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A study of the Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) in glucose and insulin homeostasis

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Abstract

Atherosclerotic cardiovascular disease (ASCVD) is thought to account for >30% of all deaths today. Patients at very high risk, who suffer from ASCVD and comorbidities, such as diabetes, are projected to have 10-year event risks between 26% to 43%. Considering that low-density lipoprotein cholesterol (LDLc) is the main driver of ASCVD and that reducing its circulating levels was demonstrated to be beneficial in reducing clinical events, new guidelines required patients to reduce their LDLc levels to very low levels. Therefore, therapeutic agents are currently being developed in order to decrease this societal predicament. One of the very promising agents is directed towards lowering proprotein convertase subtilisin/kexin type 9 (PCSK9) synthesis or circulating levels. Indeed, clinical trials on these PCSK9-inhibitors demonstrated safety and beneficial effects on cardiovascular outcomes. Several research groups are interested in determining whether PCSK9 may be important for other processes than cholesterol homeostasis. Indeed, some groups propose that PCSK9 could play a role in the development of diabetic dyslipidemia. Other groups are concerned over the possibility that targeting PCSK9 could lead to the excessive ectopic accumulation of cholesterol and lipid particles in insulin sensitive tissues, resulting in lipotoxicity, insulin resistance and/or impaired insulin secretion. Therefore, we addressed these questions in this thesis. In the first section we investigated the possible direct effect that PCSK9 could have on the function and biosynthesis of the insulin receptor in cell culture and in mouse liver. In the second section we investigated whether the absence of PCSK9 in mice could lead to impaired glucose and insulin homeostasis. Our main results demonstrate that exogenous PCSK9 decreases insulin receptor signaling and *in vitro* expression leads to decreased biosynthesis likely due to upregulation of acute endoplasmic reticulum (ER) stress. Analyses of pancreatic β-cells and hepatocytes further demonstrated that the absence of PCSK9 in 6-month old male mice does not lead to impaired glucose or insulin homeostasis. Our studies provide a new mechanism for the potential role of PCSK9 in lipid homeostasis, suggesting that by decreasing insulin signaling, PCSK9 could promote the development of dyslipidemia, although further investigations are required to validate this hypothesis. Our analysis suggests that targeting PCSK9 in diabetic or pre-diabetic patients would be safe and could even be beneficial, keeping in mind, however, the limitations of our studies which are based on mouse models that inherently have low levels of LDLc.

Résumé

La maladie cardiovasculaire athéroscléreuse (ASCVD) représenterait plus de 30% de tous les décès aujourd'hui. Les patients à très haut risque, qui souffrent d'ASCVD et de comorbidités, telles que le diabète, devraient présenter des risques d'événements sur 10 ans entre 26% et 43%. Considérant que le cholestérol à lipoprotéines de basse densité (LDLc) est le principal moteur de l'ASCVD et que la réduction de ses taux circulants s'est avérée bénéfique pour réduire les évènements cliniques, de nouvelles lignes directrices ont exigé des patients qu'ils réduisent leurs taux de LDLc à des niveaux très bas. Par conséquent, des agents thérapeutiques sont actuellement en cours de développement afin de réduire cette situation difficile pour la société. L'un des agents très prometteurs vise à abaisser la synthèse de la proprotéine convertase subtilisine / kexine de type 9 (PCSK9) ou les taux circulants. En effet, les essais cliniques sur ces inhibiteurs de la PCSK9 ont démontré leur innocuité et leurs effets bénéfiques sur les résultats cardiovasculaires. Plusieurs groupes de recherche sont intéressés à déterminer si la PCSK9 peut être importante pour d'autres processus que l'homéostasie du cholestérol. En effet, certains groupes proposent que la PCSK9 pourrait jouer un rôle dans le développement de la dyslipidémie diabétique. D'autres groupes s'inquiètent de la possibilité que le ciblage de la PCSK9 puisse entraîner une accumulation ectopique excessive de cholestérol et de particules lipidiques dans les tissus sensibles à l'insuline, entraînant une lipotoxicité, une résistance à l'insuline et / ou une altération de la sécrétion d'insuline. Par conséquent, nous avons abordé ces questions dans cette thèse. Dans la première section, nous avons étudié l'effet direct que la PCSK9 pourrait avoir sur la fonction et la biosynthèse du récepteur de l'insuline en culture cellulaire et dans le foie de la souris. Dans la deuxième section, nous avons examiné si l'absence de PCSK9 chez la souris pouvait entraîner une altération de l'homéostasie du glucose et de l'insuline. Nos principaux résultats démontrent que la PCSK9 exogène diminue la signalisation des récepteurs de l'insuline et que l'expression *in vitro* conduit à une diminution de la biosynthèse probablement due à une augmentation du stress du réticulum endoplasmique aigu. L'analyse des cellules β pancréatiques et des hépatocytes a en outre démontré que l'absence de la PCSK9 dans les souris mâles âgées de 6 mois n'altère pas homéostasie du glucose ou de l'insuline. Nos études fournissent un nouveau mécanisme pour le rôle potentiel de la PCSK9 dans l'homéostasie lipidique, suggérant qu'en diminuant la signalisation de l'insuline, PCSK9 pourrait favoriser le développement de la dyslipidémie, bien que des

investigations supplémentaires soient nécessaires pour valider cette hypothèse. Pour conclure, notre analyse suggère que le ciblage de PCSK9 chez les patients diabétiques ou pré-diabétiques serait sûr et pourrait même être bénéfique, en gardant à l'esprit, cependant, les limites de nos études qui sont basées sur des modèles murins qui ont intrinsèquement de faibles niveaux de LDLc.

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List of abbreviations

ABCA1: ATP-binding cassette transporter subfamily A member 1

ACC: acetyl-CoA carboxylase

ADH: autosomal dominant hypercholesterolemia

AnxA2: annexin A2

apo(a): apoprotein(a)

apoB: apolipoprotein B

ApoE-R2: apoE receptor 2

ARH: autosomal recessive hypercholesterolemia

ASCVD: atherosclerotic cardiovascular disease

BFA: brefeldin A

BMI: body mass index

CAD: coronary artery disease

CAP1: adenylyl cyclase-associated protein 1

CETP: cholesteryl ester transfer protein

CHCs clathrin-heavy chains

CHRD: C-terminal cysteine-, histidine rich domain

ChREBP: carbohydrate responsive element binding protein

CLCs: clathrin-light chains

CNX: calnexin

CRT: calreticulin

CV: cardiovascular

DNL: *de novo* lipogenesis

DS: Donohue Syndrome

EGF: epidermal growth factor

ERAD: ER-associated degradation

FAS: fatty acid synthase

FFAs: free fatty acids

FH: familial hypercholesterolemia

FPG: fasting plasma glucose

GOF: gain-of-function

GSIS: glucose-stimulated insulin secretion

GSK3: glycogen kinase 3

GTT: glucose tolerance test

GWAS: genome wide association studies

HBA1c: glycated hemoglobin A1

HDL: high-density lipoproteins

HEK293: human embryonic kidney cells

HepG2: human-derived hepatoma cells

HMG-CoA: hydroxymethyglutaryl co-enzyme A

HMGCR: HMG-CoA reductase

HoFH: homozygous FH

HOMA-IR: homeostasis model assessment of insulin resistance

IDL: intermediate-density lipoproteins

IGF-1 and -2: insulin-like growth factors-1 and -2

IHH: immortalized human hepatocyte

InsR: insulin receptor

InsR-A: InsR isoform A

IPGTT: intraperitoneal glucose tolerance test

IR: insulin resistance

ITT: insulin tolerance test

LBD: ligand-binding domain

LD: lipid droplets

LDL: low-density lipoproteins

LDLc: low-density lipoprotein cholesterol

LLTs: lipid lowering treatments

LOF: loss-of-function

Lp(a): lipoprotein (a)

LPL: lipoprotein lipase

LRP-1: LDL related protein-1

LXR: liver X receptor

mAb: monoclonal antibodies

MRI: magnetic resonance imaging

mTORC: mammalian target of rapamycin complex

MTP: microsomal triglyceride transfer protein

MUFAs: mono-unsaturated fatty acids

NAFLD: non-alcoholic fatty liver disease

PAD: perigonadal adipose tissue

PCs: proprotein convertases

PCSK9: proprotein convertase subtilisin/kexin type 9

PDK1: 3-phosphoinositide-dependent protein kinase 1

PERPP: post-ER pre-secretory proteolysis

PI3K: phosphoinositide-3-phosphate kinase

PIP3: phosphatidylinositol (3,4,5)-trisphosphate

proInsR: precursor of InsR

proPCSK9: precursor of PCSK9

PTEN: phosphatase and tensin homolog

PTT: pyruvate tolerance test

PUFAs: poly-unsaturated fatty acids

Q-PCR: quantitative polymerase chain reactions

RIDD: regulated Ire1-dependent decay

RMS: Rabson-Mendenhall syndrome

Scd1: stearoyl-CoA desaturase-1

sdAb: single domain antibody

sdLDL: small dense lipoproteins

SH3BD: SH3 binding domain

SREBP-2: sterol-regulatory element binding protein -2

sXBP1: spliced XBP1

T2DM : type II diabetes mellitus

TFRC: transferrin receptor

TG: triacyglycerol

TGN: *trans*-Golgi Network

TRL: TG rich lipoprotein

UPR: unfolded protein response

VLDL: very-low density lipoproteins

VLDLR: VLDL receptor

Preface

This PhD thesis was written in accordance to the manuscript-based thesis guidelines. It is presented as four chapters including an introductory literature review, two research articles in the process of publication and a concluding thesis discussion.

The work conducted during this thesis pertained to a novel protein, PCSK9, that has immediately upon its discovery gained large interest in the field of atherosclerosis. Indeed, in just over 10 years pharmacological inhibitors targeting this protein were approved in several developed countries in efforts to reduce atherogenic cholesterol to extremely low circulating levels. The societal and economic impact of such a discovery will certainly become obvious in the coming years. The focus of this thesis, however, relates to the possible roles PCSK9 may play in glucose and insulin homeostasis. Several publications in the recent years have associated either increased PCSK9 or absence of PCSK9 with insulin resistance or increased risk for diabetes. The work presented in this thesis provides both new underlying mechanisms for these associations and help improve the understanding for the role PCSK9 plays in insulin responsive tissues.

The elements of the thesis that are considered original scholarship and distinct contributions to knowledge are listed here:

- Identification of a new target of PCSK9 *in vitro*, the insulin receptor;
- Identification of a new mechanism for PCSK9 *in vitro*, upregulation of acute ER stress;
- Identification of a new regulatory mechanism for PCSK9 of the insulin receptor signaling pathway *in vivo* and *in vitro*;
- Direct implication of PCSK9 in insulin homeostasis;
- Characterization of *Pcsk9* KO mice glucose and insulin homeostasis in the pancreas and liver in comparison to WT mice;
- Characterization of liver lipogenesis in *Pcsk9* WT and KO mice following a fasting and refeeding challenge;
- Identification that the insulin receptor increases its total and cell surface levels during fasting, irrespective of PCSK9 levels.

List of publications:

- Manuscript 1 “PCSK9 a negative regulator of the insulin receptor: effects on signaling activity and biosynthesis rate” is in the process of submission to The Journal of Biological Chemistry;
- Manuscript 2 “Islet and liver insulin-related phenotypic characterization of *Pcsk9*$^{-/-}$ mice” is in the process of submission to The Journal of Biological Chemistry.

Author contributions

The experiments were designed, analyzed and conducted by me and invaluable assistance from collaborators is detailed under the author contribution sections of both Chapters II and III. Both manuscripts were written by myself with guidance from my supervisor Dr. Nabil G. Seidah. The overall thesis was overseen by committee members with profound knowledge and experience in the research subject.

Chapter I: Introduction

I.1 Literature review

In the study of PCSK9 in glucose and insulin homeostasis this thesis begins with an introduction that includes brief historical overviews of key proprotein convertase (PC) discoveries, most relevant studies elucidating PCSK9 expression, function and main interactors and PCSK9 therapeutical inhibition. Moreover, since the insulin receptor (InsR) was identified in this thesis as a new target of PCSK9, this introduction then overviews the structural and functional properties of the InsR and its importance in diabetes. Finally, the introduction to this thesis overviews the literature related to cholesterol mediated diabetes development.

I.1.1. PCSK9: the 9th member of the family of proprotein convertases

Proteins are the effector molecules of cell biology. They are the end-product of the more stable, inherited, genetic material, DNA, guardian of speciation and evolution. Modifications of the genome through gene mutations, horizontal gene transfer and sexual reproduction, allow for the creation of new pathways and regulatory mechanisms, cell differentiation, cell memory and defense – immunity, or cell invasion. There are ~20,000 protein coding genes in the human genome but taking into account alternative splicing, amino acid polymorphisms and post-translational modifications each of these genes could give rise to 100 different proteins (Ponomarenko et al., 2016). It is the post-translational modifications of proteins that drive cell kinetics. The great diversity of these rapid protein modifications enables the regulation of numerous processes including protein quality control, concentration gradients of signaling molecules, membrane tethering of cytosolic proteins, changes in three-dimensional chromatin conformation. Most of these modifications are reversible although some, like proteolytic cleavage, are irreversible and are important for the activation or inactivation of signaling proteins (such as growth factors and hormones). This section will review the mammalian family of proprotein convertases (PCs), their discovery, the regulation of their enzymatic activity, their ancestral origins and preservation across metazoan species.

I.1.1.1 Discovery of proprotein convertases

In the mid-twentieth century, extensive peptide sequencing using Sanger and Edman's methods generated polypeptide sequences from purified proteins culminating in the prohormone theory, which was simultaneously and independently enunciated by two teams in 1967, Michel Chrétien with Choh Hao Li (Chretien and Li, 1967) and Donald Steiner with Philip Oyer (Steiner and Oyer, 1967). These two groups studied the pro-opiomelanocortin gene and the proinsulin gene respectively. This theory stated that secretory bioactive peptides are generated from a common precursor propeptide by site-specific endoproteolytic cleavage at paired-basic residues, a new post-translational modification and the first indication of the existence of endogenous proprotein convertases.

Studies in yeast *Saccharomyces cerevisiae* later identified mutant strains that no longer secreted killer toxin and the biologically active mating pheromone α-factor (Leibowitz and Wickner, 1976; Wickner and Leibowitz, 1976). The absence of this activity cosegregated with the *Kex2* mutants in several genetic crosses leading to the cloning of the normal *Kex2* gene and the prediction of its coded protein "kexin" (Julius et al., 1984). Expression of wild-type kexin in yeast mutants restored the proteolysis of the prepro-α-factor and secretion of the biologically active α-factor. This study was the first validation for the biological relevance of a eukaryotic endoproteolytic enzyme (Julius et al., 1984). Hence, the identification in 1984 of the first processing enzyme began with the cloning of yeast *Kex2* and the identification of kexin (Julius et al., 1984). Roebroek *et al.* then identified a fragment of the human *Furin* gene in 1986 as a proto-oncogene with putative receptor recognition function (Roebroek et al., 1986). However, it was Fuller and colleagues in 1989 that suggested that "furin" would be the first candidate for a human prohormone-processing enzyme since the catalytic domains of furin and kexin shared 50% identity (Fuller et al., 1989). The recognition that kex2 belongs to the subtilisin-like (subtilase) family of serine proteases was first proposed by Mizuno *et al*. (Mizuno et al., 1988). This was then rapidly extended in 1990 to furin (van de Ven et al., 1990). The other kexin-like mammalian subtilases were identified in the 1990s by probing cDNA libraries with consensus sequences of the kexin-like and subtilisin-like catalytic domain of subtilases: PC1/3 (Seidah et al., 1990; Smeekens and Steiner, 1990), PC2 (Seidah et al., 1991; Smeekens et al., 1991), PC4 (Mbikay et al., 1994; Nakayama et al., 1992; Seidah et al., 1992), PC5/6 (Lusson et al., 1993; Nakagawa et al., 1993),

PACE4 (Kiefer et al., 1991), PC7 (Seidah et al., 1996), SKI-1/S1P (a pyrolysin-like PC) (Sakai et al., 1998; Seidah et al., 1999), and PCSK9 (a proteinase K-like PC) (Seidah et al., 2003).

I.1.1.2 PCs are subtilisin-like proteases

Serine proteases are one of five classes of proteolytic enzymes, which together represent ~1.7% of the mammalian genome (Lopez-Otin and Overall, 2002). Most of the serine peptidases may be grouped into six clans based on the three-dimensional fold of the catalytic domain, the two largest clans being chymotrypsin and subtilisin. Together with the carboxypeptidase C they share a common "catalytic triad" of three amino acids, serine nucleophile, aspartate electrophile and histidine base (Rawlings and Barrett, 1994). Members of the subtilisin clan are further subdivided into six families based on sequence homology of the catalytic domain and are expressed in bacteria, archaea and eukaryota. The bacterial subtilisin-like, bacterial thermitase-like, yeast kexin-like and fungus proteinase K-like families demonstrate a high degree of sequence similarity between family members (from >37% to >64% identity) (Siezen, 1996), whereas the bacterial lantibiotic-like and archaea pyrolysin-like families are characterized by low sequence similarity between members and to other subtilases (Siezen, 1996).

The catalytic core, defined by highly conserved residues and structures, contains α-helix and β-strand elements, the catalytic triad (Asp, His, Ser) and the Asn "oxyanion hole", which is important to stabilize the tetrahedral transition state (Siezen, 1996) (view Figure I.1.1). The only accepted substitute for the Asn residue is an Asp in the PC2 subgroup of the kexin family. The catalytic domain is always located at the N-terminus of the polypeptide sequence, directly after the prodomain. The majority of subtilases are synthesized as pre-pro-enzymes, with a signal peptide required for translocation of the nascent chain into the ER lumen. Cleavage of the signal peptide generates the PC-zymogen with the prodomain at the N-terminus, and further cleavage results in the mature protease. However, there are examples of subtilases that are not synthesized as zymogens, and while the large majority are secreted, some are intracellular such as tripeptidyl-peptidase II (Tomkinson, 1994). Interestingly, in most bacteria, archaea and lower eukaryotes subtilases are active extracellularly, under alkaline conditions. They cleave mostly non-specifically and are required for defense or growth from proteinaceous substrates. Whereas in

higher eukaryotes subtilases are involved in the specific processing and maturation of proproteins (Seidah, 2011).

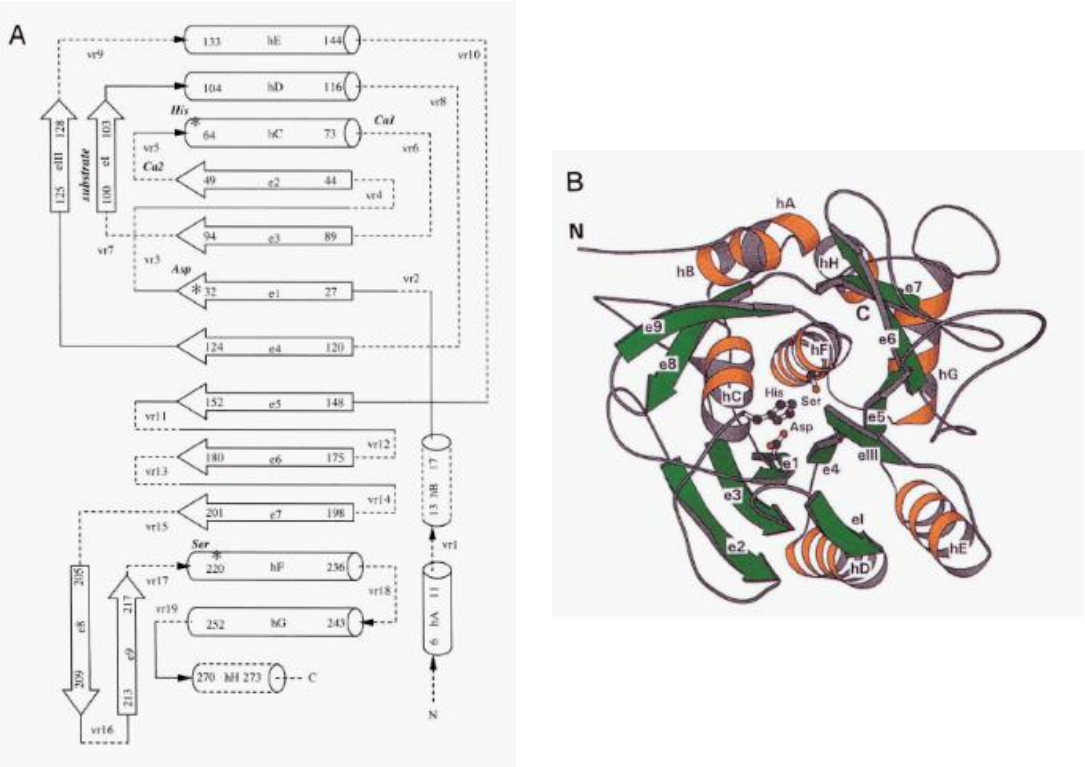

Figure I.1. 1 Secondary and tertiary structure of subtilases' catalytic domain

Schematic representation of the core subtilases' catalytic domain secondary and tertiary structures. ***A,*** topologic representation, with α-helices as cylinders and β-strands as arrows. Are also shown, catalytic triad residues, Ca^{2+} binding sites and substrate binding site. ***B,*** tertiary structure representation of subtilisin, where catalytic residues are shown in ball-and-stick representation (Siezen, 1996).

I.1.1.3 PC functional diversification

The mammalian PC-family comprises seven kexin-like PCs: PC1/3, PC2, furin, PC4, PC5/6-A, PC5/6-B (isoforms resulting from alternative splicing), PACE4 and PC7; a pyrolysin-like PC: SKI-1/S1P; and a proteinase K-like PC: PCSK9 (Seidah and Prat, 2012) (view Figure I.1.2). The kexin-like PCs cleave peptide bonds C-terminal to a consensus dibasic motif: (**K**/**R**)-X*n*-(**R/K**)↓ Pi′ where X can be any amino acid except Cys and X*n* = 0, 2, 4, or 6 residues. The

enzyme SKI-1 recognizes the motif (**R**/**K**)-X-(**L**, **I**, **V**)-Z↓, where Z is any amino acid except Pro, Cys, Glu, and Val. PCSK9 autocatalytically cleaves itself at the **VFAQ↓SIP**. Site-directed mutagenesis suggested that Val (P4) and Pro (P3') are the most critical amino acids in this motif, with lesser contributions by Phe (P3) (Benjannet et al., 2004). In addition, only proPCSK9 containing a P1 Gln, Ala, Met, Asn, Ser, and Thr allow the secretion of PCSK9, with P1 Met being the only amino acid at P1 that behaves like the wild type Gln (Benjannet et al., 2012). More recently, the partial contribution of the Ser at P6 in the sequence SSVFAQ↓SIPWNL was also suggested and the identities of P2'- P6' were shown to have little effect on cleavage (Chorba et al., 2018).

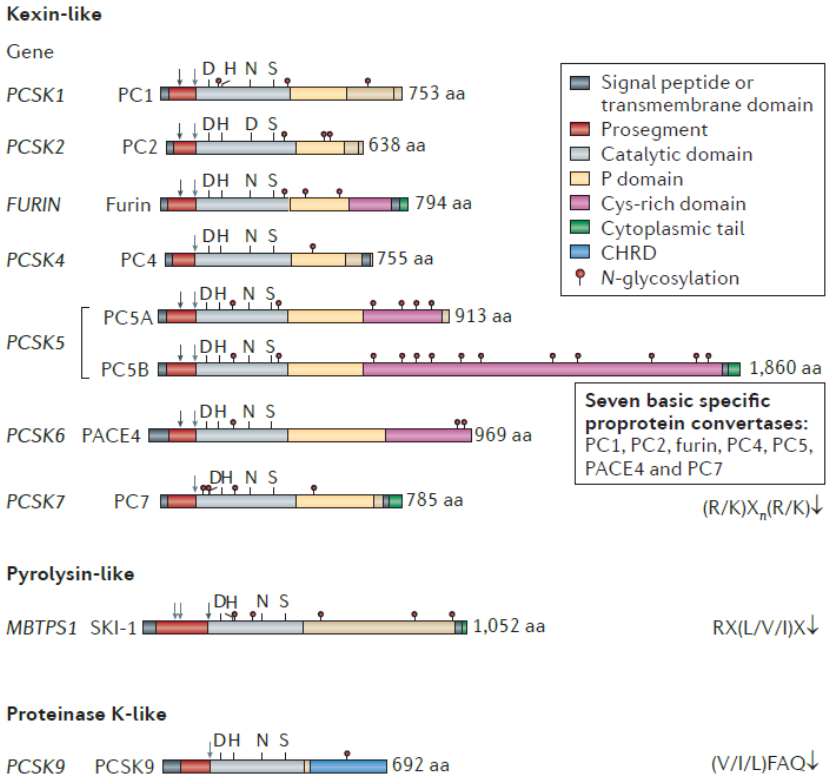

Figure I.1. 2 Primary structure of human proprotein convertases

The nine different human proprotein convertases are arranged into their subclasses, kexin-like, pyrolysin-like and proteinase K-like. Are shown, their respective domains, catalytic residues, N-glycosylation positions, cleavage sites and recognition motifs (Seidah and Prat, 2012).

Achievement of the crystal structure of the mouse furin and yeast kexin ectodomains allowed the structural modelling of the other mouse and human PCs. These three-dimensional models were key to describe the enzyme/substrate interactions, particularly within the active-site

cleft (Henrich et al., 2005; Holyoak et al., 2004; Holyoak et al., 2003). These results revealed an important clustering of negatively charged residues in the substrate binding region of all the kexin-like PCs corresponding to a requirement for positive charges of basic amino acids at the N-terminus of the substrate cleavage site (Henrich et al., 2005). Furin contains 16 acidic residues in that cluster, PACE4, PC5/6 and PC4 have 15 each, and PC7, PC1/3 and PC2 have 13. The surface distribution of these charges is also important to match substrate opposing charges. Interestingly, PC4, PC5/6 and especially furin share a preference for substrate basic residues at P6 and P7 possibly due to the positioning of their negative charges in the catalytic cleft (Henrich et al., 2005). The global charge and surface distributions patterns demonstrate that PACE4, PC4, PC5/6 resemble furin closely, whereas PC1/3, PC2 and PC7 are closer to kexin. Suggesting an early evolutionary separation into furin-like PCs subsequent to gene duplication events (Henrich et al., 2005).

PC functional diversification and specific substrate cleavage is also made possible by tightly controlled enzymatic activity. As described previously, the majority of subtilases are synthesized as proproteins, or zymogens, including the PCs. The prodomain is cleaved by a primary intramolecular autocatalysis in the ER (for all PCs but PC2) and acts as a chaperone required for the folding of the protein into its proper tertiary conformation (Ikemura et al., 1987). Subsequently, the prodomain remains non-covalently bound to the catalytic cleft with nanomolar affinity acting as a competitive inhibitor and maintaining the enzyme in an inactive state until it has reached the subcellular localization where the cognate substrates are required to be activated or inactivated (Anderson et al., 1997; Creemers et al., 1995). The C-terminal domain differs between PCs and may contain a transmembrane domain. Indeed, furin, PC7, PC5/6-B and SKI-1 are type-I transmembrane proteins, whereas the others are soluble luminal proteins. The cytosolic tail then encodes trafficking motifs which will determine their enrichment in different subcellular compartments. Furin, PC7, PC5/6, PACE4 are trafficked through the constitutive secretory pathway and are activated in the TGN/endosomes (or at the cell surface for PACE4, PC5/6). Active furin and PC7 can cycle from the plasma membrane back to the TGN through endosomes (Thomas, 2002). For example, a recently identified motif E*X*E*XXX*L^{725} in the C-terminal tail of PC7 was found to be critical for the endosomal localization and activity of PC7 mediated human transferrin receptor shedding (Declercq et al., 2012; Durand et al., 2020). PC1/3, PC2 and sometimes PC5/6-A are sorted to immature secretory granules of endocrine and neural cells where they are activated

therein (Day et al., 1992; Lusson et al., 1993). SKI-1 is active in the *cis-medial* Golgi, and PCSK9 is the only PC that remains catalytically inactive due to association of the mature enzyme with its inhibitory prodomain, and for which no enzymatic substrates but itself have been identified. The kexin-like PCs contain a P domain C-terminal to the catalytic domain, which is required for pH and calcium sensing (Henrich et al., 2003; Tangrea et al., 2002). It is important for the activation of the enzyme by inducing the second less susceptible cleavage (only required for some PCs) and release of the inhibitory prodomain in the more acidic endosomes and mature secretory granules (Anderson et al., 1997; Zhou et al., 1998). PC1/3 and PC2 activities are also regulated by natural inhibitors proSAAS and 7B2 respectively (Cameron et al., 2000; Martens et al., 1994). 7B2 is a pan neurosecretory cell protein, a chaperone of proPC2 required for the exit from the ER (Benjannet et al., 1995; Hsi et al., 1982; Marcinkiewicz et al., 1986; Seidah et al., 1983). In the secretory granules lowered pH and increased calcium concentrations induce the release of 7B2 and the prodomain resulting in active PC2.

These mechanisms could explain how the PCs developed substrate specificity in contrast to the more promiscuous prokaryotic orthologues. Limited proteolysis by PCs likely developed with multicellular organisms to enable intercellular communication.

I.1.1.4 Origins and preservation of PCs through Metazoa

In order to study the pre-vertebrate evolutionary history of PCs, Tao *et al.* identified the presence of kexin-like PCs in several metazoan (all animals having a body composed of differentiated cells into tissues and organs) genomes (Tao et al., 2019). They demonstrated that the last common ancestor of the modern bilaterian (~650 mya) had *PCSK7*, *PCSKX* (a kexin-like PC that was lost in vertebrates), *PCSK2*, *PCSK1*, *furin1* and *furin2/PCSK5* genes. They suggested that a gene duplication event occurred in the ancestral filosozoan or holozoan (premetazoan, >750 mya) to give rise to *PCSK7*, the most ancient kexin-like PC. Gene duplication in the ancestral planulozoan then gave rise to *PCSK1* and a furin-like gene. The relationship between the other PCSK orthologue groups was not resolved, however they seem to have arisen from several gene duplication events from *furin1* and *furin2* in the vertebrates forming two sister clans: *furin* (*PCSK3*) and *PCSK4*, and *PCSK5* and *PCSK6*. *PCSK2* may have arisen in the ancestral planulozoan prior to *PCSK1* and the furin-like genes. These described phylogenic relationships are

very similar to those proposed previously by Seidah *et al.* (view Figure I.1.3). This research group did not study *SKI-1/S1P* (*PCSK8* orthologue group) and *PCSK9* origins, however homologs were reported in *Drosophila* (Seegmiller et al., 2002) and in *Arabidopsis* (Liu et al., 2007) for the former and in a few metazoan species such as amphioxus, sea cucumber and gastropods for the latter (Cameron et al., 2008a; Ren et al., 2016). Suggesting that *PCSK9* was present in the nephrozoan, whereas the report of a homolog of *SKI-1/S1P* in the Kingdom Plantae suggests a premetazoan origin for this gene.

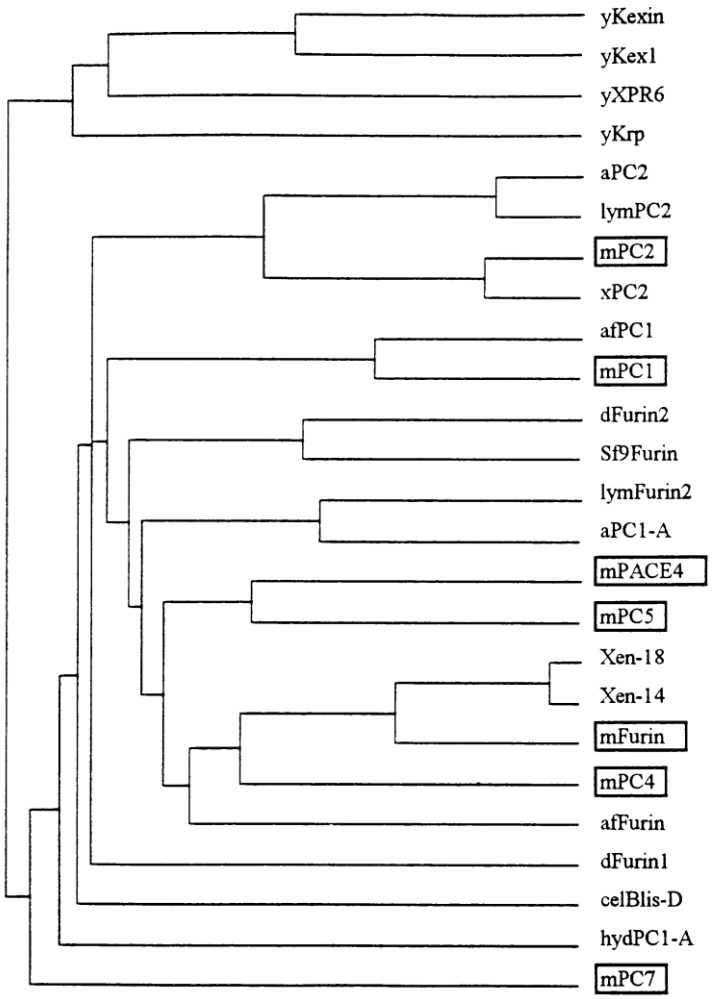

Figure I.1. 3 Phylogenic tree of the seven kexin-like mouse PCs

Mouse PCs' evolutionary tree is represented and complemented with other species' PCs (Seidah et al., 1998).

Tao *et al.* contemplated in their study whether PC diversification may be traced back to their bilaterian ancestors. They studied the leech *Helobdella austinensis*, a spiralian protostome

(common ancestor with vertebrates is the bilaterian). They demonstrated that the *PCSK1* and *PCSK2* homologs displayed tissue-specific expression, where *Hau-Pcsk1* localized to the supraoesophageal ganglion (the leech brain) and where *Hau-Pcsk2* localized to the central nervous system (CNS) and cell clusters of the gut, likely exocrine cells. Therefore, they confirmed that the *PCSK1* and *PCSK2* are likely involved in neurosecretion in the *Helobdella*. Since it is known that the mammalian *PCSK1* and *PCSK2* expressions are restricted to neuroendocrine cells, their data suggests the conservation of an ancestrally acquired functional specialization. In contrast, furin-like PCSKs, *Hau-Pcsk7*, and *Hau-Pcskx* were broadly expressed as is it also true for the mammalian *FURIN* and *PCSK7*.

In summary, Tao and colleagues demonstrated that the kexin-like PCs, the pyrolysin-like PCs and the proteinase K-like PCs have very deep origin suggesting that they have evolved independently in parallel and are characterized by functional convergence of proprotein convertase activity. Moreover, their non-redundant enzymatic activities have been essential for the preservation of their orthologue genes across metazoan species since Urbalateria (Tao et al., 2019).

I.1.1.5 PC physiological functions

Studies using gene knockout mouse models have helped identify PC specific *vs.* redundant enzymatic activities and important physiological roles (Creemers and Khatib, 2008; Scamuffa et al., 2006; Seidah and Prat, 2012). The principal phenotypes are summarized in Table I.1.1. The absence of furin, PC5/6 and SKI-1/S1P is embryonic lethal, suggesting important non-redundant activities and substrates in the developing embryo. Furin inactivation results in serious defects in the cardiovascular system and embryonic death from hemodynamic insufficiency and cardiac ventral closure defects (Constam and Robertson, 2000b; Roebroek et al., 1998). PC5/6 gene knockout results in impaired activation of numerous growth factors required for the normal development of the embryo, such as platelet-derived growth factors, bone morphogenic proteins and Lefty proteins (Essalmani et al., 2008). Conditional knockouts allowed for the determination of a PC5/6 unique enzymatic substrate, Gdf11, shown to be important for the induction of Hox genes and consequently the regionalization of the axial skeleton (Essalmani et al., 2008). PACE4 is critical during the specification of left-right axes and anterior central nervous system development. PACE4 null mice are 75% viable and display complex craniofacial malformations

(Constam and Robertson, 2000a). PC4 is predominantly expressed in reproductive organs and its absence results in decreased rates of fertilization likely due to the absence of processing of growth factors required for normal fertility (e.g. PACAP, fertilins, IGF, TGF, etc.) (Gyamera-Acheampong and Mbikay, 2009; Seidah et al., 1992). Knockout mice of PC1/3 and PC2 genes are viable, although PC1/3 disruption results in severe growth retardation where the adult mice are 60% the size of the wildtype mice, which is accounted for by the decreased processing of growth hormone releasing hormone (GHRH) (Zhu et al., 2002). PC2 KO mice have chronic fasting hypoglycemia, accounted for by the absence of glucagon processing (Furuta et al., 1997). PC1/3 and PC2 exhibit impaired processing of various hormonal and neuroendocrine precursors. Lastly, PC7 null mouse embryos show no apparent deleterious phenotype, suggesting redundant substrate processing with other PCs in the embryo (Wetsel et al., 2013). The only PC7 specific substrate known to date is the transferrin receptor (Guillemot et al., 2013), which was identified following a genome-wide association study linking a PC7 intronic SNP with plasma levels of soluble transferrin receptor (Oexle et al., 2011). The mouse transferrin gene cannot be cleaved by PCs, since the P1 site reveals a Lys instead of an Arg, and such a substitution in the human gene abrogates PC7 cleavage.

Thus, even though *in vitro* all PCs can cleave precursors at the consensus dibasic motif, *in vivo*, PCs are implicated in the processing of specific substrates particularly during embryogenesis as elegantly demonstrated by gene knockout studies. Overall, PC redundancy may have served for species' survival, but it is the differentiation in their functions that allowed for complexity.

