cardiologie

November 14, 2013

Dear Zuhier Awan,

I authorize you to use the following paper in your PhD Thesis:

The Québec Child and Adolescent Health and Social Survey: design and methods of a cardiovascular risk factor survey for youth by Paradis et al. published in the April 2003 issue of the Canadian Journal of Cardiology

Best regards,

Stanley Nattel, MD

Editor in Chief
Canadian Journal of Cardiology

Professor of Medicine
Paul-David Chair in Cardiovascular Electrophysiology
University of Montreal

Director, Electrophysiology Research Program
Montreal Heart Institute

The Québec Child and Adolescent Health and Social Survey: Design and methods of a cardiovascular risk factor survey for youth

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G Paradis, M Lambert, J O'Loughlin, et al. The Québec Child and Adolescent Health and Social Survey: Design and methods of a cardiovascular risk factor survey for youth. Can J Cardiol 2003;19(5):523-531.

BACKGROUND: Although atherosclerosis begins in childhood, there are no recent Canadian data on cardiovascular risk factors in provincially or nationally representative samples of youth.
OBJECTIVE: To describe the design and methods of the 1999 Québec Child and Adolescent Health and Social Survey, which assessed the prevalence and distribution of risk factors in a representative sample of Québec youth.
METHODS: School-based, multistage, cluster sampling survey of youth aged nine, 13 and 16 years. Measures included height, weight, subscapular and tricipital skinfolds, blood pressure (measured with the Dinamap), a fasting blood draw for assessment of lipoproteins and glucose, an age-adapted youth questionnaire on lifestyles and a parent questionnaire.
RESULTS: One thousand two hundred sixty-seven, 1186 and 1160 subjects aged nine, 13 and 16 years, respectively, responded to the questionnaire and had height, weight, skinfold thickness and blood pressure measured (response proportions of 83%, 79% and 78% respectively); 783, 818 and 874 subjects of the same age agreed to the blood draw (response proportions of 52%, 55% and 59%, respectively). Comparisons of characteristics of participants and nonparticipants in the blood draw showed few differences. The mean relative difference between lipoprotein and glucose values obtained at the study laboratory and a reference method varied from –0.3% to 6.1%. Design effects for means and proportions varied from 1.0 to 1.8.
CONCLUSION: Despite its complexity, this survey was able to achieve a high level of precision for multiple measures. It will provide the most complete data on cardiovascular risk factors ever collected among children and adolescents in Canada.

Key Words: *Epidemiology; Pediatrics; Population health; Risk factors*

L'Enquête sur la santé et la condition sociale des enfants et des adolescents du Québec: Conception et méthodologie d'une enquête sur le facteur de risque cardiovasculaire chez les jeunes

HISTORIQUE : Bien que l'athérosclérose se déclare pendant l'enfance, aucune donnée canadienne récente ne porte sur les facteurs de risque cardiovasculaire auprès d'échantillons provinciaux ou nationaux représentatifs des jeunes.
OBJECTIF : Décrire la conception et la méthodologie de l'Enquête sur la santé et la condition sociale des enfants et des adolescents du Québec, menée en 1999, qui a permis d'évaluer la prévalence et la distribution des facteurs de risque dans un échantillon représentatif de jeunes Québécois.
MÉTHODOLOGIE : Enquête en grappes, à plusieurs degrés et en milieu scolaire auprès de jeunes de 9 ans, 13 ans et 16 ans. Les mesures incluaient la taille, le poids, le pli cutané sous-scapulaire et tricipital, la tension artérielle (mesurée à l'aide du Dinamap), une prise de sang à jeun afin d'évaluer les lipoprotéines et le glucose, un questionnaire sur les modes de vie adapté à l'âge des jeunes et un questionnaire pour les parents.
RÉSULTATS : Mille deux cent soixante-sept, 1 186 et 1 160 sujets de 9, 13 et 16 ans, respectivement, ont répondu au questionnaire et ont fait mesurer leur taille, leur poids, l'épaisseur de leur pli cutané et leur tension artérielle (proportion de réponse de 83 %, 79 % et 78 %, respectivement), et 783, 818 et 874 sujets du même âge ont accepté de subir la prise de sang (proportion de réponse de 52 %, 55 % et 59 %, respectivement). La comparaison des caractéristiques des participants et des non-participants à la prise de sang a révélé peu de différences. La différence relative moyenne entre les valeurs de lipoprotéine et de glucose obtenues au laboratoire d'étude et une méthode de référence variait entre –0,3 % et 6,1 %. Les effets conceptuels relatifs aux moyennes et aux proportions oscillaient entre 1,0 et 1,8.
CONCLUSION : Malgré sa complexité, cette enquête a permis d'obtenir un taux de précision élevé pour des mesures multiples. Elle fournira les données plus complètes sur les facteurs de risque cardiovasculaire jamais compilées auprès d'enfants et d'adolescents au Canada.

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Although it has been almost 50 years since the first autopsy studies reported atherosclerosis in the arterial tree of young adults, the pediatric roots of ischemic cardiovascular disease (CVD) remain relatively understudied. Korean and Vietnam war autopsy studies identified coronary atherosclerosis in 44% to 77% of young male war casualties (mean age at death was 22 years) (1,2). Advanced lesions were found in 5% to 15%, suggesting that the atherosclerotic process had begun years earlier. Subsequent research revealed the presence of fatty streaks and atherosclerotic plaques in the aorta and coronary arteries of young children and adolescents who had died of external causes (3-5). More recent investigations have even documented fatty streaks in human fetal aortas (6).

Additional evidence supporting the pediatric origins of atherosclerosis includes statistically significant associations between CVD risk factors (RFs) and the extent of coronary atherosclerosis found at autopsy among youth who died of violent causes. In the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study, complete RF assessment by postmortem measurements were obtained from 1479 of 2876 subjects aged 15 to 34 at the time of death. Low high density lipoprotein cholesterol (HDL-C), elevated non-HDL cholesterol, smoking, obesity, hypertension and glycohemoglobin levels were associated with the extent or severity of atherosclerosis in the coronary arteries or the aorta (7-12). Among individuals with a favourable lipoprotein profile (non-HDL-C less than 4.14 mmol/L and HDL-C greater than or equal to 0.91 mmol/L) smoking, elevated glycohemoglobin, obesity in men and hypertension in African Americans were associated with atherosclerosis (13).

The Bogalusa study (14) showed strong dose-response relationships between the extent of coronary and aortic fatty streaks and fibrous plaques at autopsy of adolescents and young adults who died of violent causes, and the number of RFs measured years earlier. Studies using noninvasive methods to assess atherosclerosis among youth have also shown strong relationships between hyperlipidemia and coronary artery calcifications detected by ultrafast computed tomography (15), endothelial dysfunction measured by brachial reactivity (16-18) and carotid artery intima-media thickening assessed by ultrasonography (19,20). In addition, echocardiography has demonstrated increased left ventricular mass and diastolic dysfunction among hypertensive adolescents (21).

The importance of the study of CVD RFs in youth stems not only from their association with pediatric atherosclerosis but also from their persistence into adulthood, a phenomenon known as tracking. Both elevated levels of total cholesterol (TC) and blood pressure have been shown to track into adulthood (22-24). In addition, childhood obesity, physical inactivity and cigarette smoking are correlated with adult levels of these RFs (25).

Given the high degree of tracking of CVD RFs and the possibility that preventive efforts might be more effective if implemented during childhood and adolescence (25), obtaining reliable estimates of the population distribution of RFs is an important first step for the development of rational public policy for CVD prevention. With the exception of smoking, there are no recent representative national or provincial data on CVD RFs in Canadian youth. This report describes the design of the Québec Child and Adolescent Health and Social Survey (QCAHS), which was conducted to provide such data (26). It also presents the background, target population, sample design and recruitment, measurements and procedures, response rates, selected data analysis issues and ethical considerations for this survey, with a particular emphasis on its cardiovascular component.

OBJECTIVE OF THE QCAHS

The objective of the QCAHS was to provide a profile of general health and social well being of Québec youth. The cardiovascular component aimed to document the distribution of major CVD RFs and associated lifestyles, including blood lipoprotein levels, blood pressure, overweight and obesity status, smoking and physical inactivity. An additional objective was to assess the association between CVD RF and environmental (family, socioeconomic status, etc) and genetic factors. Because of the added measurement complexity, a separate survey was designed to assess dietary intake in another sample of Québec youth and is not considered in this paper.

METHODS

Design of the QCAHS

The QCAHS was a multistage, stratified, cluster sampling survey of in- and out-of-school youth in Québec. The survey was conducted between January and May 1999 and included an age-specific youth self-administered questionnaire, a parent questionnaire, a school director questionnaire, and biological and anthropometric measures. Because the school director questionnaire was not related to CVD, it will not be considered further.

Population

The QCAHS target population consisted of Québec youth aged nine, 13 or 16 years on March 31, 1999. Single year age categories were selected because many biological and behavioural variables can change within a narrow age range. Nine-year-old children were selected because, based on the authors' previous experience and results from the pilot study, they are the youngest group from which reliable information can be gathered from self-administered questionnaires (27); this age group was also chosen because most nine-year-olds are prepubertal. Thirteen-year-olds were selected because most will have entered puberty and because most youth begin high school at this age in Québec. Finally, by age 16, most boys and almost all girls will have completed puberty. In addition, school is obligatory up until age 16 in Québec and corresponds to the beginning of a transition period to adulthood.

Students attending federal government schools, native schools, schools in very remote regions, schools in which more than 50% of youth are handicapped and schools with fewer than 12 subjects of the desired age were excluded. The sampling frame contained 97% of all children aged nine, 13 and 16 years in Québec. About 5% of 16-year-old adolescents in Québec have dropped out of school. To maintain representativeness, a random sample of out-of-school 16-year-olds was also selected, but only to participate in the questionnaire portion of the survey.

Sampling

The sampling frame consisted of the 1998 to 1999 Québec Ministry of Education student roll, which contains the name, date of birth, home address and the name of the school attended for all students in Québec. In addition, the student roll of 1997 to 1998 was used to identify adolescents aged 16 in 1998 to 1999 who had dropped out of school or moved out of province.

Independent samples were drawn for each age. Four levels of stratification were used to optimize provincial representativity of the sample. First, the 17 administrative regions of Québec were divided into outlying (four regions) and other regions. For cost and logistic reasons, two of the four outlying regions were randomly selected with probability of selection proportional to the number of age-appropriate students. The 13 other regions were all selected for the next stage.

Within administrative regions, schools were stratified according to 1) language of instruction (French or English); 2) public or private status (only for French schools); and 3) geographic location based on metropolitan census areas. Approximately 60 schools were randomly selected for each age group. Finally, within each school sampled, random samples of approximately 25 subjects stratified by sex were selected. Because some youth aged 13 years are still in elementary school, these adolescents were sampled from the same elementary schools already sampled for nine-year-old children, as well as from a sample of high schools.

A sample size of 1500 per age was targeted to obtain coefficients of variation of less than 15% for sex-specific proportions of 10% and of 7% for both sexes combined, taking the cluster sampling design and an estimated response proportion of 80% into account.

Survey recruitment

The survey resulted from a partnership between the Institut de la statistique du Québec, the Ministère de la Santé et des Services Sociaux and the Ministère de l'Éducation du Québec, several regional public health departments and university researchers. Information letters were sent to the principal of each school and an appointment was made to explain the purpose of the study and answer questions about the protocol.

Subjects were given an envelope to be brought home to their parents. It contained a colour flyer and a letter describing the survey and a request for participation, a consent form for parents and children, a parental questionnaire and a stamped preaddressed return envelope for the parent questionnaire and consent form.