Table I.1. 1 Summary of mouse PC knockout phenotypes

Convertases	Null phenotype
Furin	Embryonic death at e10.5 due to hemodynamic insufficiency, ventral closure defect, axial rotation defect, abnormal yolk sac vasculature.
Pcsk1	Viable mice of 60% normal size with defects in prohormone processing.
Pcsk2	Viable mice, retarded growth, hypoglycemia, defects in endocrine peptide processing.
Pcsk6	Embryonic death (e10.5) 25% of mice from abnormal organ positioning and/or craniofacial malformations.
Pcsk4	Viable mice with reduced fertility.
Pcsk5	Embryonic death (e4.5-7.5).
Pcsk7	No apparent phenotype.
MBTPS1	Embryonic death (e4), abnormal epiblast formation and impaired implantation.
Pcsk9	Viable mice with hypocholesterolemia.

Table adapted from (Scamuffa et al., 2006).

Large genome wide association studies (GWAS) have established the association of several non-synonymous polymorphisms in the *PCSK1* gene with a large risk for common and extreme obesity (Benzinou et al., 2008; Choquet et al., 2013; Kilpelainen et al., 2009; Meyre et al., 2009; Speliotes et al., 2010), placing *PCSK1* as the 3rd most prevalent monogenic contributor for obesity (Choquet et al., 2013). Indeed, the variant N221D occurs at a frequency of 3-5% and the double mutant Q665E/S690T at a frequency of 24% (Benzinou et al., 2008). These associations are strongly modulated by ethnicity and age. Moreover, rare deleterious variants in *PCSK1* are also found in ~1% of obese individuals (Creemers et al., 2012) conferring ~8.7-fold increased risk for the development of obesity (Creemers et al., 2012). Patients with *PCSK1* mutations have enteric endocrinopathies manifested by lifelong diarrhea and impaired nutrient assimilation (Martin et al., 2013), however they develop hyperphagia (Farooqi et al., 2007) accompanied with strong appetite

leading to youth-associated weight gain and obesity (Stijnen et al., 2014). Interestingly, *PCSK1* knockout mice and mice homozygous for N222D LOF mutation do not show comparable phenotypes, where the later resembles the human phenotype associated with deficient PC1/3 (Lloyd et al., 2006). PC1/3 is highly implicated in the regulation of appetite, glucose homeostasis and nutrient assimilation. One example of PC1/3 substrate is α-MSH (produced from POMC in the hypothalamus), which generates an anorexigenic signal that antagonizes the Agouti-related peptide orexigenic signal. Therefore, the lack of PC1/3 peptide products could lead to an imbalance in appetite signaling. Moreover, several mutants have demonstrated ER-retention leading to toxicity manifested by increased susceptibility to ER stress (Blanco et al., 2015), the latter is a well-recognized risk factor for the development of obesity. Therefore, the pathogenesis of *PCSK1* obesity associated variants could be due to either of the effects or even to a combination of these mechanisms (for review (Ramos-Molina et al., 2016)).

The low-frequency coding variant of PC7 (R504H) was found to be associated with ~30% decreased plasma triglyceride levels in an African American population (Peloso et al., 2014). Since *PCSK7* is localized close to the gene cluster *APOA5/APOA4/APOC3/APOA1* and also because a *PCSK7* locus is in linkage disequilibrium with *APOA5* expression (Pennacchio et al., 2001), Ashraf *et al.* were interested in studying the potential regulation of ApoA5 by PC7 (Ashraf et al., 2020). Interestingly, they demonstrated an ER localized, non-enzymatic mediated degradation of ApoA5 by PC7 in acidic autophagosomes/lysosomes (Ashraf et al., 2020). This regulation was supported *in vivo* by the increased circulating apoA-V levels in WT mice compared to *Pcsk7*$^{-/-}$ mice fed a high fat diet (Ashraf et al., 2020). Non-enzymatic regulation of other proteins had only been demonstrated for PCSK9 and is a new finding for kexin-like PCs, opening a new avenue of explorations and discoveries.

In addition, PCs are known to be implicated in various pathologies such as Alzheimer's disease, tumorigenesis and infections (bacterial toxins activated by PCs, viral infectivity by the processing of surface glycoproteins). Understanding the regulation of their enzymatic activity could be advantageous for future clinical applications. An important and extremely relevant example is the new coronavirus (SARS-CoV-2) pandemic responsible for >1.5 million deaths or ~2.4% of infected patients (according to the World Health Organization weekly update). The coronavirus spike protein (S-protein) is a class-I viral fusion protein that in three human-infecting coronaviruses can be cleaved at the consensus kexin-like PC processing motif (K/R)-X*n*-(R/K)↓

(Coutard et al., 2020). Cleavage is important for the activation of the S-protein since the S1 ectodomain recognizes angiotensin converting enzyme 2 on the host cell and the S2 membrane anchored protein is important for viral entry into host cells of the lungs and other tisues. Coutard *et al.* suggested that considering its high expression in the lungs, furin is a likely candidate for proteolytic processing of the SARS-CoV-2 S-protein (Coutard et al., 2020). Interestingly, the SARS-CoV-1 from the 2002 outbreak was comparatively less pathogenic and contained a monobasic cleavage site. Importantly, the absence of a second basic amino acid within the motif would abrogate PC cleavage, suggesting that another host cell protease may be responsible for cleavage and generation of the S1 and S2 subunits, although the SARS-CoV-1 has been reported to be poorly cleaved. Alternatively, SARS-CoV-1 enters host cells by an endocytosis pathway that does not require prior priming before infection (Millet et al., 2020). Altogether, these results suggest that inhibition of PCs could be advantageous in reducing the pathogenicity of SARS-CoV-2 by preventing cleavage of its S-protein (Coutard et al., 2020).

I.1.2. PCSK9: 3rd locus for autosomal dominant hypercholesterolemia

Heritability is defined as the proportion of phenotypic variance in a population attributable to additive genetic factors (Hindorff et al., 2009). Cardiovascular disease (CVD) is one of the leading causes of mortality worldwide, accounting for ~31% of all deaths (Roth et al., 2015). Prevalent cases of total CVD cases and deaths are increasing worldwide (Roth et al., 2020). Coronary artery disease (CAD), the most prevalent form of cardiovascular disease, was estimated to be a highly heritable complex disease. Approximately 40% of CAD cases are caused by gene inheritance for both men and women, as determined from a Swedish longitudinal study of over 20 thousand twins (Peden and Farrall, 2011; Zdravkovic et al., 2002). Since genotypes are not confounded by environmental exposures, the identification disease-associated variants will thus help define the true causal molecular pathways, which are in turn crucial for the development of therapeutic strategies. Family-based linkage studies show strength in identifying disease-causing loci for 'Mendelian' disorders but show limited potential for complex diseases, which, we have learned from genome-wide association studies, are caused by modest effects from several variants (1000 Genomes 2015). Although even today with the advent of large-scale collaborative GWAS, only up to 21.2% of CAD heritability is accounted for by 243 identified causal loci (Nelson et al., 2017).

This section will illustrate the use and importance of family linkage studies through the discovery of a familial hypercholesterolemia (FH) causing gene, *PCSK9*, that furthermore, have been substantial in establishing the promises of a new and effective therapeutic target.

I.1.2.1 Introduction to FH and the formation of atherosclerotic plaques

Coronary artery disease and stroke are the two most prevalent forms of CVD and are consequences of atherosclerosis. Atherosclerosis is the complex process leading to the formation of plaques in the vessel wall of arteries. Dysfunctional endothelial cells lining the arteries, that no longer mediate proper vascular tone, allow the progressive infiltration of lipoprotein particles. Recruited monocytes differentiate into macrophages which phagocytose the cholesterol-rich particles and propagate an inflammatory signal, including the NLRP3 inflammasome. Other immune cells are involved in the plaque progression process, including dendritic cells, T cells and B cells. Smooth muscle cells will then proliferate and lead to intimal hyperplasia, thickening of the intimal layer of the vessel, thereby obstructing blood flow. Rupture of the plaque creates the formation of a blood clot which may then entirely obstruct a blood vessel and cause extensive tissue damage, or even death in the case of an important myocardial infarct. Several risk factors for CAD and myocardial infarct have been identified and are summarized in Figure I.1.4 (for reviews (Boren et al., 2020; Khera and Kathiresan, 2017; Libby et al., 2019)).

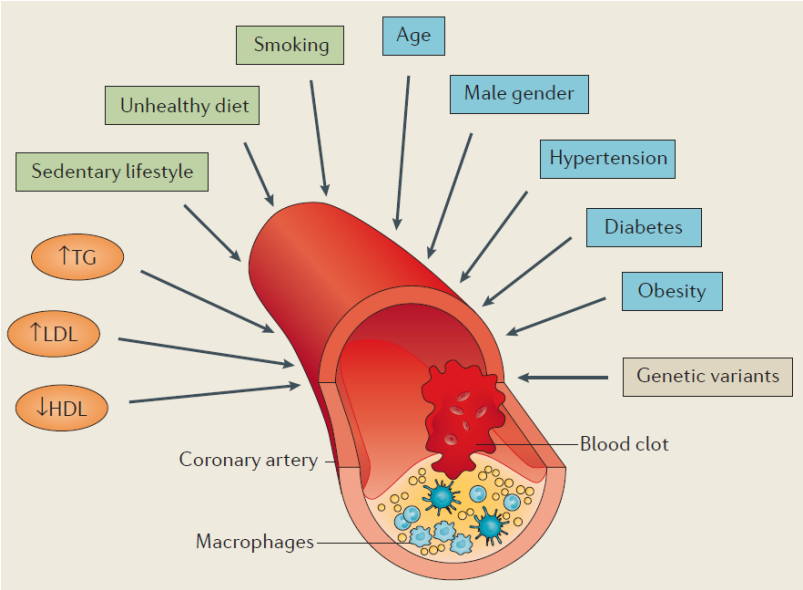

Figure I.1. 4 Risk factors for myocardial infarction

Schematic representation of the various risk factors that contribute to the development of CAD (Khera and Kathiresan, 2017).

There are four major classes of lipoprotein particles that are defined by size and density, which englobe high-density lipoproteins (HDL), low-density lipoproteins (LDL), very-low density lipoproteins (VLDL) and chylomicrons (see Figure I.1.5). Density is determined by the relative protein and lipid content and demonstrates an inverse relationship with particle diameter since the larger particles are enriched in triacylglycerol (TG) whereas smaller denser particles are enriched in cholesterol and protein content (%). Apolipoproteins provide structure to the lipoproteins, they act as scaffolds to regulate enzymes involved in the metabolism of the lipids while in the circulation, moreover, they allow binding to cell surface receptors and internalization of the particles. The backbone protein for chylomicrons, VLDL and LDL is apolipoprotein B (apoB) and is required for lipidation and maturation of the lipoproteins. LDL is a metabolic product of VLDL, which is the primary apoB-containing lipoprotein produced by the liver. In contrast, chylomicrons are synthesized by intestinal cells and contain a shorter form of apoB that does not contain the LDLR binding site present on the full form apoB of VLDL and LDL particles. While the apoB protein remains on the lipoprotein for its full life cycle, other apolipoproteins are exchangeable (AI, CI, CII, CIII, E) which for example activate or inactivate lipoprotein lipase, responsible for TG hydrolysis (for review (Doonan et al., 2018)). LDL cholesterol (LDLc) is statistically the

strongest single predictor for CAD and represents 90% of all circulating apoB-containing lipoproteins in the fasting state (for review (Ference et al., 2017)).

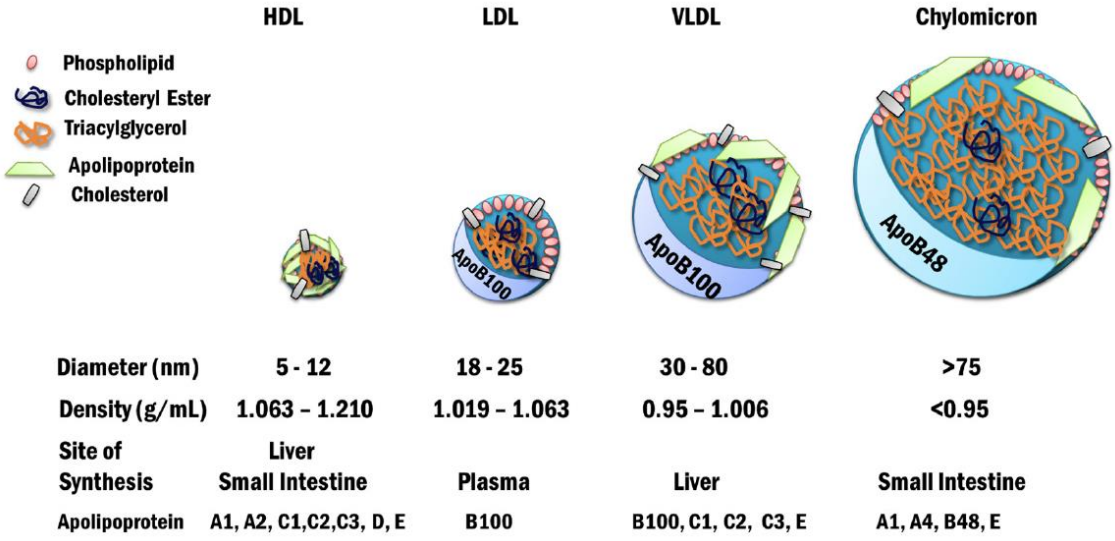

Figure I.1. 5 Four main plasma lipoprotein particle classes

HDL, LDL, VLDL and chylomicrons constitute the four main classes of lipoprotein particles of blood plasma. Lipoproteins are arranged by decreasing density and are shown particle content, diameter, density, site of synthesis and associated lipoproteins (Doonan et al., 2018).

Familial hypercholesterolemia is a common genetic disorder estimated at a prevalence of ~1:311 depending on the ethnic group and founder populations (Hu et al., 2020; Sturm et al., 2018). It is characterized by an isolated elevation in LDLc, increasing the risk for CVD by fourfold (Khera and Kathiresan, 2017). Indeed, the cumulative lifetime LDLc exposure will determine the age at which the individual will exceed the threshold beyond which myocardial infarct risk increases exponentially (Ference et al., 2018). FH is the primary monogenic driver of CAD identified to date, characterized by mutations in *LDLR, APOB* and *PCSK9* genes, principally. Although, whereas *APOE* mutants are normally associated with an increase in cholesterol and TGs (type III hyperlipoproteinemia), an *APOE* variant L167del, has also been associated with FH, since it causes autosomal dominant hypercholesterolemia (ADH) by downregulating LDLR (Awan et al., 2013; Cenarro et al., 2016; Marduel et al., 2013). Therefore, there are other genes for which damaging mutations that increase CAD risk by affecting other important processes of lipid homeostasis, in particular the lipoprotein lipase (LPL) pathway. Mutations have been identified in *LPL*, *APOA5*, and others with a prevalence of ~1 in 115, and lastly mutations in *LPA* (lipoprotein (a)) occurring with a prevalence of ~1 in 240 increasing CAD risk by fourfold (Khera and Kathiresan, 2017).

Since CAD is a complex disease, ~20% of tested individuals (prevalence of 1 in 5) may also inherit several common alleles conferring a polygenic risk increasing CAD by twofold. Therefore, among individuals with hypercholesterolemia, only 2% harbour an FH mutation, an estimation highly dependent on population study (Khera and Kathiresan, 2017).

I.1.2.2 Identification of a third locus for FH

Essential familial hypercholesterolemia had been recognized early in the twentieth century, but it was the study from Kachadurian *et al*. in 1964 that reconciled certain controversies about its mode of inheritance (Khachadurian, 1964). They described the inheritance and penetrance of clinical manifestations of xanthomatous lesions, hypercholesterolemia and atherosclerotic lesions from ten Arab sibships (consanguinity was described in 5 of these families). This allowed them to provide evidence that xanthomatosis may occur in both heterozygous and homozygous patients defining the gene as incompletely dominant for this disorder. In summary, homozygous patients have high levels of serum cholesterol (>500 mg/dL), extensive xanthomas starting in childhood, and frequently advanced heart disease in youth, whereas heterozygous patients have moderate elevation of the serum cholesterol and xanthomatosis may develop later in life. In 1974, Morganroth *et al.* described hypercholesterolemia in children with a distinct mode of inheritance, whereby increased LDLc and xanthomas were absent in parents and grandparents (Morganroth et al., 1974). The children proved to be extremely sensitive to the cholesterol lowering therapy at the time (cholestyramine) in contrast to the commonly described FH. This disorder was coined "pseudohomozygous type II hyperlipoproteinemia" and defined as autosomal recessive; the responsible gene is an adaptor protein important for LDLR function termed ARH for autosomal recessive hypercholesterolemia (Garcia et al., 2001). Also in 1974, Goldstein and Brown characterized ADH as resulting from defects in cell-surface receptor mediated LDL internalization (Brown and Goldstein, 1974), they then described the first mutations in the *LDLR* gene in 1977 (Goldstein et al., 1977) and Inneraty *et al*. described mutations in *APOB* in 1987 in patients where the LDLR activity was normal but the affinity of apoB for the LDLR was impaired (Innerarity et al., 1987). In 1999, in the context of a French national project motioned for the discovery of genes involved in FH (today is named CHOPIN and led by Dr. B. Cariou), the genetic research group led by Prof. C. Boileau identified a third locus for ADH (Varret et al., 1999). They performed

linkage analysis and exclusion mapping in a large French ADH family in which defects in *LDLR* or *APOB* had been ruled out. This family was clinically and biologically indistinguishable from FH. Co-segregation of genetic markers with the ADH phenotype identified a common region (haplotype) between affected individuals localizing *FH3* to a 9-cM interval at 1p34.1-p32 (Varret et al., 1999). These results were rapidly confirmed by Hunt *et al.* in 2000, a group that studied a Utah pedigree which also clearly excluded linkage to either the *LDLR* or the *APOB* genes, since they were able to map and associate the same region to an FH phenotype in a large family (Hunt et al., 2000).

I.1.2.3 Discovery of the *PCSK9* gene

The region that co-segregated with the *FH3* locus contained 41 genes including a newly identified protein, NARC-1, the 9th member of mammalian PCs (Seidah et al., 2003). In the search for an enzyme cleaving precursors at sites not recognized by the known PCs, Seidah *et al.* identified a yet uncharacterized polypeptide that had been patented by two pharmaceutical companies (Millenium Pharmaceuticals, Cambridge, MA, patent no. WO 01/57081 A2 and Eli Lilly, patent no. WO 02/14358 A2) (Seidah et al., 2003). NARC-1 was identified in the protein data banks by the traditional method of probing for sequence similarity with the subtilase catalytic site consensus sequences and was found to share high sequence identity (~42%) to the proteinase K family of subtilases. Since the human, mouse and rat amino acid sequences share ~92% identity, they characterized NARC-1 tissue expression in the mouse and the rat during development and in the adult rodent. Importantly, they demonstrated that NARC-1 expression is very high in the developing liver and intestine, which remain the major sites of expression in the adult, but also NARC-1 expression was increased in a similar fashion to apoB during liver regeneration. Since these tissues are the main sites of apoB expression and from the positioning of the gene on the *FH3* locus, Dr. N. G. Seidah subsequently collaborated with Dr. Boileau's genetics group in France to confirm that mutations in the NARC-1 coding gene were responsible for the FH phenotype in their affected families (Abifadel et al., 2003). The two identified mutations were missense mutations: S127R in the HC2 and the HC92 families and the second mutation F216L in the HC60 family. The *NARC-1* protein coding gene was named *PCSK9* and the protein was termed PCSK9

from then on. The affected patients from the Utah pedigree were also shortly after confirmed to carry a missense mutation in PCSK9, D374Y (Timms et al., 2004).

I.1.2.4 Description of GOF and LOF variants

PCSK9 S127R, D374Y and F216L, were rapidly defined as gain-of-function (GOF) since overexpression of these variants (S127R and F216L) and the wildtype PCSK9 in mice led to hypercholesterolemia (Benjannet et al., 2004; Maxwell and Breslow, 2004; Park et al., 2004). Importantly, these studies revealed that there was a strong decrease of liver LDLR resulting from PCSK9 overexpression with no effect on LDLR mRNA. These results suggested that PCSK9 regulates the LDLR post-transcriptionally resulting in an LDLR dependent increase in plasma LDLc. Furthermore, overexpression in LDLR knockout mice had no effect on plasma cholesterol levels (Maxwell and Breslow, 2004; Park et al., 2004). Several other GOF variants were later identified from various countries (Allard et al., 2005; Homer et al., 2008; Miyake et al., 2008; Noguchi et al., 2010). The identified GOF mutations, the country of origin, their relative position and baseline LDLc are illustrated in Figure I.1.6 (Hopkins et al., 2015).

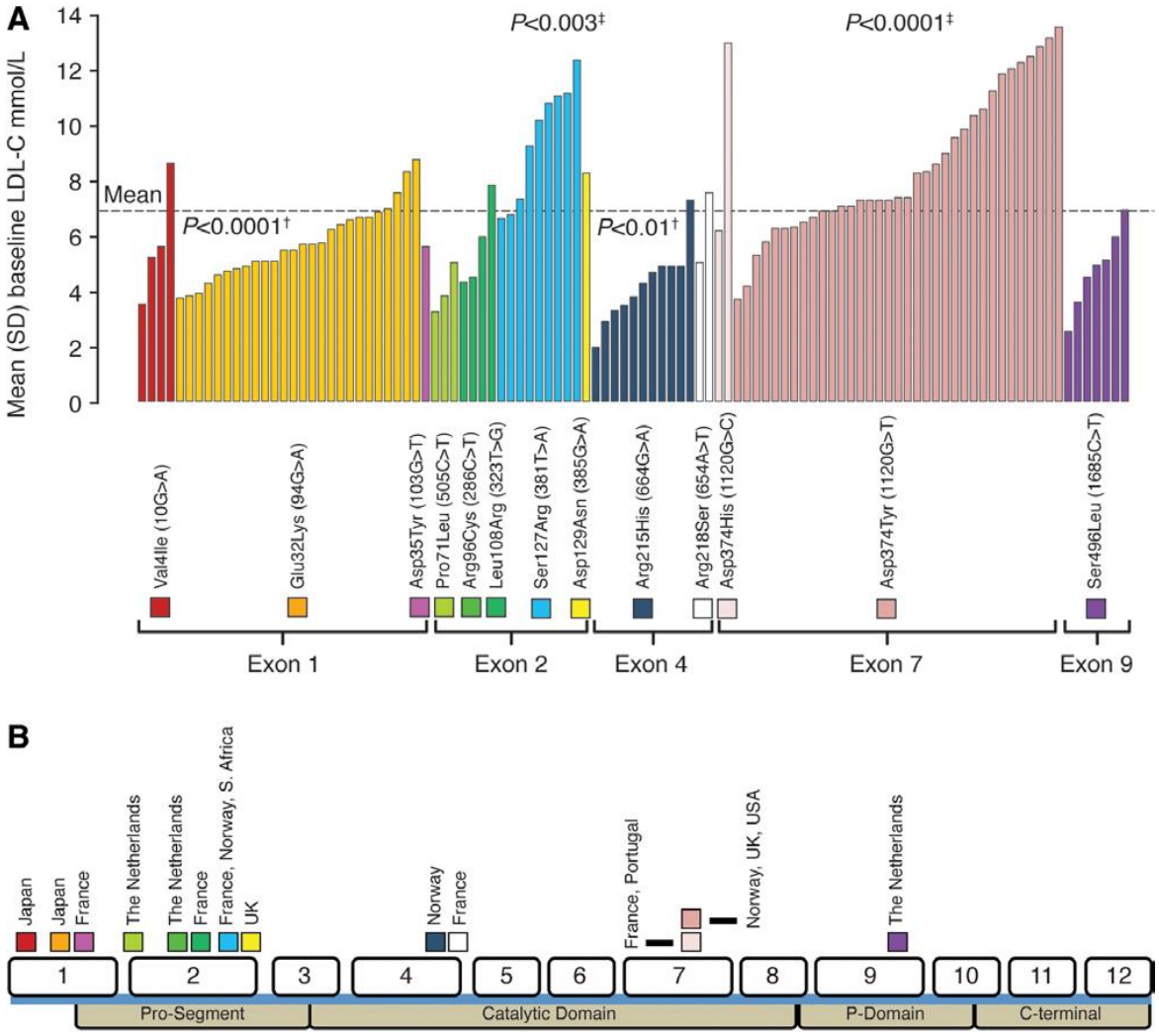

Figure I.1. 6 Origin, relative positioning and impact on baseline LDLc of *PCSK9* GOF mutations

A, Schematic positioning on *PCSK9* gene of identified GOF mutations and their effect on LDLc**.** ***B,*** illustration of mutation position in PCSK9 domains and country of origin (Hopkins et al., 2015).

Conversely, there are two genetically distinct disorders characterized by decrease or absence of plasma lipids which are caused by a variety of *APOB* gene mutations. Hypobetalipoproteinemia is an autosomal codominant disorder where the heterozygotes have low concentrations of apoB and LDLc and are clinically asymptomatic, whereas homozygotes have extremely low or undetectable levels of apoB and LDLc (Linton et al., 1993). These homozygous patients have severe clinical manifestations including: 1) fat malabsorption with lipid droplet accumulation in the intestinal and liver cells, 2) acanthocytosis, a malformation of the erythrocytes,

3) retinitis pigmentosa, a diffuse disease of the central nervous system, 4) neuromuscular degeneration, spinocerebellar degeneration and peripheral neuropathy. This severe phenotype is indistinguishable to the recessively inherited apoB deficiency state, abetalipoproteinemia (Linton et al., 1993). Additionally, homozygous knockout of apoB results in embryonic lethality in mice (Farese et al., 1995). Finally, a large number of hypobetalipoproteinemia patients do not harbor *APOB* mutations (Fouchier et al., 2005; Tarugi et al., 2007).

Therefore, it was important to identify whether *PCSK9*, similarly to the *APOB* gene, could harbor mutations causing low plasma lipids and possibly any of these clinical manifestations, in the situation of only traces or absence of plasma apoB. In 2005, in the context of the Dallas Heart Study, a large multiethnic population study of Dallas County, plasma samples were analyzed for lipid and lipoprotein content (Cohen et al., 2005). The samples with the lowest LDLc contents were subsequently sequenced for mutations in the *PCSK9* gene. The research group was able to identify two nonsense mutations in the *PCSK9* gene for plasma samples of African American descent, Y142X and C679X, which result in virtually undetectable circulating levels of PCSK9 due to the absence of PCSK9 protein and to unstable and dominant negative forms of PCSK9, respectively. Further examination of the family members demonstrated that these variants co-segregated well with hypocholesterolemia (Cohen et al., 2005). Importantly, this research group verified that rates of cholesterol synthesis and cholesterol absorption were comparable to noncarriers (measured lathosterol and campesterol, which respectively correlate with biosynthesis and absorption). Furthermore, the sizes of the LDL particles were similar that of controls suggesting that the variants result in a reduction in the number of particles in circulation (Cohen et al., 2005). These results were confirmed in a larger study in the US (ARIC) and a loss-of-function (LOF) variant was identified in individuals of European descent (R46L), although is characterized by more moderate decrease in plasma LDLc levels (21% decrease vs. 40% for the nonsense variants) (Cohen et al., 2006). Finally, since these identified LOF variants are relatively common, (nonsense variants prevalence of 2% in African Americans and missense variant 3.2% European Americans), the researchers were able to demonstrate that a life-long decrease in LDLc is strongly protective for CVD (hazard ratio 0.5 for R46L carriers and 0.11 for nonsense mutation carriers, Y142X and C679X) (Cohen et al., 2006).

I.1.3. PCSK9 variants in the study of structure/function

There are more than 100 natural *PCSK9* variants that have been identified including 73 missense/nonsense variants (Seidah et al., 2017), where GOF variants may increase the affinity to the LDLR or confer resistance to PC proteolysis, for example. While LOF result in truncations that disrupt proper tertiary folding of the protein and prevent ER exit, or in the case of R46L, for example, result in a protein more susceptible to proteolysis and decreased phosphorylation at S47 (Dewpura et al., 2008). This section will present how the study of these variants has greatly hastened the understanding of how the PCSK9/LDLR complex modulates LDL internalization.

I.1.3.1 Extracellular pathway: main pathway regulating hepatic LDLR and LDLc

The same year that PCSK9 was discovered and parented to the third FH locus, *PCSK9* had been shown to be coregulated with genes involved in cholesterol synthesis (Horton et al., 2003; Maxwell et al., 2003). Moreover, shortly after the *PCSK9* gene was associated with hypercholesterolemia, several studies quickly demonstrated that secreted PCSK9, when added exogenously to hepatic cell lines or mouse primary hepatocytes, is able to decrease cell surface LDLR and LDL internalization in 20 min as well as decrease total LDLR levels in a dose dependent manner (Cameron et al., 2006; Lagace et al., 2006; Maxwell and Breslow, 2004). Direct binding of PCSK9 to the extracellular domain of the LDLR at the cell surface is required for uptake, colocalization in the endocytic/lysosomal compartments and for LDLR degradation (Holla et al., 2007; Lagace et al., 2006; Qian et al., 2007; Zhang et al., 2007). Furthermore, parabiosis experiments of transgenic PCSK9 mice and wildtype mice demonstrated that plasma PCSK9 can actively decrease hepatic LDLR levels (Lagace et al., 2006). LDL particle receptor-mediated endocytosis by clustering of the LDLR receptors in small pockets on the cell surface called clathrin-coated pits explain the highly efficient process leading to LDL lysosomal degradation (Brown and Goldstein, 1986). Notably, in hepatocytes and lymphocytes, this process requires the binding of the cytoplasmic tail of the LDLR to an adaptor protein, ARH (but not in fibroblasts) (Eden et al., 2002; Garcia et al., 2001). Studies further demonstrated that PCSK9 mediated LDLR degradation is dependent on clathrin coated pits (Nassoury et al., 2007; Wang et al., 2012) but independent on ARH (Park et al., 2004; Wang et al., 2012) since LDLR lacking a cytoplasmic tail

is also degraded by PCSK9 (Canuel et al., 2013; Strom et al., 2010). Together these results suggest that there is a third protein (or "Protein X") involved in the complex required for PCSK9 function that would be the link to the endosomal system. Furthermore, the complex's degradation is independent from the proteasome and the autophagosome and instead requires lysosomal proteases (Benjannet et al., 2004; Maxwell et al., 2005; Wang et al., 2012). Finally, enzymatically inactive PCSK9 (expression of the prodomain and the catalytic domain in *trans*) demonstrated equivalent efficiency to WT PCSK9 in degrading the LDLR (Li et al., 2007; McNutt et al., 2007).

I.1.3.2 LDLR structure/function analysis

The LDLR exhibits an efficient and masterful mechanism for LDL and VLDL extracellular binding, endocytosis, and release (Rudenko et al., 2002). ApoB-100 and apoE bind the ligand-binding domain (LBD) of the LDLR, consisting of ~40 amino acid cysteine-rich tandem repeats. This domain is followed C-terminally by the epidermal growth factor (EGF) precursor homology domain, which contains two cysteine-rich EGF-like domains, EGF-A and EGF-B, followed by a β-propeller domain and a third EGF-C like domain. Then follows an O-linked glycosylated threonine, serine-rich region, a transmembrane domain and the short cytoplasmic tail containing the NPxY motif required for endocytosis into clathrin-coated pits (Jeon and Blacklow, 2005). Clusters of acidic residues in the cysteine-rich repeats of the LBD interact with the negative charges on apoB and apoE at neutral pH. The LDLR requires the EGF precursor homology domain for ligand release and efficient recycling. The crystal structure of the LDLR demonstrated a conformational change between neutral and acidic pH such that the elongated, extended conformation in neutral plasma pH changes to a compact shape under acidic (pH 5.3), endosomal-like conditions. Under acidic conditions, the N-terminal ligand binding repeats (modules R4 and R5) preferentially fold back to bind the β-propeller domain of the EGF precursor-like domain releasing the LDL or VLDL in the endosomes (Rudenko et al., 2002). The release through this preferential binding is required for the recycling of the LDLR to the cell surface since the LDLR lacking the EGF-A and -B may traffic normally to the cell surface but is subsequently rapidly degraded (van der Westhuyzen et al., 1991). The histidines at this binding interface between the cysteine-rich repeats R4 and R5 and the β-propeller domain form salt-bridges at acidic pH and are highly conserved between species. Additionally, mutations for these histidine residues have been

found in FH patients (Rudenko et al., 2002), consistent with the notion that impaired release of LDL by, or recycling of, the LDLR results in increased plasma LDLc.

I.1.3.3 PCSK9 structure and variants shed light on function

In 2007, the three-dimensional structure of PCSK9 was generated synchronously between three groups (Cunningham et al., 2007; Hampton et al., 2007; Piper et al., 2007). The catalytic triad of PCSK9 (D186, H226 and S386) is conserved and the domain structure is similar to that of proteinase K, subtilisin E and furin (Cunningham et al., 2007). However, when compared to the related mammalian subtilisins, the prodomain of PCSK9 seems to have acquired features that would retain the prodomain tightly bound to the catalytic site. As explained in the previous sections, the prodomain of PCs is required for proper folding of the protease. Autocatalytic cleavage in ER of the properly folded protein results in a mature protease inhibited by the prodomain. The adaptations from PCSK9 include: an extra β-strand and helical turn at the N-terminus of the prodomain which forms a "hood" over the active site; hydrogen bonding of the inhibitory P1-P6 residues to the backbone of the substrate groove; and a much shorter L-loop that is important in PCs for the second cleavage and release of the prodomain (Siezen, 1996). Therefore, these structural indications support the notion that PCSK9 catalytically inhibited by its prodomain regulates the LDLR in a non-enzymatic fashion. The C-terminal cysteine-, histidine rich domain (CHRD), consists of three pseudo-β-barrel motifs with the principal fold made of six anti-parallel β-strands held together by three internal disulfide bonds. Additionally, despite poor sequence conservation, the three pseudo-β-barrel folds show a clear overall 3-fold symmetry within the domain. The only other protein that matches this distinctive arrangement of cysteines repeated three times as in PCSK9 is resistin. Resistin is an adipokine linked with type II diabetes mellitus (T2DM) and obesity. The CHRD and resistin may share a common ancestor due to the remarkable homology in the pseudo-β-barrel (Hampton et al., 2007) (view Figure I.1.7 for sequence alignment between the CHRD subdomains and resistin). Interestingly, the C679X LOF variant causes a 14-amino acid truncation at the C-terminus and results in a protein that is retained in the ER, likely because this nonsense mutation results in misfolded protein from the loss of a disulfide bond between C679 and C608 in subdomain 3 of the CHRD (Benjannet et al., 2004).

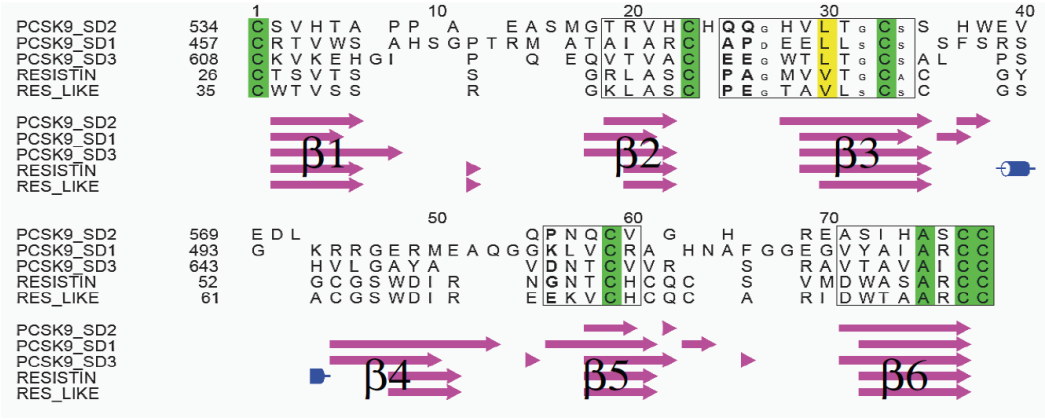

Figure I.1. 7 Sequence alignment of PCSK9 CHRD domain with resistin

The three CHRD subdomains (SD) are aligned with resistin and resistin-like monomers. Secondary structure is depicted as β-sheets (magenta) and α-helices (blue) (Hampton et al., 2007).

The EGF-A domain of the LDLR binds to the catalytic domain of PCSK9 (Kwon et al., 2008; Zhang et al., 2007). The FH mutation D374Y located at the binding interface results in 10- to 25-fold increased affinity to the EGF-A domain (Cunningham et al., 2007; Kwon et al., 2008). Several studies demonstrated that the LDLR binds PCSK9 with increased affinity under acidic conditions, such as in endosomes (Cunningham et al., 2007; Fisher et al., 2007; Piper et al., 2007; Zhang et al., 2007). Indeed, in endosomes an additional binding interaction strengthens the affinity of PCSK9 for the LDLR, where the LDLR LBD recognizes the positive charges of the cysteine-, histidine-rich domain (CHRD) of PCSK9 (Holla et al., 2011a; Tveten et al., 2012; Yamamoto et al., 2011). Therefore, the CHRD acts as a decoy for the LBD preventing its interaction with the β-propeller domain of the LDLR required for efficient recycling (view Figure I.1.8). Additionally, three PCSK9 GOF mutations (L108R, S127R, D129G) have been localized to a small binding interface between the prodomain and the β-propeller domain of the LDLR (view Figure I.1.9), suggesting that enhanced interaction at this site maintains the PCSK9/LDLR complex in an inhibitory conformation preventing LDLR LBD binding to the β-propeller domain in the endosomes (Abifadel et al., 2012; Lo Surdo et al., 2011). Interestingly, the same domain contains an inhibitory segment for the function of PCSK9. Indeed, the deletion of the mobile highly acidic N-term of the prodomain (amino acid residues 31-52) results in >7-fold increase in affinity for the LDLR (Benjannet et al., 2010; Holla et al., 2011b; Kwon et al., 2008).