Data collection

Data were collected at school in a 3 h morning session. An additional data collection period was occasionally necessary for students who were absent. Separate teams of 10 persons including pediatric nurses, kinesiologists, interviewers and a team coordinator collected data in each of 10 geographical territories in Québec. A pilot test of several survey instruments was conducted among 329 students from three elementary and three high schools in the Montréal area in 1996 and a pretest of the full survey procedures and instruments was conducted among 196 youth in six Québec schools and 50 out-of-school adolescents in March and April 1998. A two-day training session for team coordinators and a four-day training session for all staff were held before the survey. All aspects of data collection were reviewed during the training session with a particular focus on adherence to the measurement protocol. Staff were tested and certified for the blood pressure and anthropometric measures.

Measurement and variables

Blood lipids, apolipoproteins, insulin and glucose: Because subjects had fasted for at least 10 h, the blood draw was the first procedure conducted, followed by a light breakfast. Ten millilitres of blood was collected by phlebotomy in a sterile tube containing 1 mg/mL ethylenediaminetetraacetic acid, immediately put on ice, centrifuged on site within 90 min, separated into five aliquots of plasma and one cell pellet and frozen on dry ice. Blood samples were shipped within 24 h to the nutrition laboratory, Ste-Justine Hospital, where they were stored at –80°C until analyzed. The biochemical analyses were performed by the Department of Clinical Biochemistry at Ste-Justine Hospital.

Plasma TC, HDL-C and triglyceride (TG) concentrations were measured colourimetically on a Synchron Cx7 (Beckman Couter, USA) using Beckman Coulter reagents. TC was measured by the cholesterol oxidase method, HDL-C with a homogeneous assay and TG by enzymatic hydrolysis followed by the measurement of free glycerol. Low density lipoprotein cholesterol (LDL-C) concentrations were calculated according to the Friedewald equation (28). Plasma glucose was measured enzymatically using glucose oxidase on a Beckman Coulter Synchron Cx7. Insulin concentrations were determined with the ultrasensitive insulin kit on the Access immunoassay system from Beckman-Coulter. This technique consists of a double antibody sandwich assay using chemiluminescence as the detection method; the antibodies used are specific to insulin and do not cross-react with proinsulin or peptide C. Apolipoproteins A1 and B were measured by rate nephelometry using the Beckman array. Standardization was conducted according to the recommendations of the International Federation of Clinical Chemistry with reagents and standards from Beckman Coulter.

Blood pressure: Blood pressure was measured on the right arm after subjects had been sitting at rest for at least 5 min and no less than 30 min after breakfast. Blood pressure cuff size was based on arm circumference measurement and according to the Project Heartbeat Protocol (29). Measurements were obtained with an oscillometric instrument (Dinamap XL, model CR9340, Critikon Co, Tampa, USA) with demonstrated validity (30) and according to procedures developed by the Child and Adolescent Trial for Cardiovascular Health Program (31). Three consecutive measures of resting blood pressure were obtained at 1 min intervals and the average of the last two measures was used in the analyses. In addition, the mean arterial pressure and resting pulse, which are measured automatically by the Dinamap, were recorded. The 10 Dinamap instruments used for data collection were calibrated against a standard mercury manometer before the start of the survey.

Anthropometric measures: Height was measured with a standard measuring tape. Subjects removed their shoes and heavy clothing and stood straight against a wall. A triangular level with a 90° angle was placed against the wall and the head of the subject to ensure that the head remained in the Frankfort horizontal plane. Height was recorded to the nearest millimetre (0.1 cm) during maximal inspiration. Weight was measured to the nearest 0.2 kg in light indoor clothing without shoes or extra sweaters using a spring scale tested daily for accuracy and calibrated using a set of standard weights. Triceps and subscapular skinfold thickness were measured on the right side to the nearest 0.1 cm using a Lange caliper (Beta Technology, USA) and the method described by Lohman et al (32). Subjects stood with their feet together and arms at their sides. They could request that measures be taken over their clothing and when this was done the thickness of the garment was measured with the caliper and subtracted from the overall measurement (skinfold plus garment) before recording.

All measurements were repeated and, if they differed by more than 0.5 cm for height, 0.2 kg for weight and 1 mm for skinfolds, a third measure was taken. The average of the two closest measures was used in data analysis. The body mass index was computed as

weight (kg)/height^2 (m^2). Waist and hip circumferences were not measured.

Youth questionnaire

Two questionnaires were developed, one for nine-year-old children and one for 13- and 16-year-old adolescents. The questionnaire for nine-year-olds contained fewer questions and fewer response categories for certain questions. In addition, the wording of selected questions was simplified. Thirteen- and 16-year-old adolescents completed their questionnaires individually. Nine-year-old children were grouped with two interviewers; one interviewer standing in front of the group read the questions one by one providing instructions according to a manual of instructions. The second interviewer circulated among the students responding to queries and verifying that children were following instructions. The questionnaire was administered in French or English according to the language used in the school; 45 to 60 min was required for completion.

Most questions included in this survey were previously validated or used in major national or Québec-wide surveys (and, hence, available in French and English). Other questions were validated in French and English in local research. The cardiovascular component of the questionnaire included questions on smoking, physical activity and puberty. Other questions pertained to sociodemographic characteristics, school performance and well-being, social support, parent-child relationship, self-perceived body size, time spent in activities such as reading, television viewing, computer usage or video game playing, alcohol and drug consumption, health problems and use of motor vehicles. Additional questions for adolescents included participation in the workforce and sexuality. Finally, questions on the use of medication were included in the adolescent questionnaire but were asked in the parent questionnaire for nine-year-old children.

Frequency of physical activity was determined in a seven-day recall adapted from the Weekly Activity Checklist (33) and used extensively in our prior school studies (34). The original instrument correlated with an objective activity measure at $r=0.34$, $P<0.01$ (33). For each day of the preceding week (Monday to Sunday), subjects checked which of 18 physical activities they had participated in for at least 15 consecutive minutes on that day. The list of activities was slightly different for children and adolescents to reflect the different activities that interest these age groups. The list included the activities most frequently practised by the specific age groups during the winter and early spring, including physical education classes and activities during free play (34). A frequency score was computed by summing the total number of activities checked for each day of the week. Sedentary behaviours were measured using three questions that assessed the number of hours per day spent watching television (recorded separately for weekdays and weekends) and the use of home computers.

Cigarette consumption was assessed using questions from the 1994 Canadian survey of smoking among youth (35). All subjects answered two questions about lifetime use of cigarettes. Six additional questions were asked of 13- and 16-year-olds including lifetime use of at least 100 cigarettes, current use of cigarettes, and smoking by siblings and peers.

Although the wording was simplified for nine-year-olds, all subjects answered questions about puberty adapted from the Canadian National Longitudinal Study of Children and Youth (36), including questions on voice change and facial hair for boys, breast growth for girls, and underarm and pubic hair for both sexes.

Subjects aged 13 and 16 years indicated the presence of chronic health problems diagnosed by a physician or other health professional from a list of 13 items including diabetes and cholesterol or lipid problems. They also indicated medications used in the past two weeks from a list of seven categories (medication for pain or fever, for cold or allergies, for breathing problems, vitamins or minerals, antibiotics, to help concentration or calm down, or other medications). For nine-year-old children, the presence of chronic health problems and use of medication were assessed in the parent questionnaire. Finally, sociodemographic questions included age, sex, language spoken with friends, ethnicity (for 13- and 16-year-olds) and current grade.

Parent questionnaire

A parent questionnaire pertained to the family and social environment, health, use of health services, use of medication and schooling of study subjects, health behaviours of parents, family history of CVD and respiratory disease, and sociodemographic information. The instructions requested that the parent who 'knew the subject best' complete the questionnaire.

Lifestyle behaviours of parents related to CVD were assessed using questions drawn from the Canadian Heart Health Surveys (37), the Québec enquête sociale et de santé, 1998 (38) and the National Longitudinal Survey of Children and Youth (36), and included activity limitation, smoking status, self-reported height and weight, weight loss behaviour, frequency of leisure time physical activity, and consumption of alcohol and drugs. The respondent also answered questions about the smoking, alcohol use and physical activity habits of the current spouse or partner, as well as questions about the number of smokers in the household and the approximate number of cigarettes smoked daily in the household.

Additional questions assessed each biological parent's personal history of hypertension, high blood cholesterol, diabetes, heart attack or angina, stroke, cerebral vascular disease or peripheral vascular disease, asthma and hay fever, as well as the use of medication to lower blood cholesterol, hypertension medication or any medication for the heart.

Sociodemographic information included the province or country of birth of each of the subject, biological father and biological mother; language most often spoken at home; self-reported ethnocultural group; total household income; and level of education and employment status of the respondent and current spouse or partner. Copies of the questionnaires and data collection forms are available in French and English from the corresponding author or at the Institut de la statistique du Québec Website <www.stat.gouv.qc.ca>.

Data analysis: sample weights and design effect

Sample weights were calculated to reflect the number of individuals in the total population who are represented by each study subject. Study subjects were initially weighted according to the inverse of their probability of being sampled. These weights were adjusted by the inverse of the response proportions for schools and the inverse of the age-specific youth response proportions for the questionnaire, the blood draw or the parent questionnaire, respectively. The youth questionnaire weights were applied to the anthropometric and blood pressure variables because their response proportions were very similar. A final adjustment was cal-

culated to equate the age-specific sum of weights and the total number of individuals in the target population. For data analysis purposes, these population weights were transformed into sample weights such that the sum of the age-specific weights equalled the observed number of children surveyed in that age group.

The design effect reflects loss of precision that results from complex cluster sampling designs compared with a simple random sample of the same size. It is defined as the ratio of the variance under the complex design to the variance that would have been obtained under simple random sampling. Although a specific design effect can be computed for each parameter of interest, most variables are affected similarly by the design. Separate design effects were calculated for means and proportions. For means, the average of the age-specific design effect for TC, HDL-C, TG, LDL-C and glucose was computed. For proportions, the average of the age-specific design effect for over 50 variables from the child, adolescent and parent questionnaires was computed.

TABLE 1
Response proportions of the Québec Child and Adolescent Health and Social Survey, Québec, 1999

	Age			
Response (%)	9 (n=1520)	13 (n=1498)	16 In school (n=1495)	16 Out of school* (n=130)
Questionnaire	83.4	79.2	77.6	40.0
Height and weight	83.1	79.1	77.4	–
Skinfold thickness	82.6	78.3	76.6	–
Blood pressure	82.4	78.8	77.4	–
Blood draw	51.5	54.6	58.5	–
Parent questionnaire	70.1	68.8	63.7	–

**Out-of-school 16-year-olds were asked to complete a postal questionnaire only*

Ethical considerations

Approval of the study protocol was obtained from the ethics committees of the Direction Santé Québec of the Institut de la statistique du Québec, the Ministère de l'Éducation du Québec and Ste-Justine Hospital. The use of provincial lists of schoolchildren as the sampling frame was approved by the Commission d'accès à l'information du Québec. Signed informed consent was obtained from parents and their children. Separate consents were required for the questionnaire and physical measures, the blood draw, the anonymous storage of residual samples and cell pellets for future genetic analyses, and the permission to send the results of the blood pressure, TC, HDL-C, TG and glucose tests to the child's home.

The questionnaires, data collection forms and blood samples were identified by a unique identification number. The master list matching student names and identification numbers was kept locked at the Direction Santé Québec offices and destroyed after the results of the blood pressure, TC, HDL-C, TG and glucose measures were sent to the parents who had consented. An explanatory letter described the results and encouraged parents to consult their pediatrician or family physician if an abnormal result was reported.

RESULTS

Response proportions

Because independent samples were drawn for each age group, age-specific response proportions are presented. Among the 189 schools sampled (out of over 3000 schools in Québec) for nine-, 13- and 16-year-olds, 97.2% (69 of 71), 96.3% (52 of 54) and 97.0% (61 of 63; one school sampled was deemed ineligible), respectively, agreed to participate. Because of the high response proportion and to avoid the loss of precision due to school refusals, two schools each were added for the nine- and 13-year-olds and three schools for the 16-year-olds.

Table 1 presents response proportions according to measurement. The age-specific response proportion is computed by dividing the number of respondents by the number of eligible individuals. Ineligible individuals were those who had been sampled from the Québec Ministry of Education's master list but who were currently living in institutions, severely handicapped, unknown to the school or deceased. Eligible subjects included 1520 of 1564 sampled nine-year-olds, 1498 of 1556 sampled 13-year-olds, 1495 of 1560 sampled 16-year-olds and 130 of 140 sampled out-of-school 16-year-olds. Among eligible nine-, 13- and 16-year-olds, 1267, 1186 and 1160, respectively, completed the questionnaire. Similar numbers of subjects provided measures of height and weight, skinfold thickness and blood pressure. Only 783, 818 and 874 of nine-, 13- and 16-year-olds, respectively, provided blood samples and parents of 1065, 1031 and 952 subjects completed the parent questionnaire.

Response proportions were similar for both sexes except among 16-year-olds; 74% of boys and 81% of girls responded to the adolescent questionnaire, and among those who were out of school 46% of girls and 36% of boys completed the questionnaire. In addition, both 13-year-old boys and girls in elementary school had lower response proportions than those in high schools (74% versus 80%). The much lower response proportion for the blood draw raises the possibility of selection bias. However, statistically significant differences for participation in the blood draw were only noted for language spoken at home for nine-year-olds (53% for anglophone versus 67% for francophones) and physical activity for 16-year-olds (72% for those physically active versus 81% for those least active). No differences in the response proportion for blood draw were noted according to sex, pubertal status, smoking, weight category (underweight, normal weight, overweight, obesity) or parental history of CVD or CVD RFs including smoking, parent education level, household income, or rural or urban status of the school (see appendix table).

Quality control

Interviewers verified questionnaires for completeness and omissions before subjects left the data collection area. A private firm performed the coding and double data entry with careful attention to admissible codes, logic errors and filter questions. Ten per cent of anthropometric measures were repeated by a different interviewer to assess interobserver reliability. The intraclass correlations for the interrater reliability for height, weight, and subscapular and tricipital skinfolds were 0.99, 0.99, 0.96 and 0.94, respectively, indicating excellent reliability.

The Department of Clinical Biochemistry of Ste-Justine Hospital participates on a regular basis in provincial and international quality control programs and is accredited by the Canadian External Quality Assessment Laboratory (CEQAL) (Vancouver, British Columbia). CEQAL's lipid reference

TABLE 2
Interassay coefficients of variation for selected control plasma measurements

Analyte	Level (n)	CV (%)
Apolipoprotein A1 (g/L)	0.46 (46)	3.8
	0.97 (36)	3.6
	2.18 (47)	3.4
	2.54 (39)	6.5
Apolipoprotein B (g/L)	0.52 (44)	3.9
	1.02 (45)	3.5
	1.65 (35)	3.4
	1.97 (37)	6.1
Total cholesterol (mmol/L)	2.91 (24)	1.4
	3.98 (24)	1.1
	5.55 (24)	1.0
	7.45 (25)	1.3
Glucose (mmol/L)	2.2 (24)	3.8
	6.4 (24)	1.3
	13.7 (24)	1.4
High density lipoprotein cholesterol (mmol/L)	0.56 (25)	3.7
	1.63 (25)	4.3
Insulin (mmol/L)	92 (24)	4.1
	286 (23)	4.9
	694 (24)	5.0
Triglycerides (mmol/L)	1.05 (24)	4.2
	1.40 (24)	5.4
	2.13 (24)	4.0
	5.12 (25)	3.9

CV Coefficient of variation

TABLE 3
Same-day and seven-day test-retest median coefficients of variation (%) for biochemical variables

Analyte	Same-day CV % (n)	Seven-day CV % (n)
Total cholesterol (mmol/L)	0.54 (124)	1.09 (122)
HDL-C (mmol/L)	1.03 (124)	2.21 (122)
Triglycerides (mmol/L)	1.16 (124)	1.77 (122)
Glucose (mmol/L)	0.00 (124)	1.37 (122)
Insulin (mmol/L)	1.49 (116)	3.13 (116)
Apolipoprotein A1 (g/L)	0.62 (125)	2.21 (123)
Apolipoprotein B (g/L)	0.97 (122)	2.16 (120)

CV Coefficient of variation; HDL-C High density lipoprotein cholesterol

methods are directly traceable to the methods of the Centers for Disease Control and Prevention – National Heart, Lung, and Blood Institute Lipid Standardization Program. Additional laboratory reference methods have been sanctioned by the National Reference System for the Clinical Laboratory and the International Federation of Clinical Chemistry. Calibration was performed extemporaneously with each lot of samples analyzed for plasma glucose, insulin, TC, TG, HDL-C, and apolipoproteins A1 and B. For each measure, controls at different analyte levels were included with each batch and were used to compute interassay coefficients of variation (Table 2).

TABLE 4
Mean relative difference in plasma lipid and glucose values between Ste-Justine Hospital and the reference method of the Canadian External Quality Assessment Laboratory (CEQAL)*

Analyte level	Total cholesterol (n=75) (%)	HDL-C (n=75) (%)	Triglycerides (n=75) (%)	Glucose (n=50) (%)
Tertile				
1st	1.2	1.0	6.1	–
2nd	0.9	–0.3	0.8	–
3rd	1.9	–3.1	–1.3	–
Below median	–	–	–	3.2
Above median	–	–	–	3.4

**Calculated as [(Ste-Justine Hospital – CEQAL) / CEQAL] × 100. HDL-C High density lipoprotein cholesterol*

TABLE 5
Average design effects of the Québec Child and Adolescent Health and Social survey

Variables	Age (years) 9	13	16
Means			
Sex-specific analyses	1.2	1.5	1.1
Both sexes combined	1.5	1.8	1.0
Proportions			
Sex-specific analyses	1.3	1.3	1.2
Both sexes combined	1.4	1.4	1.3

The results show very low coefficients of variation between assays for the control samples indicating the high precision of the laboratory measurements. A systematic 5% sample of plasma specimens was reanalyzed on the same day and another 5% was reanalyzed one week apart. The test-retest reliability coefficients, presented in Table 3, show that the Ste-Justine Hospital biochemistry laboratory largely exceeded the performance recommendations of the National Cholesterol Education Program (NCEP) and that achieved by the College of American Pathologists Comprehensive Chemistry Survey of 5500 laboratories in the United States (39).

To assess the accuracy of the lipid measurements, 75 random plasma samples (25 per age group) were sent to CEQAL for measurement of TC, HDL-C and TG. An additional 50 random samples were sent to CEQAL for assessment of the glucose measurements. Table 4 presents the mean relative difference between the Ste-Justine Hospital laboratory values and those from CEQAL for each tertile levels of analyte, or in the case of glucose for analyte levels above or below the median. The maximal relative difference is 6.1% indicating a high level of accuracy of the plasma lipid and glucose values. When the tertiles were collapsed into a single category, the overall mean difference was 1.4%, –0.8% and 1.9% for TC, HDL-C and TG, respectively, well below the 3% for TC and 5% for HDL-C and TG recommended by the NCEP.

Design effects

Table 5 presents the average design effects for means and proportions for the QCAHS for sex-specific analyses and for analyses with both sexes combined. The design effects for means range from 1.0 to 1.8. Sex-specific design effects are lower for nine- and 13-year-olds than for both sexes combined. In addition, the 13-year-olds show the largest design effects. The design effects for proportions range from 1.2 to 1.4 and show little difference for sex-specific or sex-combined analyses and little difference by age. Design effects greater than unity reflect the loss of precision of estimates of parameters of interest due to the cluster sampling design. They will not affect the validity or point estimates but will increase the standard errors and, hence, the confidence intervals around these estimates.

DISCUSSION

The QCAHS will provide the first CVD RF data from a representative sample of youth in a Canadian province in over 20 years. Although several recent provincial and national surveys provide information on smoking behaviour, very little data are available on other RFs. This is particularly distressing because many of these RFs are associated not only with CVD but also with many other chronic diseases including diabetes, osteoporosis and several cancers.

The only other survey that provided CVD RF data in youth was the Canada Health Survey of 1978 to 1979 (40). Over 20,000 individuals responded to a lifestyle questionnaire and one-quarter of these also had physical measures. Height, weight, skinfold thickness and blood pressure were measured in children aged two and above and total blood cholesterol was measured beginning at age 15. Unfortunately, blood cholesterol was measured in several laboratories with little standardization, and blood pressure values were based on single measures obtained by nurses using the auscultatory method.

National data on body size and physical activity were provided by the 1981 Canada Fitness Survey (CFS) and the Campbell Lifestyle Survey of 1988, which included both adults and children (41,42). Both used similar procedures, the former in 13,500 randomly sampled households including 4080 subjects aged seven to 16 years and the latter in approximately one-fifth of the original participants in the CFS. In addition, these surveys measured physical fitness.

Although the QCAHS achieved high response proportions for the questionnaire, anthropometric measures and blood pressure, only 51.5% to 58.5% of youth participated in the blood draw, thus raising the possibility of bias if important characteristics associated with lipid, apolipoprotein, insulin or glucose levels are distributed differently among those who agreed and those who did not agree to phlebotomy. Among the variables compared among respondents and nonrespondents to the blood draw, only English-speaking nine-year-olds and very physically active 16-year-olds were less likely to provide a blood sample.

The QCAHS demonstrates the feasibility of conducting a youth survey with a complex design and of achieving a high degree of precision for multiple types of measures. The survey required over 100 field staff, in addition to laboratory technicians, data entry staff, data analysts and a budget of over a million dollars. The conduct of the survey during the winter added to the complexity of the field operations. Winter data collection was inevitable because the Ministry of Education student list that served as the sampling frame becomes available only in November of each academic year and because data collection had to end before the year-end examinations. Because of this, data that are influenced by season such as physical activity will have to be interpreted with caution.

The QCAHS provides important methodological information for future surveys targeting youth. Because cluster sampling results in a loss of precision, sample sizes of future surveys using similar designs would need to be increased proportionally to the design effect to achieve desired levels of precision. For example, the sampling strategy produced only moderate clustering attested by design effects ranging from 1.0 to 1.8. This reflects the careful selection of clusters and stratifying variables and, in particular, the fact that the selection of subjects within schools was accomplished through simple random sampling, avoiding cluster sampling of classrooms. In addition, small number of subjects were selected from each school and were spread over 189 schools.

One of the important results of the survey will be to provide reference values for blood pressure and blood lipids previously unavailable for the Canadian pediatric population. The large sample size will permit precise estimation of important percentile values for these parameters. These values will be useful for clinicians in the management of pediatric patients, and for public health and policy planners as a baseline against which to evaluate preventive efforts. The QCAHS will also provide valuable information on pediatric obesity, physical activity, smoking, and the association between behavioural and biological CVD RF and between social, parental and personal characteristics of subjects and CVD RF. A particular strength of the QCAHS is the collection of data not only from children and adolescents but also from their parents, which will permit more detailed characterization of the influence of the family environment on youth behaviour and RF. In addition, the distribution and prevalence of RFs will help forecast the future population burden of CVD.

The preservation of stored plasma and cell pellets will enable the study of novel or emerging RFs in a representative pediatric population. This is particularly relevant for the study of gene-environment interactions in Québec's French-Canadian population, which has a relatively homogeneous genetic inheritance. For example, we are currently exploring the expression and the correlates of the insulin resistance syndrome in our population.