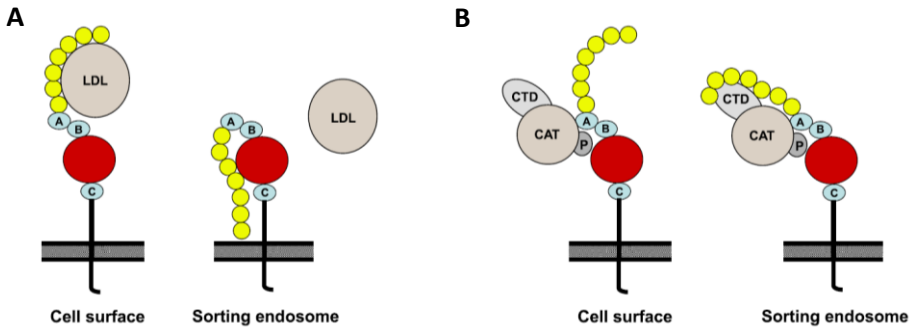

Figure I.1. 8 Four LDLR conformations adopted under neutral or acidic pH in the absence or presence of PCSK9

The LDLR is depicted with its ligand binding domain (yellow), EGF-A, -B, -C (blue), β-propeller domain (red). PCSK9 is depicted in grey with the prodomain (P), catalytic domain (CAT) and C-terminal domain (CTD). ***A,*** Under neutral pH, the LDLR binds LDL, internalization into endosomes leads to pH-mediated folding into closed conformation and LDL release. ***B,*** Upon binding of PCSK9, the ligand-binding domain folds over the PCSK9 CHRD domain preventing adoption of closed conformation (Leren, 2014).

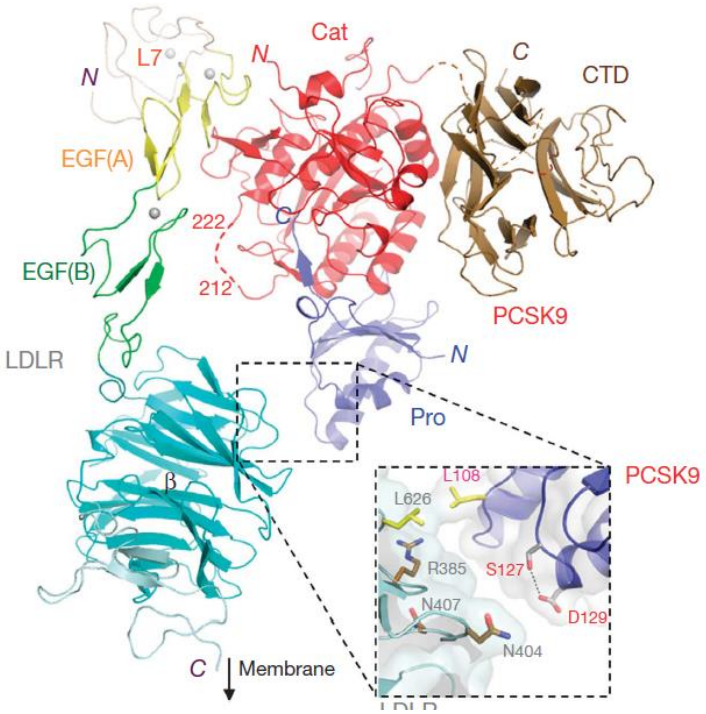

Figure I.1. 9 Tertiary structure of the PCSK9/LDLR complex

The catalytic domain of PCSK9 (red) interacts with the EGF-A domain of the LDLR (yellow). The box depicts the interactions of the pro domain of PCSK9 (blue) with the β-propeller domain of the LDLR where GOF residues of PCSK9 are in red (Lo Surdo et al., 2011).

PCSK9 activity is also regulated by post-translational modifications such as cleavage and phosphorylation. A furin and PC5/6-A cleavage site is located in an exposed and flexible loop of the catalytic domain of PCSK9. Cleavage at this site results in the loss of PCSK9 activity due to unfolding of the protein and detachment of the prosegment (Benjannet et al., 2006). GOF FH variants (F216L, R218S ad R215H) have helped to validate this regulatory mechanism, as impaired cleavage in these patients results in longer half-life of PCSK9. It has been shown that the secretory pathway kinase Fam20C phosphorylates PCSK9 at four identified Ser-residues (47, 666, 668 and 688) potentiating PCSK9 secretion and LDLR degradation. Indeed, mutations at critical residues of the recognition motif reduces phosphorylation and confers LOF to PCSK9, for example R46L, S668R and E670G (Ben Djoudi Ouadda et al., 2019).

I.1.3.4 Intracellular pathway: evidence from variants and extrahepatic tissues

Some variants (S127R and D129G) confer GOF activity despite poor autocatalytic processing and secretion from cells (Benjannet et al., 2004; Homer et al., 2008), and therefore would seem to indicate the possibility of an intracellular *vs*. an extracellular pathway for LDLR degradation. In contrast to clathrin-heavy chains (CHCs), clathrin-light chains (CLCs) are not required for clathrin-mediated endocytosis but are critical for clathrin mediated trafficking between the *trans*-Golgi Network (TGN) and the endosomal system (Poirier et al., 2009). Since the knockdown of the CLCs results in impaired LDLR degradation (Poirier et al., 2009), PCSK9 could target the LDLR for degradation directly from the TGN to lysosomes.

Recently, a new class of LDLR mutations has been described (Susan-Resiga et al., 2017), pertaining to a defect in LDL delivery to lysosomes and resistance to PCSK9-induced degradation. Accordingly, the FH patient described in the study was resistant to PCSK9 monoclonal antibody treatments. In the study, the R410S variant demonstrated resistance to extracellular PCSK9 mediated degradation but surprisingly intact intracellular mediated LDLR degradation, in turn suggesting differential regulators for both pathways. Interestingly, this mutation is in the β-propeller domain at the interaction interface with the PCSK9 prodomain (β1-β6 propeller blades) and on the opposite side from the interaction with the LBD of the LDLR (β3-β4 propeller blades) (Rudenko et al., 2002). The authors determined that there are two principal consequences from this mutation, the first is decreased sensitivity to extracellular PCSK9 and the

second is the impaired LDL release in the endosomes leading to increased "retro-endocytosis" or "regurgitation". The latter was at least in part explained by the loss of flexibility of the β3 and β4 blades under acidic conditions, which contain the residues forming the pH sensitive salt-bridges with the LBD of the LDLR (described above), important for the release of LDL (Rudenko et al., 2002). This variant when contrasted with the wildtype LDLR could be a useful tool to identify the interacting partners of the extracellular pathway. Nevertheless, two observations contrast sharply with previous studies. First, it is puzzling that this variant does not allow for the release of the ligand and still is able to recycle to the cell surface. Second, the R410S does not have a modified half-life than the wildtype while previous studies had demonstrated that impaired binding to the β-propeller domain by the LBD and failure to adopt a compact tertiary conformation leads to the rapid degradation of the LDLR (van der Westhuyzen et al., 1991).

The two pathways described preferentially take place in specific tissues, such that the liver LDLR is targeted by the extracellular pathway *vs.* the intestine, for example, where the LDLR is mainly regulated by the intracellular pathway. Indeed, endogenous inhibition of extracellular PCSK9 by annexin A2 (AnxA2) has been described (Mayer et al., 2008). Annexins are receptors that bind membrane phospholipids on the extracellular surface, such that membrane tethered AnxA2 binds to the CHRD of PCSK9 and inhibits its activity on the LDLR (Ly et al., 2014; Mayer et al., 2008). Interestingly, AnxA2 expression is particularly enriched in the intestine, adrenals, lungs, and others but not liver (Seidah et al., 2012), suggesting that this receptor could participate in determining which tissues are susceptible to extracellular PCSK9. Indeed, annexin A2 knockout mice demonstrate ~50% and ~40% decrease in LDLR in adrenals and colon respectively, suggesting functional evidence for this inhibitory mechanism (Seidah et al., 2012).

I.1.4. Other targets or ligands of PCSK9

PCSK9 has been reported to interact with several proteins and to have several targets for degradation, however, this section will only review those with greater relevance to this thesis.

I.1.4.1 Other lipid receptors (VLDLR, ApoER2, LRP-1)

The LDLR is the first member of a large and ancient family of receptors divided into subgroups based on structural homology. They are involved in endocytosis of a variety of ligands including lipoproteins, proteases and vitamins, some receptors are also important for signaling events of neuronal development, synaptic transmission, neuronal migration, etc. (Gent and Braakman, 2004; Howell and Herz, 2001). The LDLR family members are type I transmembrane receptors which are characterized by modular domain organisation containing the previously described ligand-binding cysteine rich repeats among other conserved domains between subgroups (view Figure I.1.10). The VLDL receptor (VLDLR) and the apoE receptor 2 (ApoE-R2) share the closest structural homology to the LDLR (59% and 46% identity respectively) while also contain the EGF-like precursor homology domain, the O-linked glycan domain and the NPxY motif on the cytosolic tail. These receptors are involved in the extracellular protein reelin (Reln) developmental signaling cascade and the double knockout of the ApoER2 and VLDLR is essentially identical to that of Reln knockout phenotype, which is characterized by neurological defects of the central nervous system, including the cerebral cortex, hippocampus, cerebellum, olfactory bulb and spinal cord. The giant LDL related protein-1 (LRP-1) belongs to the second subgroup, and contains the EGF-like repeat, β-propeller domain and two NPxY motifs. This receptor is known to bind and internalize multiple ligands and LRP-1 knockout is embryonic lethal in mice (Howell and Herz, 2001).

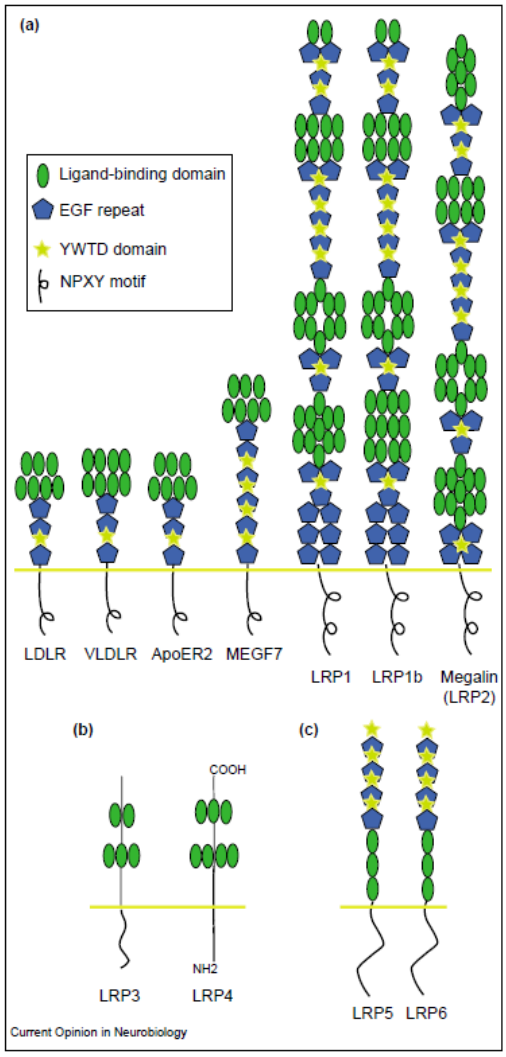

Figure I.1. 10 Structural representation of LDL receptor gene family

A, Core family members. ***B,*** poor homology of LRP3 and LRP4 with core family members. ***C,*** LRP5 and LRP6 share extracellular domains with different organisation to core family members (Howell and Herz, 2001).

Several research groups have demonstrated the regulation of ApoER2, VLDLR and LRP-1 by PCSK9, including the ability of PCSK9 wildtype and GOF D374Y to degrade VLDLR and ApoER2 in various cell lines (Canuel et al., 2013; Poirier et al., 2008). This regulation was supported by the demonstration that PCSK9 binds to VLDLR and apoER2 specifically and may be competitively inhibited by EGF-A (Shan et al., 2008). More studies have functionally validated this regulation. Firstly, PCSK9 knockout mice have shown 80% increased visceral adipose tissue, characterized by adipocyte hypertrophy, increased fatty acid uptake, and increased *ex vivo* TG synthesis. This phenotype was accounted for by the increase in VLDLR at the cell surface of

adipocytes which is regulated by circulating PCSK9 (Roubtsova et al., 2015; Roubtsova et al., 2011). On the other hand, ApoER2 is increased in neuronal cells upon silencing of PCSK9 expression, further protecting cells from apoptosis, while silencing ApoER2 reversed the protective effect (Kysenius et al., 2012; Wang et al., 2018). Additionally, findings from studies of tumor cells have implicated the regulation of LRP-1 by PCSK9 (Canuel et al., 2013). Interestingly, in these melanoma cells of variable metastatic potential, the LDLR was either resistant or sensitive to PCSK9 mediated degradation, whereas, LRP-1 was sensitive to PCSK9 mediated degradation in both tumor cell lines. The study showed that even though the LDLR and LRP-1 show similar requirement for the catalytic domain of PCSK9, it is suggested that there is a distinct degradation machinery required for LDLR and LRP-1 PCSK9 mediated degradation.

I.1.4.2 Regulation of CD36

CD36 is a membrane glycoprotein that was initially identified in platelets, then characterized as a scavenger receptor in monocytes, macrophages and other phagocytic cells and as a negative regulator of angiogenesis in specialized epithelia (Silverstein and Febbraio, 2009). It's role in hepatocyte TG metabolism was only revealed relatively recently (Koonen et al., 2007). Scavenger receptors recognize molecular patterns presented by pathogens; in particular, CD36 recognizes specific lipid and lipoprotein components of these pathogens leading to their internalization. CD36 may also recognize and lead to the internalization of endogenously derived ligands including β-amyloid peptide, glycated proteins, oxidatively modified lipoproteins, etc. (Silverstein and Febbraio, 2009), implicating CD36 in Alzheimer's disease and atherosclerosis. CD36 resides in cholesterol-rich lipid-rafts important for colocalization with signaling receptors to mediate downstream effects and may be internalized by both caveolin and clathrin-coated pits. It has a hairpin-like structure that is mainly composed of a large glycosylated extracellular loop domain (the multiligand binding domain), two transmembrane domains and short cytoplasmic domains which are palmitoylated (view Figure I.1.11).

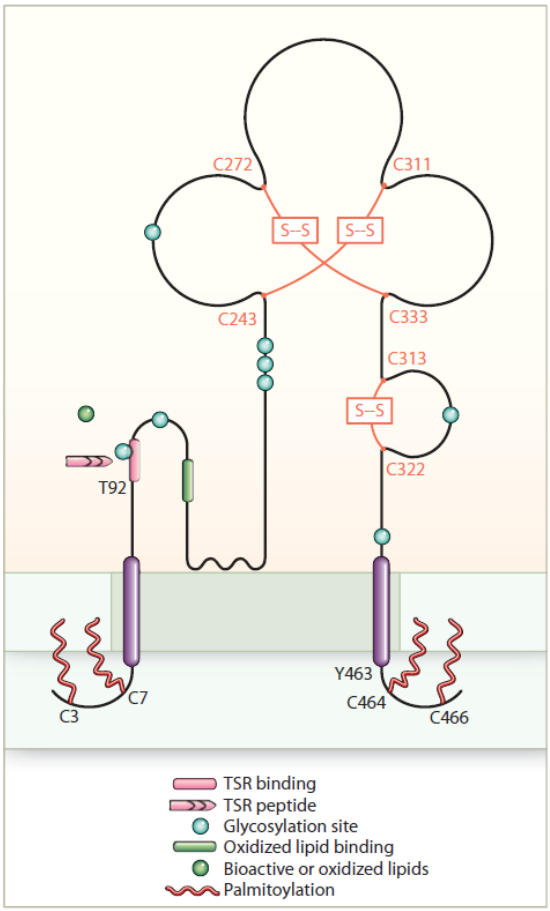

Figure I.1. 11 Structural organization of CD36

Schematic representation of CD36 topology and domains including post-translational modifications (Silverstein and Febbraio, 2009).

Koonen *et al*. demonstrated that CD36 increases in hepatocytes in response to high fat diets, contributing to increased free fatty acid internalization, TG synthesis and subsequent dyslipidemia. Moreover, overexpression of CD36 in lean mice is sufficient to affect hepatic lipid storage and secretion (Koonen et al., 2007). Therefore, CD36 may contribute to the pathogenesis of metabolic disorders, such as insulin resistance, obesity and non-alcoholic hepatic steatosis.

Demers *et al*. studied the potential functional relationship between PCSK9 and CD36 since PCSK9 knockout mice demonstrated increased CD36 levels in visceral adipose tissue (80% increase) and in the liver (300% increase) (Demers et al., 2015). They demonstrated in human and mouse cell lines that PCSK9 can induce the degradation of CD36 through a mechanism involving both the proteasomal and lysosomal systems. Moreover, PCSK9 was shown to interact directly with CD36 within similar binding affinity as PCSK9 binding to LDLR EGF-A domain at neutral

pH. Although this interaction relies on the catalytic domain, D374Y GOF variant does not confer increased activity towards CD36, however, another variant within the catalytic domain does (F379A). Moreover, the monoclonal antibody (mAb) to PCSK9 prevented CD36 degradation. Upon characterization of PCSK9 knockout mice, this research group demonstrated that the long-term resulting increase in CD36 resulted in enhanced hepatic TG content. The authors further suggested that the absence of PCSK9 could confer greater susceptibility to non-alcoholic fatty liver disease (NAFLD). In 2019, Lebeau *et al*. challenged PCSK9 knockout mice with a high fat diet to verify this hypothesis. The mice developed an enhanced NAFLD phenotype in the absence of PCSK9 in contrast to wildtype mice. The study reported increased total protein and cell surface localization of CD36 in the livers of those mice (Lebeau et al., 2019).

I.1.4.3 ApoB: extracellular interactions

The direct binding of PCSK9 to apoB in serum acts as an important inhibitor of PCSK9 activity on LDLR degradation. LDL was rapidly demonstrated to affect the binding affinity of PCSK9 to the LDLR (Fisher et al., 2007). Shortly after, it was demonstrated that PCSK9 may elute and associate with LDL in normolipidemic subjects (Kosenko et al., 2013) and with both LDL and HDL lipoproteins in mouse serum (Fan et al., 2008; Fisher et al., 2007; Tavori et al., 2013). Indeed, in human plasma, > 40% of PCSK9 is associated with LDL but not VLDL or HDL (Kosenko et al., 2013). These research groups demonstrated direct binding between PCSK9 and apoB (Hori et al., 2015; Kosenko et al., 2013; Tavori et al., 2013). Therefore, it is possible that apoB epitopes in VLDL are masked. Indeed, in contrast to LDL, VLDL binding to the LDLR requires TG lipolysis that decreases particle size and induces a conformational change of apoB (Boren et al., 1998). Moreover, the identified apoB/PCSK9 binding domain is likely to be the same highly negatively charged N-term region of the prodomain of PCSK9 (a.a. 31-52) (Kosenko et al., 2013; Sarkar et al., 2020) previously described in Section I.1.3.3 that inhibits PCSK9 binding to the LDLR by ionic repulsion (Kwon et al., 2008). Recently it was found that this intrinsically disordered region (IDR) may also form a transient α-helix that however is not critical for the PCSK9 binding affinity to the LDLR (Kirchhofer et al., 2020; Sarkar et al., 2020; Ultsch et al., 2019). The authors further suggested that binding to LDL was stabilized by interdomain interaction of the prodomain and the CHRD (Sarkar et al., 2020). This is functionally supported by the identification of several GOF

mutations that are located at this interface and greatly diminish this interdomain interaction (Sarkar et al., 2020). In summary, the association of PCSK9 to apoB in plasma inhibits PCSK9 activity and mutations impairing this association may result in FH.

I.1.4.4 ApoB: intracellular interactions

ApoB is constitutionally synthesized in the liver and intestine and its expression is regulated post-transcriptionally and post-translationally. The rate of apoB-containing lipoprotein secretion is controlled by apoB degradation since the apoB polypeptide provides the backbone structure for lipoprotein lipidation and maturation. Interestingly, the apoB mRNA can be processed into a shorter isoform, termed apo48, that does not contain the ligand-binding domain to the LDLR. This processing is accomplished by the apoB mRNA editing complex-1 (Apobec-1), which is only expressed in the intestine in humans but in both liver and intestine in rodents and makes the chylomicron apoB equivalent. The apoB mRNA is translated by the ER ribosomes where a first co-translational regulation of apoB takes place through ER-associated degradation (ERAD). Indeed, the apoB nascent polypeptide is normally lipidated by the MTP (microsomal triglyceride transfer protein) complex upon translocation into the ER lumen through the Sec61 translocation channel. These lipids confer apoB tertiary structure stability. Therefore, under conditions of low lipid supplies, the protein misfolds allowing for the ubiquitination machinery to bind to the misfolded loops further inducing retro-translocation through Sec61 and degradation in the cytosol by the proteasome. Therefore, apoB-containing lipoprotein secretion is under the regulation of ER lipid content (Doonan et al., 2018), the intracellular organelle responsible for cellular lipid content sensing and homeostasis. The second post-translational regulation of apoB intracellular levels is a specific form of autophagy. It was initially characterized as post-ER pre-secretory proteolysis (PERPP), which sequesters pre-VLDL particles that have not been properly matured in the Golgi to autophagosomes, the contents of which are subsequently degraded by the lysosomes. Acute insulin signaling, increased sortilin expression or activity and a diet rich in fish oil N-3 fatty acids are the main inducers of this pathway and further correlate with decreased VLDL secretion and decreased plasma TG (Doonan et al., 2018). Moreover, insulin resistance, such as in T2DM, results in increased VLDL secretion, likely due to impaired insulin-mediated apoB-degradation by PERPP. Additionally, it is known that the LDLR may target under-lipidated VLDL particles for degradation from the secretory pathway from a post-ER compartment that requires apoB and apoE

ligands on the VLDL particle to take place (Blasiole et al., 2008; Twisk et al., 2000). This was well supported by studies in humans, mice and cell lines demonstrating conversely that the loss of LDLR leads to an increase in VLDL secretion due to a decrease in degradation of apoB (Horton et al., 1999; Tremblay et al., 2004; Williams et al., 1990) and that those particles were small and were poorly enriched in TGs (James et al., 1989; Larsson et al., 2004; Nassir et al., 2004).

On the other hand, very soon after the discovery of PCSK9, several GOF mutations (S127R, D374Y) were associated with an increase in apoB secretion (Herbert et al., 2010; Ouguerram et al., 2004; Sun et al., 2005). This effect of PCSK9 on apoB-containing lipoproteins was further demonstrated *in vivo* and *in vitro* from PCSK9 overexpression systems from hepatocytes (Benjannet et al., 2004; Lambert et al., 2006; Sun et al., 2012; Tavori et al., 2013; Tavori et al., 2016) and from intestinal cells (Levy et al., 2013; Rashid et al., 2014). Decreased biosynthetic rates of apoB accompanied by decreased apoB-containing lipoprotein secretion (both hepatic VLDL secretion and postprandial triglyceridemia), was also observed from PCSK9 knockout mouse models (Le May et al., 2009; Rashid et al., 2005; Sun et al., 2018). Furthermore, direct intracellular binding of PCSK9 to apoB was shown to decrease apoB autophagic degradation in an LDLR independent manner *in vitro* and *in vivo* while negatively modulating the autophagy signaling pathway (Sun et al., 2018; Sun et al., 2012). In summary, these studies suggest that PCSK9 may favor TG-rich, apoB-containing lipoprotein secretion via affecting autophagy and directly binding to apoB, overall unraveling an LDLR-independent mechanism whereby PCSK9 increases TG rich particles in the circulation (view section I.1.9).

I.1.4.5 Modulation of Lp(a) secretion and internalization by PCSK9

Lipoprotein(a) (Lp(a)) is an atherogenic LDL-like particle characterized by the presence of a covalently bound apoprotein(a) (apo(a)) to apoB. Lp(a) confers an independent risk for CAD (Meireles-Brandao et al., 2019). Apo(a) is a high molecular weight protein, is uniquely expressed by the liver and shares high structural homology to plasminogen kringle IV, a key proenzyme of the fibrinolytic cascade (Marcovina and Koschinsky, 1999). Indeed, apo(a) may inhibit fibrinolysis *in vitro*, suggesting that inhibition of fibrinolysis at sites of plaque rupture may cause thrombosis, myocardial infarct and ischemic stroke (Wilson et al., 2019). Lp(a) plasma concentrations are determined by synthesis rather than catabolism and are inversely correlated to the number of

tandemly arranged kringle IV type 2 repeats, which varies among subjects ranging from 11 to >50. Indeed, the larger apo(a) isoforms are relatively inefficiently secreted from the hepatocytes (Marcovina and Koschinsky, 1999). Studies have demonstrated that apo(a) alleles with a low kringle IV copy number (<22) and high Lp(a) concentration were significantly more frequent in CAD patients and conferred a hazard ratio of 4.6 for CAD *vs.* a hazard ratio of 0.3 in individuals with >25 repeats (Kraft et al., 1996). Additionally, in contrast to LDL, the plasma concentrations of Lp(a) are not modulated by diet and physical activity and have been resistant to traditional LDL-lowering therapies.

Recently, PCSK9 targeted mAb have demonstrated in addition to a >35% reduction in LDLc, a surprising 25-30% reduction in Lp(a) (Shapiro et al., 2019). In addition, the FOURIER cardiovascular disease clinical outcomes trial demonstrated that patients with elevated Lp(a) received a greater benefit from PCSK9 targeted mAb (evolocumab); and that those with the lowest achieved Lp(a) and LDLc had the lowest event rate (Boffa and Koschinsky, 2019; O'Donoghue et al., 2019). These results were confirmed by a meta-analysis from ten phase-3 alirocumab trials (PCSK9 mAb) although significance of association for Lp(a) lowering was dependent on LDLc lowering (Ray et al., 2019b).

With the advent of PCSK9 mAb, there have been numerous studies examining the interaction between PCSK9 and Lp(a) and the mechanism for PCSK9 regulation of plasma Lp(a). Importantly, however, statins (which also increase hepatic LDLR) have not demonstrated lowering effects of Lp(a) (Hernandez et al., 2011) and may even cause a significant increase (Tsimikas et al., 2020). In addition, treatment with PCSK9 mAb have demonstrated both increased fractional catabolic rate of Lp(a) and decreased Lp(a) production rate (Reyes-Soffer et al., 2017; Watts et al., 2018). This was supported by studies that have demonstrated LDLR mediated clearance and degradation of Lp(a) (Romagnuolo et al., 2015), as well as PCSK9 mediated enhanced Lp(a) production (Villard et al., 2016). Therefore, PCSK9 mAb may increase Lp(a) clearance through enhanced expression of LDLR and/or decreased Lp(a) synthesis or assembly (Villard et al., 2016; Watts et al., 2018) at the plasma membrane (Kostner and Kostner, 2017), ultimately lowering plasma Lp(a). Additionally, it is possible that Lp(a) is not singly internalized by LDLR but also by LRP-1, CD36, TLR2 (toll-like receptor 2), SR-B1 (scavenger receptor-B1) and plasminogen receptors (Shapiro et al., 2019), which may be in turn more abundant in the absence of PCSK9. In

conclusion, more studies are required to elucidate the underlying mechanism(s) for Lp(a) regulation by PCSK9.

I.1.4.6 CAP1-dependent PCSK9 activity

A recent article presented a new protein implicated in cholesterol homeostasis termed adenylyl cyclase-associated protein 1 (CAP1) (Jang et al., 2020). The results from this article are critical because CAP1 is proposed to be the long searched 'Protein X' that is required to link the LDLR/PCSK9 complex at the cell surface to the endocytosis machinery. However, the findings from this article were surprising considering firstly that this protein had only just been identified as a receptor mediating resistin-dependent inflammatory signaling of monocytic cells and is poorly expressed in the liver (Lee et al., 2014). Moreover, this protein's structural definition does not correspond to the anticipated features for 'Protein X' since CAP1 is a cytosolic protein that does not contain a transmembrane domain and cytosolic tail, nor is part of the secretory pathway. Therefore, the mechanism transporting the receptor to the extracellular membrane remains unresolved. CAP1 is composed of an adenylyl cyclase binding domain, a proline-rich SH3 binding domain (SH3BD) and an actin binding domain. CAP1 expression pattern is similar to that of resistin with enrichment in peripheral blood mononuclear cells and bone marrow, although it appears to be ubiquitously expressed (Lee et al., 2014). It is highly conserved between species and the mouse homozygous knockout gene is embryonic lethal (Lee et al., 2014), suggesting it plays important yet uncharacterized roles during development. The interaction with CAP1 takes place through the SH3BD with the multimeric resistin complex. This complex forms a homotrimer with elevated structural homology to PCSK9 CHRD (Hampton et al., 2007) (view section I.1.3.3). CAP1 and resistin binding results in increased cAMP concentration and PKA activation, as well as NFκB related transcription of inflammatory cytokines in monocytes (Lee et al., 2014). Similarly to the resistin/CAP1 complex, the CAP1/PCSK9 binding interface was identified at the CHRD/SH3BD (Jang et al., 2020). CAP1 heterozygous knockout mice resulted in an approximately 3-fold increase in liver LDLR, and on a high fat diet resulted in decreased total plasma cholesterol and decreased LDLc (Jang et al., 2020). The *in vitro* knockdown of CAP1 prevented the degradation of the LDLR by PCSK9 and adenoviral overexpression of PCSK9 in the heterozygous CAP1 knockout mice only partially decreased liver LDLR levels in comparison

to wildtype mice (Jang et al., 2020). Moreover, the authors indicated that PCSK9 can internalize the LDLR through the clathrin and the caveolin-1 endocytic pathways, where only the caveolin-1 mediated pathway targeted the LDLR for degradation while the clathrin pathway allowed for efficient recycling of the LDLR. Furthermore, CAP1 was found to only bind to caveolin-1, and its knockdown only prevented caveolin-mediated endocytosis (view Figure I.1.12). These results contrast with previous studies that had demonstrated the requirement for clathrin heavy chain for PCSK9 mediated LDLR degradation (Nassoury et al., 2007; Poirier et al., 2009). It is interesting to note that in their model, the authors provide evidence for PCSK9 binding to CAP1 with subsequent degradation of the LDLR, but they do not examine the effect of a reduction in CAP1 levels on circulating levels of PCSK9. Moreover, it is known that the internalization rate of PCSK9 depends on cell surface LDLR, yet the authors do not study the role of the LDLR in this CAP1 dependent internalization and degradation.

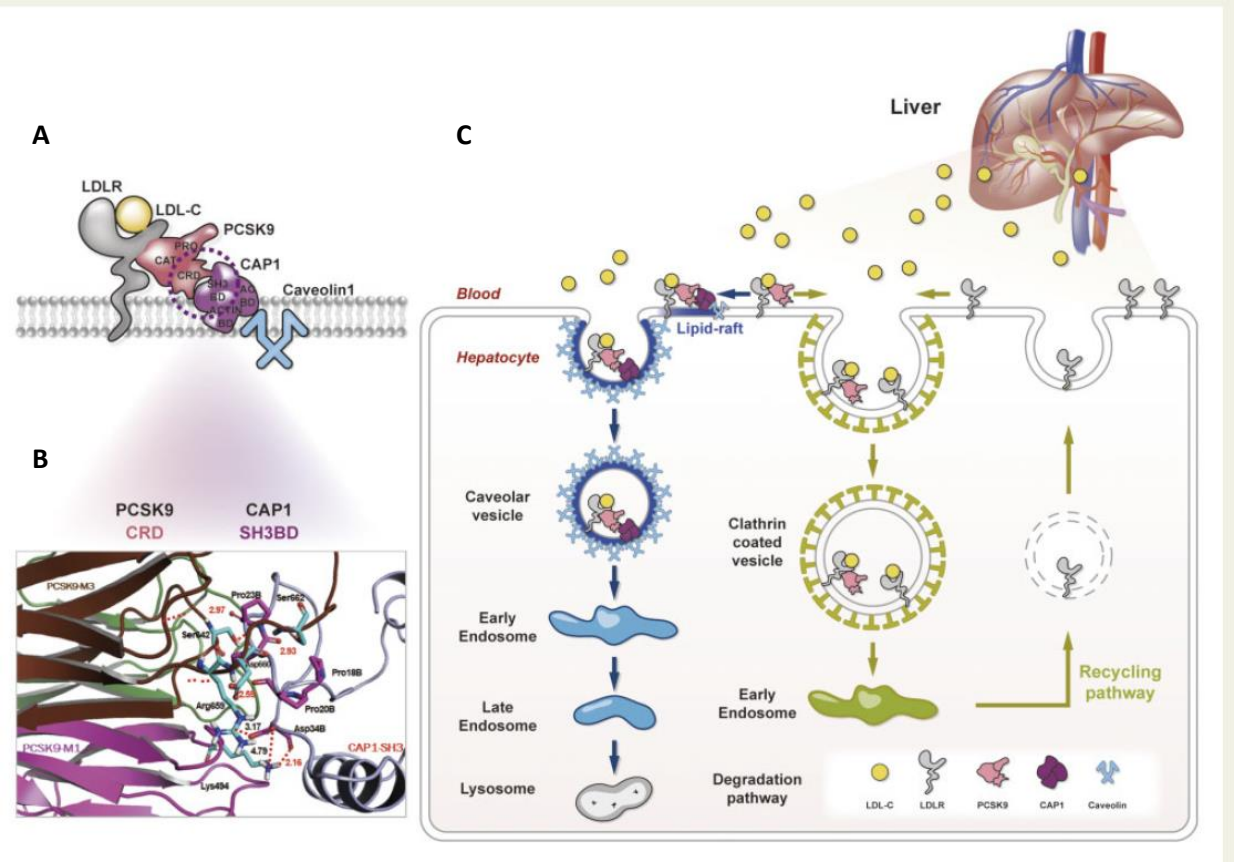

Figure I.1. 12 CAP-1 dependent LDLR/ PCSK9 complex internalization

A, schematic representation of the LDLR/PCSK9/CAP1 binding complex. ***B,*** tertiary structure depicting PCSK9 CHRD domain interaction with SH3BD domain of CAP1. ***C,*** caveolin-dependent degradation of the LDLR (Jang et al., 2020).

I.1.4.7 Inhibition by GRP94

It is known that liver LDLR is mostly sensitive to extracellular PCSK9, whereas in cell lines, an intracellular pathway has been demonstrated (Poirier et al., 2009; Seidah et al., 2018). Nassoury *et al.* characterized the trafficking of PCSK9 in the presence or absence of the LDLR in both cell lines and primary hepatocytes (Nassoury et al., 2007). Interestingly, in the absence of the LDLR, PCSK9 was mainly localized to the ER, however, under co-expression with the LDLR, both proteins localized to the degradation endosomal/lysosomal system. In contrast, the proteins colocalized to the Golgi apparatus in primary hepatocytes, correlating with the notion that liver cells are not able to degrade the LDLR through an intracellular pathway. Moreover, in both cell lines and in primary hepatocytes, proPCSK9 immunoprecipitated with the LDLR. Therefore, the interaction in the ER of immature LDLR and proPCSK9 is not the limiting event to induce colocalization to the endosomal/lysosomal system and intracellular mediated degradation of the LDLR. Therefore, Poirier *et al*. searched for an intracellular PCSK9 interacting partner which could prevent targeting of the LDLR from the *trans*-Golgi network directly to the lysosomes for degradation (Poirier et al., 2015). Indeed, they were able to identify directly interacting ER resident chaperones, GRP94, and somewhat less so, GRP78 (BiP). They demonstrated that this binding with GRP94 was not necessary for PCSK9 maturation and secretion and was competitively inhibited by EGF-A. Moreover, in the absence of GRP94, they observed an increase in PCSK9 secretion and degradation of the LDLR. Therefore, this study suggests that GRP94 is an endogenous inhibitor of PCSK9 mediated intracellular degradation of the LDLR. This was functionally confirmed by liver specific GRP94 knockout mice that demonstrated increased circulating PCSK9, decreased hepatocyte surface LDLR and increased LDLc (Poirier et al., 2015).