Limitations

Although no important differences were noted between participants and nonparticipants in the blood draw, the two groups might have differed in unmeasured characteristics. In addition, no information is available on the 20% of the target population who refused to participate in any aspect of the survey. Previous studies have shown that nonrespondents generally differ from respondents in motivation and attitudes toward health as well as RF status (43).

Except for genes, the cross-sectional nature of the survey will limit the study of new RF in this population. We were unable to obtain approval from the Direction Santé Québec to follow up respondents.

Another limitation involves the data collection procedures and measurements. Although most questionnaire items were drawn from previous instruments, misclassification may have

occurred because of memory bias, social desirability or variable reading skills particularly among nine-year-old children. Additional measurement error might have been introduced in the few cases where subjects asked that skinfold measures be taken over their clothing. Measurement error might also have been introduced in the parent questionnaire if the respondent did not know the child well or was unaware of the medical history of the biological parents. Finally, no dietary data were collected in the QCAHS.

CONCLUSION

The QCAHS will provide the best data on CVD RFs in children and adolescents ever collected in Canada. However, these data need to be collected by similar surveys in other provinces to permit the creation of a national database. In addition, repeating these surveys over time is crucial to characterize the evolution of RF and behaviours in a future generation of young Canadians. Atherosclerosis begins during childhood and adolescence and a comprehensive strategy to control CVD must include the pediatric population. A national CVD RF database for youth is urgently needed in Canada to anchor public policy and clinical efforts on evidence and a clear understanding of the pediatric roots of mass CVD.

ACKNOWLEDGMENTS: The survey was funded by the Ministère de la Santé et des Services Sociaux du Québec and by the Bureau of Nutritional Sciences, Health Canada. The first pilot study was funded by the Direction de la santé publique de Montréal-Centre. Part of the data analyses is funded by the Canadian Institutes for Health Research (MOP-44027). Dr Jennifer O'Loughlin is an Investigator in the Canadian Institutes of Health Research. The QCAHS required the contribution and support of many individuals. We wish to thank Dr Andres Petrasovits (deceased), Dr Richard Lessard, Dr Sylvie Stachenko, Dr Darwin Labarthe, Mr Daniel Tremblay, Mr Michel Beauchemin, Dr David Seccombe, Dr Elinor Wilson, Mrs Jocelyne Mercier, Mrs Ginette Lagacé, Mrs Louise Thibault, Mrs Manon Chartré, Mrs France Gareau, Dr Luc Berghmans, Dr Yvon Dieu, Mr Freddy Flandroit (deceased), Dr Françoise Narring, Dr Paul-André Michaud, Mr Alain Trugeon, Mr Philippe Lorenzo and Dr Hassen Ghannem. We also thank members of the Committee of Principal Investigators of the Canadian Heart Health Initiative and the Heart and Stroke Foundation of Canada for their support. We thank Mrs Nathalie Théorêt for typing the manuscript.

APPENDIX

Percentage of eligible subjects who participated in the blood draw according to certain characteristics. Québec Child and Adolescent Health and Social Survey, 1999

	Age (years)				Age (years)		
Variables	**9**	**13**	**16**	**Variables**	**9**	**13**	**16**
Sex				Family history of CVD			
Male	61	71	74	No	64	71	83
Female	63	69	76	One parent	72	73	81
Language spoken at home				Two parents	70	76	79
French	67*	73	80	Puberty status			
English	53*	76	83	Prepubertal	62	78	–
French and English	61*	57	89	Pubertal	63	70	75
Other	79*	75	79	Post pubertal	–	65	73
Ethnic origin				Smoking status of parent‡			
French Canadian	67	73	80	Regular	69	78	79
Other	64	73	81	Occasional	82	71	91
Relative household income†				Former	67	73	79
Poor to very poor	66	75	83	Never	62	70	81
Medium	67	74	81	Weight category of child§			
High to very high	67	74	80	Normal	63	70	74
Residence				Overweight	60	68	80
Metropolitan census area	62	72	73	Obese	61	74	77
Other	62	67	77	Physical activity level (quartile)			
Parent education‡				I	68	69	81*
Elementary school only	62	69	78	II	63	68	70*
High school	65	76	79	III	61	72	78*
Vocational school	68	73	82	IV	57	71	72*
College	68	73	79				
University	67	73	82				

**P<0.05 for comparison of the different categories. †Based on total household income and the number of people living in the household and computed according to the methods of Statistics Canada (Statistique Canada. Répartition du revenu au Canada selon la taille du ménage, 1992, Ottawa, Division des enquêtes-ménages, catalogue no. 13-207, 1993). ‡Parent who completed the parent questionnaire. §Normal: body mass index (BMI) greater than fifth but less than or equal to 85th percentile of the age-sex specific distribution; Overweight: BMI greater than 85th but less than or equal to 95th percentile of the age-sex specific distribution and tricep skinfold less than or equal to 95th percentile and subscapular skinfold less than 90th percentile; Obesity: BMI greater than 95th and triceps skinfold greater than 95th and subscapular skinfold greater than 90th percentile. CVD Cardiovascular disease*

REFERENCES

1. Enos WF, Holmes RG, Beyer J. Coronary disease among United States soldiers killed in action in Korea: Preliminary report. JAMA 1953;152:1090-3.
2. McNamara JJ, Molot MA, Stremple JF. Coronary artery disease in combat casualties in Vietnam. JAMA 1971;216:1185-7.
3. Holman RL, McGill HC, Strong JP, Geer JC. The natural history of atherosclerosis: the early aortic lesions as seen in New Orleans, in the middle of the 20th century. Am J Pathol 1958;34:209-2035.
4. Strong JP, McGill HC. Pediatric aspects of atherosclerosis. J Atheroscler Res 1969;9:251-65.
5. Strong JP, Malcom GT, McMahan CA, et al. Prevalence and extent of atherosclerosis in adolescents and young adults. Implications for prevention from the Pathobiological Determinants of Atherosclerosis in Youth Study. JAMA 1999;281:727-35.
6. Napoli C, D'Armiento FP, Mancini FP, et al. Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. J Clin Invest 1997;100:2680-90.
7. McGill HC Jr, Strong JP, Tracy RE, McMahan CA, Oalmann MC. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) research groups. Relation of a postmortem renal index of hypertension to atherosclerosis in youth. Arterioscler Thromb Vasc Biol 1995;15:2222-8.
8. McGill HC, McMahan CA Jr, Malcom GT, Oalmann MC, Strong JP. Effects of serum lipoproteins and smoking on atherosclerosis in young men and women. Arterioscler Thromb Vasc Biol 1997;17:95-106.
9. McGill HC, McMahan CA Jr, Malcom GT, Oalmann MC, Strong JP. Relation of glycohemoglobin and adiposity to atherosclerosis in youth. Arterioscler Thromb Vasc Biol 1995;15:431-40.
10. McGill HC Jr, McMahan CA, Zieske AW, et al. Youth Research Gf. Associations of coronary heart disease risk factors with the intermediate lesion of atherosclerosis in youth. Arterioscler Thromb Vasc Biol 2000;20:1998-2004.
11. McGill HC Jr, McMahan CA, Zieske AW, et al. Association of Coronary Heart Disease Risk Factors with microscopic qualities of coronary atherosclerosis in youth. Circulation 2000;102:374-9.
12. McGill HC Jr, McMahan CA, Herderick EE, et al. Effects of coronary heart disease risk factors on atherosclerosis of selected regions of the aorta and right coronary artery (PDAY) research group. Pathobiological Determinants of Atherosclerosis in Youth. Arterioscler Thromb Vasc Biol 2000;20:836-45.
13. McGill HC Jr, McMahan A, Zieske AW, Malcom GT, Tracy RE, Strong JP. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) research group. Effects of non-lipid risk factors on atherosclerosis in youth with a favorable lipoprotein profile. Circulation 2001;103:1546-50.
14. Berenson GS, Srinivasan S, Bao W, et al. Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. N Engl J Med 1998;338:1650-6.
15. Mahoney LT, Burns TL, Stanford W. Coronary risk factors measured in childhood and young adult life are associated with coronary artery calcification in young adults: The Muscatine Study. J Am Coll Cardiol 1996;27:277-84.
16. Celermajer DS, Gooch VM, Spiegelhalter DJ, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 1992;340:1111-5.
17. Sorensen GK, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge DJ, Deanfiled JE. Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein (a) level. J Am Coll Cardiol 1994;93:50-5.
18. Mietus-Snyder M, Malloy MJ. Endothelial dysfunction occurs in children with two genetic hyperlipidemias: improvement with antioxidant vitamin therapy. J Pediatr 1998;133:35-40.
19. Pauciullo P, Iannuzzi A, Sartorio R, et al. Increased intima-media thickness of the common carotid artery in hypercholesterolemic children. Arterioscler Thromb Vasc Biol 1994;14:1075-9.
20. Virkola K, Pesonen E, Akerblom HK, Siimes MA. Cholesterol and carotid artery wall in children and adolescents with familial hypercholesterolaemia: a controlled study by ultrasound. Acta Paediatr 1997:86:1203-7.
21. Daniels SR, Meyer RA, Loggie JMH. Determinants of cardiac involvement in children and adolescents with essential hypertension. Circulation 1990;82:1243-8.
22. Bao W, Srinivasan SR, Wattigney WA, Berenson GS. Usefulness of childhood low-density lipoprotein cholesterol level in predicting adult dyslipidemia and other cardiovascular risks. The Bogalusa Heart Study. Arch Intern Med 1996;156:1315-20.
23. Lauer RM, Clarke WR. Use of cholesterol measurements in childhood for the prediction of adult hypercholesterolemia. JAMA 1990;264:3034-8.
24. Mahoney LT, Lauer RM, Lee J, Clarke WR. Factors affecting tracking of coronary heart disease risk factors in children: the Muscatine Study. Ann N Y Acad Sci 1991;623:120-32.
25. Resnicow K, Robinson TN. School-based cardiovascular disease prevention studies: Review and synthesis. Ann Epidemiol 1997;7(Suppl 7):S14-31.
26. Aubin J, Lavallée C, Camirand J, et al. Enquête sociale et de santé auprès des enfants et adolescents québécois 1999. Québec: Institut de la statistique du Québec, 2002.
27. O'Loughlin J, Renaud L, Paradis G, Meshefedjian G, Zhou X. Prevalence and correlates of early smoking among elementary schoolchildren in multiethnic, low-income, inner-city neighborhoods. Ann Epidemiol 1998;8:308-18.
28. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.
29. Labarthe DR. Project Heartbeat! Blood Pressure, Manual of Operations. Houston: University of Texas Health Science Center at Houston, 1992.
30. Park MK, Menard SM. Accuracy of blood pressure measurement by the Dinamap monitor in infants and children. Pediatrics 1998;8:308-18.
31. Webber LS, Osganian V, Luepker RV, et al. Cardiovascular risk factors among third grade children in four regions of the United States: The CATCH Study. Am J Epidemiol 1995;141:428-39.
32. Lohman TG, Roche AF, Martorell R, eds. Anthropometric Standardization Reference Manual. Champaign: Human Kinetics Publishers Inc, 1988:177.
33. Sallis JF, Condon SA, Goggin KJ, Roby JJ, Lolody B, Alcaraz JE. The development of self-administered physical activity surveys for 4th grade students. Res Q Exerc Sport 1993:64:25-1.
34. O'Loughlin J, Paradis G, Kischuk N, Barnett T, Renaud L. Prevalence and correlates of physical activity behaviours among elementary schoolchildren in multiethnic, low-income, inner-city neighbourhoods. Ann Epidemiol 1999;9:397-407.
35. Stephen ST, Morin M, eds (Health Canada). Youth Smoking Survey, 1994: Technical Report. (Catalogue No. H49-98/1-1994E). Ottawa: Ministry of Supply and Services Canada, 1996.
36. National Longitudinal Survey of Children and Youth. Survey Material for the 1994-1995 Data Collection. Cycle 1. (Catalogue No. 95-01E). Ottawa: Statistics Canada, 1996.
37. MacLean DR, Petrasovits A, Nargundkar M, et al. Canadian Heart Health Surveys: a profile of cardiovascular risk. Survey methods and data analysis. Canadian Heart Health Survey Research Group. CMAJ 1992;146:1969-74.
38. Questionnaire sur les habitudes de vie et la santé. ESS98/QAA.2. Montréal: Institut de la statistique du Québec, Direction Santé Québec, 1998.
39. Rifai N, Bachorik PS, Albers JJ. Lipids, lipoproteins and apolipoproteins. In: Burtis CA, Ashwood E, eds. Tietz Textbook of Clinical Chemistry, 3rd edn. Philadelphia: WB Saunders Company, 1999.
40. Canada Health Survey. The Health of Canadians. Report of the Canada Health Survey. (Publication No. 82-538E). Ottawa: Statistics Canada, Health and Welfare Canada, 1981.
41. Canada Fitness Survey. Fitness and lifestyle in Canada. Ottawa: Fitness and Amateur Sport Canada, 1983.
42. Stephen ST, Craig CL. The Well Being of Canadians. Highlights from the 1988 Campbell Survey. Ottawa: Canadian Fitness and Lifestyle Research Institute, 1990.
43. Hennekens CH, Buring JE. Epidemiology in Medicine. Boston: Little, Brown and Company, 1987.

SANTÉ QUÉBEC

LE GROUPE LÉGER & LÉGER
RECHERCHE MARKETING

parle-nous de ta santé
ENQUÊTE SOCIALE ET DE SANTÉ
auprès des enfants
et des adolescents québécois

Montréal, janvier 1999

Madame
Monsieur

La santé des enfants et des adolescents québécois et les facteurs qui l'influencent sont mal connus. Santé Québec, en collaboration avec le ministère de la Santé et des Services sociaux, le ministère de l'Éducation et Santé Canada, mène une enquête qui vise à combler cette lacune. L'information obtenue permettra de mieux répondre aux besoins réels de nos enfants. Les avantages de leur participation sont donc communautaires plutôt que personnels. Le dépliant ci-joint vous permettra de mieux comprendre la nature de l'enquête.

Pourquoi votre jeune?

L'école de votre jeune a été choisie au hasard à partir de la liste des écoles primaires et secondaires du Québec. Le ministère de l'Éducation a fourni cette liste avec la permission de la Commission d'accès à l'information. La commission scolaire et le directeur ou la directrice de l'école appuient entièrement ce projet et ont accepté que tous les élèves sélectionnés qui auront donné leur consentement y participent. Votre enfant a été choisi au hasard parmi les jeunes de son âge pour participer au volet sociosanitaire de l'enquête. D'autres ont été choisis pour le volet alimentation.

Quelle sera votre participation?

Dans un premier temps, nous sollicitons votre collaboration pour remplir le coupon-réponse ci-joint. Il s'agit d'un formulaire de consentement que nous vous demandons de signer après avoir pris connaissance de la présente lettre et de le retourner à l'école dans l'enveloppe-réponse dès demain si possible. Nous vous encourageons à discuter de tout ceci avec votre jeune afin qu'il comprenne bien de quoi il s'agit et puisse ajouter sa signature à la vôtre en toute connaissance de cause. Nous vous demandons aussi de répondre au questionnaire confidentiel adressé au parent qui connaît le mieux l'enfant. Votre participation et celle de votre jeune sont entièrement volontaires et vous pouvez retirer votre consentement sans pénalité et à tout moment si vous le désirez.

.../2

LE GROUPE LÉGER & LÉGER
RECHERCHE MARKETING

parle-nous de ta santé

N° de dossier : _____

COUPON RÉPONSE (à retourner à l'école, dès que possible)

J'ai lu et compris l'information ci-jointe, je reconnais que ma participation et celle de mon jeune est tout à fait volontaire et que je suis libre de changer d'avis en tout temps.

Quel que soit votre choix, pourriez-vous compléter les quatre (4) questions suivantes, signer et demander à votre jeune de signer aussi.

1. J'accepte ◯ Je refuse ◯

que _____ participe à l'Enquête sociale et de santé
Nom de votre jeune
auprès des enfants et adolescents québécois (questionnaire, poids, taille, pression artérielle, pli cutané).

2. J'accepte ◯ Je refuse ◯

qu'il/elle soit soumis à une prise de sang.

3. J'accepte ◯ Je refuse ◯

que l'on me communique les résultats des tests de cholestérol et de la pression artérielle.

4. J'accepte ◯ Je refuse ◯

que l'on conserve les échantillons de sang **anonymes** pour des recherches ultérieures sur les facteurs de risque de maladies cardiovasculaires et leurs marqueurs génétiques.

Signature du parent _____

Signature du jeune _____

Date _____

Suite au verso...

Afin que l'équipe de Santé Québec puisse vous envoyer les résultats des analyses, veuillez indiquer votre adresse complète.

Nom du parent (en lettres moulées)

_____

Adresse complète

_____

_____

Même si vous refusez, veuillez retourner ce coupon-réponse à l'école.

SANTÉ QUÉBEC

LE GROUPE LÉGER & LÉGER INC.
RECHERCHE MARKETING

Enquête sociale et de santé auprès des enfants et des adolescents québécois

ENTENTE DE CONFIDENTIALITÉ

Je m'engage à prendre les mesures nécessaires pour que tous les renseignements dont je serai détenteur ou détentrice par le biais de mon travail dans l'enquête demeurent confidentiels et anonymes pour toute personne qui n'est pas assujettie à la présente entente de confidentialité.

L'identité de tout enfant ou des ses parents, le nom de l'école ou la commission scolaire participant à l'enquête ne seront jamais révélée à qui que ce soit et les résultats ne seront discutés avec personne d'autre que ma supérieure ou tout autre membre assujetti à la présente entente.

En foi de quoi j'ai signé _____

Date _____

APPENDIX: II

The Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) study

PMID: 14609996

Partners Human Research Committee
Partners Human Research Office
116 Huntington Avenue, Suite 1002
Boston, MA 02116
Tel: (617) 424-4100
Fax: (617) 424-4199

Continuing Review: Notification of IRB Approval/Activation

Protocol #: 2004-P-000644/14; BWH

Date: 02/22/2013

To: Paul Ridker, MD, MPH
Medicine
900 Commonwealth Avenue,

From: Fausta M Figueroa
PHS Research Management
116 Huntington Ave Suite 1002

Title of Protocol:	JUPITER
Version Date:	03/02/2004
Sponsor/Funding Support:	President and Fellows of Harvard College
IRB Continuing Review #:	10
IRB Review Type:	Expedited - (8)(c)
IRB Approval Date:	02/20/2013
Approval Effective Date:	02/22/2013
IRB Expiration Date:	02/20/2014

This Project has been reviewed and approved by the BWH IRB. During the review of this Project, the IRB specifically considered (i) the risks and anticipated benefits, if any, to subjects; (ii) the selection of subjects; (iii) the procedures for securing and documenting informed consent; (iv) the safety of subjects; and (v) the privacy of subjects and confidentiality of the data.

NOTES: Closed to Enrollment: Data Analysis Only.

As Principal Investigator you are responsible for the following:

1. Submission in writing of any and all changes to this project (e.g., protocol, recruitment materials, consent form, study completion, etc.) to the IRB for review and approval prior to initiation of the change(s), except where necessary to eliminate apparent immediate hazards to the subject(s). Changes made to eliminate apparent immediate hazards to subjects must be reported to the IRB.
2. Submission in writing of any and all adverse event(s) that occur during the course of this project in accordance with the IRB's policy on adverse event reporting.
3. Submission in writing of any and all unanticipated problems involving risks to subjects or others.
4. Use of only IRB approved copies of the consent form(s), questionnaire(s), letter(s), advertisement(s), etc. in your research. Do not use expired consent forms.
5. Informing all physicians listed on the project of changes, adverse events, and unanticipated problems.

The IRB can and will terminate projects that are not in compliance with these requirements. Direct questions, correspondence and forms (e.g., continuing reviews, amendments, adverse events, safety reports) to Fausta M Figueroa, (617) 424-4119.

ADULT STUDY SUBJECT INFORMATION AND CONSENT FORM AND AUTHORIZATION TO USE AND DISCLOSE MEDICAL INFORMATION PART I (Screening)

Title: A Randomized, Double-Blind, Placebo-Controlled, Multicenter, Phase III Study of Rosuvastatin (CRESTOR®) 20 mg in the Primary Prevention of Cardiovascular Events Among Subjects with Low Levels of LDL-Cholesterol and Elevated Levels of C-Reactive Protein (JUPITER)

Study Code: 4522US/0011 Subject Initials: << >>

Enrollment No: << >>

Sponsor: AstraZeneca Pharmaceuticals LP Wilmington, DE 19850-5437

Section 1 Introduction

You are being asked to take part in a screening visit to determine whether you are eligible to participate in the JUPITER clinical research study. The JUPITER research study is called the main study. If you are found to be eligible, you may decide to continue beyond the screening visit and participate in the main study. If so, the main study will be discussed with you and a full informed consent form will be signed at that time. This screening consent form describes the purpose, procedures, benefits and risks of the screening visit and how your medical information will be used and who may see it. If you are in any other study, you cannot take part in this screening visit.

Your study doctor is an investigator for this screening visit, and as an investigator, is interested both in your health and the conduct of this study. The study doctor is being paid by AstraZeneca (the study sponsor) to conduct this screening visit. Before participating in this screening visit, you may seek the opinion of another doctor who is not connected to the study. You do not have to take part in any clinical study offered by any doctor. Your decision whether or not to take part in this study will not affect the medical care you receive from your doctor.

After this screening visit has been explained to you and if you decide to take part, you will be asked to sign this consent form, which gives permission for the study doctor to proceed with the screening test. You will be given a copy of this consent for your records.

Read this information carefully and please ask the study doctor or the study staff if you have any questions.

Section 2 Purpose of the Screening Visit and the Study

The screening part of the study is to determine your eligibility to participate in the main study. To be eligible, you must have low to normal levels of low-density lipoprotein cholesterol (LDL-C), and also have elevated levels of C-Reactive Protein (CRP). Lipoproteins are "fat" molecules in the blood. It is thought that increased levels of LDL-C may be associated with the blockage of arteries, and may place individuals at risk for future cardiovascular events such as a heart attack, stroke and angina.

AstraZeneca, the sponsor of this study, has developed a drug called rosuvastatin calcium that has been approved by the United States Food and Drug Administration (FDA) for the treatment of high cholesterol (a fatty-like substance in the blood). However, rosuvastatin has not been approved for use in reducing the risk of heart attack, stroke or angina in individuals with low to normal LDL-C and elevated CRP. This is the question being addressed in this study.

Section 3 Description of the Screening Visit Procedures

At the screening visit, you will: 1) be asked questions about your past and/or present health, and any medical conditions that you may have; 2) be evaluated by a member of the research team and asked to read and sign this consent form after all your questions have been answered; 3) have a sample of your blood taken (about 2 teaspoons) after fasting (nothing to eat or drink, except water, for the previous 8 hours). The blood sample will be sent to the laboratory to test your blood cholesterol levels (including LDL-C) and CRP level.

Section 4 Risks, Discomforts and Inconveniences

During the procedure to collect a sample of your blood, you may experience discomfort, pain, bleeding and/or bruising at the site of needle insertion. While unlikely, you may feel dizzy or faint during the blood draw. There is also a risk of infection called phlebitis (on rare occasion) or a hematoma (blood clot) that could develop at the site where the blood was collected.