I.1.4.8 GRP94 and GRP78/BiP: Role of PCSK9 in ER stress

In most mammalian cells, one third of all proteins are synthesized in the ER, but in certain cells, like hepatocytes, it may rise to up to 70% (Benyair et al., 2011). High-quality protein folding is essential for cell survival and function, additionally, protein folding is the most error-prone step in gene expression. Therefore, various control mechanisms have evolved to preserve protein-folding homeostasis through rigorous quality control processes, ER compartmentalization and

through sensors of ER homeostasis, which impact cell fate. Chaperone association of nascent polypeptides prevents aggregation through hydrophobic patches, are required for proper protein folding and coordinate quality control processes. Two of the main chaperone systems are BiP (GRP78) and calnexin (CNX)/calreticulin (CRT). BiP acts in concert with its co-chaperones of the ERdj family (ERdj1-7) and the nucleotide exchange factors Sil1 and GRP170 (Benyair et al., 2011; Ellgaard et al., 2016). BiP functions with different ERdj proteins for: 1) recruitment to translating ribosomes and for protein translocation; 2) for protein folding; and 3) ERAD of misfolded proteins. The lectin chaperones, CNX/CRT, assist glycoprotein folding, where folding state is revealed by specific modifications of their N-glycans. Nascent polypeptides are co-translationally conjugated to a branched oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$ at asparagine residues of the Ans-Xxx-Ser/Thr motif. Trimming of the glucose residues of the attached oligosaccharide by glucosidases (GlucI and GlucII) yields recognition signals for lectins that may serve to inhibit the secretion of misfolded proteins, prevent their aggregation and/or promote formation of di-sulfide bonds through the recruitment of ERp57 (thiol-disulfide oxidoreductase). Lectin binding may also be important for sorting of substrates for ERAD (Benyair et al., 2011). GRP94 is another important folding factor, which has recently been shown to be recruited by BiP in its ADP-bound, "client-bound" state (Sun et al., 2019). GRP94, in contrast to BiP interacts with a narrow range of substrates (Argon and Simen, 1999; Melnick et al., 1994). When the ER becomes overwhelmed or stressed with high protein load, a signaling pathway, termed the unfolded protein response (UPR) pathway, will initiate several adaptive mechanisms, through the activation of sensor proteins PERK, IRE1α, and ATF6. These will promote ER protein folding and ERAD by upregulating the expression of ER chaperones BiP, GRP94 and others (Wu and Kaufman, 2006), inhibit global translation, stimulate autophagy and an antioxidant response, altogether favoring cell survival. In turn, severe stress and sustained activation of the PERK pathway will induce the maladaptive UPR and apoptosis (Wang and Kaufman, 2016) (view Figure I.1.13).

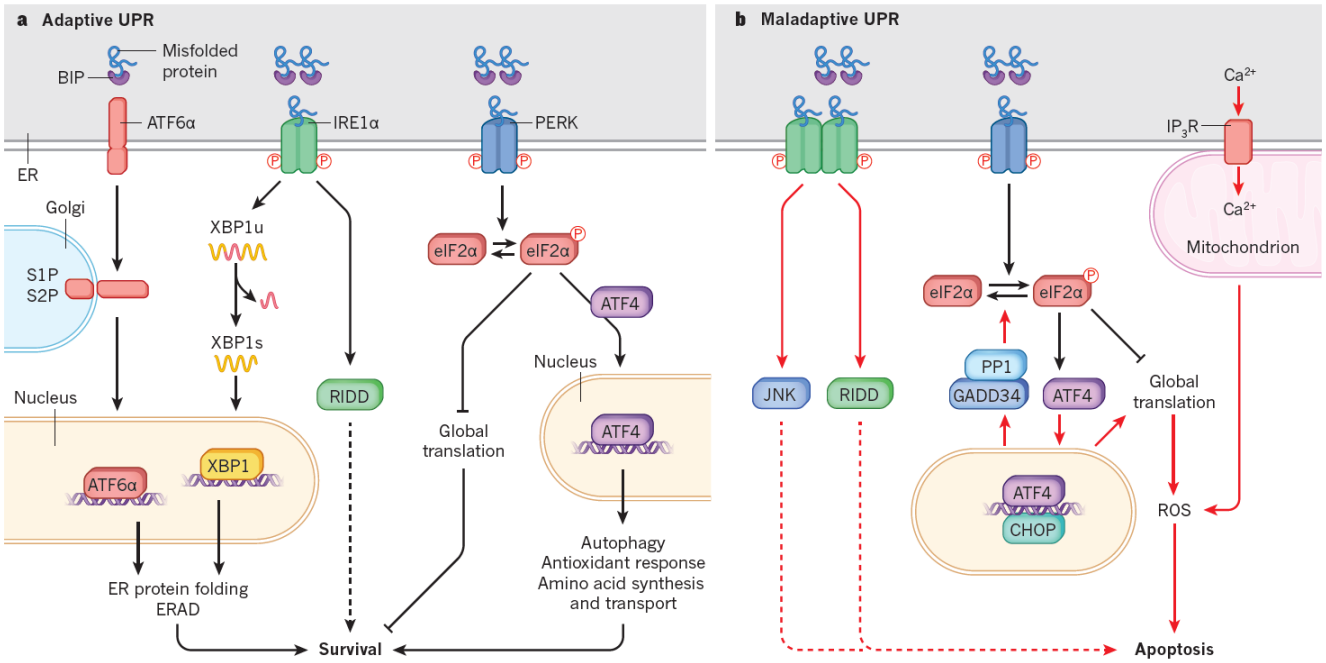

Figure I.1. 13 The UPR signaling pathways

A, Adaptive UPR leads to the upregulation of ER protein folding, ERAD, inhibition of global translation, and autophagy. It is initiated upon disinhibition of the ER sensors ATF6α, IRE1α and PERK from BiP. ATF6α transcription factor translocates to the Golgi apparatus where it is processed and activated. IRE1α and PERK oligomerize and are activated by *trans*-autophosphorylation. These will induce the activation of transcription factors XBP1 and ATF4. The regulated Ire1-dependent decay (RIDD) downstream of IRE1α mediates mRNA decay alleviating ER protein load. ***B,*** Sustained activation of PERK leads to apoptosis mainly through the activation of CHOP (Wang and Kaufman, 2016).

PCSK9 expression is upregulated by ER stress inducing agents in several hepatic cell lines, primary hepatocytes and in mice treated with these agents (Lebeau et al., 2017). In accordance with previous publications, ER stress also upregulated LDLR and activated sterol-regulated element binding protein -2 (SREBP-2), suggesting SREBP-2 mediated PCSK9 and LDLR stimulated expression (Lebeau et al., 2017; Pai et al., 1996; Werstuck et al., 2001). However, under these conditions, PCSK9 secretion was suppressed by ER retention of the mature PCSK9, thereby allowing for an increase in cell surface LDLR (Lebeau et al., 2017). The authors suggested that PCSK9 interaction with GRP94 could favor this retention, in particular because GRP94 is also upregulated during ER stress. Moreover, ER stress may have a general effect on protein retention in the ER since it has been reported to impair anterograde COPII vesicular trafficking (Chen et al., 2013). ER resident PCSK9 variants (uncleaved, proPCSK9) oligomerize causing dominant negative effects on WT PCSK9 (Benjannet et al., 2012; Cariou et al., 2009; Mayne et al., 2011).

Although, these do not normally induce ER stress (Benjannet et al., 2012), knockdown of GRP94 in the presence of PCSK9 induces an upregulation of UPR markers and allows binding of proPCSK9 to BiP (Lebeau et al., 2018; Poirier et al., 2015). Moreover, by preventing remnant particle internalization, the presence *vs.* the absence of PCSK9 was found to be protective from palmitic acid induced ER stress and cytotoxicity in hepatic cell lines (Lebeau et al., 2019). This protective effect of PCSK9 was further validated *in vivo* where high fat diet caused ER stress, hepatic steatosis and inflammation in *Pcsk9*$^{-/-}$ mice. These results suggest that PCSK9 participates in ER homeostasis particularly under ER toxic conditions.

I.1.5. Regulation of PCSK9 expression

I.1.5.1 Cholesterol and SREBP-2

In the plasma, PCSK9 levels are most reproducibly associated with LDLc (Lakoski et al., 2009; Lambert et al., 2008; Mayne et al., 2007), although the association is modest since variations in fasting plasma PCSK9 levels in a large cohort (>3000 probands) only accounted for 7% of the variations in LDLc (Lakoski et al., 2009). Therefore, circulating levels of PCSK9 poorly represent its regulatory activity of LDLc. This weak correlation could in part be explained by the significant proportions of inhibited circulating PCSK9 resulting from either furin/PC5-A-cleavage (Benjannet et al., 2006) or by association to LDL particles (~30% of plasma PCSK9) (Kosenko et al., 2013; Tavori et al., 2013).

PCSK9, LDLR and cholesterol synthesis are upregulated under conditions of low intracellular cholesterol by a common transcription factor, SREBP-2 (Horton et al., 2003; Maxwell et al., 2003). Several groups have demonstrated that PCSK9 is upregulated along with the LDLR by hydroxymethyglutaryl co-enzyme A (HMG-CoA) reductase inhibitors (statins) which enhance SREBP-2 activity (Awan et al., 2012; Careskey et al., 2008; Dubuc et al., 2004; Mayne et al., 2008). Additionally, an SRE element was identified in the PCSK9 promoter, almost identical to that of the LDLR (Jeong et al., 2008). Therefore, under conditions of low intracellular cholesterol, the cell paradoxically upregulates both the LDLR and PCSK9 to allow for cholesterol internalization and subsequent degradation of the LDLR and clearance of PCSK9 from the circulation ensuring both intracellular cholesterol homeostasis and constant LDLc concentrations.

It is known that hepatic cholesterol synthesis follows a diurnal rhythm with a nadir in the afternoon and a peak at night, and may be monitored by measuring lathosterol, a marker for cholesterol synthesis (Galman et al., 2005; Miettinen, 1982). In a human study by Persson *et al.* PCSK9 and lathosterol were both strongly correlated with normal diurnal rhythm and short-term fasting conditions, suggesting synchronous regulation (Persson et al., 2010). This study also showed that after 18 hours of fasting, PCSK9 levels were reduced by ~35% and after 66 hours, they were lowered by 64%-97% (Persson et al., 2010), these observations were closely paralleled by lathosterol. In contrast, plasma LDLc remained stable for all these time points. The suppression of cholesterol synthesis and plasma PCSK9 would likely have been accompanied by a reduction in LDLR at the mRNA level, considering that these genes are known to be coregulated by SREBP-2 and, it is known from mice, that SREBP-2 is suppressed during fasting in the liver (Horton et al., 1998). Altogether, PCSK9 and LDLR transcriptional suppression would result in stabilized LDLR protein levels and in turn plasma LDLc, due to the resulting absence of PCSK9 mediated degradation of LDLR. Very similar results were reported by another research group where fasting induced a strong decline in secreted PCSK9 and also lathosterol-to-cholesterol ratio in healthy individuals (Browning and Horton, 2010).

Cholesterol-rich diets (2-3% content) alter hepatic SREBP-2 and PCSK9 expression in rodent models. Indeed, these diets resulted in an increase in hepatic cholesterol content and a decrease in SREBP-2 and PCSK9 protein expression (Chong et al., 2011; De Smet et al., 2015; Persson et al., 2009). Enterocyte PCSK9 mRNA and protein is also sensitive to incubation with cholesterol (Leblond et al., 2009). In contrast, high-fat and high-fat, high-protein short term diets in young healthy volunteers increased lathosterol synthesis but did not significantly alter PCSK9 concentrations and trended towards an increase (Cariou et al., 2013). Moreover, an acute oral fat load in healthy subjects did not lead to changes in plasma PCSK9 concentrations (Cariou et al., 2013). Therefore, in humans, high fat diets have little to no impact on PCSK9 concentrations, however, these diets contained other fatty acids which may have an impact on PCSK9 expression as well.

I.1.5.2 Insulin, glucagon and SREBP-1c

PCSK9 also correlates well with TGs, fasting insulin and glucose levels and insulin resistance in non-diabetic cohorts (Baass et al., 2009; Cui et al., 2010; Dubuc et al., 2010; Ferri et al., 2020; Ghosh et al., 2015; Lakoski et al., 2009). Accordingly, studies in mice had previously demonstrated that PCSK9 expression is positively regulated by nutritional status, suppressed by fasting and stimulated by re-feeding, further suggesting that insulin drives PCSK9 expression through the transcriptional activity of SREBP-1c (Costet et al., 2006). In another early study that further strengthens the role for insulin driven PCSK9 expression, a model of type 1 diabetes in rats (diabetes induced by streptozotocin resulting in destruction of pancreatic β-cells and suppression of insulin secretion), demonstrated strongly downregulated hepatic LDLR and PCSK9 mRNA levels, which were accompanied with undetectable PCSK9 protein levels and unchanged LDLR protein levels, likely due to the suppression of PCSK9 synthesis (Niesen et al., 2008).

Glucagon has been demonstrated to suppress SREBP-1c, -2 and suppress PCSK9 expression in hepatocytes (Miao et al., 2015; Persson et al., 2009). Glucagon regulation of PCSK9 independently from the effects on SREBPs is highly probable and was suggested by Persson *et al.* in their study in rats that showed that glucagon induced a 30% decrease in SREBP-2 *vs*. a 75% decrease in PCSK9 mRNA (Persson et al., 2009). In order to better understand the role of insulin and glucagon in the regulation of PCSK9, Miao *et al.* suppressed insulin signaling by either inducing the hepatocyte specific knockout of the insulin-receptor (LIRKO), destruction of the β-cells of the pancreas (streptozotocin treatment), or anti-sense knockdown against the insulin receptor in *ob/ob* mice (mouse model for type II diabetes) (Miao et al., 2015). All these *in vivo* conditions resulted in strongly decreased PCSK9 levels, reproducing observations mentioned above (Costet et al., 2006; Niesen et al., 2008). However, the acute knockdown of the insulin receptor in lean, wild-type mice did not significantly decrease PCSK9 levels, similarly to the study by Ai *et al* (Ai et al., 2012). The authors also confirmed the observations from Persson *et al.* that glucagon may downregulate PCSK9 *in vitro* and further showed that LIRKO mice demonstrate an additional 80% decrease in hepatic PCSK9 when fasted in comparison to non-fasted, even though they have abolished insulin signaling, suggestive of *in vivo* glucagon regulation of PCSK9 (Miao et al., 2015). Therefore, under *in vivo* fasting conditions, the regulation of PCSK9 expression in hepatocytes is defined by the activities of glucagon and insulin.

In the same study by Cariou *et al.* described above, the short-term high-fructose diet increased PCSK9 significantly but did not alter LDLc or lathosterol synthesis (Cariou et al., 2013). Under these conditions, plasma PCSK9 concentrations associated strongly with liver steatosis and increased TG in plasma in addition to decreased hepatic and whole-body insulin sensitivity. Since the authors did not observe any changes in LDLc, they suggested that the PCSK9 increase was independent from cholesterol synthesis pathway and regulation by SREBP-2, while most likely resulted from increased SREBP-1c activity, particularly since a high fructose diet is known to increase SREBP-1c activity (Haas et al., 2012), as fructose inhibits hepatic lipid oxidation and favors VLDL TG synthesis (Tappy and Le, 2010).

A highly relevant study directed on overweight or obese adolescents and young adults as an exploration of the effects of an intensive dietary intervention on the levels of circulating PCSK9 demonstrated that performing caloric restriction (deficit of ~25% in total calories) for eight weeks significantly decreased PCSK9 (Levenson et al., 2017a). Interestingly, the strongest predictors of changes in PCSK9 were improvements in fasting insulin and homeostasis model assessment of insulin resistance (HOMA-IR), independently from weight loss. Similarly to the study by Cariou *et al*., this study showed no significant association of PCSK9 with LDLc, which could be accounted for by the relatively small number of participants. PCSK9 is therefore closely associated with insulin sensitivity and fatty acid metabolism, however a functional link between these associations is not yet elucidated and requires further investigation (Richard et al., 2012).

I.1.5.3 HNF1α

The last most recognized nuclear transcription factor regulating PCSK9 expression is HNF1α. Indeed, a plant-derived hypocholesterolemic compound, berberine, upregulates LDLR levels through the downregulation of PCSK9. HNF1α, has been demonstrated to be inhibited by berberine and required for the latter's effects on PCSK9 (Cameron et al., 2008b). Moreover, liver-specific knockdown of HNF1α results in decreased PCSK9 levels (Shende et al., 2015). In contrast, Ai *et al*., treated mice with rapamycin, an inhibitor of mammalian target of rapamycin (mTORC)-1, which resulted in an increase in PCSK9 by increasing HNF1α levels (Ai et al., 2012).

I.1.5.4 PPARα

Finally, the nuclear transcription factor PPARα is known to be upregulated during fasting state and is required for the expression of genes involved in fatty acid catabolism. Accordingly, fenofibrates, which upregulate PPARα, also reduce PCSK9 in mice (Kourimate et al., 2008; Lambert et al., 2006). PPARα has been shown to decrease PCSK9 expression through the liver X receptor and liver X receptor agonists decrease PCSK9 in rat hepatocytes as well (Kourimate et al., 2008; Ou et al., 2001). Moreover, in humans, mono-unsaturated and poly-unsaturated fatty acids (MUFAs, PUFAs) or a Mediterranean diet (which is rich in MUFAs and PUFAs) decrease PCSK9 by ~12% (Bjermo et al., 2012; Richard et al., 2012). These FAs are ligands for SREBP-1c and PPARα (Sekiya et al., 2003).

I.1.5.5 Variations with sex and age

One of the strongest covariates associated with circulating levels of PCSK9 is sex. This association has been confirmed by several large studies from the United States, China and Europe (Cui et al., 2010; Ferri et al., 2020; Lakoski et al., 2009). Interestingly, the measured plasma PCSK9 levels reported between these large studies varies over a wide range, while the mean concentrations between studies vary as well. In the Dallas Heart Study in the US, the African-Americans and European-Americans have very similar distributions, median and mean PCSK9, mean 510 ng/mL *vs.* 529 ng/mL, with exclusion of the common LOF mutations (Lakoski et al., 2009). From the study of Prediction, Prevention and Intervention for type 2 diabetes in Nanjing, in China the mean concentration of serum PCSK9 was 69.35 ng/mL (Cui et al., 2010). From the IMPROVE study including individuals from five different European countries the mean levels were of 310.8 ng/mL (Ferri et al., 2020). Therefore, considering the levels were all measured from overnight fasted individuals with equivalent distributions of adult men and women, possible reasons for such large variations could be genetic and/or environmental factors conferring confounding effects. All these studies, however, reported higher levels of PCSK9 in women than in men, which remained significant after adjustments for covariates. Moreover, PCSK9 levels were greater in post-menopausal women than in pre-menopausal women, and are independent of estrogen status; finally, both men and women demonstrate increased LDLc with aging, however

this may be explained by a paralleled increase in PCSK9 only for women and not men, since PCSK9 does not associate significantly with age in men. The direct effect of estrogens on PCSK9 is controversial since some studies show inverse correlation between PCSK9 and endogenous estrogens (decrease of 14% PCSK9 when endogenous estrogens are high) (Ghosh et al., 2015; Persson et al., 2012) while another did not (Guo et al., 2015). In the rat, however, pharmacological administration of estrogens decrease PCSK9 mRNA (Persson et al., 2009). Another possible effect of estrogens on PCSK9 function is the effect they may have on the trafficking of the LDLR, as evidenced in mice by a sex- and tissue-specific subcellular distribution of the LDLR, which is determined by E2 levels (Roubtsova et al., 2015). Altogether, these results suggest sex-dependent differential modulation of PCSK9 mediated degradation of the LDLR in rodents and humans (Fu et al., 2019).

I.1.6. Therapeutic inhibition of PCSK9

I.1.6.1 ASCVD is largely LDLc driven

In an eloquent assessment of the causal role of LDLc in the development of atherosclerotic cardiovascular disease (ASCVD) by Ference *et al*. that considers meta-analyses of prospective epidemiologic studies, Mendelian randomization studies and randomized trials, two important conclusions are drawn (Ference et al., 2017). The first is the consistent dose-dependent association between the absolute magnitude of exposure of the endothelium vasculature to high LDLc and the risk for ASCVD, and the second is the apparent increase in risk with longer LDLc exposure. Through their assessment they provide and summarize the evidence to prove that these associations between LDLc and ASCVD risk satisfy the criteria for causality. Their assessment is summarized in Table I.1.2. They do not exclude that there are emerging evidence that other apoB-containing lipoproteins are also directly and independently causal of ASCVD.

Table I.1. 2 Evidence for LDL causality of ASCVD

Criterion (modified from reference[5])	Evidence grade	Summary of the evidence (references)
1. Plausibility	1	LDL and other apolipoprotein (apo) B-containing lipoproteins (very low-density lipoprotein their remnants, intermediate-density lipoprotein and lipoprotein(a)) are directly implicated in the initiation and progression of ASCVD; experimentally induced elevations in plasma LDL and other apoB-containing lipoproteins lead to atherosclerosis in all mammalian species studied.[2,5–12]
2. Strength	1	Monogenic and polygenic-mediated lifelong elevations in LDL lead to markedly higher lifetime risk.[13–20,27–31,40,43]
3. Biological gradient	1	Monogenic lipid disorders, prospective cohort studies, Mendelian randomization studies, and randomized intervention trials uniformly demonstrate a dose-dependent, log-linear association between the absolute magnitude of exposure to LDL and risk of ASCVD[13–22,27–36,38–40,42–47]
4. Temporal sequence	1	Monogenic lipid disorders and Mendelian randomization studies demonstrate that exposure to elevated LDL precedes the onset of ASCVD[13–20,27–31,40,43]
5. Specificity	1	Mendelian randomization studies and randomized intervention trials both provide unconfounded randomized evidence that LDL is associated with ASCVD independent of other risk factors[28,31–33,40,43]
6. Consistency	1	Over 200 studies involving more than 2 million participants with over 20 million person-years of follow-up and more than 150 000 cardiovascular events consistently demonstrate a dose-dependent, log-linear association between the absolute magnitude of exposure to LDL and risk of ASCVD[13–22,27–36,38–40,42–47]
7. Coherence	1	Monogenic lipid disorders, prospective cohort studies, Mendelian randomization studies, and randomized intervention trials all show a dose-dependent, log-linear association between the absolute magnitude of exposure to LDL and risk of ASCVD[15–18,21,22,28,30–32,35,36,43,44,47]
8. Reduction in risk with intervention	1	More than 30 randomized trials involving over 200 000 participants and 30 000 ASCVD events evaluating therapies specifically designed to lower LDL (including statins, ezetimibe, and PCSK9 inhibitors) consistently demonstrate that reducing LDL cholesterol (LDL-C) reduces the risk of ASCVD events proportional to the absolute reduction in LDL-C[32–34,38,39,42,45–47]

Criteria are graded by a modification of the quality criteria adopted by the European Society of Cardiology system.
For reference, see http://www.escardio.org/Guidelines-&-Education/Clinical-Practice-Guidelines/Guidelinesdevelopment/Writing-ESC-Guidelines (31 January 2017).
These are defined as follows:
Class 1: Evidence and/or general agreement that the criterion for causality is fulfilled.
Class 2: Conflicting evidence and/or a divergence of opinion about whether the criterion indicated causality.
Class 3: Evidence or general agreement that the criterion for causality is not fulfilled.

Adapted from (Ference et al., 2017).

Indeed, atherosclerosis is mainly driven by LDL intimal penetration in the artery wall, phagocytosis by macrophages, and oxidation of the lipids (phospholipids, cholesteryl esters, and cholesterol) and apoB by myeloperoxidase and/or by reactive oxygen species, which when released initiates immuno-inflammatory responses preventing efferocytosis of necrotic and apoptotic cells (removal of dead cells) and formation of a necrotic core which together with smooth muscle cell proliferation lead to hyperplasia of the artery wall and subsequent obstruction of the vessel (Boren et al., 2020). Other apoB-containing lipoproteins contribute to this process, and include Lp(a), damaging due to its properties of carrying proinflammatory oxidized phospholipids. The classic TG-rich particles, in contrast, require hydrolysis of TGs and modification to cholesterol-enriched remnant particles before being efficiently up-taken by receptors of the LDLR family at the arterial wall (intermediate-low density lipoprotein, IDL) (Boren et al., 2020).

Certain factors may increase the atherogenicity of LDL particles by stimulating the initial steps of atherosclerosis: transcytosis and retention of LDL in the subendothelial space of the arterial wall (Boren et al., 2020). Transcytosis occurs through secretory/endocytic pathway, involving caveolae, scavenger-receptor B1, activin receptor-like kinase 1 and LDLR. However, transcytosis of LDL across the endothelial cell monolayers of the arterial wall is thought to be independent on the LDLR since FH patients carriers of an *LDLR* null allele in contrast to a defective allele show increased number of diseased vessels, among other phenotypes (Vohl et al., 1997). Additionally, PCSK9 mediated degradation of the LDLR does not affect transcytosis (Armstrong et al., 2015), whereas the LDLR is important for transcytosis across the blood-brain barrier. Activation of the NLRP3 inflammasome by lipid loaded macrophages leads to the production of interleukin-1 family of cytokines in turn increasing transcytosis of LDL. Additionally, hyperglycemia has been demonstrated to prevent the degradation of caveolae through the inhibition of AMPK-MTOR-PIK3C3 pathway, thereby leading to increased available caveolin-1 for transcytosis (Bai et al., 2020). Finally, decreasing hypercholesterolemia improved the endothelial barrier to LDL transcytosis in mice (Bartels et al., 2015).

Genes that encode arterial wall proteins regulating the susceptibility to LDL retention have been found to be strongly associated with CAD (Klarin et al., 2018). Moreover, changes in the core and surface LDL particle affect the ability of apoB to bind to proteoglycans of the arterial wall, for example, enrichment of LDL with cholesteryl oleate enhances binding and atherogenesis. In addition, apoE and apoC-III increase the affinity of the LDL for arterial wall proteoglycans.

Insulin resistant states such as the metabolic syndrome and T2DM are characterized by VLDL overproduction and secretion by the liver and intestine (Boren et al., 2020). These conditions of high plasma TG also result from decreased TG hydrolysis and delay in VLDL clearance due to the saturation of circulating lipoprotein lipases in addition to conditions of high apoC-III. Cholesteryl ester transfer protein (CETP) will exchange TG particles of VLDL for cholesteryl esters to the core of LDL. In turn, these will be hydrolysed by hepatic lipases leading to small dense low-density lipoproteins (sdLDL). Therefore, the formation of sdLDL are dependent on plasma TGs and are favoured when plasma TG levels exceed 1.7 mmol/L. Increased plasma TG, sdLDL and an accompanied decrease in high density lipoproteins are the key attributes of atherogenic dyslipidemia. In contrast, a healthy state is characterized by lower levels of plasma

TGs, resulting from highly active lipases and low hepatic TG content, smaller VLDL and larger, lighter LDL (Boren et al., 2020).

sdLDL biological features increase the atherogenicity of LDL. They demonstrate prolonged plasma residence time due to decreased binding to LDLR resulting from the conformation changes of apoB induced by decrease particle size. sdLDL demonstrate enhanced arterial wall infiltration due to smaller size. Furthermore, they show increased binding affinity to arterial wall proteoglycans favouring enhanced retention and increased susceptibility to oxidation of lipids and glycation which promote an immune response, inflammation, and a necrotic core formation.

I.1.6.2 Review of clinical trials outcomes

The recent discovery and understanding of these mechanisms that render the LDL particle atherogenic come to emphasize and reinforce the importance of rapidly and effectively decreasing LDL in patients of high risk for CV events. Indeed, according to the Cholesterol Treatment Trialists meta-analysis, each 39 mg/dL (1 mmol/L) reduction was associated with a 21% reduction in major cardiovascular events (Baigent et al., 2005). To reach the LDLc target, lifestyle modifications and treatment with high-intensity statins are recommended. However, only approximately 30% of patients with established ASCVD achieve the LDLc goals that are considered as acceptable in treatment guidelines (Steen et al., 2017). Therefore, there remains great necessity for the pursued development of other lipid lowering treatments (LLTs).

Inhibitors of the cholesterol synthesis pathway as a means to decrease plasma lipids was first appreciated by Endo and others with the discovery of compactin in 1976 (Endo et al., 1976; Tobert, 2003). Lovastatin was then developed for clinical trials (Alberts et al., 1980; Tobert, 2003). However, it was not until 1987 that statins became available for prescription and until 1994, with the Simvastatin Survival Study (4S) trial, in which all controversies against the safety for the use of statins were cleared and evidence towards reduction in coronary deaths and reduction in major coronary events and revascularization procedures were demonstrated (Pedersen et al., 2000). Statins went through a long period of controversies particularly because there was significant toxicity at high doses noted in long-term animal toxicology studies, which were not reproduced in human trials. Moreover, until the 4S trial, there was no evidence for a reduction in mortality rates.

The principal risks associated with the administration of statins are myopathy, muscle pain and rarely rhabdomyolysis, particularly when taken with other drugs, such as fibrates and niacin (Wood et al., 2020). Additionally, several meta-analyses have shown that intensive-dose statins compared with moderate-dose statin therapy are associated with an increase risk for new-onset diabetes (Preiss et al., 2011; Sattar et al., 2010).

The way statins effectively decrease circulating LDLc was unexpected by those who discovered compactin and lovastatin in the mid-1970s (Alberts et al., 1980; Endo et al., 1976). It was the Nobel Prize winners Brown and Goldstein that demonstrated that statins by inhibiting the rate limiting enzyme of the cholesterol synthesis pathway result in the depletion of mevalonate which results in the upregulation of the enzymes of the mevalonate pathway, as well as the LDLR, through SREBP-2 (Bilheimer et al., 1983). Importantly, it is the upregulation of the LDLR by hepatocytes that is crucial for statins' protective effects. This was confirmed in animals and human liver biopsies, which demonstrated a two-fold increase of LDLR protein in the livers of patients (Tobert, 2003). One of the effects of upregulating SREBP-2 is the upregulation of PCSK9 expression, accordingly, studies have demonstrated that statins upregulate PCSK9 in human hepatocarcinoma cells (Mayne et al., 2008) and in patients (Careskey et al., 2008), where a 42% reduction in LDLc was accompanied by a 34% increase in PCSK9, suggesting firstly that statins are only partly efficient at reducing LDLc due to this effect and secondly that targeting PCSK9 would provide an additive and complementary effect towards statin treatment.

Achieving lower LDLc levels with a lipid lowering drug as an adjuvant to statins has been difficult, however with the reports of the IMPROVE-IT trial testing the efficacy of ezetimibe, the authors demonstrated that further reductions in LDLc improved clinical outcomes (Cannon et al., 2015). Ezetimibe decreases LDLc by inhibiting cholesterol absorption from the intestine (Kosoglou et al., 2002; Sudhop et al., 2002). When added to statins, Ezetimibe further reduces LDLc levels by ~23% and reducing by 6% the risk for cardiovascular events.

Rapidly after the discovery of PCSK9 in 2003 there was high interest in developing inhibitors particularly for the development of monoclonal antibodies because it is a circulating protein. The "humanization" of monoclonal antibodies generated against PCSK9 in mice is necessary for administration to human patients and involves the modification of the protein sequences of the antibody light and heavy chain to antibody variants produced naturally in humans. Evolocumab by AMGEN, alirocumab by SANOFI/REGENERON and bococizumab by Pfizer

were developed in 2009, initiating the first clinical trials. The development of the not completely humanized bococizumab and its clinical trials were interrupted because the treatment triggered the development of anti-drug antibodies, thereby decreasing the effectiveness of the treatment. Moverover, 1 in 10 patients showed no reduction in LDLc levels. In contrast to bococizumab, which is a partially humanized antibody, alirocumab and evolocumab are fully humanized antibodies, thereby preventing immunogenicity.

Results of the phase-III clinical trials of alirocumab, the ODYSSEY LONG TERM, and of evolocumab, OSLER-1 and -2, were published in 2015 allowing for their rapid approval by the Food and Drug Administration in the US the same year, as an adjuvant to maximally tolerated statins (Robinson et al., 2015; Sabatine et al., 2015). Importantly, these studies reported efficient lowering of LDLc by -61% for both alirocumab and evolocumab consistently over a period of 1 and 1.5 years, for OSLER-1 and -2 and ODYSSEY LONG TERM respectively. Adverse events that occurred more frequently with the PCSK9 inhibitors than placebo were injection-site reactions, myalgia, neurocognitive disorders and ophthalmologic disorders.

Longer term studies designed to assess the effect of these inhibitors towards improving clinical outcomes were published in March 2017, termed the FOURIER trial for evolocumab and in March 2018, termed the ODYSSEY OUTCOMES trial for alirocumab (Sabatine et al., 2017; Schwartz et al., 2018). In both trials, the administration of the PCSK9 inhibitor further reduced LDLc by >50% from baseline achieved with statin therapy, without major safety concerns, over the course of the studies (2.2 and 2.8 years for FOURIER and ODYSSEY OUTCOMES, respectively). Importantly, while there were some differences in the study design and recruitment of patients, the relative risk reduction in CV events achieved per 1 mmol/L of decreased LDLc were consistent with the previously reported LLT studies for statins and ezetimibe (Cannon et al., 2015; Cholesterol Treatment Trialists et al., 2010). The main characteristics for the study designs and the differences between both studies are illustrated in Table I.1.3. Patients recruited in both trials were high-risk patients with established ASCVD, ~80% of all patients had had a history of myocardial infarct and all patients had >70 mg/dL (1.8 mmol/L) LDLc despite maximal tolerated statins as background therapy. Among important differences between the studies, it can be noted that the patients in the FOURIER trial were required to have an additional risk factor besides the acute coronary syndrome (such as diabetes mellitus, hypertension…). Additionally, in the ODYSSEY OUTCOMES trial, patients were required to have had a recent acute coronary

syndrome (in the last 4 to 52 weeks), whereas it was a criterion for exclusion in the FOURIER trial. Moreover, because of safety concerns regarding lowering LDLc levels to extremely low levels (<15 mg/dL, 0.38 mmol/L) due previous concerns about a possible association with hemorrhagic stroke (Amarenco et al., 2006), patients from the ODYSSEY OUTCOMES study for which levels were lowered to <15 mg/dL were blindly switched to placebo. Mean LDLc levels for both trials are illustrated in Figure I.1.14.

Table I.1. 3 Comparison between the FOURIER and ODYSSEY trial study designs

Trial details	FOURIER trial	ODYSSEY OUTCOMES trial
Enrolled population (*N*)	27,564	18,924
Age entry criteria	≥ 40 years and ≤ 85 years	≥ 40 years
Inclusion criteria	Prior MI, stroke or symptomatic PAD plus additional high-risk features[a]	Prior acute coronary syndrome (between 1 and 12 months)
Lipid entry criteria	LDL-C ≥ 70 mg/dL or non-HDL-C ≥ 100 mg/dL	LDL-C ≥ 70 mg/dL or non-HDL-C ≥ 100 mg/dL or ApoB ≥ 80 mg/dL
Allowed baseline lipid-lowering therapy	Moderate or high-intensity statin	High-intensity statin[c]
Primary endpoint	CV death, MI, stroke, unstable angina or coronary revascularization	CHD death, MI, unstable angina or stroke
Dedicated study for neurocognitive evaluation	Within the main trial (EBBINGHAUS study)[b]	Ongoing in a separate study[d]
Therapy down-titration when LDL low	No	Yes

Adapted from (Furtado and Giugliano, 2020).

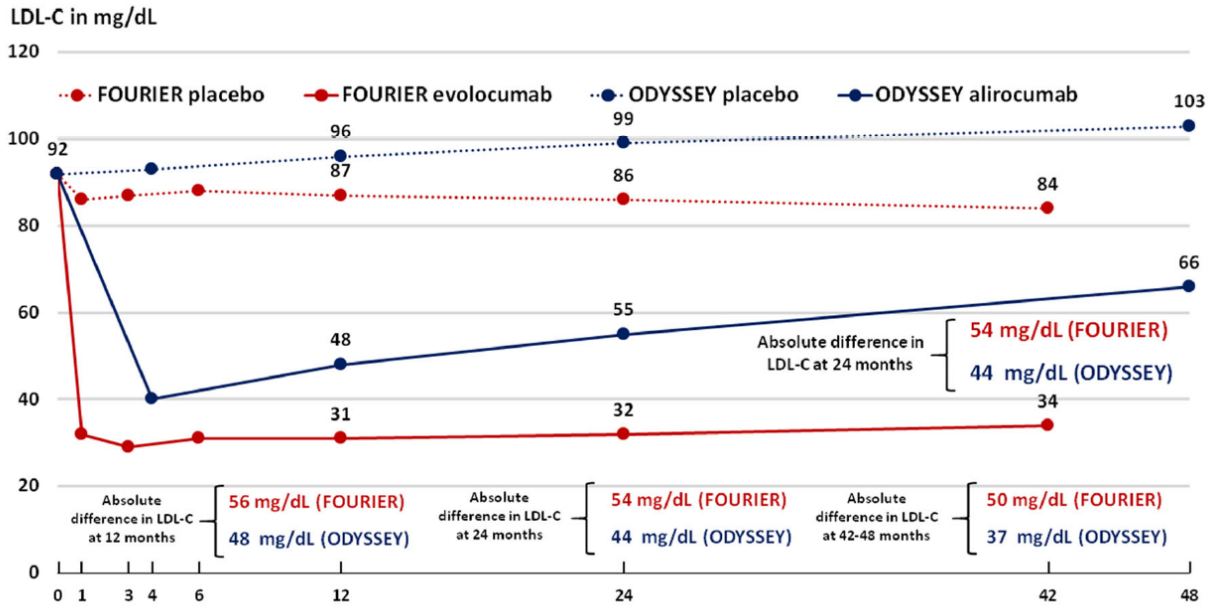

Figure I.1. 14 Efficiency of evolocumab and alirocumab in maintaining sustained reduction in LDLc during the FOURIER and ODYSSEY trials.

Monthly or bi-monthly injections of either placebo or PCSK9 mAb efficiently reduces LDLc levels in both FOURIER and ODYSSEY trial (Furtado and Giugliano, 2020).