Section 5 Possible Benefits to Participants

You may be given free cholesterol education materials and you may be counseled on the benefits of diet and moderate exercise. Your study doctor cannot guarantee that you will benefit from participation in this screening visit.

Section 6 Alternative Treatments

The alternative is not to be screened for this study.

Section 7 Treatment and Compensation for Injury Resulting from this Screening Visit

If any physical injury occurs from the blood drawing procedure, you will receive appropriate medical treatment. You will not be reimbursed for lost wages, other damages or losses, or for

medical expenses that have been covered by your medical or hospital insurance or other programs providing such coverage. No other form of compensation is available from AstraZeneca except remedies under the law. Compensation for medical expenses is not an admission of fault or liability by AstraZeneca or anyone else. The above section does not restrict your right to seek legal assistance. You do not waive any legal rights by signing this screening visit Informed Consent Form.

Section 8 Payment for Taking Part in the Screening Visit

You will not receive any compensation for participation in the screening visit.

Section 9 Expenses (Cost of Taking Part in the Screening Visit)

The study doctor's visit and the above laboratory tests will be provided at no cost to you or your insurance company.

Section 10 Taking Part is Voluntary

It is up to you whether to take part in this screening visit. You may refuse to take part without penalty or loss of benefits to which you are otherwise entitled.

Section 11 Confidentiality and Authorization to Collect, Use and Disclose Your Medical Information

This section ("the Authorization") explains how your medical information will be collected, used and shared with certain other persons involved in the study and describes your rights, including the right to see your medical information.

Purpose of this Authorization

You are being asked to permit to the collection, use and sharing of your medical information in order to see if you qualify to participate in the main study.

What Does Medical Information Mean?

Your medical information is information about your physical or mental health or condition. It includes:

- any medical information about you created or collected during this study visit
- information about you that the study doctor may need in order to be able to see if you qualify to participate in this study

This information may identify you because it may contain, for example, your name, address, telephone number, date of birth, race or ethnic origin or other unique identifiers.

Use and Disclosure of Your Medical Information

If you sign this form, you allow the study doctor to collect and use your medical information to see if you qualify for the main study. You also allow the study doctor to share your medical information with:

- the study sponsor, including its affiliates, its representatives and its contractors who work on behalf of the study sponsor to conduct the study
- other doctors and health care professionals who are involved in the study
- the Institutional Review Board (IRB) that watches over the study; and
- government agencies overseeing this study or the study drug, including the Food and Drug Administration (FDA), other Department of Health and Human Services agencies, and government agencies in the United States and other countries

Will Persons Looking At Your Medical Information Be Able to Identify You?

A screening number and your initials will be recorded on your blood sample. The results of your blood sample are considered protected information. However, the study sponsor, its representatives and contractors, regulatory authorities and other supervising bodies may look at this at the study doctor's site. The reason these persons may look at your medical information is to make sure the study has been done properly and that study data have been collected correctly, or for other reasons allowed by law.

Notice on Redisclosure of Your Medical Information and Confidentiality

Federal law provides that the study doctor can only share your medical information with those persons whom you have permitted to see it. However, if you sign this form, those persons may share your medical information with other persons. Federal law does not protect you against this. (The laws of your state may provide additional privacy protection.)

Publication of Study Results

Except as explained in section 11, your medical information will be kept confidential. If you enter the main study, the data and results from the study may be presented at meetings or in publications, but in those presentations people taking part in the study will not be identified by name.

Your Right to See and/or Copy Your Medical Information

You have the right to see and copy your medical information related to the screening visit for as long as the study doctor holds this information. However, you may not be able to see some of your records related to the study until after the study has been completed, because it could spoil the study.

Withdrawing Your Authorization

You may withdraw your authorization (permission) regarding your participation in the screening visit at any time.

If you withdraw your authorization, you cannot continue to take part in the study.

Expiration of Your Authorization

Your authorization will expire at the end of the main study, following expiration of any required record retention period.

Section 12 Whom to Ask If You Have Questions

You have the right to ask questions about this study at any time and are encouraged to do so. If you have any questions about this research study, ask the research study doctor or the research staff .

If you experience an injury you believe is related to this screening visit, contact <<*person*>> at <<*telephone # >>*.

If you have any questions about your rights as a research subject, you may contact <<*name of IRB* >> at << *telephone # >>*.

Section 13 Right to Withdraw and Termination Procedures

You may withdraw from participation in this screening visit at any time without penalty or loss of benefits to which you are otherwise entitled.

Section 14 INFORMED CONSENT STATEMENT AND AUTHORIZATION:

I, _____ (name of subject), **HAVE READ AND I UNDERSTAND ALL THE INFORMATION IN THIS INFORMATION AND CONSENT FORM. I HAVE BEEN GIVEN THE CHANCE TO DISCUSS IT AND ASK QUESTIONS. ALL MY QUESTIONS HAVE BEEN ANSWERED TO MY SATISFACTION. I VOLUNTARILY CONSENT TO TAKE PART IN THIS SCREENING VISIT. I UNDERSTAND I WILL RECEIVE A COPY OF THIS INFORMATION AND CONSENT FORM.**

BY SIGNING THIS INFORMATION AND CONSENT FORM, I HAVE NOT GIVEN UP ANY OF THE LEGAL RIGHTS, WHICH I OTHERWISE WOULD HAVE AS A SUBJECT IN A RESEARCH STUDY.

I AUTHORIZE THE COLLECTION, USE AND DISCLOSURE OF MY MEDICAL INFORMATION IN ACCORDANCE WITH THIS FORM.

Signature of Subject	**Date of Signature**
Printed name of Subject	
Signature of Person Administering this Consent	**Date of Signature**
Printed Name of Person Administering this Consent	

ADULT STUDY SUBJECT INFORMATION AND CONSENT FORM AND AUTHORIZATION TO USE AND DISCLOSE MEDICAL INFORMATION PART II

Title: A Randomized, Double-Blind, Placebo-Controlled, Multicenter, Phase III Study of Rosuvastatin (CRESTOR®) 20 mg in the Primary Prevention of Cardiovascular Events Among Subjects with Low Levels of LDL-Cholesterol and Elevated Levels of C-Reactive Protein (JUPITER)

Study Code: 4522US/0011 Subject Initials: << >>

Enrollment No: << >>

Sponsor: AstraZeneca Pharmaceuticals LP Wilmington, DE 19850-5437

Section 1 Introduction

You have been asked to take part in a clinical research study. For you to be able to decide to be part of this study or not, you should understand enough about its risks and benefits to make an informed decision. This process is known as informed consent. This consent form describes the purpose, procedures, possible benefits and risks of the study. This form will also explain how your medical information will be used and who may see it. Once you have been fully informed about the study and had any questions answered, you will be asked to sign this form if you wish to take part and to allow your medical information to be collected, used and shared with certain persons involved in the study. If you do not sign this form, you will not be able to take part in the study. If you are in any other study, you cannot take part in this study.

Your study doctor is an investigator for this study, and as an investigator, is interested both in your health and in the conduct of this study. The study doctor is being paid by AstraZeneca (the study sponsor) to conduct this study. Before entering this study or at any time during the study, you may ask for a second opinion about your care from another doctor who is in no way connected with this study. You do not have to take part in any clinical study offered by any doctor. Your decision whether or not to take part in this study will not affect the medical care you receive from your doctor.

After the study has been explained to you and if you decide to take part, you will be asked to sign this consent form, which gives permission for the study doctor to proceed with the tests, procedures and medications administered in this study. You will be given a copy of this consent for your records. If you decide to take part, you are free to withdraw (leave the study) at any time without giving a reason. Likewise, your study doctor may decide that it may not be in your best interest to continue taking the study medication. If you have been asked to

stop taking the study medication, you are still in the study and will complete all your clinic visits until the study is over. If you are not taking study drug and are unable to attend your scheduled visit, the study staff will contact you to complete your scheduled visits via telephone.

Read this information carefully and please ask the study doctor or the study staff if you have any questions.

Section 2 Purpose of the Study

AstraZeneca, the sponsor of this study, has developed a drug called rosuvastatin calcium that has been approved by the United States Food and Drug Administration (FDA) for the treatment of high cholesterol (a fatty-like substance in the blood). However, rosuvastatin has not been approved to be used in reducing the risk of cardiovascular events in individuals with LDL < 130 mg/dL but with elevated levels of CRP as is being investigated in this study.

Rosuvastatin belongs to a group of drugs called "statins" (statins are medications that may help lower cholesterol levels). Rosuvastatin has already been given to more than 25,000 subjects in other clinical studies, in doses up to 80 mg. Previous studies have shown that the side effects of this drug were generally mild to moderate in intensity and short lived.

We are doing this study to determine the safety and effectiveness of long-term therapy with rosuvastatin compared with a placebo (inactive pill). We want to learn if this treatment might be effective in reducing the risk of cardiovascular events such as a heart attack, stroke, angina and other medical conditions. A daily dose of 20 mg of rosuvastatin or placebo will be taken by men and women like you, who have low to normal levels of low density lipoprotein (LDL) cholesterol, but with elevated levels of C-Reactive Protein (CRP). Lipoproteins are "fat" molecules in the blood. It is thought that increased levels of lipoproteins may be associated with the blockage of arteries. CRP is a clinical marker of generalized body inflammation without symptoms. It is thought that if you have high levels of CRP, you may be at risk for future cardiovascular events such as a heart attack, stroke, angina and other medical conditions that are associated with "hardening or blockage of the arteries" known as atherosclerosis or arteriosclerosis.

About 15,000 other subjects will be recruited from the US, Canada, Europe and Latin America.

Section 3 Description of the Study and Procedures

Your participation in this study will be for a minimum of 3 1/2 years or until the study has completed. During this time you will be asked to visit the clinic for approximately 11 scheduled visits. The number of procedures will vary at each visit. Your participation will end when the study is over.

There are certain medications and food supplements that you must not take during the study. The research staff will review these with you. If you are taking any of these medications, you will be asked to stop taking them while participating in the study. If necessary, an acceptable

substitute will be suggested. If there is no acceptable substitute you will not be able to take part in the study. Please make sure to talk to your study doctor or the research staff before you take any new medication or new supplement during the study.

You must be willing to attend all scheduled visits, take the study drug as directed and have the required tests and procedures done for each visit. You will be asked not to eat or drink anything (referred to as fasting) except water for eight hours before certain visits. If you do not fast, you may be asked to return to the clinic within 3 days after fasting for 8 hours. Having your visit rescheduled will allow you to fast before your blood is drawn.

This study drug is yours and should not be taken by anyone else. If your study doctor instructs you to stop taking the study drug at any time during the study, you are still in the study and will continue to come back to the clinic for your remaining scheduled visits until the study is over. If you are not taking study drug and are unable to return to the clinic, you will receive a phone call from the clinic according to your scheduled visits to gather necessary follow-up information.

Midway between your scheduled visits and one month after your final visit, the study staff will contact you to ask you about your overall health since your previous visit to the clinic. If the study doctor looses contact with you during or after the study, the study doctor or study sponsor will:

- contact the people listed on your contact list
- employ the service of an outside agency to obtain necessary follow-up information
- do a search of the National Death Index

You are to continue with your normal exercise routine for the entire length of the study. You should consult with the study doctor prior to beginning any new exercise routine.

At the Screening Visit (Visit 1), you were found eligible to be in this research study.

At Visit 2 (2 weeks after Visit 1) you will:

- provide fasting blood and urine samples (no food or drink 8 hours before your visit)
- undergo a physical examination that includes height, weight, blood pressure, heart rate and waist measurement
- provide your complete medical history including current medical conditions, medications (including vitamins and herbal supplements), allergies, tobacco use, and family history of heart disease
- receive one bottle of study drug

You will be assigned to one of two treatment groups: rosuvastatin (20mg) or placebo (inactive pill). The treatment group to which you will be assigned will be decided randomly (by chance). You have an equal chance (50%) of receiving rosuvastatin or a placebo. You should return all bottles and unused tablets to the clinic at every visit.

At Visit 3 (4 weeks after Visit 2) you will:

- return any unused study drug and the bottle. A new bottle will be given to you with enough study drug for the next 3 months
- report on your overall health and let the study staff know if you are taking any new medications

At Visits 4 (3 months after Visit 3), 5, 7 and 9 (every 6 months) you will:

- provide blood and urine samples
- report on your overall health and let the study staff know if you are taking any new medications
- return any unused study drug and the bottles (even if empty). You will be given new bottles with enough study drug for you to take once a day until your next visit

Visits 6, 8 and 10 (every 6 months) you will:

- provide <u>fasting</u> blood and urine samples (no food or drink 8 hours before your visit)
- report on your overall health and let the study staff know if you are taking any new medications
- return any unused study drug and the bottles (even if empty). You will be given 2 new bottles with enough study drug for you to take once a day until your next visit

If extended visits are needed to complete the study, you will be asked to return at 6 month intervals until the study ends. At these visits you will:

- provide blood and urine samples
- report on your overall health and let the study staff know if you are taking any new medications
- return any unused study drug and the bottles (even if empty). You will be given 2 new bottles with enough study drug for you to take once a day until your next visit

At the Final Visit you will:

- provide <u>fasting</u> blood and urine samples (no food or drink 8 hours before your visit)

- undergo a physical examination that includes height, weight, blood pressure, heart rate and waist measurement
- report on your overall health and let the study staff know if you are taking any new medications
- return any unused study drug and bottles (even if empty)

Section 4 Risks, Discomforts and Inconveniences

The study drug, rosuvastatin, like all medications, may cause side effects. You may experience none, some or all of those listed below. There may be unknown risks involved in taking this drug. Please advise the study doctor immediately of any unusual symptom(s) that you might experience.

The side effects that have been previously reported with the study drug rosuvastatin have been usually mild and short-lived. The reported side effects are:

myalgia (muscle pain or weakness)	upset stomach (nausea)	liver abnormalities (increased liver enzymes)
abdominal (belly pain)	constipation	muscle abnormalities (increased muscle enzymes)

The study drug, rosuvastatin, may involve other risks, including possible life-threatening reactions, which are unknown at the present time. A rare side effect associated with statin medications is rhabdomyolysis (a rare life-threatening condition that occurs as a result of death of the muscle), which most often occurs with higher statin doses. Symptoms of rhabdomyolysis are muscle pain and weakness and, in some cases, rhabdomyolysis is associated with partial or complete shutdown of the kidneys. If the kidneys are affected, the urine output is reduced or absent and the color of the urine is dark. Another possible side effect associated with statin medications is myopathy (a condition that affects the muscles). The symptoms of myopathy include muscle weakness in the arms, legs, neck, muscle pain or tenderness, fatigue, and dark urine. You should discuss these side effects in more detail with your study doctor.

You should contact your study doctor immediately if you develop unexplained muscle pain or muscle weakness, fever, fatigue, or if you don't feel well. You should stop your study medication if this occurs.

A very small number of subjects developed kidney failure while taking rosuvastatin 80 mg/day (the dose for this study is only 20 mg). While these subjects had other illnesses and were on additional medications which could have caused kidney damage, it is not possible to exclude the possibility that rosuvastatin may have contributed to the kidney failure in these subjects.

A very rare side effect is jaundice or hepatitis. Hepatitis is a form of liver disease in which the eyes or skin become yellow due to the disease. If you notice jaundice or are told you have liver disease, please tell your study doctor immediately so he/she can perform a test to check your liver. If there are any problems that make it unwise for you to continue, the study medication will be stopped.

As with any medication, there may be other unknown side effects. If during the course of the study you experience any side effects, uncommon events or worsening of a condition you already have, you should immediately report them to the study doctor or research staff. If in the judgment of the doctor, there is any problem(s) related to the study drug, the study doctor may decide that the study drug should be stopped. If this should occur, you are still in the study and will return to the clinic for your remaining scheduled visits until the study is over.

Blood Collection

Over the course of the study, no more than 200 milliliters of your blood (approximately 16 tablespoons) will be collected for testing. During this procedure you may experience discomfort, pain, bleeding and/or bruising at the site of needle insertion. Occasionally, you may feel dizzy and/or faint during the blood collection procedure. There is also a risk of infection called phlebitis (on rare occasion) or a hematoma (blood clot) that could develop at the site where the blood was collected.

Possible Drug Interactions

Potential reactions with blood thinning medications such as Coumadin® (warfarin)

The study drug, rosuvastatin, is known to sometimes interact with warfarin (often called by the brand name Coumadin®), a medication that slows down the blood's ability to clot and is sometimes referred to as a "blood thinner". If you are taking warfarin, there is a slight possibility that the study drug may increase the effect of warfarin, causing your blood to become too thin. You could have an episode of bleeding, which may take the form of bruising, a nosebleed, bleeding gums, or rarely, internal bleeding. If you are currently taking warfarin, or if you begin to take warfarin at any time during this study, you must inform the study doctor and your family doctor. If you notice any increase in bleeding or bruising, you should report this to the study doctor immediately. In order to reduce the risk of abnormal bleeding, the study doctor may order extra blood tests called the International Normalized Ratio (INR) to see if the effect of warfarin has been increased, causing your blood to become too thin. If this is the case, the study doctor may adjust your dosage of warfarin. INR blood tests will also be done just before and just after you receive rosuvastatin for the first time. INR testing uses one-teaspoon of blood each time the test is performed.

Any Antacid such as Maalox® or Amphojel® should be taken at least 2 hours after the dose of study drug to avoid the possibility of reducing the effectiveness of rosuvastatin.

New Information

You will be given any new information about the study drug that becomes known during the course of this study that the study doctor or the study sponsor believes might reasonably affect your willingness to continue to take part in the study.

Section 5 Possible Benefits to Participants

You may or may not directly benefit from participating in this research study. It is possible that the risk of your chances of having a cardiovascular event may be decreased unless you receive placebo (inactive pill). Your study doctor cannot guarantee that you will benefit from your participation in this study. In the future, other people like you could benefit from the results of this research. Information gained from this research could lead to improved medical care for them. However, your study doctor will not know whether there are benefits to people with low to normal levels of LDL cholesterol but with elevated levels of CRP until all of the information obtained from this research has been collected and analyzed.

Section 6 Alternative Treatments

You do not have to take part in this study to be treated for elevated levels of CRP. However, there currently is no FDA-approved drug treatment for elevated CRP levels. You may discuss other options and their related risks/side effects with your primary care doctor.

Section 7 Treatment and Compensation for Injury Resulting from this Study

If you suffer any side effect or injury, notify your study doctor immediately so that you can receive appropriate medical treatment. If you suffer any side effect or other physical injury resulting directly from the study drug, the study sponsor will pay for the reasonable costs of medical treatment if:

- you took the study drug as directed by the study doctor;
- your injury was not deliberately caused;
- the study doctor was immediately notified about your injury; and
- you followed the medical advice of the study doctor.

You will not be paid for lost wages or other damages or losses or for medical expenses that have been covered by medical or hospital insurance or by third party or governmental programs providing such coverage. No other form of compensation is available from the study sponsor except remedies available under the law. In signing this form, you do not give up any legal rights. Payment of medical expenses is not an admission of fault or liability by the study sponsor or anyone else.

Section 8 Payment for Taking Part in the Study

You must complete Visit 3 to receive reimbursement for your travel expenses as follows: $60.00 after the completion of Visit 3; and $25.00 for each visit to the clinic completed until the study is over. Payment is not contingent on completing the entire study. No additional costs will be reimbursed.

Section 9 Expenses (Cost of Taking Part in the Study)

The study drug, study doctor's visits, and laboratory tests related to this study will be provided to you at no cost. Neither you, nor your insurance company will be charged for the study-related services provided during the course of the study.

Section 10 Taking Part is Voluntary

It is up to you whether to take part in this study or not. You may refuse to take part in the study or stop taking study drug at any time without penalty or loss of benefits to which you are otherwise entitled.

Section 11 Confidentiality and Authorization to Collect, Use and Disclose Your Medical Information

This section ("the Authorization") explains how your medical information will be collected, used and shared with certain other persons involved in the study and describes your rights, including the right to see your medical information.

Purpose of this Authorization

You are being asked to permit the collection, use and sharing of your medical information so that the safety and effectiveness of the study drug can be evaluated as described in section 2 above.

What Does Medical Information Mean?

Your medical information is information about your physical or mental health or condition. It includes:

- your previous medical records and information about you created or collected during the study (for example, the dates or results of various tests or examinations); and
- information about you that the study doctor may need in order to be able to monitor, and report to regulatory authorities on, the safety of the study drug and is created or collected by any physician, hospital or other healthcare provider that treats you outside of the study.

This information may identify you because it may contain, for example, your name, address, telephone number, photograph, date of birth, social security number, race or ethnic origin or other unique identifiers.

Use and Disclosure of Your Medical Information

If you sign this form, you allow the study doctor to collect and use your medical information to carry out this study. You also allow the study doctor to share your medical information with:

- the study sponsor, including its affiliates, its representatives and its contractors who work on behalf of the study sponsor to conduct the study
- other doctors and health care professionals who are involved in the study
- the Institutional Review Board (IRB) that watches over the study; and
- government agencies overseeing this study or the study drug, including the Food and Drug Administration (FDA), other Department of Health and Human Services agencies, and government agencies in the United States and other countries.

If you sign this form, you also authorize any physician, hospital or other healthcare provider that treats you outside of the study to disclose to the study doctor any medical information about you that the study doctor may need in order to be able to monitor, and report to regulatory authorities on, the safety of the study drug during the period required for this study. This authorization includes disclosure in the event of your death.

Will Persons Looking At Your Medical Information Be Able to Identify You?

That part of your medical information sent by the study doctor to the study sponsor ("study data") usually does not identify you personally (for example, by name, address, or social security number). Instead, the study doctor uses your initials and a code number on the study data sent to the study sponsor. However, the study sponsor, its representatives and contractors, regulatory authorities and other supervising bodies may look at all your medical information at the study doctor's site. The reason these persons may look at your medical information is to make sure the study has been done properly and that study data have been collected correctly, or for other reasons allowed by law.

Notice on Redisclosure of Your Medical Information and Confidentiality

Federal law provides that the study doctor can only share your medical information with those persons whom you have permitted to see it. However, if you sign this form, those persons may share your medical information with other persons. Federal law does not protect you against this. (The laws of your state may provide additional privacy protection.)

Publication of Study Results

Except as explained in this section 11, your medical information will be kept confidential. The data and results from this study may also be presented at meetings or in publications, but in those presentations people taking part in the study will not be identified by name.

Your Right to See and/or Copy Your Medical Information

You have the right to see and copy your medical information related to the study for as long as the study doctor holds this information. However, you may not be able to see some of your records related to the study until after the study has been completed, otherwise it could spoil the study.

Withdrawing Your Authorization

You may withdraw your Authorization (permission) regarding your medical information at any time by writing to the study doctor at the following address: << >>. If you withdraw this Authorization, the study doctor will no longer use your medical information or share it with others under the Authorization for this study, unless the study doctor needs to do so to protect the study data. However, the study sponsor may still use information about you that was shared with the study sponsor before you withdrew your Authorization.

If you withdraw your Authorization, you cannot continue to take part in the study.

Expiration of Your Authorization

Your Authorization will expire at the end of the study, following expiration of any required record retention period.