Both trials demonstrated a benefit for PCSK9 inhibitors for their defined endpoints, which were coronary death, myocardial infarct, ischemic stroke or unstable angina in the ODYSSEY OUTCOMES trial, and cardiovascular (CV) death, myocardial infarct, stroke, unstable angina or coronary revascularization in the FOURIER trial. While both trials demonstrate benefit in lowering LDLc with baseline LDLc <80 mg/dL, the ODYSSEY OUTCOMES trial demonstrated stronger benefits of mAb to PCSK9 for patients with LDLc >100 mg/dL, supporting the concept that the absolute change in LDLc is proportional to the relative risk reduction (Silverman et al., 2016). The FOURIER trial showed no significant advantages of PCSK9 inhibitors for CV or all cause death. While even though the ODYSSEY OUTCOMES trial did demonstrate a significant decrease in all-cause death, there was no effect on CV related death. These were expected since trials looking at the efficiency of statins in reducing mortality had demonstrated a significant time-dependent effect, where reduction by 12% was only observed after 5 years of on-going study. Therefore, longer clinical trials are required for PCSK9 mAb to achieve incremental reductions in mortality. Randomized control trials have demonstrated a proportional decrease in atheroma burden with decreases in LDLc in patients treated with statins (Nicholls et al., 2011; Nicholls et al., 2007;

Nissen et al., 2006; Nissen et al., 2004). The most potent statins, atorvastatin and rosuvastatin limited the progression and induced regression in coronary disease, where regression was observed in two thirds of the study patients during the 104 weeks of therapy. In a recent study investigating the effect of evolocumab on progression of coronary disease in statin-treated patients, GLAGOV randomized control trial, serial intravascular ultrasonography imaging demonstrated that over the course of 18 months, the patients with evolocumab treatment on background intensive statin or moderate statin therapy showed atherosclerosis regression, whereas patients on placebo and background statins did not show plaque regression (Nicholls et al., 2016). Moreover, a recent and highly relevant study examined the expression of PCSK9 in healthy *vs.* calcified aortic valves and whether it may contribute to valve calcification (Perrot et al., 2020). Indeed, PCSK9 expression was detectable in extracellular matrix of stenotic valves and *in vitro* valve interstitial cells were able to secrete PCSK9. Importantly, inhibition of extracellular PCSK9 significantly reduced calcium deposition (Perrot et al., 2020). Therefore, these results suggest that modulating PCSK9 levels influence plaque progression. Moreover, they demonstrate that patients achieving very low levels of LDLc with combination therapy benefited from incremental plaque regression, supportive of more stringent LDLc level recommendations for high risk patients.

Patients at very high risk, that have ASCVD and comorbidities, such as diabetes, chronic kidney disease, recent acute coronary syndrome, poorly controlled risk factors of smoking and hypertension have between 26% to 43% projected 10-year ASCVD risk, compared to ASCVD patients without comorbidities for which the risk was 16% to 20% (Robinson et al., 2016). Consequently, new guidelines were released in 2019 by the European Society of Cardiology (ESC)/European Atherosclerosis Society (EAS) for the management of dyslipidemias. Patients at high risk that have had a recent myocardial infarct should decrease their LDLc $\geq$50% from baseline and <1.4 mmol/L (<55 mg/dL). According to simulations, Allahyari *et al*. predicted that even with maximal dose of statins and ezetimibe, around half of the patients with a recent myocardial infarct would require additional treatment from PCSK9 inhibitors (Allahyari et al., 2020). However, prices for treatment with PCSK9 mAb are currently too expensive to reach accepted cost-effectiveness ratio.

In addition to high risk patients described above, patients with FH should also benefit from treatment with PCSK9 inhibitors, since many patients do not reach recommended LDLc levels despite being on maximal LLTs. Clinical trials demonstrated substantial reductions (~60%) in

LDLc accompanied by a significant decrease in Lp(a) in heterozygous FH patients treated with alirocumab or evolocumab (McCullough et al., 2018; Raal et al., 2015). In a longer-term study, they showed that this reduction is sustained over the course of 3 years (Dufour et al., 2017). Homozygous FH (HoFH) patients with no residual functional LDLR on the other hand are resistant to PCSK9-reducing therapy and hence are more difficult to treat. In these patients, apheresis is the most effective treatment option to reduce LDLc. A study investigated the potential benefit of evolocumab for these patients (Raal et al., 2017). Among patients in this cohort, 34 were receiving apheresis at the start of the study, 100% were on statins and 95% on ezetimibe. The study demonstrated a significant 20.6% reduction in LDLc (absolute reduction of 1.5 mmol/L) throughout the 1.7 years of the study duration for the patients receiving apheresis and those without. However, there is only an effect for the patients that had LDLR receptor defective mutations or double heterozygotes with one *LDLR* and one *PCSK9* or *APOB* mutation, in contrast to receptor null patients which showed no response to mAb similarly to a previous proof of concept study (Stein et al., 2013). Therefore, residual LDLR activity is key for the effect of PCSK9 mAb inhibitors.

As a promising alternative to mAb is the siRNA against PCSK9, inclisiran, which was developed by American Medicines Co. and bought by Novartis after promising clinical trials demonstrated safety and effectiveness of the drug. Inclisiran was carefully designed in order to target liver cells by conjugating the double-stranded siRNA to triantennary N-acetylgalactosamine, which will undergo receptor-mediated endocytosis by binding to asialoglycoprotein (almost exclusively expressed by hepatocytes) (Stoekenbroek et al., 2018). Moreover, in order to prevent immunogenic reactions and processing by nucleases, overall increasing the half-life of the siRNA, chemical modifications to the RNA duplex and its termini were conducted. Phase III clinical trials ORION-10 and ORION-11 were conducted on high risk patients with ASCVD, ORION-11 included patients with an additional or a combination of additional risk factors such as diabetes, renal impairment… Patients were also on maximal tolerated statin therapy and did not reach recommended LDLc levels (>70 mg/dL). Injections were done on day 1, day 90 and then every 6 months for a period of 18 months (Ray et al., 2020). Inclisiran resulted in >50% reductions in LDLc and -80% PCSK9 levels for both trials. Moreover, PCSK9 siRNA resulted in statistically significant decreases in apoB, non-HDL cholesterol, TGs, Lp(a). Adverse events were reported to be mild or moderate and occurred with similar frequency

in inclisiran and placebo groups, moreover, development of antidrug antibodies occurred in 2% and 2.5% of patients from ORION-10 and ORION-11, respectively. To conclude, inclisiran is a promising new therapeutic that may be comparable to mAb in its efficiency in reducing LDLc and clinical outcomes, however such a prospective study has not yet been conducted. Some of the advantages of siRNA to PCSK9 are the infrequent administrations (2-3 times yearly), which would ensure much better treatment adherence than injections 1-2 times monthly. Moreover, such a treatment would significantly improve the lifestyle of hoFH patients receiving apheresis, or hoFH children for example (investigated in ORION-2 and -5 clinical trials). This treatment might be more cost-effective than mAb treatment considering the infrequent administrations that are required. Lastly, inclisiran prevents PCSK9 biosynthesis, which in addition to inhibiting extracellular PCSK9 function, would also inhibit intracellular PCSK9. This thesis has described several intracellular roles for PCSK9, among those are the binding to apoB intracellularly and increase of VLDL secretion. Therefore, a possible advantage of inclisiran could be reducing overproduction of VLDL particles in patients with insulin resistance such as in T2D and metabolic syndrome.

I.1.7. Insulin receptor

The insulin receptor (InsR) is a type I transmembrane protein, part of the receptor tyrosine kinase superfamily and is expressed in all mammalian cells. Alternative splicing gives rise to two isoforms which differ by the inclusion or exclusion of exon 11. InsR isoform A (InsR-A) is the shortest isoform with a deletion of a few amino acids after the ligand binding site that increases its affinity for insulin-like growth factors-1 and -2 (IGF-1 and -2). Interestingly, InsR-A is mostly present during development, whereas InsR-B is the main isoform expressed in the liver during adulthood. This alternative splicing may therefore be seen as a mechanism to reduce promiscuity of the InsR, thereby also reducing the growth promoting effects of IGF-1 and -2, for which high levels may lead to tumor development, for example (Brahmkhatri et al., 2015). The InsR and IGF-1 receptor share ~60% sequence homology and are therefore highly conserved (Kushi et al., 2020). However, it is clear that these receptors do not exert redundant functions since mutations in the *INSR* may lead to critical diseases with poor prognosis and premature death.

I.1.7.1 *INSR* related insulin resistance

INSR-related severe syndromic insulin resistance is a rare and untreatable autosomal recessive disease that leads to death during infancy or adolescence, while some patients may live through until their third decade (Kushi et al., 2020). The phenotype ranges from the more severe form of Donohue Syndrome (DS) (or leprechaunism) to the milder Rabson-Mendenhall syndrome (RMS) (Kushi et al., 2020). DS is characterized by severe insulin resistance, fasting hypoglycemia, and postprandial hyperglycemia, severe growth defects, hypotonia, characteristic facies, organomegaly and death occurs before one year of age. RMS is more variable in phenotype severity and is characterized by severe insulin resistance, diabetic ketoacidosis and microvascular complications. Other characteristics may include growth retardation, intellectual disability, and facial features. Carriers of the pathogenic variants associated with *INSR*-related severe syndromic insulin resistance are usually asymptomatic, however, some may demonstrate type A insulin resistance, which is the third phenotype known to be associated with *INSR* germline mutations and is characterized by hyperandrogenism, insulin resistance, including acanthosis nigricans and diabetes without obesity. Type A insulin resistance is usually autosomal dominant. DS may not be treated with insulin sensitizers such as Metformin or with insulin, however RMS and type A insulin resistance may. Human recombinant IGF-1 and leptin are being evaluated for potential treatment options. IGF-1 stimulates glucose uptake in muscle and reduces hepatic glucose production, however potential side effects include severe hypoglycemia and soft-tissue overgrowth (tumors). Leptin is known to improve lipid profiles (reduce TGs and increase HDL) and plasma glucose levels. More than 70 disease causing genetic variants have been identified in the *INSR* gene and have been classified into five categories according to their effect: they may affect insulin receptor synthesis, transport, ligand affinity, tyrosine kinase activity and half-life (Porter and Barrett, 2005). There are other less frequent single-gene mutations that cause further conditions related to insulin signaling. These have been identified in kinases and phosphatases of the insulin signaling pathway and may either lead to insulin resistance or on the contrary potentiate insulin signaling (causing overgrowth, cancer and hypoglycemia). Lastly, type B insulin resistance describes an extremely rare autoimmune disease where the patient develops antibodies that antagonize the insulin receptor and is usually associated with other autoimmune conditions (Willard et al., 2016). Patients

demonstrate hyperglycemia and elevated levels of circulating insulin due to insulin hypersecretion, moreover some autoantibodies may act as agonists and lead to hypoglycemia (Willard et al., 2016).

I.1.7.2 InsR structure/function studies

The insulin receptor was first purified in 1979 by Jacobs *et al.* using by affinity chromatography with insulin-agarose and run on sodium dodecyl sulfate/polyacrylamide gel to verify the purity of the eluate (Jacobs et al., 1979). Several studies from these early years had confirmed that the receptor is composed of two subunits migrating at 135 kDa (α subunit) and 95 kDa (β subunit) that are disulfide linked in the plasma membrane. Massague *et al*. proposed that the major subunit (non-denaturing condition) M_r 350,000 is composed of $(\alpha)_2(\beta)_2$ heterotetramer and that a minor subunit M_r 210,000 (denaturing conditions) is composed of αβ (Massague et al., 1980). Hedo *et al.* further postulated that the receptor with apparent molecular mass of 210 kDa undergoes proteolytic cleavage to generate the α and β subunits (Hedo et al., 1983). In 1985, the gene sequence of the insulin receptor confirmed that the 210 kDa subunit was indeed the precursor of the α and β subunits (Ebina et al., 1985; Ullrich et al., 1985). Therefore, the insulin receptor is synthesized as a single polypeptide chain, which is cleaved after transport to the late Golgi compartment (Hedo et al., 1983) at a dibasic cleavage site (Ebina et al., 1985; Ullrich et al., 1985). It was still unknown, how insulin, by binding to the α subunit, could propagate the intracellular signal, even though it was known that insulin induced phosphorylation of downstream proteins. Kasuga *et al*. first demonstrated that InsR is phosphorylated on the β subunit (Kasuga et al., 1982), then since phosphotyrosine had just been discovered as a growth factor signal downstream from growth factors such as the epidermal growth factor, this group and others hypothesized and demonstrated that insulin induced InsR tyrosine autophosphorylation (Kasuga et al., 1983; Roth et al., 1983; Van Obberghen et al., 1983). Cloning of the *INSR* allowed for site-directed mutagenesis of the ATP binding site further proving that tyrosine kinase activity of the InsR is necessary for propagation of the insulin signal. Finally, identification in Japan of a young Japanese type A insulin resistant patient harboring a mutation in the ATP binding site allowed for *in vivo* validation of the requirement for InsR tyrosine kinase activity for insulin activity (Odawara et al., 1989); note that

0.05% of the general population in Japan is estimated to be a carrier of *INSR* pathologic mutation (Kushi et al., 2020).

Additionally, identification in two sisters with type A insulin resistance of a single nucleotide substitution in codon 735 (AGG → AGT) in the proteolytic cleavage site, resulting in the replacement of Arg with Ser and changing the cleavage recognition sequence from Are-Lys-Arg-Arg to Arg-Lys-Arg-Ser, stirred physiological interest for the processing of proinsulin receptor (proInsR) (Kobayashi et al., 1988; Yoshimasa et al., 1988). This gene was normally expressed, with proper glycosylation, transport and insertion into the plasma membrane, however, mature insulin receptor was undetectable. It appears however that this mutation is not solely responsible for the disease phenotype since several studies have demonstrated that the proreceptor is efficiently autophosphorylated upon insulin binding with equivalent sensitivity and affinity as to the normal receptor (Arakaki et al., 1989; Dardevet et al., 1991; Hedo et al., 1983; Salzman et al., 1984; Yoshimasa et al., 1990). Thus, the sisters may be affected by coexistence of other genetic defects at downstream steps in the insulin transduction pathway implicated in the pathogenesis of insulin resistance (view Table I.1.4) for a list of affected genes involved in insulin signaling).

Table I.1. 4 Identified mutations in genes of the InsR signaling pathway and their phenotype

Gene	Effect on protein function	Effect on insulin action	Frequency	Condition caused	Other characteristics
INSR	Loss	Resistance	Relatively common (> 200)	Type A insulin resistance, Donohue, and Rabson–Mendenhall syndromes	Acanthosis nigricans, polycystic ovary, hirsutism (in type A insulin resistance); early death (in Donohue and Rabson–Mendenhall)
PIK3R1	Loss	Resistance	Rare (> 30)	SHORT syndrome	Short stature, facial characteristics
PIK3R2	Gain	Sensitivity	Rare (> 10)	None	Segmental overgrowth or megalencephaly
PIK3CA	Gain	Sensitivity	Rare (> 60)	None	Segmental overgrowth or megalencephaly
AKT1	Gain	Not known	Rare (> 20)	Proteus syndrome	Overgrowth of various tissues, mosaic mutation
AKT2	Loss	Resistance	Very rare (3)	None	Hypertension
	Gain	Sensitivity	Very rare (1)	None	Overgrowth, hypoglycemia
AKT3	Gain	Not known	Very rare (~ 3)	None	Megalencephaly
TBC1D4	Loss	Resistance	Very rare (1)	None	Acanthosis nigricans, postprandial hyperinsulinemia
PTEN	Loss	Sensitivity	Relatively common (> 300)[a]	Cowden syndrome	Hamartoma, cancer predisposition
PTPN11	Gain	Resistance	Relatively common (> 800)[b]	Noonan syndrome	Short stature, congenital heart disease, skeletal malformation
PRKCE	Loss	None[c]	Very rare (1)	SHORT syndrome	Short stature, facial characteristics

Numbers in parentheses for frequency indicate the number of cases or families described in published reports

[a]Fifteen families have been evaluated for glucose tolerance or insulin sensitivity

[b]One family has been evaluated for glucose tolerance or insulin sensitivity

[c]The patient might have been too young to develop apparent insulin resistance

Adapted from (Kushi et al., 2020).

The insulin receptor monomers are N-glycosylated in the ER, which have been shown to be required for binding to ER folding chaperones calnexin and calreticulin. Indeed, these chaperones ensure proper three-dimensional conformation allowing for dimerization, disulfide bond formation and ER exit. In the absence of glycosylation and binding to ER lectin chaperones, misfolded monomer binding to BiP allows for ER retention and refolding until they reach proper conformation for dimerization or degradation of receptors with prolonged retention (Bass et al., 1998; Olson and Lane, 1987). Mutations of cysteines important for subunit dimerization prevent efficient dimerization and lead to rapid receptor degradation (Wu and Guidotti, 2002).

I.1.7.3 InsR signaling in the liver

Genetic mutations causing loss-of-function or gain-of-function, as we have seen with PCs and *INSR*, reveal information about the function of the protein. In the case of *INSR* it is more difficult to decipher the direct *vs.* indirect effects of insulin signaling due to insulin's pleiotropic effects. In general, insulin is an anabolic hormone for which effects on metabolism may be summarized depending on the target tissue: in muscle, insulin promotes glucose uptake and protein synthesis; in adipose tissue, insulin promotes glucose and FA uptake while suppressing lipolysis; in liver insulin promotes TG synthesis, glucose utilization and suppresses glucose production; and in neurons, insulin promotes anorexigenic and locomotor signals (Haeusler et al., 2018). Insulin signals through kinase cascades and its effects are reversible as they may be antagonized by Ser/Thr phosphorylation or terminated by phosphatases. Interestingly, one the most important mechanisms to downregulate insulin signaling is the degradation of InsR, which occurs under conditions of high insulin concentrations. Indeed, reduced availability of InsR under hyperinsulinemic conditions is associated with insulin resistance, such as demonstrated in obese mice and humans (Grunberger et al., 1989).

Studies suggest that insulin signaling in the liver is the main determinant on insulin's whole-body action. This is supported by the observation that mouse liver specific knockout of *InsR* recapitulates the human being phenotype with lack of functional InsR, i.e. failure to inhibit glucose production and induce de novo lipogenesis, such that even under high-fat diets, mice fail to accumulate lipids in hepatocytes (Michael et al., 2000). Moreover, liver-targeted exogenous expression of InsR (through adeno-associated viral expression) in T2DM rodent models rescues the majority of the whole-body diabetic phenotype (Wang et al., 2019), such as restored euglycemia, improved hepatic and systemic insulin sensitivity with observed decreased circulating FFA, suggesting decreased adipocyte lipolysis (Wang et al., 2019).

Insulin binding to InsR stimulates InsR autophosphorylation and subsequent recruitment of insulin-receptor substrates which then activate the phosphoinositide-3-phosphate kinase (PI3K)/Akt pathway. PI3K recruits Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK1) at the cell membrane by generating phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PDK1 then activates Akt through via phosphorylation of Thr308. Phosphatase and tensin homolog (PTEN) antagonizes PI3K by dephosphorylating and PIP3 to PIP2. Full activation of Akt requires

further phosphorylation by mTORC2 at Ser473. While there are three isoforms of Akt, Akt2 is the main isoform for metabolic regulation as is suggested by mouse studies and by human genetic mutations (Kushi et al., 2020), where inactivation of Akt2 leads to insulin resistance and diabetes and GOF Akt2 mutations in humans lead to insulin hypersensitivity, overgrowth and hypoglycemia (Kushi et al., 2020). Akt activates mTORC1, that will switch the cell from a catabolic to an anabolic state stimulating protein, lipid and nucleic acid synthesis. One of the key lipogenic transcription factors that is activated by mTORC-1 is SREBP-1c, which is required for the development of a fatty liver. Akt also phosphorylates FoxO1 transcription factor – that is thought to antogonize SREBP-1c – resulting in its inactivation by nuclear exclusion and inhibition of the expression of gluconeogenesis genes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase. By phosphorylating glycogen kinase 3 (GSK3), will also inhibit gluconeogenesis and suppress glycogen synthesis. Hepatic insulin signaling is briefly illustrated in Figure I.1.16. (for review (Santoleri and Titchenell, 2019)).

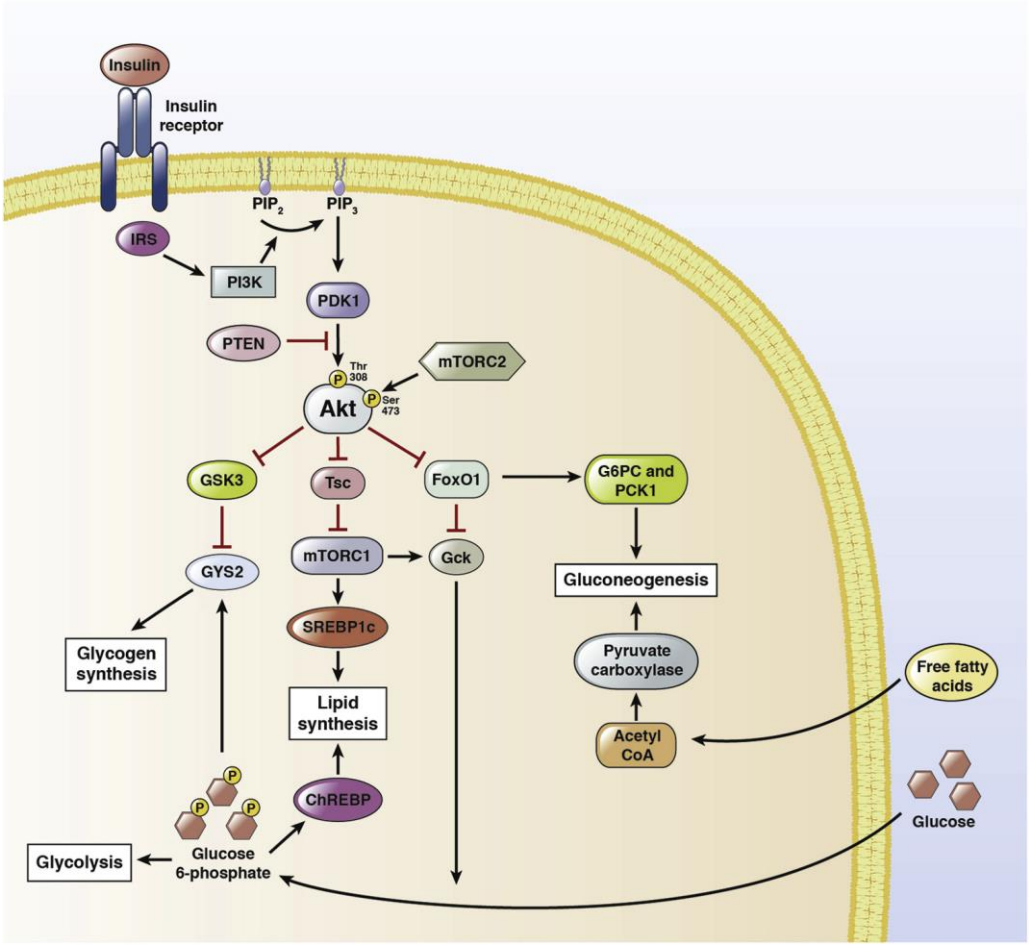

Figure I.1. 15 Insulin receptor signaling pathway in the liver

In the liver, insulin activates Akt through phosphorylation by PDK1 and mTORC2 leading to glycogen synthesis, inhibition of gluconeogenesis, and lipid synthesis (Santoleri and Titchenell, 2019).

I.1.8. Increased risk for diabetes upon LDLc lowering

I.1.8.1 Association of LDLc lowering gene variants with diabetes risk

Statins had been found to increase the risk for new-onset T2DM (Sattar et al., 2010) in a dose-dependent manner (Preiss et al., 2011). In order to identify the underlying mechanism for this association, Swerdlow *et al*. identified natural variants in the gene of HMG-CoA reductase associated with decreased LDLc to use them as proxy for HMG-CoA reductase (HMGCR)

inhibition by statins (Swerdlow et al., 2015). Therefore, this mendelian randomization paradigm allowed them to perform an unconfounded, unbiased analysis of the variants' associations with bodyweight, body mass index (BMI), waist circumference, plasma insulin and glucose, and risk of T2DM. This analysis included the genetic associations with biomarkers and outcomes from up to 43 studies for the principle analysis (SNP rs17238484), which consisted in 195 444 individuals, and from up to 21 studies for the subsidiary analysis (SNP rs12916), which consisted of 94 652 individuals. This study demonstrated the association of these *HMGCR* variants associated with lower LDLc were also associated with increased risk of T2DM, increased bodyweight and BMI (Swerdlow et al., 2015). These findings were concurred by the subsidiary analysis and by a third analysis from a large GWAS of T2DM. Finally, the authors obtained bodyweight data from 20 large statin trials in order to verify the association of statin treatment with bodyweight gain. Interestingly, this study was the first to identify an effect of statins on bodyweight gain. Since the latter is one of the strongest risk factors for the development of insulin resistance and T2DM, it may explain this association with diabetes. Moreover, while the *HMGCR* SNPs were positively associated with bodyweight, BMI and waist and hip circumference, the SNPs' associations with increased risk for T2DM were attenuated to null after adjustment for BMI, further supporting a potential role for weight gain in the development of T2DM upon treatment with statins. Finally, the authors did not identify an association between LDLc lowering and the risk for T2DM (Swerdlow et al., 2015).

From the FH screening program registry in the Netherlands, Besseling *et al.* demonstrated a 38% decreased risk of developing T2DM in FH patients compared with their unaffected relatives (Besseling et al., 2015). This association for a protective effect of FH for T2DM has been also found in two other studies, one small observational study in Quebec in 1996 (Vohl et al., 1997) and one more recent observational study from Spain (Climent et al., 2017). Interestingly, in the study by Besseling *et al.* the association with T2DM showed an inverse relationship between severity of the FH mutation on cardiovascular risk with T2DM prevalence. This research group put forward the hypothesis for an LDLR-mediated transmembrane cholesterol transport causal association with T2DM, arguing that excessive cholesterol inside the β-cells of the pancreas could impair insulin secretion (Roehrich et al., 2003; Rutti et al., 2009) and is detrimental to the cell (Cnop et al., 2002). Adjusting for BMI, however, appeared to attenuate the relationship between FH and T2DM, supporting the hypothesis that BMI may also be a mediator (Besseling et al., 2015).

In support of this hypothesis, Ference *et al.* demonstrated the associations of several LDLc-lowering variants in both *HMGCR* and *PCSK9* genes with increased risk of diabetes per unit decrease in LDLc (Ference et al., 2016). However, these associations were limited to patients with impaired fasting plasma glucose, additionally, neither genes were associated with T2DM risk among patients with normal fasting glucose levels. Nevertheless, because PCSK9 and HMGCR LDLc-lowering variants demonstrated very similar effects on the risk of cardiovascular disease and on the risk of diabetes per unit decrease of LDLc, the authors proposed that the underlying mechanism is shared between both associations and is likely to involve the LDLR receptor-mediated pathway, as proposed by Besseling *et al* (Besseling et al., 2015).

However, it seems that both statin treatment and variants associated with *HMGCR* increase body weight and BMI, which are primary risk factors for the risk of developing diabetes as mentioned above (Swerdlow et al., 2015), whereas PCSK9 LDLc-lowering variants were not associated with an increase in body weight or BMI, suggesting that PCSK9 variants and HMGCR variants in their associations with an increased risk for diabetes may imply alternative mechanisms. On that account, in a meta-analysis of genetic association studies, Lotta *et al.* investigated the association of LDLc lowering genetic variants with T2DM, including *NPC1L1, HMCGR, PCSK9, ABCG5/G8, and LDLR* (Lotta et al., 2016). From this analysis they confirmed the previous positive associations of *HMCGR* with T2DM and identified a positive association between the PCSK9 R46L variant and the risk of diabetes, whereas the association with *ABCG5/G8* with T2DM did not reach statistical significance. Moreover, there was no association with T2DM for the missense variants in *HMGCR, PCSK9, ABCG5/G8* and *LDLR* genes. They identified an inverse association of the LDLc lowering variants at the *NPC1L1* locus with CAD and a direct association with T2DM risk, which was unchanged after adjustment for variations in the known neighboring association signal for T2DM in the glucokinase gene. However, due to heterogeneity between the association of the LDLc lowering genes with metabolic biomarkers and anthropometric traits, the authors suggested that the risk for T2DM was likely caused by gene-specific underlying mechanisms. For example, the variants at the *PCSK9* locus associated with fasting plasma glucose levels and 2-hour glucose levels (from a glucose tolerance test), suggesting impaired glucose tolerance. Whereas, similarly to the studies described above, the *HMGCR* variants associated with increased fasting insulin, BMI and waist circumference, which are more suggestive of insulin resistance. The effect of *NPC1L1* inhibition on glucose metabolism is not well understood yet due to different expression

levels in rodents and humans of the protein in liver and intestine. In the mouse, suppressing NPC1L1 improves the metabolic phenotype by suppressing gluconeogenesis (Kurano et al., 2015). Moreover, for a similar reduction in LDLc, the association with T2DM differed by gene, suggesting that LDLc levels is not a common denominator for the development of T2DM. Finally, the association of the PCSK9 R46L LOF function variant with T2DM was confirmed in other studies (Nelson et al., 2019).

I.1.8.2 Cholesterol lipotoxicity to β-cells

T2DM is caused by chronic peripheral insulin resistance and impaired insulin secretion due to progressive decline in β-cell function. Pancreatic β-cells are endocrinal cells responsible for insulin secretion and maintaining plasma glucose within the physiological range. During adulthood, β-cells have low regenerative capacity and are susceptible to cellular stressors. Under conditions of insulin resistance such as the metabolic syndrome and obesity the lipid profile is atherogenic with elevated non-HDL cholesterol, increased TGs and sdLDL, additionally, fasting plasma free fatty acids (FFAs) are elevated, indicating chronically increased levels of plasma FFA. These have been associated to an increased risk for impaired β-cell function, or lower insulin secretion, and the development of T2DM (Charles et al., 1997; Johnston et al., 2018; Paolisso et al., 1995; Salgin et al., 2012). Elevated FFAs under acute conditions will allow for compensation for insulin resistance by further stimulating glucose-stimulated insulin secretion (GSIS) of the β-cells, however, under chronic conditions, FFAs will lead to lipotoxicity of insulin sensitive tissues, such as muscle and adipose tissue, as well as lipotoxicity of the β-cells. Elevated FFAs, such as palmitic acid, will induce apoptosis in the β-cells via the generation of cytotoxic metabolites and activation of deleterious interlinked intracellular mechanisms, such as ER stress, oxidative stress and mitochondrial dysfunction, impaired autophagy and inflammation (view Figure I.1.16). Therefore, lipotoxicity usually refers to the deleterious effects of FFAs on glucose homeostasis through impairment of insulin production, secretion and progressive decline in β-cell mass (for review (Lytrivi et al., 2020; Oh et al., 2018)).

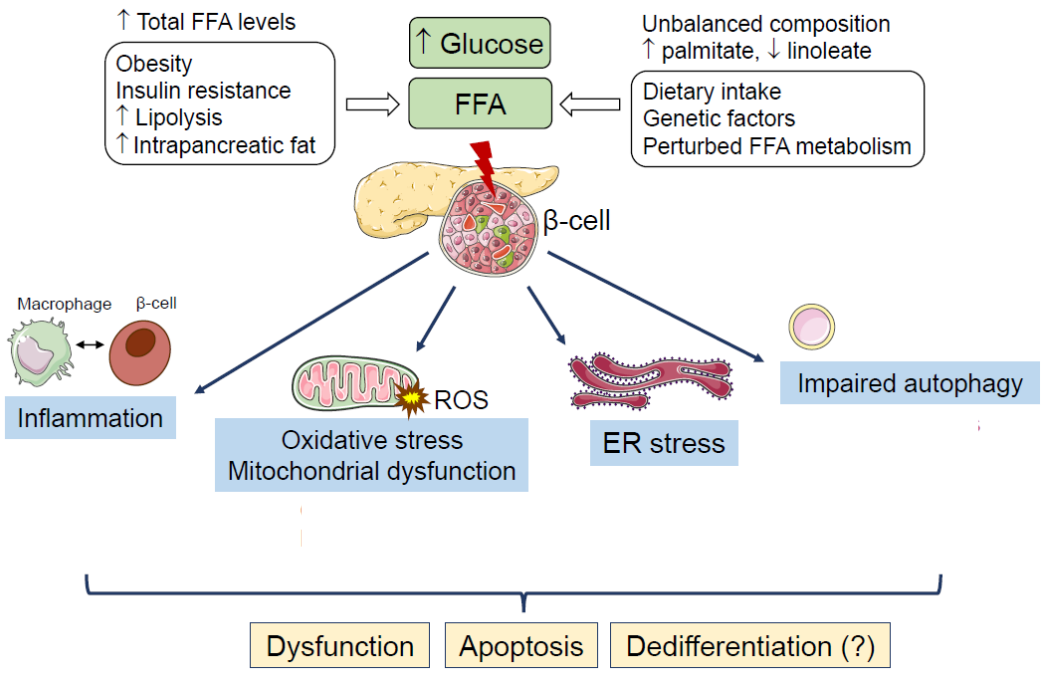

Figure I.1. 16 Risk factors and molecular mechanisms leading to β-cell toxicity

Increased FFA under conditions of obesity, insulin resistance for example will cause inflammation, oxidative stress and mitochondrial dysfunction, ER sress and/or impaired autophagy altogether leading to loss of β-cell mass and function (Lytrivi et al., 2020).

Cholesterol is an integral component of cell membranes that is essential to define curvature and fluidity as well as being required for formation of lipid rafts, which in turn serve as microdomains for signaling molecules, protein-protein interactions and vesicle trafficking. Excessive cholesterol may impact several of the steps required for GSIS. For example, excessive cholesterol may reduce the production of ATP required for the coupling of glucose sensing to vesicle fusion and insulin secretion. Indeed, these conditions decrease glucose uptake by GLUT2 transporters, decrease translocation and activation of glucokinase from insulin granules to the cytoplasm and affect mitochondrial membrane fluidity, in turn impairing the electron transport chain (Lee et al., 2011). Moreover, excessive cholesterol reduces voltage-gated Ca^{2+} channels' density and decreased extracellular Ca^{2+} influx resulting in decreased vesicle fusion to the plasma membrane (Lee et al., 2011). Excessive cholesterol may also cause ER stress (Kong et al., 2017). Islets from T2DM patients show ER expansion (morphometric sign of ER stress) as well as increased susceptibility for the increased expression of ER stress markers (including BiP, XBP1 and CHOP) (Marchetti et al., 2007). Importantly, chronic ER stress is an important pathway

leading to β-cell dysfunction and eventually cell death. Several studies have demonstrated that pancreatic islet β-cells may uptake LDL and VLDL (Cnop et al., 2002; Grupping et al., 1997; Rutti et al., 2009). Those may induce a dose-dependent decrease in insulin synthesis and increase in apoptosis, importantly HDL may prevent the toxic effects of LDL and VLDL, possibly because the HDL particle is enriched in antioxidant proteins in contrast to LDL (Tomas et al., 2004).

Intracellular islet cholesterol is tightly regulated *in vivo* in mice models and results from the cell's adaptation through the regulation of expression of different receptors responsible for cholesterol internalization (LDLR) and efflux (ABCA1) as well as from cholesterol biosynthesis. ATP-binding cassette transporter subfamily A member 1 (ABCA1) mediates the efflux of cellular cholesterol to HDL apolipoprotein acceptor. Mice with specific inactivation of the ABCA1, in the β-cells have impaired glucose tolerance and normal insulin sensitivity, suggesting β-cell dysfunction (Brunham et al., 2007). Moreover, the islets demonstrate accumulation of cellular cholesterol and impaired insulin secretion, providing *in vivo* evidence that islet cholesterol is key for proper β-cell function. Although the absence of LDLR leads to hypercholesterolemia in mice, it did not lead to increased islet cholesterol levels or β-cell disfunction, highlighting the importance of the LDLR for LDLc internalization (Kruit et al., 2010b). Moreover, WT islets with functional LDLR transplanted into hypercholesterolemic *Ldlr*$^{-/-}$ mice did not result in impaired β-cell function or cholesterol accumulation. This was showed to be due to the compensation from ABCA1 cholesterol efflux since a similar transplantation of islets with impaired ABCA1 did result in impaired β-cell function (Kruit et al., 2010b). Furthermore, islets from *ApoE*$^{-/-}$ mice showed decreased ABCA1 levels and increased islet cholesterol. The authors suggested that cholesterol efflux is rate limiting in maintaining balanced intracellular cholesterol in β-cells.

Several studies have investigated the effect of the absence of PCSK9 on glucose homeostasis in murine models (Da Dalt et al., 2019; Langhi et al., 2009; Mbikay et al., 2015; Mbikay et al., 2010). These studies showed that the absence of PCSK9 results in ~2-3-fold increase in β-cell LDLR. Although Langhi *et al*. found no effect on glucose homeostasis (Langhi et al., 2009), the other studies show impaired insulin secretion accompanied by decreased plasma insulin and altered islet morphology, altogether suggesting lipotoxicity from the resulting increase in β-cell LDLR (Da Dalt et al., 2019; Mbikay et al., 2015; Mbikay et al., 2010). It is unclear, however, whether *Pcsk9*$^{-/-}$ islets have an increase (Da Dalt et al., 2019) or a decrease (Mbikay et al., 2010)

in islet insulin content. Importantly, Da Dalt reported a ~3-fold increase in intracellular esterified cholesterol, and ~2-fold increases in fatty acids, accompanied by a significant decrease in targets of SREBP-2 transcription factor *HMGCR*, *LDLR* mRNA expression (Da Dalt et al., 2019). However, the authors did not investigate whether the protein levels of ABCA1 were altered, although mRNA were unchanged.

To conclude there have been several convincing mechanistic and *in vivo* murine studies demonstrating the importance of balancing the intracellular cholesterol for the proper function of β-cells, however, there have yet to be clinical evidence associating increased plasma cholesterol with an increased risk of impaired glucose homeostasis and T2DM (von Eckardstein et al., 2000).