Section 12 Whom to Ask If You Have Questions

You have the right to ask questions about this study at any time and are encouraged to do so.

If you have any questions about this research study, ask the research study doctor or the research staff at *<<telephone # >>*.

If you experience an injury you believe is related to this study, contact *<<person>>* at *<<telephone # >>*.

If you have any questions about your rights as a research subject, you may contact *<<name of IRB >>* at *<< telephone # >>*.

Section 13 Right to Withdraw and Termination Procedures

You may withdraw from this study at any time without penalty or loss of benefits to which you are otherwise entitled. Your participation may be ended without your consent if you become ineligible to continue in the study, if you fail to follow the instructions of the study doctor, if you experience a study-related injury, or for any other reason.

When you stop taking part in the study, you must go through the termination procedures the study doctor considers necessary for your safety.

You must return all unused study drug to the study doctor.

Section 14 INFORMED CONSENT STATEMENT AND AUTHORIZATION:

I, _____ (name of subject), HAVE READ AND I UNDERSTAND ALL THE INFORMATION IN THIS INFORMATION AND CONSENT FORM. I HAVE BEEN GIVEN THE CHANCE TO DISCUSS IT AND ASK QUESTIONS. ALL MY QUESTIONS HAVE BEEN ANSWERED TO MY SATISFACTION. I VOLUNTARILY CONSENT TO TAKE PART IN THIS STUDY. I UNDERSTAND I WILL RECEIVE A COPY OF THIS INFORMATION AND CONSENT FORM.

BY SIGNING THIS INFORMATION AND CONSENT FORM, I HAVE NOT GIVEN UP ANY OF THE LEGAL RIGHTS, WHICH I OTHERWISE WOULD HAVE AS A SUBJECT IN A RESEARCH STUDY.

I AUTHORIZE THE COLLECTION, USE AND DISCLOSURE OF MY MEDICAL INFORMATION IN ACCORDANCE WITH THIS FORM.

_____ _____

Signature of Subject **Date of Signature**

_____

Printed name of Subject

_____ _____

Signature of Person Administering this Consent **Date of Signature**

_____

Printed Name of Person Administering this Consent

Genetic Research Addendum to Informed Consent Form

Title: A Randomized, Double-Blind, Placebo-Controlled, Multicenter, Phase III Study of Rosuvastatin (CRESTOR®) 20 mg in the Primary Prevention of Cardiovascular Events Among Subjects with Low Levels of LDL-Cholesterol and Elevated Levels of C-Reactive Protein (JUPITER)

Study Code: 4522US/0011 Subject Initials: << >>

Enrollment No: << >>

Sponsor: AstraZeneca Pharmaceuticals LP Wilmington, DE 19850-5437

An optional part of this study involves the collection of a blood sample from you for genetic (DNA) research. Before you decide whether you would like participate in this part of the study we would like to give you some more information.

Section 1 What is the Background to and Purpose of the Genetic Research?

Cells in your body contain a type of molecule called deoxyribonucleic acid, or DNA for short. DNA is what your genes are made of. Genes are inherited and direct growth, development, and how the body functions. For example, some genes control the color of your hair or eyes. Scientists have learned a lot about how genes work. There are many differences, or variations, in DNA from one person to another. These variations may affect a person's chance of suffering from a particular disease or the way a person responds to a particular drug. You are being asked to donate a blood sample for genetic research that will determine part of the structure of your DNA and enable us compare it to medical information about you. You are being asked to do this because you are already taking part in the JUPITER study, which we will call "the main study" in the rest of this document. We will use the words "this genetic research" for the genetic research we are now asking you to take part in.

We are asking you and other subjects participating in the main study to donate a blood sample because we want to study genetic differences in people who have low to normal levels of LDL and cholesterol but elevated levels of CRP. We also want to study and better understand the relationship between genetic markers and diseases. We will not use the blood sample you donate for other purposes.

The purpose of this genetic research is not to provide you with test results. The study sponsor (AstraZeneca) will not make any results available to you, any insurance company, your employer, your family, the study doctor, or any other physician who treats you now or in the future. You should be aware that the study sponsor has no obligation to conduct this genetic research, or any additional research on your blood sample or DNA.

You should be aware that the study sponsor does not have to conduct this genetic research, or any additional research on your blood sample or DNA.

Section 2 Study Procedure

If you decide to donate a sample, we will draw about 2 teaspoons (10 mL) of blood from you at Visit 2. DNA will be extracted from your blood sample. In this process, most of the original blood sample will be used up but a small amount will be kept as a "backup" in case of problems in the testing of your DNA.

The DNA sample and the remaining blood sample will be stored with similar samples from other people at a secure location. DNA and blood samples from this genetic research will be destroyed 15 years after the main study is completed. Your DNA may be studied at any time before this. The pattern of variations in your DNA may be compared with medical information collected in the main study, but only as this information relates to the research goals described above. The results from this genetic research may be analyzed along with results from other research.

Section 3 Risks and Inconveniences

These are no different from those experienced by you in having blood taken in the main study.

Section 4 Possible Benefits

There is no direct benefit to you in taking part in this genetic research. However, this research may contribute to our understanding of whether or not people with low to normal levels of LDL cholesterol but elevated CRP are at risk for developing atherosclerosis.

Section 5 Taking Part is Voluntary

It is up to you whether to donate a sample for genetic research or not. You may refuse to donate a sample at any time without penalty or loss of benefits to which you are otherwise entitled. You will receive the same treatment and care in the main study whether or not you donate a blood sample for genetic research as described in this document. **If you decide not to donate a sample, you can still take part in the main study.**

Section 6 No Payment for Taking Part

You will not be paid for donating a sample for use in this genetic research.

Section 7 Rights to the Results of Genetic Research

Any information derived directly or indirectly from this genetic research, as well as any patents, diagnostic tests, drugs, or biological products developed directly or indirectly as a result of this genetic research, are the sole property of the study sponsor (and its successors, licensees, and assigns) and may be used for commercial purposes. You have no right to this property or to any share of the profits that may be earned directly or indirectly as a result of this genetic research. However, in signing this form and donating a blood sample for genetic

research, you do not give up any rights that you would otherwise have as a participant in research.

Section 8 Confidentiality and Authorization to Collect, Use and Disclose Your Protected Information

In this section ("the Authorization") you are being asked for permission to use and share certain information about you so that the genetic research described in this document may be carried out. This section describes:

- how your protected information will be collected, used, and shared with certain other persons involved in this genetic research,
- how your confidentiality will be protected,
- your rights.

What is Your "Protected Information"?

In a file at the study site, the code number used to label your blood sample will be recorded next to your name. For this reason, information obtained from your blood sample, including the results of research on your DNA, are considered protected information. Special precautions will therefore be taken to ensure that the genetic research described in this document will be carried out with a very high degree of confidentiality.

Will Anyone be Able to Identify You?

Your blood sample will not be labeled with your name, but, as mentioned above, only with the same code that is given to you in the main study. As an added level of security your DNA, when it is extracted from your blood sample, and the results of any research on your DNA will receive a second code number. A file linking the first and second codes will be kept in a secure place at AstraZeneca with restricted access. If you change your mind about participating in this genetic research, this link will allow us to locate your sample and destroy it.

The coding of samples and results is to ensure that genetic research results are kept confidential by keeping your identity and these results separate. Very few people will be able to connect your identity and the results of research on your DNA, and only for special reasons. If there should be a medical emergency, an AstraZeneca doctor whose job it is to find out what caused the problem might know your identity and might also have access to the results of research on your DNA. AstraZeneca staff whose job it is to make sure the research has been done properly by checking the records at the study doctor's site, will also be able to identify you from your medical files but will not have access to the results of this genetic research. Apart from these persons, other research staff at AstraZeneca will not know your identity. Regulatory authorities, who also may wish to check that this genetic research has been done properly, will also have access to your files and know your identity.

The data and results of this genetic research may be reviewed with collaborators and published. Neither your name nor any other information that identifies you personally will appear in any publications or reports.

Use and Disclosure of Your Protected Information

If you sign this form and donate a blood sample, you allow the study doctor to use your protected information to carry out the genetic research described in this document. As noted above, there are special precautions taken to ensure that persons looking at the results of research on your DNA should not be able to identify you personally. Subject to those precautions, the study doctor may nonetheless share your protected information with:

- the study sponsor, including its affiliates, its representatives, and its contractors who work on behalf of the study sponsor to carry out this genetic research;
- other doctors and health care professionals who are involved in this genetic research;
- the Institutional Review Board (IRB) that watches over the main study; and
- government agencies overseeing this genetic research.

The blood samples that are donated to the study sponsor, and the DNA prepared from them, will not be given or sold to anyone else, nor will they be used for purposes other than the genetic research described in this document.

Notice on Redisclosure of Protected Information

The study doctor can only share your protected information with persons whom you have permitted to see it, and only in the ways you have permitted. However, if you sign this form those persons may share your protected information with other persons. Federal law does not protect you against this, but the laws of your state may provide additional protection. However, the study sponsor will only share your protected information with its and its affiliates' staff involved in this genetic research and with the persons whom you permit to receive it under this form.

Section 9 Withdrawing your Consent and Authorization

You may withdraw your consent to the use of your sample in genetic research at any time. If you withdraw your consent **before** your blood sample is sent for genetic research, the study doctor will arrange to have it destroyed. If you withdraw your consent **after** your blood sample has been sent for genetic research the study sponsor and the study doctor will ensure that your blood sample and any DNA that has been extracted from it are destroyed. However, if genetic research has already been performed the study sponsor is not obliged to destroy results of this research. In this case only the blood sample and any extracted DNA will be destroyed.

You may withdraw your Authorization (permission) regarding the use and disclosure of your protected information at any time by writing to the study doctor at the following address: << >>. If you withdraw this Authorization, the study doctor will no longer use your protected information or share it with others under the Authorization for this genetic research, unless the study doctor needs to do so to protect the research results. However, the study sponsor may still use information about you that was shared with the study sponsor before you withdrew your Authorization.

If you withdraw your Authorization, you can continue to take part in the main study unless you withdraw your Authorization for the main study.

Expiration of Your Authorization

In signing this form, you authorize the use and disclosure of your protected information as necessary to carry out the genetic research described in this document. Your Authorization will expire 15 years after the main study is completed.

Section 10 Whom to Ask if You Have Questions

If you have questions about donating a sample, any study-related injury, or your rights as a subject please contact:

Dr *<<name>>*

Phone No. << >>

Nurse *<<name>>*

Phone No. << >>

Address *<<<>>*

If you have questions about your rights as a research subject, you may contact:

<<name>>

<<Phone No.>>

Section 11 Informed Consent Statement and Authorization

I, _____ (name of subject), have read and I understand all the information in this informed consent and authorization form. I have been given the chance to discuss it and ask questions. All my questions have been answered to my satisfaction. I voluntarily consent to take part in this genetic study. I understand I will receive a copy of this informed consent and authorization form.

By signing this informed consent and authorization form, I have not given up any of the legal rights, which I otherwise would have as a subject in a research study. I authorize the collection, use and disclosure of my medical information in accordance with this form.

_____ _____

Signature of **Subject** Date of Signature

To be signed and dated by the subject

_____

Printed name of **Subject** (BLOCK CAPITALS)

_____ _____

Signature of person conducting the informed consent discussion Date of Signature

_____

Printed name of person conducting the informed consent discussion (BLOCK CAPITALS)

I do not consent to have my blood used for genetic (DNA) testing under the conditions described in this section.

_____ _____

Signature of Subject Date of Signature

_____

Printed name of Subject (BLOCK CAPITALS)

_____ _____

Signature of person conducting the informed consent discussion Date of Signature

_____

Printed name of person conducting the informed consent discussion (BLOCK CAPITALS)