I.1.8.3 Results of PCSK9 mAb clinical trials and new onset of diabetes or changes in glycemic markers

The FOURIER trial prespecified analysis demonstrated safety and efficacy in patients with diabetes or prediabetes (Sabatine et al., 2017). In the placebo group patients with diabetes were at significantly greater risk for the defined cardiovascular endpoints even though baseline LDL concentrations were equivalent between patients with diabetes and those with normoglycemia. Treatment with evolocumab significantly reduced non-HDL cholesterol, apoB and TGs by equivalent amounts in both non-diabetics and diabetics. Moreover, evolocumab significantly reduced cardiovascular outcomes consistently between both groups. Importantly, these results suggest that since the diabetic group had increased risk at baseline, the absolute risk reduction in cardiovascular endpoints with evolocumab is greater for the diabetes group. The treatment with evolocumab did not increase the risk for new-onset diabetes when compared to placebo, likewise the levels of fasting plasma glucose (FPG) and glycated hemoglobin A1 (HBA1c) were similar between diabetics, prediabetics and normoglycemics either on placebo or evolocumab treatment. The study from the alirocumab ODYSSEY OUTCOMES trial prespecified analysis for the risk of developing diabetes reported very similar results with a greater risk for diabetics for cardiovascular events, equivalent reductions in lipid parameters for diabetics, prediabetes and normoglycemics, and greater absolute benefit for cardiovascular outcomes for the diabetic patients (Ray et al., 2019a). Moreover, they demonstrated no increase in new-onset diabetes in alirocumab when compared to placebo (Ray et al., 2019a).

A meta-analysis from 2018 of 20 random controlled trials but which excluded the only recently published data from alirocumab in ODYSSEY OUTCOMES prespecified study on the risk of developing T2DM, however, suggested a small but significant increase in FGP and HBA1c, particularly in patients that reached very low levels of LDLc (de Carvalho et al., 2018). OSLER-1 study went on for ~4 years and showed no signs of increased risk for T2DM (Sabatine et al., 2015). Lastly, inclisiran was demonstrated to be as effective in diabetic patients as in normoglycemic patients without affecting HBA1c (Leiter et al., 2019). Considering that these clinical trials and their analyses are so recent it is difficult to conclude that there is a definite risk to be anticipated from the long-term use of PCSK9 mAb inhibitors for the treatment of CAD. All studies, however, argue that the benefit against cardiovascular events largely outweighs the potential risk for T2DM, particularly since these treatments in diabetic patients and prediabetic patients have shown significant benefit equivalent or greater to that from non-diabetics.

I.1.9. PCSK9 and diabetic dyslipidemia

Patients with T2DM and ASCVD are at very high risk of cardiovascular events and as detailed earlier a major cause is high prevalence of atherogenic dyslipidemia, or diabetic dyslipidemia, despite treatment with statins and other LLTs. One of the main characteristics of diabetic dyslipidemia is elevated plasma TG and TG rich lipoprotein (TRL) remnants. These are determined by LPL activity (hydrolyze chylomicrons and VLDL particles to produce chylomicron remnants and intermediate-density lipoproteins (IDL)) and clearance of TRL remnants by LDLR, heparan sulfate proteoglycan (HSPG) syndecan-1 (Williams and Chen, 2010). Moreover, production and secretion of VLDL from the liver is also an important predictor of plasma chylomicron remnants, as IDL particles will compete with chylomicron remnants for the same receptors for clearance, as well as compete for LPL.

In T2DM, several processes are impaired due to peripheral insulin resistance and contribute to elevated plasma TGs. Diabetic patients demonstrate postprandial lipemia, which could in part be explained by the normally inhibited chylomicron production and secretion by intestinal cells by insulin and glucagon-like peptide -1 (GLP-1) (Veilleux et al., 2014; Xiao et al., 2014; Xiao and Lewis, 2012). Under normal conditions, insulin suppresses adipose tissue hormone sensitive lipase and in the liver the production of large buoyant VLDL particles as well as glucose production.

Insulin resistance in adipose tissue will lead to lipolysis and release of FFAs into the circulation which are taken up by the liver. Selective hepatic insulin resistance refers to impaired action of insulin to suppress liver gluconeogenesis, by impaired inhibition of FoxO1 (Kamagate and Dong, 2008), but maintained stimulation of *de novo* lipogenesis (DNL), by activation of mTORC1 (Leavens and Birnbaum, 2011), thereby resulting in overproduction of TGs (view Figure I.1.17). Since the liver is not a lipid storage organ, accumulation of TGs in lipid droplets (from both increased substrate influx and DNL) characterizes a disorder termed NAFLD. In order to balance hepatic TGs, the hepatocytes will upregulate VLDL secretion and produce large, buoyant VLDL. Another mechanism that is important to regulate lipid overload in hepatocytes is FA β-oxidation in the mitochondria. However, *de novo* lipogenesis inhibits FA β-oxidation as the malonyl-Co-A intermediate will inhibit the activity of carnitine palmitoyltransferase (CPT1), a transporter that is required for the shuttle of long-chain FAs to the mitochondrial matrix. Therefore, insulin resistant livers demonstrate impaired suppression of VLDL secretion, impaired β-oxidation and overproduction of TGs, and are associated with NALFD (Younossi et al., 2019). Finally, a consequence of the production of large, buoyant VLDL particles is the formation of sdLDL (as described previously in section I.1.6.1) and small dense HDL (sdHDL) (Adiels et al., 2006), which due to their altered composition are catabolized more rapidly leading to their overall decrease (Rashid et al., 2003).

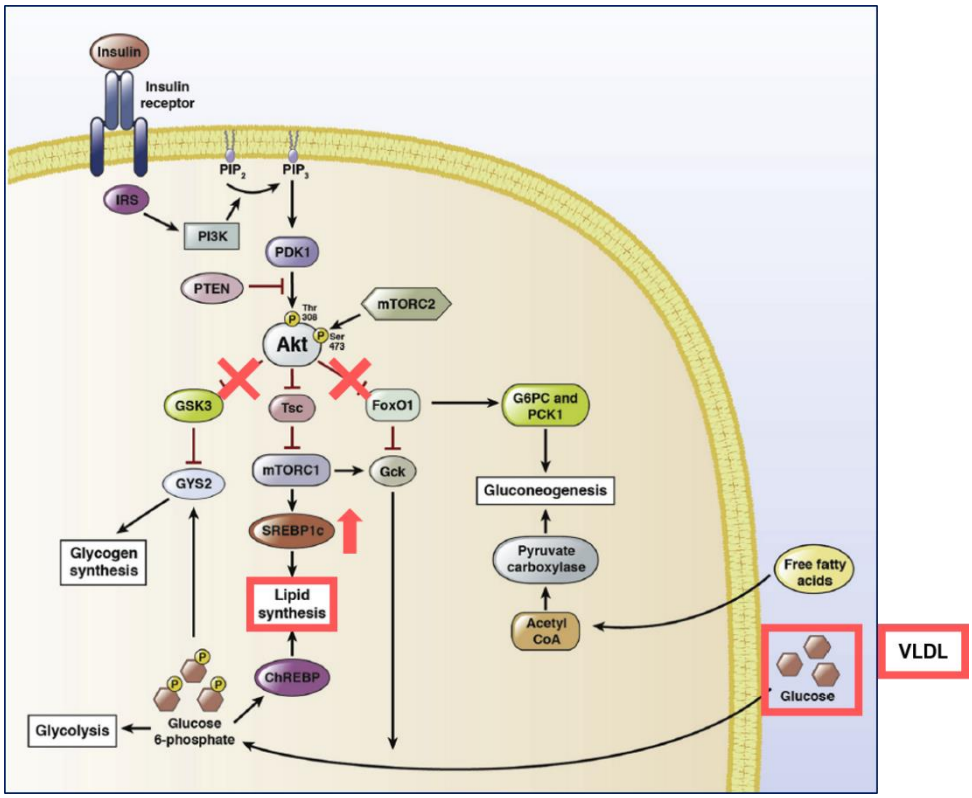

Figure I.1. 17 Selective hepatic insulin resistance

Hepatic selective insulin resistance is an early event in the development of T2DM, where Akt stimulation of glycogen synthesis and inhibition of gluconeogenesis is impaired and lipid synthesis is maintained. As a result, glucose substrate accumulates leading to TG synthesis and liver steatosis (Santoleri and Titchenell, 2019).

Several studies have demonstrated an association between PCSK9 with fasting plasma glucose, insulin and TGs (described in section I.1.5.2) (Awan et al., 2014; Baass et al., 2009; Brouwers et al., 2011; Cui et al., 2010; Ferri et al., 2020; Lakoski et al., 2009). Interestingly, Brouwers *et al*. demonstrated that plasma PCSK9 association with TG, non-HDL cholesterol, apoB is stronger in T2DM than in normoglycemic patients (note: all patients were asked to stop all LLTs for the study and any glucose lowering drugs on the day of blood withdrawal), suggesting that the degree of glucose metabolism may modify the association between plasma PCSK9 and non-HDLc and apoB (Brouwers et al., 2011). The authors speculated that elevated PCSK9 could take part in decreased apoB-containing lipoprotein particle clearance, thereby acting synergistically with VLDL overproduction in the development of diabetic dyslipidemia (Brouwers et al., 2011). PCSK9 associates positively with markers of insulin resistance, such as fasting insulin, fasting plasma glucose and HOMA-IR (Awan et al., 2014; Baass et al., 2009; Cui et al.,

2010; Lakoski et al., 2009) supporting the hypothesis that PCSK9 could play a role in the development of dyslipidemia associated with the metabolic syndrome. Interestingly, plasma PCSK9 has also been demonstrated to correlate with T1DM and HbA1c (Bojanin et al., 2019; Laugier-Robiolle et al., 2017; Levenson et al., 2017b) and also insulin resistance in T1DM – regardless of glycemic control, assessed by estimated glucose disposal rate (eGDR) (Bojanin et al., 2019). PCSK9 has also been found to associate directly with liver fat accumulation in animal and human studies (Cariou et al., 2013; Ruscica et al., 2016). Circulating PCSK9 was associated with hepatic expression of SREBP-1c, levels increased with steatosis severity independently of metabolic confounders and liver damage (Paquette et al., 2020; Ruscica et al., 2016). The authors suggested that PCSK9 synthesis and secretion may be involved in NAFLD pathogenesis.

In summary, PCSK9 may be associated with plasma TGs, insulin resistance and NALFD either by preventing efficient clearance of TRL remnants (direct downregulation of LDLR), or by common regulation and stimulation by insulin (through SREBP-1c). Moreover, PCSK9 has been shown to regulate other receptors important for lipid homeostasis (such as VLDLR, apoER2 and CD36) and intracellular PCSK9 has been demonstrated to regulate apoB secretion (developed in section I.1.4). Therefore, the role of PCSK9 – whether it be causal or correlational – for diabetic dyslipidemia is not yet well understood and may be independent from LDLR regulation.

I.2 Working hypothesis and research objectives

There is no evidence yet for the direct implication of PCSK9 in the regulation of plasma insulin or plasma glucose, however epidemiological studies show an association of PCSK9 plasma levels with markers of hepatic insulin resistance (HOMA-IR, fasting plasma glucose, fasting plasma insulin) (described in section I.1.9); moreover, Mendelian randomization studies suggest an implication for the inhibition of PCSK9 activity towards the development of T2DM (described in section I.1.8). We hypothesize that PCSK9 may regulate InsR signaling and that the absence of PCSK9 leads to increased LDLR mediated LDLc internalization into insulin responsive tissues affecting glucose homeostasis. Furthermore, evidence from PCSK9 LOF variants demonstrate that proPCSK9 leads to ER-retention of WT PCSK9 (described in section I.1.4.8). Therefore, we hypothesize that proPCSK9 variants may affect other secretory pathway protein biosynthesis. Hence, in this thesis we were interested in investigating:

(i) the effect of PCSK9 on the direct modulation of InsR signaling and InsR biosynthesis; and

(ii) the effect of the absence of PCSK9 on glucose and insulin homeostasis.

Chapter II: Manuscript 1 (To be submitted to the Journal of Biological Chemistry)

Title: "PCSK9 a negative regulator of the insulin receptor: effects on signaling activity and biosynthesis rate"

Authors: Julie Cruanès, Alexandra Evagelidis, Jennifer L. Estall, Annik Prat, Nabil G. Seidah

Journal: The Journal of Biological Chemistry, to be submitted

II.1. Preface

While the focus of this thesis is the impact of PCSK9 on glucose and insulin homeostasis, in this chapter we were more specifically interested in investigating whether through two possible independent regulatory pathways, PCSK9 could result in impaired insulin signaling. The first is the regulation by extracellular PCSK9 of InsR downstream signaling, whereas the second is the effect of intracellular, ER-retained, proPCSK9 on InsR biosynthesis and levels. From this study, we could further hypothesize that PCSK9 may be directly implicated in the development of insulin resistance and diabetic dyslipidemia through the modulation of InsR signaling and/or through ER proteotoxic effects.

PCSK9 a negative regulator of the insulin receptor: effects on signaling activity and biosynthesis rate

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Keywords: Insulin receptor signaling, PCSK9, insulin receptor, ER stress

II.2. Abstract

PCSK9, a recent therapeutic target for hypercholesterolemia, targets the LDLR for degradation in the lysosomes. PCSK9 has both extracellular and intracellular activities, where PCSK9 also targets other cell surface receptors for degradation and interacts with other secretory proteins within the cell. Its reported positive association with markers of hepatic insulin resistance in patients merits further investigation. Here we show that exogenous PCSK9 decreases insulin signaling *in vivo* in mice and *in vitro* in immortalized human hepatocytes. This process is inhibited *in vitro* by mAb but not by single-domain Ab (sdAb) to PCSK9 supporting the implication of the catalytic domain. Furthermore, we demonstrate that co-expression of PCSK9 and InsR leads to strong downregulation of InsR levels through the upregulation of acute ER stress, likely by the activation of IRE1α and the RIDD pathway. These data are the first evidence for a direct involvement of PCSK9 on InsR levels and function, further opening future studies to the role of PCSK9 on diabetic dyslipidemia.

II.3. Introduction

Insulin resistance is a systemic disorder characterized by reduced insulin signaling despite increased insulin concentrations. The metabolic syndrome, in turn, is described as a clustering of typical cardiovascular risk factors, many for which the pathophysiology is driven by resistance to insulin. Therefore, insulin resistance is intricately linked to the metabolic syndrome and together they may precede the development of diabetes and cardiovascular disease. The role of the InsR in the etiology insulin resistance has been somewhat underappreciated even though alterations in the metabolic fate of the InsR largely influences insulin signaling pathways (Chen et al., 2019). Indeed, more than 70 pathogenic variants have been identified throughout the gene that are causative for severe insulin resistance, classified under five pathogenic categories impacting the receptor's biosynthesis, trafficking, function and half-life (Taylor et al., 1991). Furthermore, the InsR levels are strongly decreased in obese and diabetic mice and patients, corroborating the physiological relevance of studying the InsR in the context of insulin resistance (Grunberger et al., 1989).

PCSK9 is the 9th member of the mammalian proprotein convertases (Seidah et al., 2003), a family of serine proteases involved in the activation or inactivation of a broad spectrum of proteins, such as precursor growth factors and hormones, their receptors, transcription factors, viral and bacterial proteins, etc. (Seidah and Prat, 2012). Shortly after its discovery, natural *PCSK9* variants were identified in families with autosomal dominant hypercholesterolemia without any mutations in the known FH genes (Abifadel et al., 2003). Importantly, as the variants segregated well with the disease, the *PCSK9* gene was confirmed as the third locus for FH, after the *LDLR* and the *APOB* coding genes (Abifadel et al., 2003; Timms et al., 2004). PCSK9 is strongly expressed in the liver and intestine and is secreted by hepatocytes into the circulation. PCSK9 strongly downregulates the protein levels of the LDLR of target tissues, resulting in increased levels of LDLc. Natural variants of *PCSK9* are classified into GOF variants associated with increased cardiovascular event risk or conversely to LOF variants, associated with low to very-low protective levels of plasma cholesterol (Cohen et al., 2005; Cohen et al., 2006).

Several epidemiological studies have demonstrated positive associations of plasma PCSK9 with markers of increased diabetes risk, including insulin resistance, increased fasting plasma glucose and insulin (Baass et al., 2009; Cui et al., 2010; Ferri et al., 2020; Lakoski et al., 2009).

PCSK9 also correlates with insulin resistance and/or diabetic dyslipidemia in patients with type I and type II diabetes mellitus (Baass et al., 2009; Bojanin et al., 2019; Brouwers et al., 2011; Laugier-Robiolle et al., 2017; Levenson et al., 2017a; Levenson et al., 2017b). The mechanisms underlying these associations and the possible causal role for PCSK9 are gaining avid interest, particularly since the recent demonstration that PCSK9 inhibitors do not increase the risk for diabetes and importantly show significant benefit to diabetic patients (Ray et al., 2019a; Sabatine et al., 2017).

On the other hand, several LOF variants are retained in the ER as unprocessed precursors (proPCSK9) and have been demonstrated to exert a dominant negative effect on the wild-type allele counterpart. These missense mutations code for the single-allele double mutant R104C/V114R and the Q152H (Cariou et al., 2009; Mayne et al., 2011). In carrier probands, the wild-type PCSK9 was undetectable in the plasma. It is known that proPCSK9 forms a tertiary structure distinct from the processed form (Garvie et al., 2016) which in contrast to the matured PCSK9, oligomerizes with itself in the ER (Seidah et al., 2003), suggesting that the dominant negative effect of proPCSK9 could at least in part be explained due to this. The Q152H mutation involves the replacement of the P1 residue Gln of the autocatalytic cleavage site VFAQ$_{152}$↓SIP by His, thereby preventing processing into the mature enzyme (Mayne et al., 2011). Additionally, PCSK9$_{Q152H}$ also binds to and retains wild-type PCSK9 in the ER reducing intramolecular processing (Benjannet et al., 2012; Mayne et al., 2011). The impact of these variants on other secretory pathway proteins has not been demonstrated.

In the present study, we show that that secreted PCSK9 decreases insulin signaling in mouse liver and in immortalized human hepatocytes. We also show that PCSK9$_{Q152H}$ (or proPCSK9) decreases the levels of nascent receptors, including InsR and transferrin receptor (TFRC). It interacts with the precursor of InsR (proInsR) and significantly decreases both the proInsR and matured InsR protein levels in cell lines. Therefore, although PCSK9$_{Q152H}$ was very recently shown not to induce ER stress under normal physiological conditions (Lebeau et al., 2020) it may have other ER-related functions such as the fine tuning of nascent receptor levels.

II.4. Methodology

II.4.1. Animal procedures

All experimental procedures were approved by the IRCM care committee. C57BL/6J background mice were studied and housed under 12 hr light and dark cycles. Mice were fed a chow diet (Teklad Global). Insulin and PCSK9 injection experiments were conducted on 4 hr-fasted, 3 months-old male mice. Recombinant human PCSK9 (BMS) (10 μg) was injected in the tail vein, whereas human insulin (Humulin R by Lilly) (0.2 U/kg body weight) was injected in the inferior vena cava. Liver samples were collected and immediately frozen with dry ice and stored at -80ºC until analysis.

II.4.2. Cell culture and inhibitors

All cells were cultured at 37°C under 5% CO_2. HepG2 human cells (ATCC # HB-8065) and IHH (Han Moshage UH) were grown in EMEM medium (Wisent bioproducts) supplemented with 10% FBS (GIBCO BRL). These cell lines were transfected between at ~60% confluency using Fugene HD reagent (Promega). HEK293 cells (ATCC #CRL-1573) were grown in DMEM medium (Wisent bioproducts) with 10% FBS and transfected at ~70% confluency using jetPRIME reagent (Polyplus). Gene knockdown was achieved by transfecting cells with a pool of four siRNAs against human LDLR or a scrambled siRNA (Dharmacon; siGENOME SMARTpool) at ~50% confluency using INTERFERin (Polyplus). Cells were collected 48 hrs post-transfection after an overnight incubation in serum-free medium or conditioned media prepared from stable cell lines. To inhibit proteasomal degradation, cells were treated with MG132 (Sigma-Aldrich) (5 μM, 16 hrs) and to inhibit lysosomal or autophagosomal degradation, cells were treated with chloroquine (Sigma-Aldrich) (150 μM, 16 hrs).To inhibit the exit of secretory proteins from the ER, cells were treated with brefeldin A (Sigma-Aldrich) (2 μg/mL, 16 hrs).

II.4.3. Conditioned media production for media swap assays

PCSK9-V5 enriched conditioned medium was prepared from HEK293 stable cell lines expressing PCSK9-V5. Control medium was prepared from HEK293 stable cell lines expressing the empty vector control. For the $PCSK9_{R46L}$ media swap experiment, HEK293 cells were transfected with the control plasmid, PCSK9-V5 expressing plasmid or $PCSK9_{R46L}$ expressing plasmid. Conditioned media is prepared with serum free DMEM incubated on the cells for 18 hrs, and PCSK9 is quantified in the medium with the previously described home-made ELISA (Dubuc et al., 2010). The media were adjusted to ~1 µg/mL or ~300 ng/mL and aliquoted at -80ºC. Human insulin (Lilly) was added to the different conditioned media at a final concentration of 1 µM overnight before cell collection for analysis.

II.4.4. Plasmids

The untagged pcDNA3 vector (Invitrogen) was used to express InsR, the pIRES vector (Invitrogen) was used to express PCSK9, and its mutants, TFRC, LDLR, FGF21 and the empty pIRES vector was used as a control, all express a C-terminal V5 tag. PCSK9 mutants were described previously, $PCSK9_{Q152}$ (Benjannet et al., 2012), $PCSK9_{H226A}$ (Seidah et al., 2003), $PCSK9_{C679X}$ (Nassoury et al., 2007), $PCSK9_{KDEL}$ (Nassoury et al., 2007), Pro (Nassoury et al., 2007), LX (Nassoury et al., 2007), CHRD (Nassoury et al., 2007), $PCSK9_{R46L}$ (Benjannet et al., 2006), $PCSK9_{G316C}$ (Di Filippo et al., 2017).

II.4.5. Immunoblotting and immunoprecipitation

Proteins were extracted in RIPA 1X buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 0.1% SDS, 1% Nonidet P40, and 0.25% Na deoxycholate) with a cocktail of protease inhibitors (cOmplete, Roche) and a cocktail of phosphatase inhibitors (PhosSTOP, Sigma-Aldrich) for insulin signaling experiments. Bradford assay was used to evaluate the protein concentrations. For immunoblotting, 30 µg of proteins were resolved on SDS/PAGE (8% to 10% Tris-glycine) and transferred onto nitrocellulose membranes. Membranes were blocked with fat-free 5% milk powder dissolved in Tris buffer saline (0.1 M Tris/HCL pH 8 and 1.5 M NaCl) containing 0.1% Tween-100 (TBS-T).

Incubations with primary and secondary antibodies coupled to horseradish peroxidase (HRP) were done in 5% milk overnight at 4ºC and over 2 hrs at room temperature, respectively, except for Cell Signaling Technology antibodies which were revealed in 5% fatty acid free BSA dissolved in TBS-T. For immunoprecipitations, cell lysates were incubated with ProtA/G beads (Santa Cruz, Sc-2003) in RIPA 0.5X, with rotation, overnight at 4ºC with primary. Following washes, pull-downs were revealed by SDS-PAGE as described. All membrane proteins were detected by chemiluminescence (Bio-Rad), revealed and analysed using the ChemiDoc MP imaging system (Bio-Rad).

II.4.6. Antibodies

List of primary antibodies for Western Blotting:

- α-InsRβ (Enzo Life Sciences, ADI-905-603-100;
- α-P-IGF1Rβ/P-InsRβ (Cell Signaling Technology, 9021);
- α-mLDLR (R&D Systems, AF2255);
- α-hLDLR (R&D Systems, AF2148);
- α-hPCSK9 (homemade, Nassoury 2007);
- α-P-S473 Akt (Cell Signaling Technology, 9271);
- α-Akt (total) (Cell Signaling Technology, 9272);
- α-P-S9 GSK3β (Cell Signaling Technology, 9323);
- α-GSK3β (total) (Cell Signaling Technology, 9315);
- α-P-S51-eIF2α (Invitrogen, 44-728);
- α- β-actin (Sigma-Aldrich, A2066);
- α- α-tubulin (Sigma-Aldrich, T4026).

List of primary antibodies for immunoprecipitation:

- α-V5 tag (Invitrogen, PIN 46-0705);
- α-hTFRC (Abcam, ab84036);
- α-hInsR (Millipore, MAB1138);
- α-hPCSK9 (homemade described in (Nassoury et al., 2007).

List of secondary antibodies for Western Blotting:

- α-V5-HRP (Sigma-Aldrich, V2260);
- α-mouse-HRP (VWR, CA95017-556L);
- α-rabbit-HRP (VWR, CA95017-332L);
- α-goat-HRP (Santa Cruz, Sc 2020).

II.4.7. Metabolic labeling

HEK293 cells were transiently transfected as described above. At 48 hrs post-transfection, the cells were washed in PBS medium and preincubated in DMEM without methionine and cysteine (Wisent) for 1 hr. Cells were then incubated for 30 min in radioactive medium, DMEM without methionine and cysteine with 100 mCi/mL of [^{35}S]Met/Cys (Perkin Elmer, EasyTag EXPRESS 35S Prot labeling mix, NEG 77202MC). Cells were lysed as described, proteins were quantified by Bradford, immunoprecipitated as described and run on SDS-PAGE as described. The gels were dried and autoradiographed as described (Benjannet et al., 2001). Total cell lysate radioactivity was counted by trichloroacetic acid precipitation (10%) (Sigma-Aldrich) and measured using a scintillator.

II.4.8. RNA extraction and gene expression

Total RNA from cells was extracted with TRIzol (Invitrogen). cDNA was generated from 250 ng of total RNA using a SuperScript II cDNA reverse transcriptase (Invitrogen). Quantitative PCR was done using the SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA) and the following sense and antisense primers: housekeeping gene human TATA-box binding protein (TBP) (exon4-5), 5'- CGAATATAATCCCAAGCGGTTT and 5'- GTGGTTCGTGGCTCTCTTATCC; h*s*XBP (splice site) 5'- TGCTGAGTCCGCAGCAGGTG and 5'- GCTGGCAGGCTCTGGGGAAG; hInsR (exon 8-9) 5'- GGGCCAAGAGTGACATCATTTA and 5'- GGGTGGTTTCCACTTCAGAATA.

II.4.9. Statistical analysis

Cell and mouse result values are expressed as mean ± SEM. Significant differences between groups were evaluated with an unpaired two-tailed Student's t-test.

II.5. Results

II.5.1. Exogenous PCSK9 decreases insulin signaling in adult male mice

PCSK9 expression is regulated by SREBPs, where SREBP-2 is activated by decreased ER-membrane cholesterol concentrations (<5%) (Radhakrishnan et al., 2008; Sakai et al., 1996) to increase intracellular cholesterol concentrations and SREBP-1c is activated by mTORC1 downstream from insulin in order to stimulate *de novo* lipogenesis. Accordingly, insulin has been demonstrated to upregulate PCSK9 expression (Costet et al., 2006). However, whether PCSK9 affects insulin signaling has, to our knowledge, not yet been investigated. Therefore, we attempted to verify this possible regulatory effect by administering recombinant PCSK9 acutely on short-term insulin induced InsR signaling in adult male C57BL/6J mice. Mice were fasted for 4 hours to normalize insulin signaling, then at 5 min following acute tail-vein injection of 10 μg of recombinant PCSK9 (BMS-LVL) or PBS control, an insulin (0.2 U/Kg body weight) or PBS bolus were injected into the inferior vena cava. 5 min later, mice (7 mice/condition) were sacrificed, their livers extracted and the lysates separated on SDS-PAGE to analyze by western blot the levels of LDLR as well as InsR and its downstream signaling (Figure II.5. 1A-D). At 10 min following PCSK9 injection, we observed a significant ~30% decrease in LDLR levels (in comparison to control PBS) (Figure II.5. 1C). Interestingly, at 5 min post-insulin injection, the activity of PCSK9 on the LDLR is blunted, suggesting that on the short-term insulin interferes with PCSK9-mediated LDLR degradation. We also noticed that PCSK9 does not affect the levels of InsR (Figure II.5. 1C) or its insulin-induced auto-phosphorylation into P-InsR as evidenced by an unchanged P-InsR/InsRβ ratio (Figure II.5. 1D). However, PCSK9 blunted the downstream insulin signalling, as evidenced by significantly reduced ratio of P-Akt/Akt (-30%) (Fig. 1D). These data suggest that even though PCSK9 does not significantly affect InsR autophosphorylation/activation, it does reduce its downstream signaling capacity, and hence its insulin sensitivity, possibly via diminished recruitment of IRS-1 and -2 by the P-InsR.

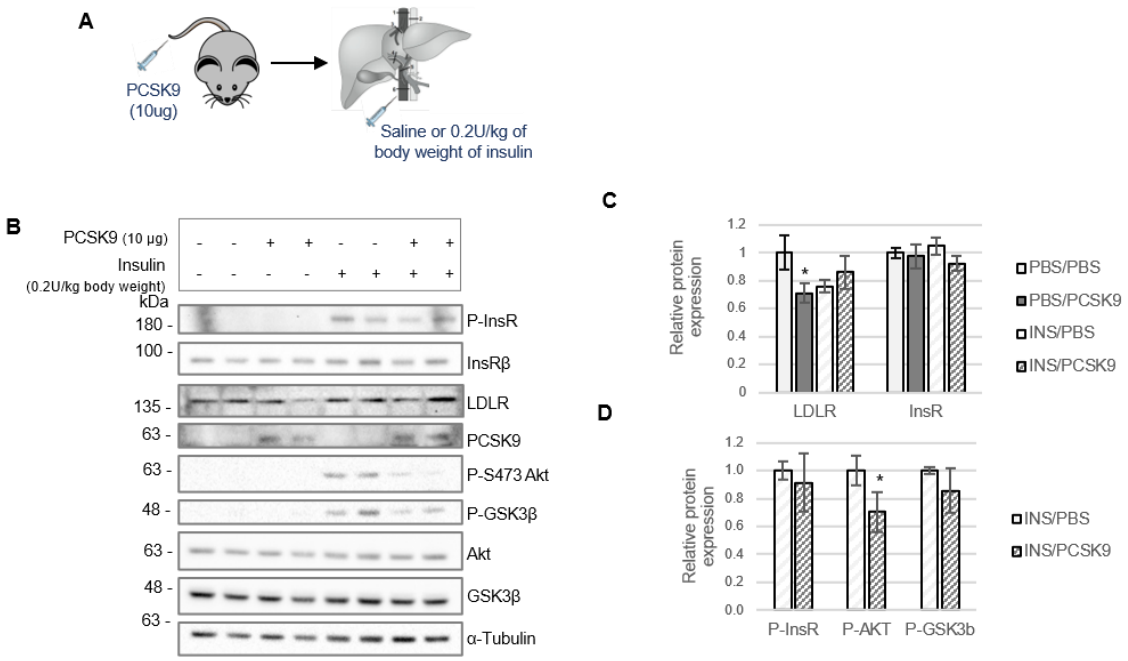

Figure II.5. 1 PCSK9 downregulates insulin signaling acutely in adult male mice

A, Three-months old male mice were fasted for four hours, reconstituted PCSK9 (10 µg) or control saline solution was injected into the tail vein, the mouse was then put to sleep for five minutes, insulin (0.2 U/kg of body weight) or saline control was then injected in the inferior vena cava. After five more minutes, the liver was extracted for protein isolation. The mouse liver samples were analyzed by WB for downstream insulin signaling, including the activation of Akt and GSK3β, ***B, C*** and ***D***. The experiment was performed on B6J (n=7 mice/condition, left panel). The data are presented as the mean ± standard error of the mean. Statistical comparisons with controls were performed by an unpaired two-tailed Student's t-test. *P < 0.05.

II.5.2. Exogenous PCSK9 decreases insulin signaling in immortalized human hepatocytes

We next investigated the effect of overnight exogenous PCSK9 (~1 µg/mL) in the presence of insulin (1 µM) on InsR downstream signaling using the immortalized human hepatocyte (IHH) cell line, an insulin responsive cell line (Schippers et al., 1997). Serum free control conditioned media or PCSK9 enriched conditioned media were prepared from human embryonic kidney cells (HEK293) stably expressing empty-vector or hPCSK9. As expected, in the presence of insulin we observed: (i) enhanced insulin-mediated degradation of the InsR; (ii) increased phosphorylation of Akt (P-Akt) that occurs downstream of InsR activation; and (iii) increased LDLR levels, likely as a response to the activation of SREBP-1c by insulin (Brown and Goldstein, 1997) (Figure II.5. 2A, lane 3). In addition, under PCSK9 media swap conditions we observed: (i) an expected significant

~60% decrease in LDLR protein levels (Figure II.5. 2A, lanes 2 and 4 *vs.* lanes 1 and 3), (ii) a similar ~70% enhanced insulin-mediated degradation of InsR (Figure II.5. 2A, lane 4); and (iii) ~70% attenuated levels of P-Akt compared to control conditions in absence of PCSK9 (Figure II.5. 2A, lane 4 *vs.* lane 2) in the absence or presence of insulin. A previous study demonstrated an interaction between the LDLR and the InsR at the plasma membrane, inhibiting the LDL internalization activity of the LDLR (Ramakrishnan et al., 2012). Thus, we hypothesized that PCSK9 could inhibit InsR signaling by the formation of the trimeric complex LDLR/InsR/PCSK9. Therefore, to test whether this PCSK9 effect on P-Akt is mediated by its ability to bind to and degrade the LDLR, we reproduced the experiment under knockdown conditions of the LDLR (Figure II.5. S1 in Appendices). Our data showed that in the absence of LDLR the effect of PCSK9 on the insulin-mediated P-Akt was maintained. Therefore, PCSK9 mediated inhibition of P-Akt is LDLR-independent.

We next investigated whether neutralizing exogenous PCSK9 with the use of a mAb (AMG 145) or a single domain antibody (sdAb) developed by our lab (P1.40) (Weider et al., 2016) to PCSK9 would rescue its effect on P-Akt. The mAb targets the catalytic domain of PCSK9, whereas the sdAb targets the CHRD of PCSK9. Therefore, we performed the same experiment described above but included mAb or sdAb with the PCSK9 enriched conditioned medium or the control medium. Our results demonstrate that incubation with mAb but not with sdAb inhibits PCSK9 mediated downregulation of P-Akt (Fig. II.5. 2B and C), suggesting that the catalytic domain is important and that the CHRD is not implicated in this process.

Lastly, we were interested in comparing the effect of WT PCSK9 with a known PCSK9 LOF variant, reported to be associated with an increased risk for type 2 diabetes (Lotta et al., 2016; Nelson et al., 2019), $PCSK9_{R46L}$, on insulin signaling. Therefore, we reproduced the same experiment while incubating the IHH cells with conditioned medium enriched in WT PCSK9 or in $PCSK9_{R46L}$ or with control conditioned medium. Under these experimental conditions the effects of WT and $PCSK9_{R46L}$were comparable: ~80% decrease in P-Akt (Figure II.5. 2D and E), suggesting that the prodomain is not critical in this process.

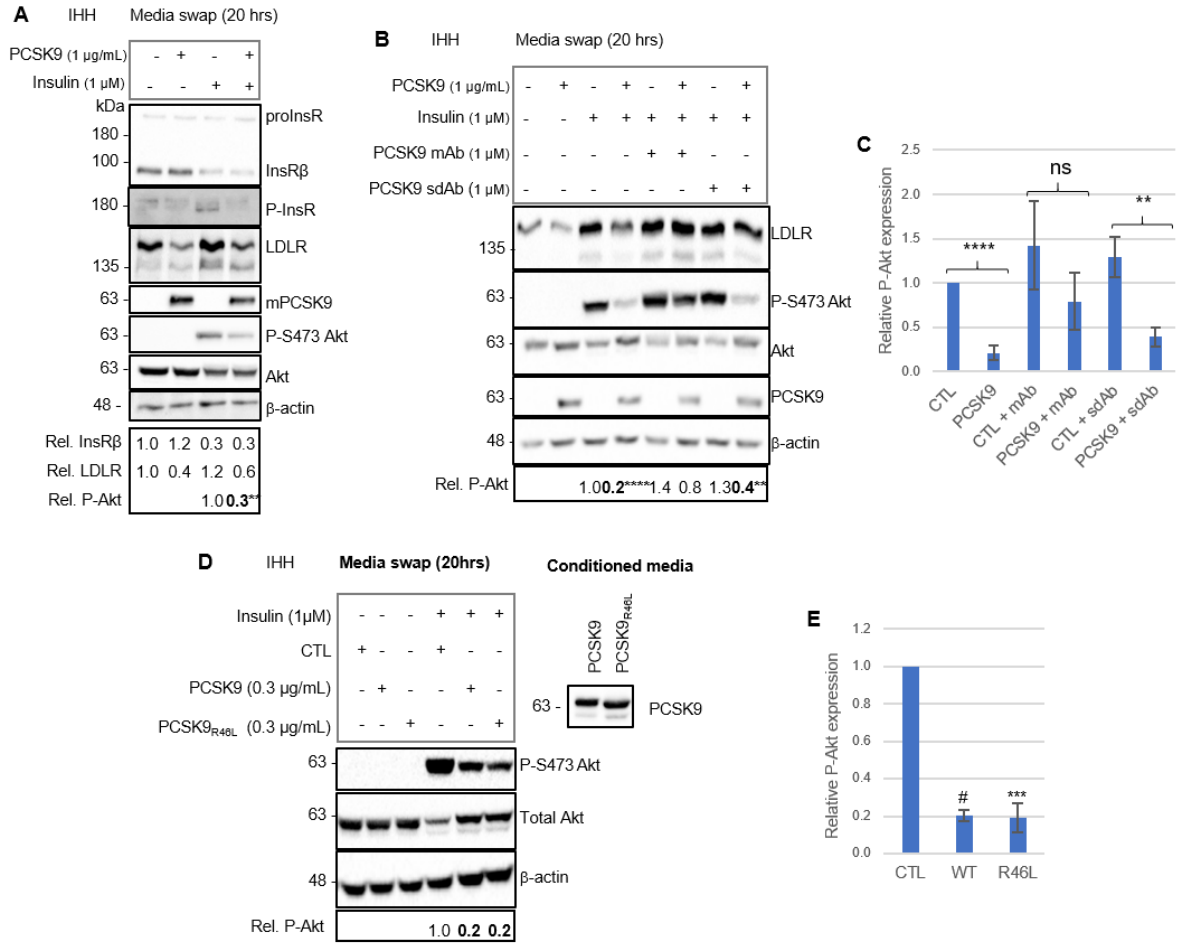

Figure II.5. 2 Exogenous PCSK9 decreases insulin signaling *in vitro*

IHH cells were incubated O/N with serum-free conditioned media lacking or containing 1 µg/mL of PCSK9-V5 and in the presence or absence of 1 mM insulin. Cell lysates were analyzed for the activation of Akt (pS473) by WB in ***A***. ***B*** and ***C***, the effect of inhibiting exogenous PCSK9 by mAb or sdAb (1 µM) on the activation of Akt (pS473) was analyzed by WB. ***D*** and ***E***, IHH cells were incubated O/N with serum-free conditioned media lacking or containing 0.3 µg/mL of PCSK9-V5 or $PCSK9_{R46L}$-V5 and in the presence or absence of 1 µM insulin and analyzed by WB. The input conditioned medium was analyzed by WB (right panel). The data are representative of at least three independent experiments and are presented with mean ± standard deviation. Statistical comparisons with controls were performed by an unpaired two-tailed Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, $^{\#}$P < 0.00001.

II.5.3. Overexpression of proPCSK9 induces the downregulation of InsR early in the secretory pathway

PCSK9 and InsR are both synthesized as zymogen proteins (proPCSK9 and proInsR) with a signal peptide allowing them to enter the ER and traffic to the cell surface and/or media through the secretory pathway. ProInsR requires the formation of a homodimer and proper folding in order to exit the ER (Lu and Guidotti, 1996). Upon reaching the cell surface, a proprotein convertase, such as furin, cleaves proInsR into disulfide-linked α and β domains, generating a heterotetramer (Bravo et al., 1994; Robertson et al., 1993).

In order to verify that $PCSK9_{Q152H}$ may affect the maturation process of other precursor proteins in the ER, human-derived hepatoma cells (HepG2) and HEK293 were transfected with WT PCSK9, $PCSK9_{Q152H}$, $PCSK9_{H226A}$ and InsR expressing cDNA plasmids (1:1 ratio). $PCSK9_{Q152H}$ and $PCSK9_{H226A}$ are known natural and selected mutations respectively, which result in an unprocessed PCSK9 that is retained in the ER (Benjannet et al., 2012; Benjannet et al., 2004; Cariou et al., 2009). Under these conditions, we observed a non-significant decrease in InsR in the presence of WT PCSK9 and a significant 30% and 60%, in HepG2 and HEK293 cells respectively, decrease in total InsR levels in the presence of the ER-retained variants (Figure II.5. 3A and B). The regulation of InsR by ER-retained proPCSK9 was further confirmed by the significant 50% decrease in endogenous InsR under conditions of $PCSK9_{Q152H}$ overexpression in HEK293 cells (Figure II.5. 3C).

In order to verify whether the regulation is indeed taking place in ER, and whether retaining matured PCSK9 in this subcellular localization might increase the effect on InsR, we treated cells with Brefeldin A (BFA). The latter results in the inhibition of trafficking past the ER by fusing the ER and *cis*, *medial* Golgi compartments away from the TGN, and hence we expected an accumulation of mature PCSK9 in the ER (Lippincott-Schwartz et al., 1991). Under the co-transfection of $PCSK9_{WT}$, $PCSK9_{Q152H}$ and InsR, we first confirmed that the BFA treatment was effective since PCSK9 is no longer secreted into the medium (Figure II.5. S2A in Appendices). In addition, we also observed that BFA treatment results in a large reduction in InsRβ and a moderate increase in proInsR (Figure II.5. S2A in Appendices), likely due to the lack of its processing by the convertases, which are activated in the TGN and beyond in the secretory pathway (Seidah and Prat, 2012). Interestingly, BFA treatment of cells expressing $PCSK9_{WT}$ or $PCSK9_{Q152H}$ resulted in a comparable effect on InsR than in the control DMSO treated cells (Figure II.5. S1B in

Appendices). Importantly, these results confirmed that the effect of PCSK9 takes place early in the secretory pathway but also suggest that retaining mature PCSK9 in the ER does not lead to an increased effect on Akt.

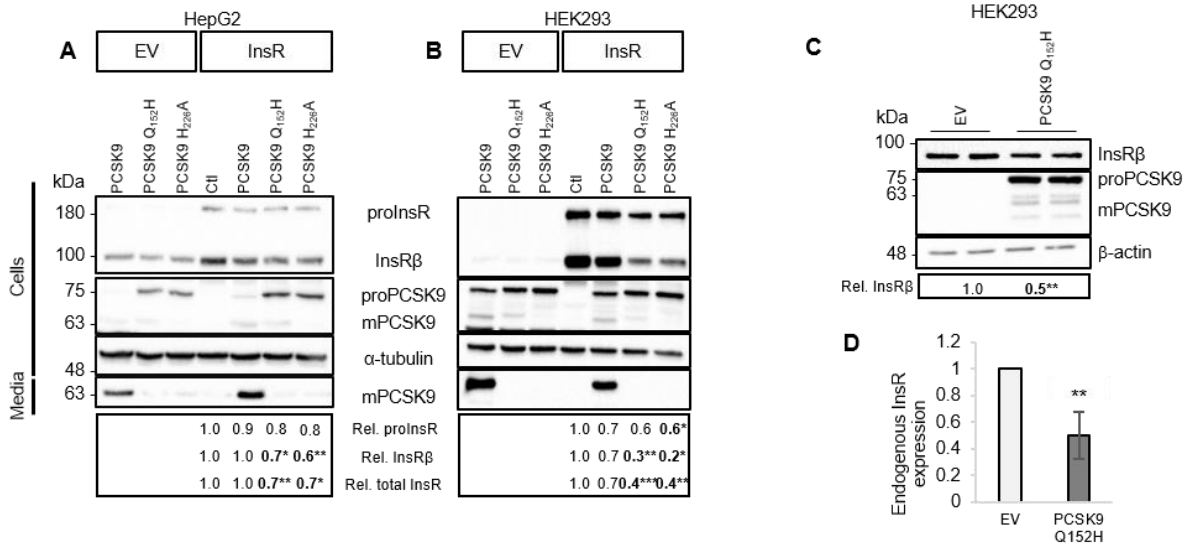

Figure II.5. 3 Overexpression of proPCSK9 induces the downregulation of InsR in the secretory pathway.

HepG2 (***A***) and HEK293 (***B***) cells transiently co-expressing InsR with a control empty vector plasmid (EV), wild-type PCSK9-V5, and ER-retained proPCSK9 forms: either naturally occurring $PCSK9_{Q152H}$-V5 or catalytically inactive mutant $PCSK9_{H226A}$-V5 were analyzed by WB. ***C***, Endogenous InsR from HEK293 cells transiently expressing either EV or $PCSK9_{Q152H}$-V5 was analyzed by WB, ***D***. The data are representative of at least three independent experiments and are presented with mean ± standard deviation. Statistical comparisons with controls were performed by an unpaired two-tailed Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

II.5.4. ProPCSK9 interacts with proInsR

ProPCSK9 has been shown to interact with the immature LDLR in the ER (Nassoury et al., 2007). Interestingly, the InsR has previously been reported to interact with the LDLR all throughout the secretory pathway, including the ER (Ramakrishnan et al., 2012). Therefore, we first confirmed the interaction of the LDLR with the InsR by co-immunoprecipitation of the co-transfected proteins in HepG2 cells (Figure II.5. 4A). The interaction between the LDLR and mature InsR was detected under control conditions (*not shown*), however, treating the cells with MG132, a proteasomal inhibitor, would appear to rescue much of the interactions for both the proInsR and the mature InsR with the LDLR (Figure II.5. 4A). Similarly, the interaction of PCSK9

with the proInsR was also detected under co-expression and MG132 treated conditions in these cells (Figure II.5. 4A). Co-transfection in HEK293 cells of the InsR with WT or PCSK9$_{Q152H}$ under non-treated conditions and performing an immunoprecipitation with either α-PCSK9 or with α-InsR antibodies confirmed the association between both proteins (Figure II.5. 4B). Moreover, the association appears to be strongest between the zymogens (Figure II.5. 4B).

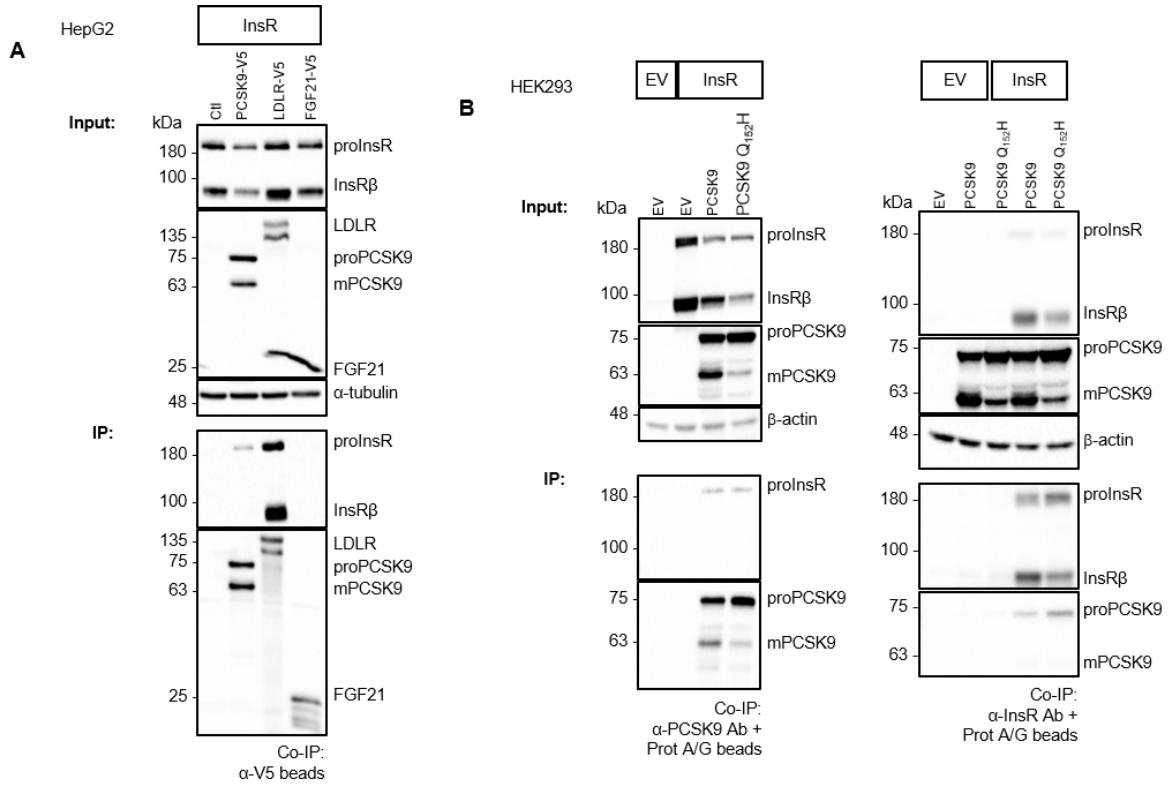

Figure II.5. 4 ProPCSK9 interacts with proInsR.

A, HepG2 cells transiently co-expressing InsR with the control EV plasmid, wild-type PCSK9-V5, LDLR-V5 or FGF21-V5 were treated with MG132 (2.5 mM, O/N). Cell lysates were immunoprecipitated with α-V5 beads and analyzed by immunoblot. ***B***, HEK293 cells were transiently co-transfected with the indicated plasmids and cell lysates were immunoprecipitated with α-PCSK9 Ab or α-InsR Ab and analyzed by immunoblot. The data are representative of at least three independent experiments.

II.5.5. Natural ER-retained PCSK9 variants decrease InsR

We next tested whether the regulation of the InsR by PCSK9 in the ER implicates the action of immature proPCSK9 and/or its mature PCSK9 form. To test this, we used ER-retained proPCSK9 mutants, PCSK9$_{Q152H}$, PCSK9$_{H226A}$, PCSK9$_{G316C}$, PCSK9$_{C679X}$, and compared them

with ER-retained $PCSK9_{KDEL}$ for the efficiency of proInsR downregulation (Figure II.5. 5A). The KDEL motif is fused to the C-terminus of PCSK9 to allow for retention of the mature PCSK9 in the ER through binding to KDEL receptors (Figure II.5. 5A). When transfected in HEK293 cells, the ER-retained proPCSK9 mutants demonstrate significantly stronger degradation effects on total InsR levels than the $PCSK9_{KDEL}$ construct (70-60% decrease *versus* 30% non-significant decrease) (Figure II.5. 5B-C). Therefore, these results confirmed that proPCSK9 is required *vs.* mature PCSK9, similarly to the previous observation that retaining mature PCSK9 in the ER through BFA treatment did not induce a stronger decrease in the InsR. While co-transfecting the InsR with the prodomain, the LX construct – which includes both the pro and the catalytic domains – or the CHRD demonstrated that the LX downregulates the InsR as efficiently as the WT, whereas the pro and the CHRD domains have a small and no effect, respectively (Figure II.5. 5B, D). Therefore, the pro and catalytic domains together are required, whereas the CHRD is dispensable for PCSK9 mediated InsR downregulation. Accordingly, even though both the pro and CHRD domains only weakly affect InsR levels, the pro domain shows stronger binding efficiency (Figure II.5. 5B). Moreover, $PCSK9_{KDEL}$ also demonstrated efficient binding to proInsR while its effect on InsR levels did not reach statistical significance (Figure II.5. 5C). Therefore, these results suggest an absence of correlation between association and downregulatory activity.

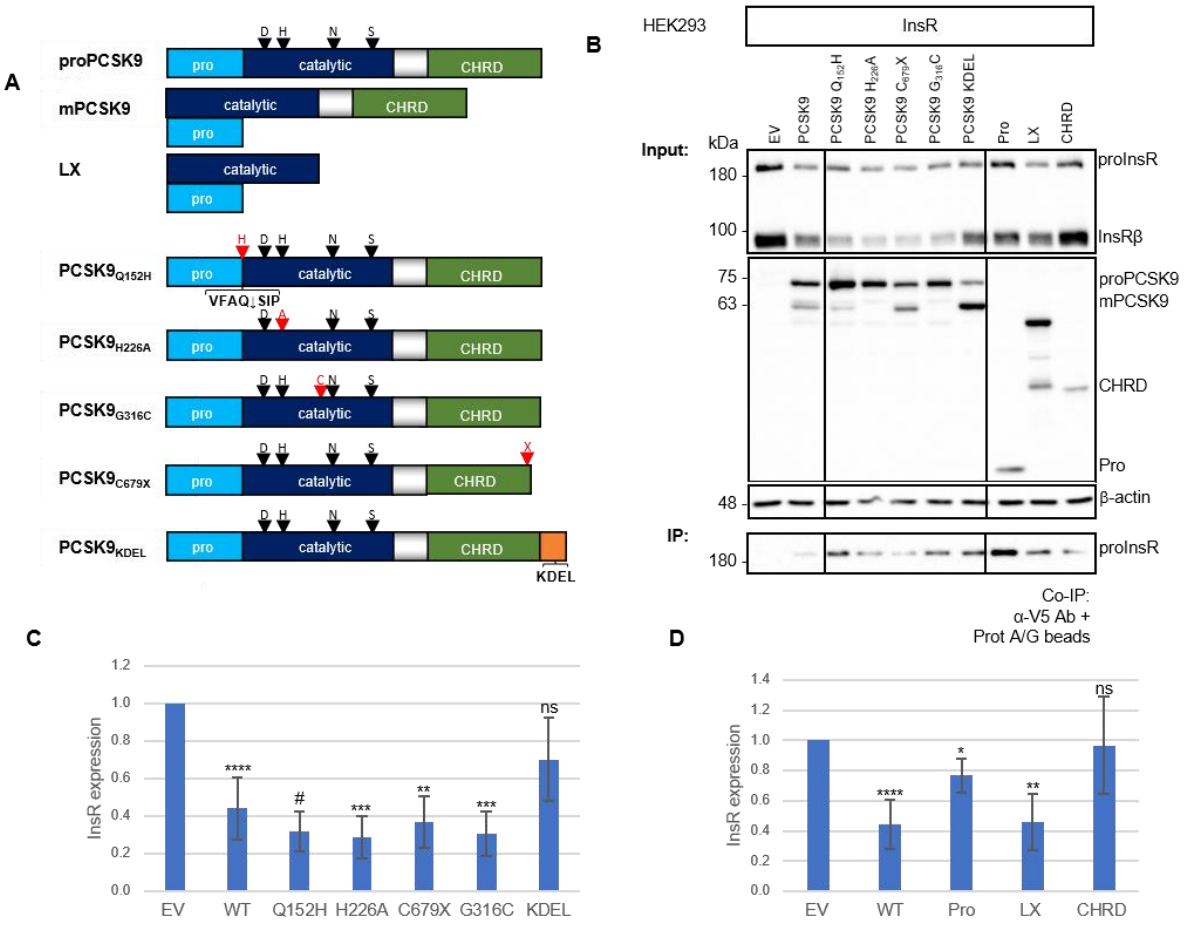

Figure II.5. 5 Natural ER-retained PCSK9 variants decrease InsR

A, Schematic diagram of PCSK9 WT proprotein and mature forms, variants and domain constructs fused with V5 tag. ***B***, HEK293 cells were transiently co-transfected with the indicated plasmids and cell lysates were immunoprecipitated with α-V5 Ab and analyzed by immunoblot. ***C*** and ***D***, Relative quantifications of total InsR co-expressed with variants or with domain constructs by WB analysis. The data are representative of at least 3 independent experiments and are presented with mean ± standard deviation. Statistical comparisons with controls were performed by an unpaired two-tailed Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

II.5.6. PCSK9$_{Q152H}$ decreases nascent receptors in the ER

Since the association of the PCSK9 various constructs to the InsR was not determinant for InsR downregulation, but rather it is the expression of an un-cleaved, ER-retained PCSK9 zymogen that consistently showed a strong decrease in InsR levels, we next examined the specificity of this effect for another receptor. Therefore, we examined the effect of PCSK9$_{Q152H}$ on the human

transferrin receptor 1 (TFRC), a type II transmembrane protein, which similarly to the InsR, is ubiquitously expressed (Terng et al., 1998). The co-transfection in HEK293 cells of these proteins demonstrated equivalent downregulatory activity of $PCSK9_{Q152H}$ on either the InsR or the TFRC (Figure II.5. 6A), suggesting that $PCSK9_{Q152H}$ regulates the levels of several receptors in the ER.

Then, in order to verify whether the biosynthesis rates of these receptors were affected by $PCSK9_{Q152H}$, we performed metabolic labelling experiments. HEK293 cells co-expressing $PCSK9_{WT}$, $PCSK9_{Q152H}$ or FGF21 with InsR were metabolically labelled with a 30 min pulse of ^{35}S-Met/Cys to immunoprecipitate either InsR or the PCSK9 (Figure II.5. 6B top *vs.* bottom panel, respectively). Our results demonstrated a non-significant decrease in proInsR in the presence of FGF21, used as a negative control here for the effect of PCSK9, and a strong reduction in proInsR in the presence of $PCSK9_{WT}$ and $PCSK9_{Q152H}$, by 50% and 80% respectively (Figure II.5. 6D). These data strongly suggested that the downregulatory effect is very rapid, such as by ERAD, as has been shown for ER transmembrane proteins (Scheffer et al., 2019), a co-translational degradation of the nascent polypeptide, or a decrease in the InsR mRNA stability. We next co-transfected $PCSK9_{WT}$ and $PCSK9_{Q152H}$ with TFRC and performed a similar pulse to metabolically label and pull-down TFRC or PCSK9 (Figure II.5. 6C top *vs.* bottom panel, respectively). Our results confirmed a significant ~40% decrease in TFRC levels in the presence of $PCSK9_{Q152H}$, and no effect by $PCSK9_{WT}$. These results suggest that PCSK9 exerts a stronger and more rapid effect on the InsR in contrast to TFRC, however, under steady state conditions these differences are not discernable. Furthermore, the un-cleaved, ER-retained PCSK9 has a strong propensity to oligomerize and to exert a dominant negative effect on the WT allele in heterozygous patients (Benjannet et al., 2012), could induce more a general effect in the ER, resulting in decreased biosynthesis of several proteins.

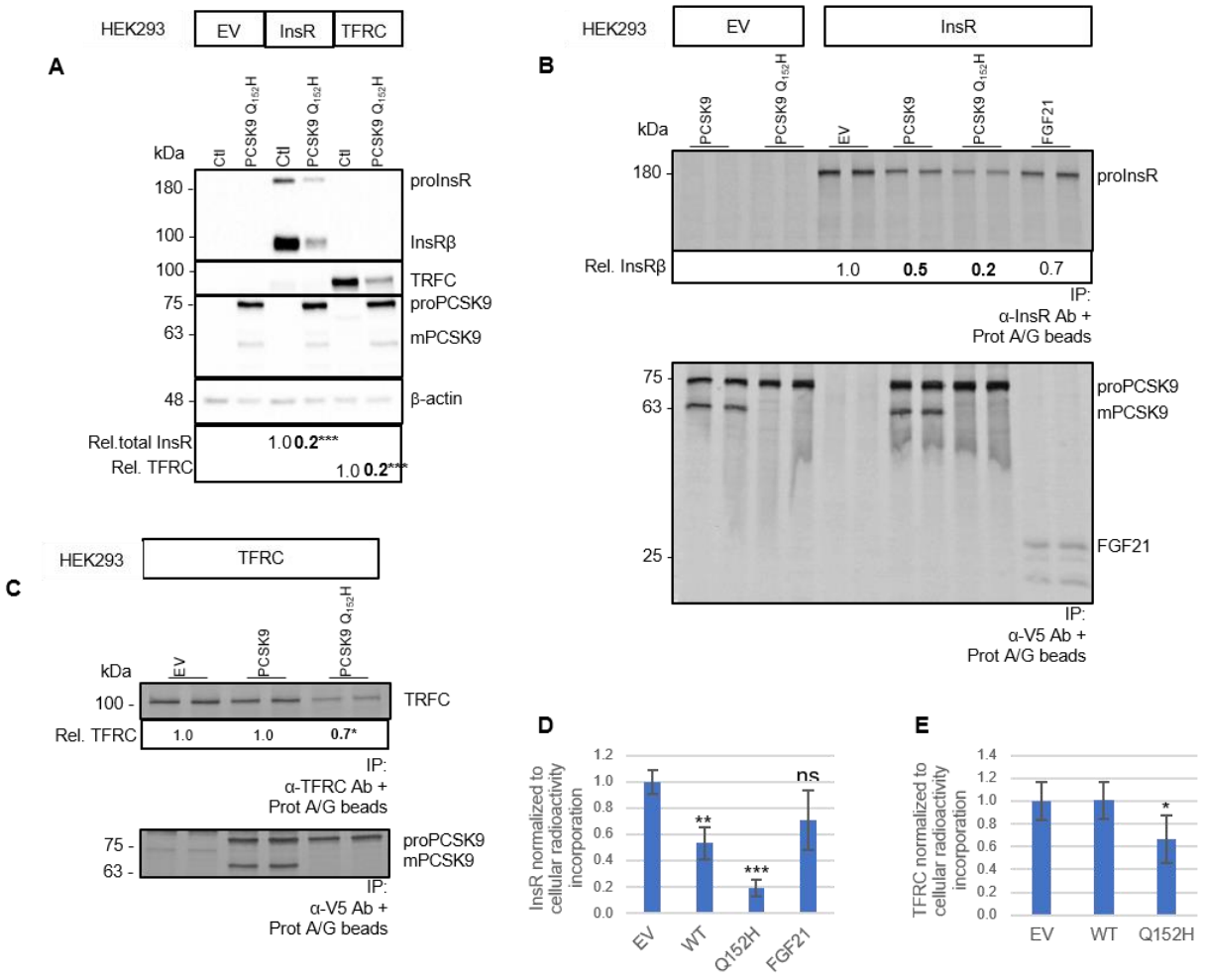

Figure II.5. 6 Rapid downregulatory effect of proPCSK9 on newly synthesized receptors.

A, HEK293 cells were transiently co-transfected with EV, InsR or TFRC and control EV or $PCSK9_{Q152H}$-V5 variant. Cell lysates were analyzed by WB. ***B*** and ***C***, HEK293 cells were transiently co-transfected with InsR and control EV, wild-type PCSK9-V5, $PCSK9_{Q152H}$-V5 variant or FGF21-V5 (panel *A*), or TFRC with control EV, wild-type PCSK9-V5, $PCSK9_{Q152H}$-V5 variant. 48 h post-transfection, the cells were pulse-labeled with [^{35}S]Met/Cys for 15 min and immunoprecipitated with α-InsR Ab or α-V5 Ab. Cell lysates were analyzed by SDS/PAGE on 8% Tris-glycine gels then autoradiographed for imaging and relative quantification in ***D*** and ***E***. Band intensity was normalized to cellular radioactivity incorporation. Data is presented as mean ± standard deviation of three independent experiments. Statistical comparisons with controls were performed by an unpaired two-tailed Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

II.5.7. $PCSK9_{Q152H}$ decreases proInsR mRNA levels through the activation of IRE1α

Misfolded proteins are retained in the ER through their binding with lectin chaperones or in the case of misfolded InsR, for example, to BiP/GRP78 (Bass et al., 1998), the other main protein folding chaperone system of the ER. Accumulating misfolded proteins may overwhelm the ER activating a signaling pathway, termed the UPR pathway, further inducing ER adaptation. These processes allow for the adaptation of the ER by the upregulating of ER chaperone synthesis, activating ERAD, inhibiting global translation and/or stimulating autophagy, altogether favoring cell survival (Wang and Kaufman, 2016). Therefore, we first verified whether the downregulation of InsR could be rescued by inhibiting protein degradation through ERAD or autophagy by treating the cells with MG132 or chloroquine (Omari et al., 2018). The treatments showed no differences in total InsR levels when compared to control conditions (Figure II.5. 7A) suggesting that the effect was not mediated by protein degradation. In order to verify whether general translation is inhibited by the activation of PERK, one of the three main arms of UPR activated under chronic ER stress, we verified the activation status of eIF2α (Wang et al., 2012). Indeed, PERK phosphorylates eIF2α to inhibit translation (Zhang et al., 2002). However, there was no increase in eIF2α phosphorylation when the InsR is co-transfected with un-cleaved, ER-retained mutants when compared to the control conditions (Figure II.5. 7B), suggesting that PERK is not activated, and general translation is not inhibited. Lastly, we show by quantitative polymerase chain reactions (Q-PCR) that the InsR mRNA was decreased under co-transfection conditions with WT PCSK9 and $PCSK9_{Q152H}$ (Figure II.5. 7C). This decrease in InsR mRNA was paralleled by a significant increase in spliced XBP1 (sXBP1). XBP1 is spliced by IRE1α, an exonuclease activated under acute ER stress (Calfon et al., 2002; Yoshida et al., 2001). Therefore, co-transfection of InsR with either WT or uncleaved, ER-retained PCSK9 results in acute ER stress, leading to an adaptation of the cell, which prevents the activation of PERK, a chronic ER stress sensor.

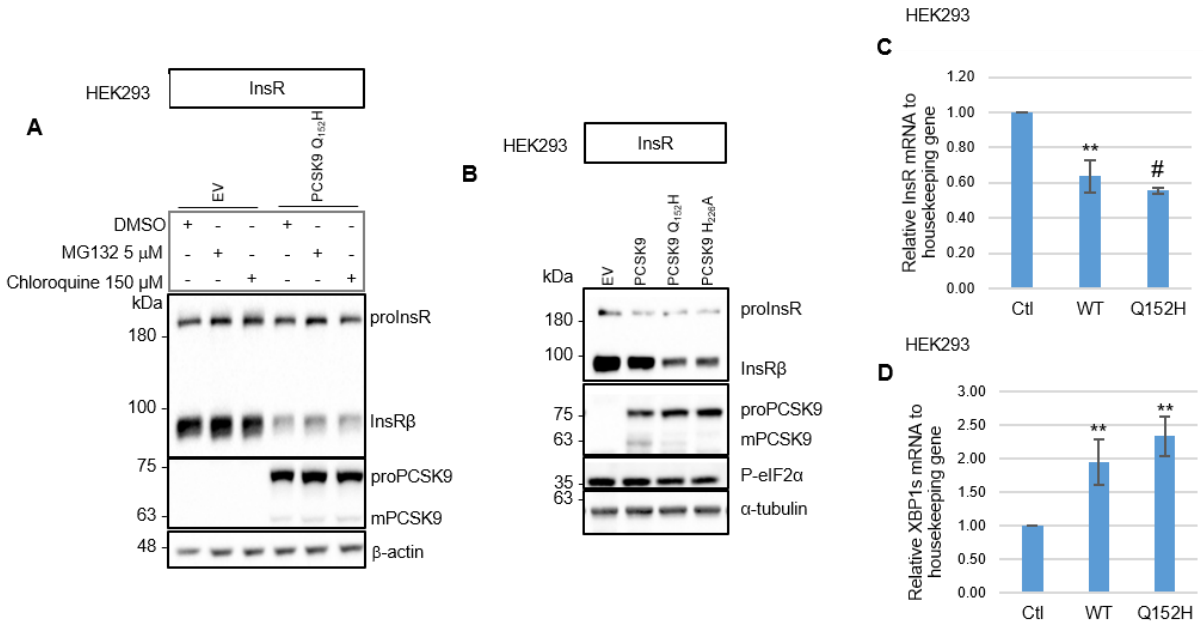

Figure II.5. 7 The downregulatory effect of proPCSK9 is likely due to ER stress and acute UPR.

A, HEK293 cells were transiently co-transfected with control EV or InsR and control EV or $PCSK9_{Q152H}$-V5 variant then treated with proteasomal and lysosomal inhibitors MG132 and chloroquine (5 mM, 150 mM O/N respectively). Cell lysates were analyzed by WB. ***B***, HEK293 cells transiently co-expressing InsR with a control empty vector plasmid (EV), wild-type PCSK9-V5, $PCSK9_{Q152H}$-V5 or $PCSK9_{H226A}$-V5 were analyzed by WB for the activity of eIF2α, a global regulator of translation in response to cellular stress (left panel).***C*** and ***D***, HEK293 cells were transiently co-transfected with InsR and control EV, wild-type PCSK9-V5 or $PCSK9_{Q152H}$-V5 variant. RNA was extracted for qPCR analysis of spliced XBP1 mRNA levels, upregulated upon ER stress, and InsR mRNA levels. Data is representative of three independent experiments.

II.6. Discussion

In light of several studies demonstrating positive associations of PCSK9 with impaired insulin and lipid homeostasis, we were interested in investigating the possible regulatory effect of PCSK9 directly on the insulin receptor and on insulin signaling. Indeed, insulin signaling in the liver plays a crucial role in maintaining whole body lipid homeostasis, including lipid production and lipid clearance. Insulin also regulates the expressions of the LDLR and PCSK9 thereby further modulating lipoprotein internalization kinetics and fractional catabolic rates.

We firstly demonstrate in this study that exogenous PCSK9 decreases InsR signaling *in vivo* in adult male mice and *in vitro* in immortalized human hepatocytes. This effect was prevented by monoclonal antibodies targeting the catalytic domain of PCSK9 but not by single domain antibodies targeting the CHRD domain of PCSK9 (results are summarized in Figure II.6.A). Whether this process may be causal in the relationship between PCSK9, liver insulin resistance and diabetic dyslipidemia requires further investigation. However, we also demonstrate in this study that the R46L variant also decreases insulin signaling *in vitro*. This variant has been associated with an increased risk for the development of T2DM by multi-centered epidemiological studies (Lotta et al., 2016; Nelson et al., 2019). Interestingly, this variant was associated with higher fasting plasma glucose and 2-hr post meal plasma glucose, which are primarily determined by hepatic glucose production and either impaired glucose stimulated insulin secretion or skeletal muscle sensitivity, respectively (Abdul-Ghani et al., 2008). The R46L variant as well as other gene lipid lowering variants (*HMGCR, NPC1L1*) have been proposed to be mechanistically causal for diabetes by increasing LDLR mediated LDLc clearance resulting in pancreatic β cells cholesterol lipotoxicity (Besseling et al., 2015; Ference et al., 2016). Therefore, decreasing PCSK9 upon treatment for hypercholesterolemia has been surveyed closely. However, the large-scale longer-term clinical trials have demonstrated no increased new onset diabetes or worsening of diabetes associated with PCSK9 inhibition, and on the contrary, have demonstrated increased absolute beneficial cardiovascular outcomes for diabetes *vs.* normoglycemic patients (Ray et al., 2019a; Sabatine et al., 2017). In order to verify this hypothesis, longer term follow-up is required, as was the case for the causal association of statin treatment with increased risk for T2DM (Preiss et al., 2011; Sattar et al., 2010). Importantly, new recommendations to lower LDLc to very low levels in high risk patients will require treatment with PCSK9 inhibitors (Allahyari et al., 2020). Therefore,

if PCSK9 alters insulin receptor signaling, treatment with these inhibitors will be even more beneficial for prediabetic and diabetic patients in improving liver insulin sensitivity.

We further demonstrate that PCSK9 downregulates the expression of the InsR under co-expression conditions with stronger reduction observed with unprocessed, ER-retained mutants (denoted as proPCSK9 in Figure II.6. B). Our study suggests that PCSK9 mediated the retention of proInsR in the ER while also increased InsR mRNA decay induced by acute ER stress activation.

Two independent studies demonstrated that carriers of unprocessed, ER-retained PCSK9 variants show significant decrease in circulating PCSK9, from virtually undetectable levels to ~50% reduction (Cariou et al., 2009; Mayne et al., 2011). *In vitro* these studies prevented secretion and demonstrated retention of WT PCSK9 in the ER as unprocessed proprotein, without affecting total PCSK9 levels. Similarly, in the present study we observe an increase in the ratio of proInsR/mature InsR, suggesting retention of proInsR in the ER by proPCSK9.

Moreover, the missense mutants, the prosegment alone ($PCSK9_{pro}$) and $PCSK9_{L455X}$ ($PCSK9_{LX}$) used in this study, have all been demonstrated to oligomerize (Benjannet et al., 2012). Whereas the CHRD domain, which did not affect InsR levels or retention in the ER, does not oligomerize (Benjannet et al., 2012). Variants in genes leading to protein oligomerization, or protein aggregation, upregulated UPR and ER storage disease (Callea et al., 1992). An example is *PCSK1* N221D variant which leads to childhood obesity and nutrient malabsorption. The etiopathology associated with this disease may be explained by both the lack of crucial active PC1 substrates in the hypothalamus and in the intestine but also from the proteotoxicity of misfolded $PC1_{N221D}$, which further retains WT PC1 by aggregation in the ER (Ramos-Molina et al., 2016).

The canonical UPR response is modulated by the levels and duration of the stress (Rutkowski and Kaufman, 2004). It is triggered upon dissociation of BiP from three ER membrane bound sensors and binding to the exposed hydrophobic residues of misfolded proteins (Boot-Handford and Briggs, 2010). IRE1α is one of the three membrane bound ER sensors, along with PERK and ATF6. Acute ER stress activates IRE1α, an exonuclease that promotes protein remodeling. It alleviates the translocation and folding machinery through a process termed Regulated IRE1-Dependent Decay (RIDD), which leads to the rapid degradation of mRNAs that are targeted to the ER for protein translation (Hollien et al., 2009; Hollien and Weissman, 2006). IRE1α also activates XBP1 by splicing XBP1 mRNA, allowing the product to reach the nucleus and upregulate the

expression of chaperones important to adapt to ER protein aggregation as well as lead to ERAD and/or autophagy (Pirog et al., 2019). In the present study, we observe an upregulation of the activity of the exonuclease IRE1α through the increased splicing of XBP1 upon expression of WT PCSK9 or uncleaved, ER-retained PCSK9 with InsR in comparison to InsR with control. Acute ER stress under these conditions could be induced by the PCSK9 oligomers. Indeed, ER stress has been demonstrated to increase PCSK9 expression, while retaining it in the ER (Lebeau et al., 2018). Moreover, even though $PCSK9_{Q152H}$ has been demonstrated to elude the induction of the UPR (Benjannet et al., 2012; Lebeau et al., 2018; Lebeau et al., 2020), in alignment with the results from our study, this variant has nevertheless demonstrated increased susceptibility to ER stress activation in comparison to $PCSK9_{WT}$ (Lebeau et al., 2018). On the other hand, acute ER stress could also be induced by misfolded proInsR. Accordingly, the InsR has been demonstrated to bind BiP when misfolded, allowing it to be retained in the ER for proper refolding (Bass et al., 1998). Additionally, many studies have demonstrated ER stress and IRE1α regulation of insulin receptor expression and activity. ER stress downregulated InsR expression ((Xu et al., 2010), our unpublished data) as well as trafficking from the ER to the cell surface (Brown et al., 2020), which is accompanied by strong inhibition of InsR signaling (Brown et al., 2020; Ozcan et al., 2004; Xu et al., 2010). Finally, studies reported RIDD activity in the downregulation of genes involved in lipid and lipoprotein biogenesis (So et al., 2012). Therefore, we report through our experiments that proPCSK9 may retain nascent receptors in the ER and that increased susceptibility to ER stress leads to the downregulation of their levels.

This work brings new perspectives on how PCSK9 could modulate the development of T2DM, either by affecting InsR biosynthesis and increasing susceptibility to ER stress or by decreasing insulin receptor downstream signaling. These results are promising for patients with and without diabetes or prediabetes since PCSK9 mAb have shown greater absolute benefit than patients without diabetes. If PCSK9 plays a role in insulin signaling, perhaps this mechanism could take part in the development of diabetic dyslipidemia. Therefore, PCSK9 could be a driver of dyslipidemia by: (i) decreasing LDLR levels thereby resulting in competition from TG rich lipoprotein remnants with LDLc for LDLR and result in decrease in fractional catabolic rates (Boren et al., 2020); (ii) increasing apoB-secretion; (iii) hepatic or even possibly muscle and adipose tissue impaired insulin receptor signaling.

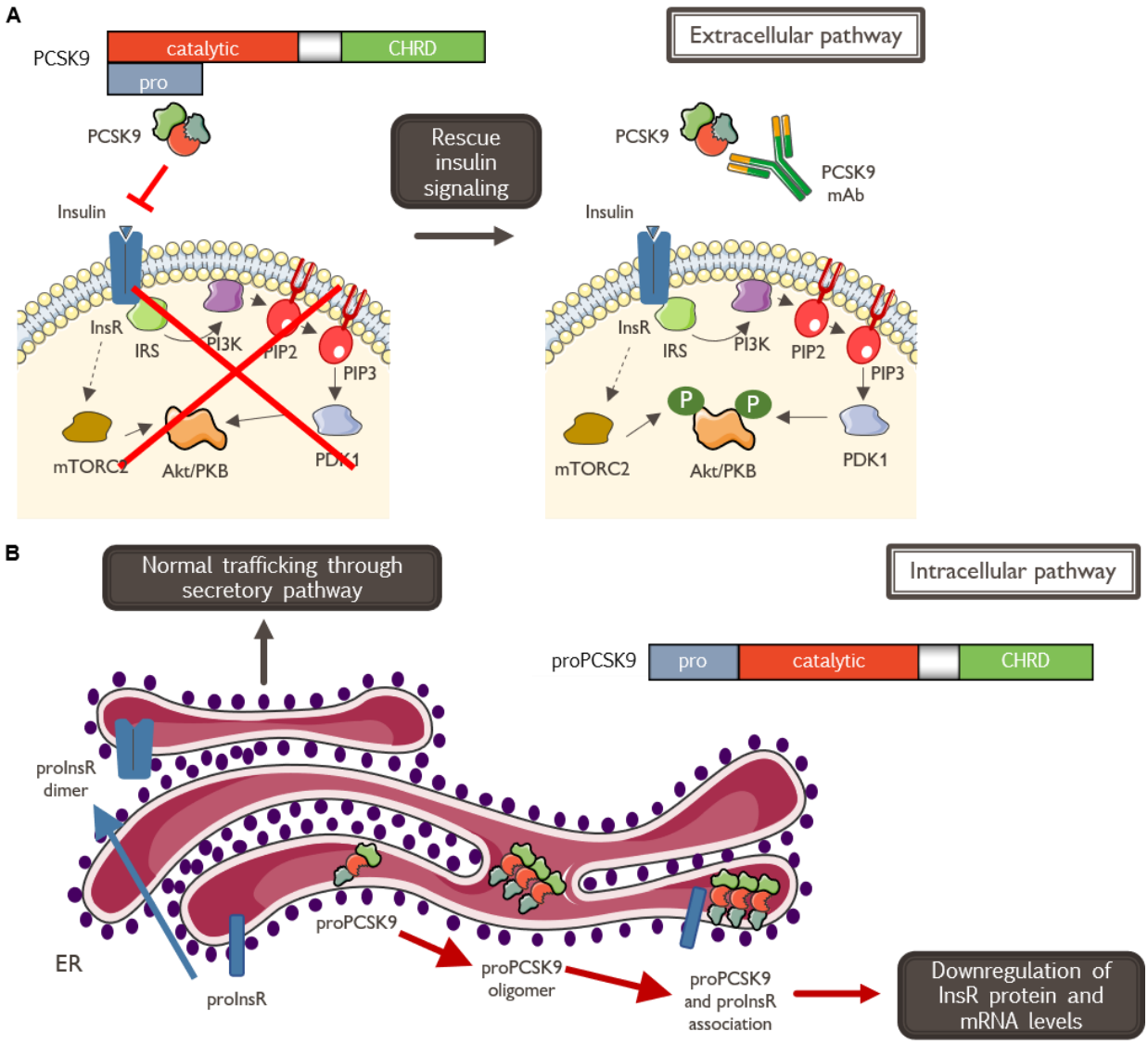

Figure II.6. 1 Schematic diagram illustrating the extracellular and intracellular pathways through which PCSK9 regulates insulin signaling and InsR levels respectively

The graphic illustrations used in this figure are from Servier Medical art (https://smart.servier.com).

II.7. Additional Information

II.7.1. Funding

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II.7.3. Conflict of interest

The authors declare no conflict of interest with the contents of this article.

II.7.4. Author contributions

JC conducted all of the experiments, analyzed the results and wrote the article. AE contributed to the cloning work. JE contributed to experimental design and scientific discussions. AP maintained the colonies for the mice in the C57BL/6J background. NGS contributed to the conception of the experiments, analysis of the data and writing of the article.

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Chapter III: Manuscript 2 (to be submitted to The Journal of Biological Chemistry)

Title: "Islet and liver insulin-related phenotypic characterization of *Pcsk9*$^{-/-}$ mice"

Authors: Julie Cruanès, Anna Roubtsova, Jadwiga Marcinkiewicz, Annik Prat, Nabil G. Seidah

Journal: in the process of submission to The Journal of Biological Chemistry

Keywords: T2DM, PCSK9, LDLR, insulin resistance, steatosis, glucose homeostasis

III.1. Preface

In the third chapter we turned towards the effect of the absence of PCSK9 on increased risk for T2DM. Under chronic conditions of increased LDLR-dependent LDLc internalization into peripheral tissues, lipotoxicity may result in impaired tissue function. In section I.1.8 we reviewed Mendelian randomization studies and large genetic epidemiologic studies which concurrently demonstrate an association between the loss of PCSK9 function and an increased risk for T2DM. Therefore, we present in this section the use of genetic mouse models in order to investigate the underlying mechanisms for these associations.

Islet and liver insulin-related phenotypic characterization of *Pcsk9*$^{-/-}$ mice

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Keywords: T2DM, PCSK9, LDLR, insulin resistance, steatosis, glucose homeostasis

III.2. Abstract

PCSK9 is a promising new therapeutic target for the prevention of cardiovascular disease, where its inhibition has led to the decrease of atherogenic LDLc to unprecedented levels. Concerns over the safety of clearance of LDLc into peripheral tissues have led to population studies identifying a potential risk for the development of T2DM upon long-term inhibition of PCSK9. Cholesterol and fatty acids are known to induce lipotoxic effects when intracellular levels are unbalanced leading to the progressive decline of tissue function. We demonstrate in the present study that in older PCSK9 KO mice, the chronic absence of PCSK9 does not result in impaired β-cell function, increased fat mass or hepatic insulin resistance and steatosis. Furthermore, upon over-night fasting and 24 hr refeeding challenges, the livers of these mice showed very similar regulation of lipogenic genes and lipid droplet accumulation. The LDLR, in contrast to the InsR, demonstrated differential regulation in the PCSK9 KO mice *vs.* WT mice upon the fasting/refeeding challenge. Our study suggests that in mice, the absence of PCSK9 does not lead to deleterious effects on insulin metabolism, however, further studies in humanized plasma lipid mouse models would be helpful for these investigations.

III.3. Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) was identified as the last member of the proprotein convertase family of enzymes that activate or inactivate various proteins, such as hormones, growth factors and their receptors, bacterial and viral proteins, transcription factors and secretory pathway proteins (Seidah et al., 2003). This family of proteases is therefore implicated in several diseases (degenerative, oncogenic, metabolic and inflammatory, for instance) (Seidah and Prat, 2012). Family linkage studies interested in the discovery of FH causing genes defined the critical role of PCSK9 in the modulation of LDLc in their identification of the *PCSK9* gene as the third locus for FH (Abifadel et al., 2003). Polymorphisms in the gene allowed for the sequencing of many GOF and LOF variants which have been associated with increased or decreased risk for cardiovascular disease, respectively (Allard et al., 2005; Cohen et al., 2005; Homer et al., 2008; Miyake et al., 2008; Noguchi et al., 2010). Further studies demonstrated that PCSK9 is secreted by hepatocytes into the circulation exclusively (Zaid et al., 2008), targets the LDLR and other receptors for degradation in the lysosome and/or the proteasome in a non-enzymatic way (Seidah et al., 2017). The mechanism may be extracellular driven, such as for hepatocytes, or intracellular driven such as for the adrenal glands and the intestine (Seidah et al., 2012).

Mendelian randomization studies may use GOF or LOF variants as proxies for the identification of long-term effects from treatment with certain drugs. Statins, which are currently the main treatment avenue for hypercholesterolemia, target the rate limiting step of the mevalonate pathway, the HMG-CoA reductase enzyme, and have been associated with a dose-dependent increased risk for the development of T2DM (Preiss et al., 2011; Sattar et al., 2010). This association was also true for LOF variants from the gene (*HMGCR*) (Swerdlow et al., 2015). Interestingly, both the variants and the drug demonstrate reduced association when corrections for the confounding BMI or waist-to-hip ratio are done, further suggesting that the mechanism for the development of T2DM upon inhibition of the mevalonate pathway may in part be explained by insulin resistance (Ference et al., 2016; Lotta et al., 2016; Swerdlow et al., 2015). In turn, the discovery of PCSK9, as a central element regulating cholesterol homeostasis, arose much interest in targeting the circulating protein for the treatment of ASCVD. However, because of the known predisposition that statins confer for the risk of T2DM, studies have also looked through Mendelian

randomization whether the inhibition of PCSK9 could, through a similar mechanism, result in these adverse and detrimental effects (Ference et al., 2016; Lotta et al., 2016). Indeed, these large epidemiological studies have shown that LOF variants in PCSK9 confer an increased risk by association with elevated fasting plasma glucose or insulin and elevated 2hr plasma glucose post-meal. These in turn suggest either impaired GSIS, muscle insulin resistance or increased hepatic glucose production (upon selective insulin resistance, a priming event in the development of T2DM) (Abdul-Ghani et al., 2008). Accordingly, LDLc has been demonstrated in mouse genetic models and in cells to be damaging to β-cell function – due to several mechanisms including excess cholesterol uncoupling of glucose sensing and metabolism with granule fusion to the plasma membrane for secretion (Oh et al., 2018). Therefore, because both statins and PCSK9 inhibition result in increased clearance of plasma LDLc and TG-rich lipoprotein particle remnants, the increased activity of the LDLR would lead to lipotoxicity to cells and tissues.

Hence, we investigated whether in whole body PCSK9 KO mice: (i) increased LDLR in pancreatic β-cells leads to impaired insulin secretion; and (ii) in hepatocytes, the absence of PCSK9 leads to fatty liver either from excess lipoprotein internalization (LDLc and TG-rich lipoprotein remnants) or from selective hepatic insulin resistance – increased *de novo* lipogenesis and gluconeogenesis.

We show that PCSK9 deficiency in 6-months old male mice does not lead to impaired β-cell function, muscle of liver insulin sensitivity, or modulated hepatic *de novo* lipogenesis. We further show that the absence of PCSK9 leads to a differentially regulated hepatic LDLR but not InsR upon fasting and refeeding metabolic challenge.

III.4. Methodology

III.4.1. Animals, treatments and plasma analyses

All procedures were approved by the IRCM animal care committee. WT (wild-type), and PCSK9 KO (Zaid et al., 2008) mice were on the C57BL/6 background, housed under 12-h light/dark cycles, and fed chow (Teklad Global) diet. Mice were sacrificed at 3 months of age after fasting for 3 h, unless specified. Pancreas insulin was extracted by acid-ethanol (1.5% HCl in 70% ethanol) and neutralized with Tris pH 7.5. For glucose tolerance and in vivo insulin secretion tests, tail blood samples were collected from 4 h-fasted, 6-7 months old male mice after an i.p. injection of 1.5 g/kg body weight_D-glucose. Plasma glucose levels were measured using an automatic glucose monitor (Bravo, EndoMedical). Insulin tolerance tests were with human insulin (Lilly, 1.5 U/kg) injected i.p. in 4 h-fasted mice. Pyruvate tolerance test were performed by i.p. injection (Sigma-Aldrich, 2 g/kg body weight) in 4 h-fasted mice. Fasting and refeeding experiments were conducted on 3 months old male mice. Mice were fasted for 18 h and refed for 24 h, blood and liver samples were collected before the fast, after the fast and after the refeeding. Serum was collected after the blood samples were allowed to clot then stored at -80°C, liver samples were immediately frozen with dry ice or ice-cold isopentane, embedded in OCT compound (Scigen) to be stored at -80°C. Insulin and C-peptide was measured by ELISA (Alpco). Serum PCSK9 was also measured by ELISA (CircuLex mouse/rat PCSK9 ELISA kit, MBL). HOMA-IR calculations: Glc(mmol/L) x Ins (mU/L) / 22.5 (Friedewald et al., 1972).

III.4.2. Mouse islet isolation

Mouse pancreas was enzymatically digested following bile duct perfusion with 0.4 u/ml Liberase TL (Roche) in Hank's Balanced Salt Solution (HBSS) buffer (Ca^{2+}/Mg^{2+}) followed by mechanical digestion by shaking in buffer without Ca^{2+}/Mg^{2+} containing 0.1% BSA and 20 mM HEPES pH 7.4. The digested cell pellet was washed in HBSS buffer and resuspended in Histopaque 1077 (Sigma-Aldrich) overlaid with RPMI 1640 without glucose (Thermo Fisher Scientific) prior to gradient separation by centrifugation. Handpicked islets were cultured

overnight in recovery medium, 11 mM glucose RPMI 1640, (10% FBS and penicillin/streptomycin) (Wisent).

III.4.3. Static islet glucose-stimulated insulin secretion

18 h post islet extraction, ten islets of similar size from each mouse/well were equilibrated for 2 × 20 min in KRBH buffer containing 0.01 mM fatty-acid free BSA and 2.8 mM glucose. Islets were sequentially cultured for 1 h each in KRBH buffer containing 0.04 mM fatty-acid-free BSA and 2.8 mM glucose, 28 mM glucose. Insulin content was determined following lysis in 1.5%/70% HCl/EtOH.

III.4.4. Immunoblotting and antibodies

Tissue proteins were extracted with RIPA 1X buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 0.1% SDS, 1% Nonidet P40, and 0.25% Na deoxycholate) with a cocktail of protease inhibitors (cOmplete, Roche) and a cocktail of phosphatase inhibitors (PhosSTOP, Sigma) for insulin signaling experiments. Bradford assay was used to evaluate the protein concentrations. For immunoblotting, 30 µg of proteins were resolved on SDS/PAGE (8% to 10% Tris-glycine) and transferred onto nitrocellulose membranes. Membranes were blocked with fat-free 5% milk powder dissolved in Tris buffer saline (0.1 M Tris/HCL pH 8 and 1.5 M NaCl) containing 0.1% Tween-100 (TBS-T). Incubations with primary and secondary antibodies coupled to horseradish peroxidase were done in 5% milk overnight at 4ºC and over 2 hrs at room temperature, respectively, except for Cell Signaling Technology antibodies which were revealed in 5% fatty acid free BSA dissolved in TBS-T. All membrane proteins were detected by chemiluminescence (Bio-Rad), revealed and analysed using the ChemiDoc MP imaging system (Bio-Rad) or using films followed by ImageJ quantification.

Primary antibodies used:

- α-InsRβ (Enzo Life Sciences, ADI-905-603-100);
- α-mLDLR (R&D Systems, AF2255);
- α-P-S473 Akt (Cell Signaling Technology, 9271);
- α-Akt (total) (Cell Signaling Technology, 9272);

- α- β-actin (Sigma-Aldrich, A2066);

Secondary antibodies used:

- α-mouse-HRP (VWR, CA95017-556L);
- α-rabbit-HRP (VWR, CA95017-332L);
- α-goat-HRP (Santa Cruz, Sc 2020).

III.4.5. Gene expression

Total RNA from liver was extracted with TRIzol (Invitrogen). Total RNA from islets was extracted using RNeasy Mini Kit (Qiagen). cDNA was generated from 250 ng of total RNA using a SuperScript II cDNA reverse transcriptase (Invitrogen). Quantitative PCR was done using the SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA) and the following sense and antisense primers: mPCSK9, 5′-CAGGGAGCACATTGCATCC and 5′-TGCAAAATCAA-GGAGCATGGG; LDLR, 5′-GGAGATGCACTTGCCATCCT and 5′-AGGCTGTCCCCCCAAGAC; and mHMGCR, 5′-GTACGGAGAAAGCACTGCTGAA and 5′-TGACTGCCAGAATCTGCATGTC; mSREBF2, 5'- CTGCAGGACTTGAAAGCTGG and 5'-GTCTCTCCTACCCCACCAAG; mGck 5'- CAGCTCGCCCATGTACTTT and 5'-ACCGGATGGTGGATGAGA; mIns1, 5'- CACCAGCATCTGCTCCCTCTA and 5'-GTCGAGGTGGGCCTTAGTTG; mIns2, 5'- GGCTTCTTCTACACACCCATGTC and 5'-AGCTCCAGTTGTGCCACTTGT; mChREBP, 5'- CGGTGCTCATCTGCTTCA and 5'-TCTGGAGAGCCTGGTACAT; mSREBF1, 5'- GTCACTGTCTTGGTTGTTGATG and 5'-AGATGTGCGAACTGGACAC; mFasn, 5'- TCTCGGGATCTCTGCTAAGG and 5'-GCTGTTGGAAGTCAGCTATGA. Each gene expression was normalized to that of mHPRT (hypoxanthine phosphoribosyl transferase) 5′-CCGAGGATTTGGAAAAAGTGTT and 5′-CTTCATGACATCTCGAGCAAGT.

III.4.6. Immunohistochemistry

For InsR staining, OCT embedded cryosections (6 μm thick) were immediately fixed in 4% paraformaldehyde in PBS for 1 h, rinsed three times in PBS, and blocked in 2% BSA (Sigma-Aldrich, Oakville, ON) in PBS for 1 h at room temperature. Sections were then incubated overnight

at 4°C with a goat antibody against mouse InsR (1:150; Abcam, ab5500) in PBS containing 1% BSA and washed three times for 10 min in PBS. Labeling was visualized by incubation with Alexa Fluor 488-labeled anti-goat IgGs (1:150; Invitrogen, Burlington, ON) for 1 h at room temperature in PBS. After three 10 min washes, nuclei were counterstained with Hoechst dye (Sigma-Aldrich). After two short washes, the sections were dipped in 70% ethanol and closed using 90% glycerol. Images were acquired as described previously (Roubtsova et al., 2011).

III.4.7. Oil Red O

To detect neutral lipids from liver, OCT embedded cryosections rehydrated and fixed for 1h in 4% paraformaldehyde, pH 7.4, washed in PBS1x. Sections are stained in 0.5g of Oil Red O (Allied Chemicals) dissolved in 100 ml of isopropanol (everything done on ice) then diluted in distilled water (3:2 ratio). Slides closed with VectaMount AQ Aqueous Mounting Medium (Vector Laboratories). Imaging was done using Osteomeasure Imaging software (Osteometrics).

III.4.8. Statistical analysis

Data comparing only two groups were analyzed by unpaired student *t*-tests. For data sets with two variables or multiple measurements, two-way ANOVA followed by post-hoc analysis (Holm–Sidak or Fisher's LSD test) determined significance of individual points. Analysis performed using Microsoft Excel and GraphPad Prism. Unless indicated, values are mean ± SEM.

III.5. Results

III.5.1. PCSK9 deficiency does not affect insulin homeostasis in 6 months old male mice.

Previous studies have demonstrated that the absence of PCSK9 in mice leads to the upregulation of LDLR expression in pancreatic islets, including at the surface of β-cells (Da Dalt et al., 2019; Langhi et al., 2009; Mbikay et al., 2010). Therefore, we firstly confirmed this regulation by extracting protein from whole pancreas and from freshly isolated islets from *Pcsk9* WT and KO mice and analyzing the relative expression of LDLR by immunoblot. Our results demonstrate a significant ~2-fold and ~1.67-fold increase in LDLR protein content PCSK9 KO mice *vs.* to WT mice in the whole pancreas and islets, respectively (Figure III.5.1.A and B). These ratios are consistent with previously published data (Da Dalt et al., 2019; Langhi et al., 2009; Mbikay et al., 2010). Whether PCSK9 KO islets contain similar, increased or decreased insulin levels is controversial. Therefore, we solubilized insulin from pancreata of PCSK9 WT and KO mice and measured insulin by ELISA. Our results demonstrate no significant difference in total pancreatic insulin (Figure III.5.1.C). In addition, *Insulin 1* and *2* gene expression is not significantly affected in these mice, demonstrated by mRNA relative quantification by Q-PCR (Figure III.5.1.D). The previous studies have moreover investigated the effect of absence of PCSK9 on islet intracellular cholesterol in order to verify whether the observed increased LDLR expression would lead to increased activity and LDLc internalization. Langhi *et al.* demonstrated equivalent intracellular cholesterol, in accordance with studies that demonstrated that increased LDLc internalization does not lead to increased intracellular cholesterol unless *Abca1* gene is impaired (Brunham et al., 2007; Kruit et al., 2010b; Langhi et al., 2009). However, Da Dalt *et al.* demonstrated a significant increased intracellular cholesterol accompanied by significantly increased fatty acids (Da Dalt et al., 2019). In order to determine whether β-cells were under excess cholesterol, we analyzed the activity of the SREBP-2, which senses intracellular cholesterol levels such that upon <5 mol% endoplasmic reticulum membrane cholesterol translocated to the Golgi apparatus where the transcription factor is activated by proteolysis, allowing it to upregulate genes involved in cholesterol synthesis and internalization (Das et al., 2013). We measured the expression of these target genes by Q-PCR. Even though the *Ldlr* in PCSK9 KO *vs.* WT was significantly decreased by ~20%, *Srebf2* and *Hmgcr* were not significantly altered (Figure

III.5.1.E), suggesting that cholesterol biosynthesis is not significantly altered between both genotypes, in accordance with Langhi *et al* (Langhi et al., 2009). Therefore, in our study mice lacking PCSK9 showed increased LDLR levels in the islets of Langerhans, however there was no significant effect on intra-islet insulin content or cholesterol content.

We then investigated whether the absence of PCSK9 decreases glucose tolerance and insulin secretion, as has been previously demonstrated (Da Dalt et al., 2019; Lebeau et al., 2019; Mbikay et al., 2010). We conducted a glucose tolerance test (GTT) in older mice (6 months-old male mice) by injecting glucose intraperitoneally (1.5 g/kg mouse body weight) after a 4 hour fast. Plasma glucose and insulin were measured at different time points to analyze the first and second phases of insulin secretion (Figure III.5.1.F and G). Our results demonstrated no significant differences in the glucose tolerance and insulin secretion between PCSK9 WT and KO, in accordance with Langhi *et al.* (Langhi et al., 2009). The rate of insulin internalization was compared between both genotypes by measuring the ratio of plasma insulin to C-peptide at $t = 0$ min and $t = 15$ min post glucose intraperitoneal injection. C-peptide is the domain in proinsulin that is cleaved and secreted from insulin granules in equal amounts to proteolytically matured insulin but, in contrast with insulin, it is not rapidly internalized by hepatocytes and displays a much longer plasma half-life than insulin. Our results suggest equivalent hepatocyte uptake rates of insulin between WT and KO mice pre- and post-glucose challenge (Figure III.5.1.H). Finally, in order to investigate the islets' insulin secretion capacity directly, we isolated islets from mice of both genotypes and performed *ex vivo* GSIS. Our results suggest a non-significant trend towards increased insulin secretion by KO islets (Figure III.5.1.I), confirming that the absence of PCSK9 does not impair GSIS. Overall, we observe no defects in glucose or insulin homeostasis from PCSK9 KO islets.

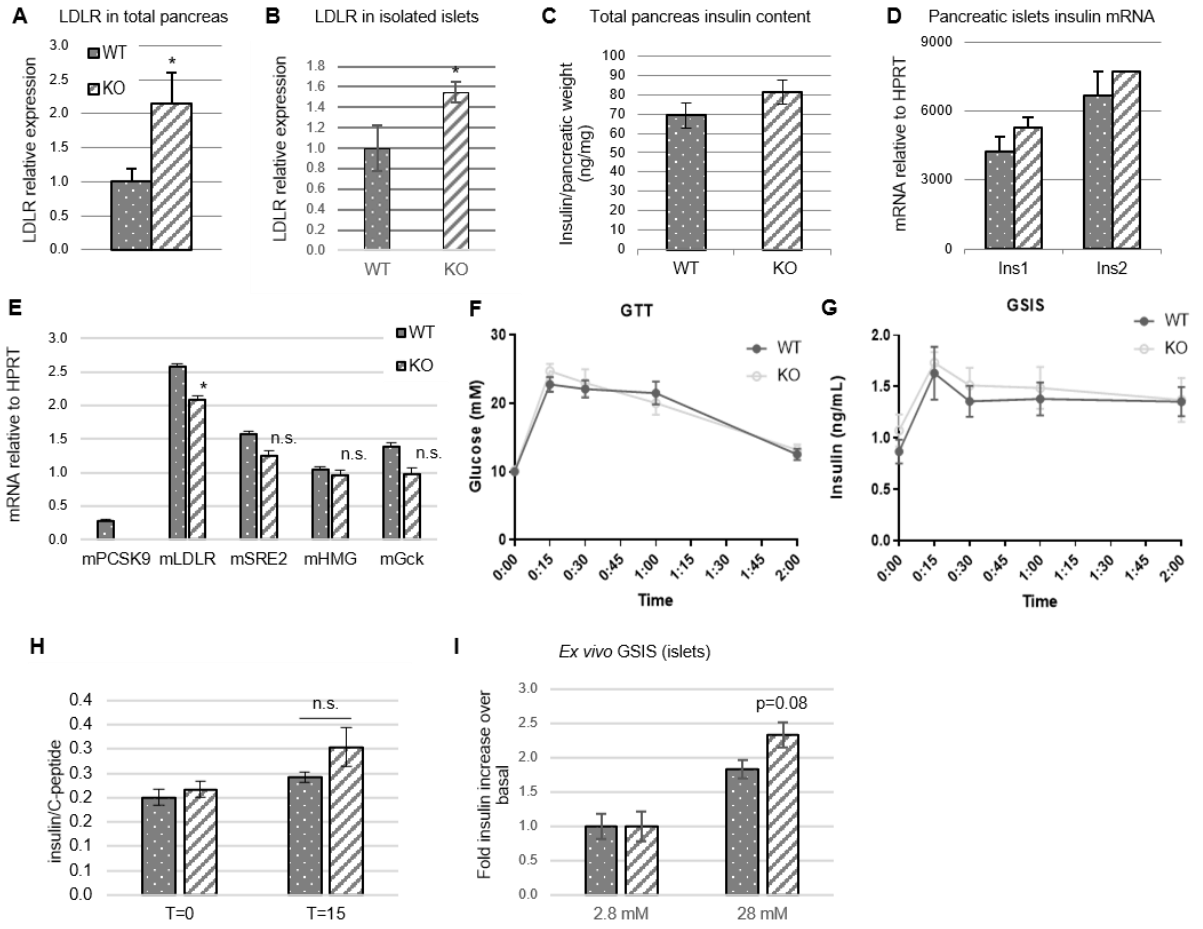

Figure III.5. 1 PCSK9 deficiency does not affect insulin homeostasis in 6 months old male mice.

A and ***B,*** Immunoblot analysis of LDLR from total pancreas and freshly isolated islets of PCSK9 WT and KO mice; *n* = 3 mice/genotype. ***C,*** Insulin solubilized from pancreas of PCSK9 WT and KO mice measured by ELISA; *n* = 4-5 mice/genotype. ***D*** and ***E***, islet mRNA isolated from both groups analyzed by Q-PCR; *n* = 3 mice/genotype. ***F*** and ***G,*** Blood glucose and insulin, respectively, were monitored during an intraperitoneal glucose tolerance test (IPGTT) (1.5 g/kg body weight); *n* = 9 or 10 mice/genotype. ***H,*** plasma insulin and C-peptide were measured by ELISA. The insulin to C-peptide ratio is represented for *Pcsk9* WT and KO mice at t = 0 min and t = 15 min of the IPGTT. ***I,*** 24 hrs after islets from both groups were isolated a GSIS test was done. Secreted insulin was measured by ELISA; *n* = 6-8 mice/genotype. Data represented by mean ± SEM. *p<0.05.

III.5.2. PCSK9 deficiency does not affect peripheral organ insulin sensitivity in 6 months old male mice

A critical publication from our group demonstrated that the absence of PCSK9 in 6 months old mice leads to a marked significant increase in visceral fat, and that this increase is likely due to the upregulation of VLDLR expression at the surface of adipocytes (Roubtsova et al., 2011). These, in turn, demonstrated hypertrophy and a significant increase in the incorporation of ^{3}H-oleate. Both liver and fat demonstrated increased *de novo* lipogenesis, evidenced by increased ^{14}C-glucose incorporation into triglycerides. Importantly, in obesity, visceral fat (abdominal fat) is the strongest predictor associated with an increased risk in diabetes or prediabetes independently of overall adiposity. Other predictors include BMI, waist circumference, and waist to height ratio (Despres, 2006; Jung et al., 2016). Indeed, visceral fat plays a direct role in the pathophysiology of insulin resistance and T2DM by secreting diabetogenic and inflammatory proteins (Gabriely et al., 2002). Therefore, we were interested in investigating whether increased visceral fat could lead to insulin resistance in PCSK9 KO mice. We first analyzed fat mass and lean mass of PCSK9 WT and KO 6 months old male mice by magnetic resonance imaging (MRI). There was no significant difference in the relative masses to body weight ratios (Figure III.5.S.1 in Appendices). In order to look more closely into visceral fat, perigonadal adipose tissue (PAD) was collected and weighed. According to Roubtsova *et al.*, we were expecting an increase in ~77% for *Pcsk9* KO 6 months old male mice (Roubtsova et al., 2011), however we observed a ~23% non-significant increase (Figure III.5.2.A). Increased fasting plasma glucose has also been reported to be associated with the loss of PCSK9 in previous mouse genetic studies and in Mendelian randomization studies were (Ference et al., 2016; Lotta et al., 2016; Mbikay et al., 2010). We observed no differences in fasting glucose or insulin in PCSK9 KO *vs.* WT mice (Figure III.5.2.B-C). Moreover, the insulin tolerance test (ITT) (1.5 U/kg body weight) demonstrated no changes between both genotypes, suggesting no differences in muscle insulin sensitivity to upregulate glucose receptors and internalize the plasma glucose (Figure III.5.2.D). The pyruvate tolerance test (PTT) (2 g/kg body weight) demonstrated a significant increase in plasma glucose for PCSK9 KO mice compared to WT mice, likely due to increased liver gluconeogenesis (2-way Anova, p = 0.008), however, the area under the curve of the PTT revealed no significant differences between both groups (Mann Whitney test) (Figure III.5.2.E). These suggest that there may be a trend for

PCSK9 KO mice for increased gluconeogenesis, but more experiments are required to confirm this. The response to insulin was also examined directly by the liver through an injection of insulin (0.5 U/kg body weight) into the inferior vena cava of mice from both genotypes, the livers were collected and P-Akt was analyzed by immunoblot as a measure of insulin downstream signaling. No significant differences were observed, perhaps due to high variability in the response from KO mice (Figure III.5.2.G). In summary, the absence of PCSK9 did not reproduce previous findings for increased visceral fat mass, no evidence for hepatic or muscle insulin intolerance was detected in these studies.

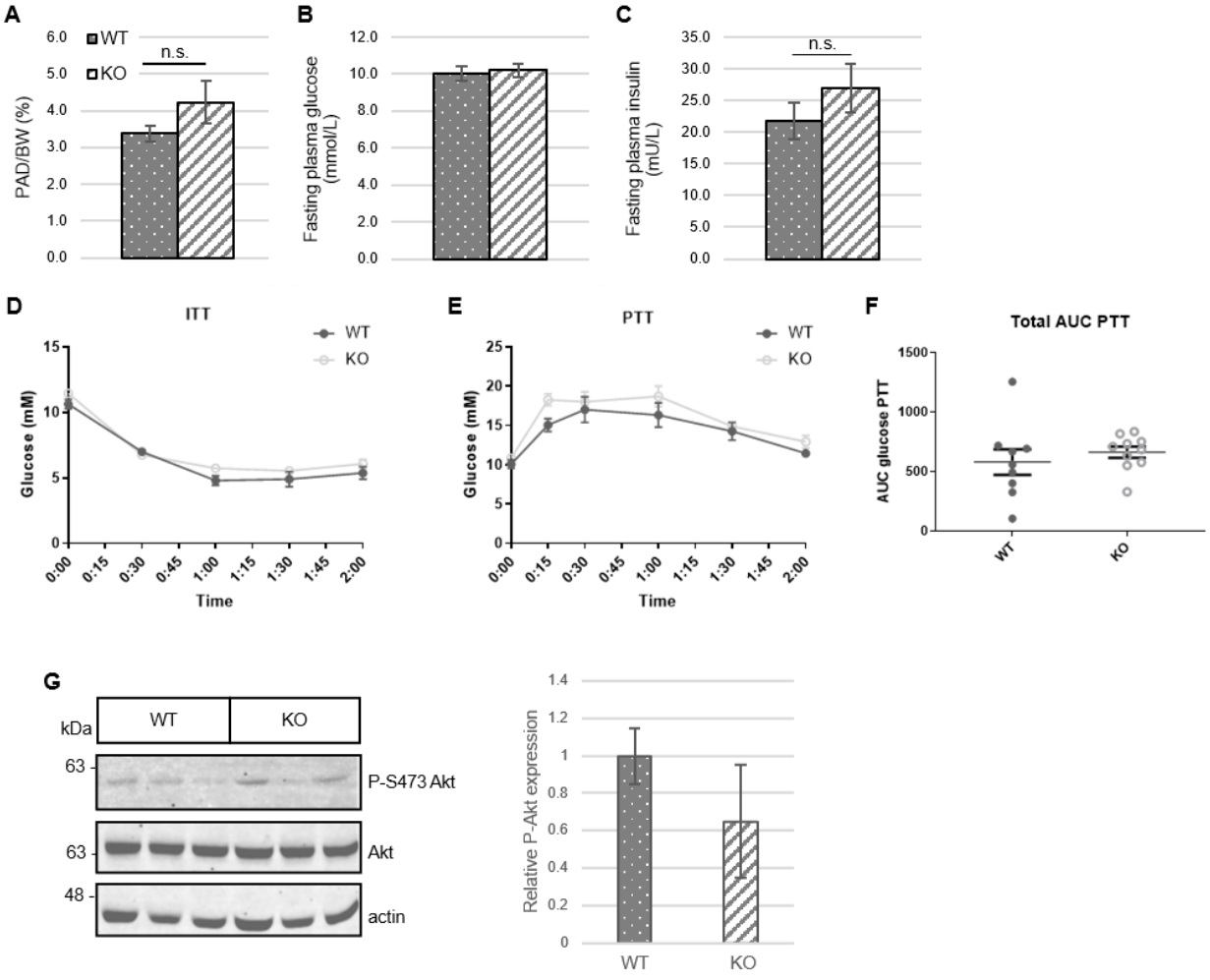

Figure III.5. 2 PCSK9 deficiency does not affect peripheral organ insulin sensitivity in 6 months old male mice.

A, Perigonadal adipose tissue and mice from both PCSK9 WT and KO groups were weighed. The ratio is expressed as a percentage; *n* = 9-10 mice/genotype. ***B,*** and ***C,*** plasma glucose and insulin were measured after a 4 hr fast for both groups; *n* = 9-10 mice/genotype. ***D*** and ***E,*** an ITT (1.5 U/kg body weight) and a PTT (2 g/kg body weight) were performed on PCSK9 WT and KO mice; *n* = 9-10 mice/genotype. ***F,*** AUC of the PTT was measured for both groups; *n* = 9-10 mice/genotype. ***G,*** Liver insulin sensitivity was assessed by injection of insulin (0.5 U/kg mouse body weight) into inferior vena cava of PCSK9 WT and KO mice; *n* = 3-6 mice/genotype. Data represented by mean ± SEM.

III.5.3. PCSK9 deficiency does modulate liver fatty acid synthesis response to O/N fasting and refeeding

Even though the 6 months old cohort of PCSK9 WT and KO mice did not show any evidence of impaired glucose and insulin homeostasis, these mice may be susceptible for the development of insulin resistance. This may be because previous studies have demonstrated that the livers of PCSK9 KO mice accumulate triglycerides in lipid droplets (LD), likely due to the regulatory activity of PCSK9 on both the LDLR and the fatty acid transport protein, CD36 (Demers et al., 2015; Lebeau et al., 2019). The accumulation of ectopic lipids in insulin responsive tissues may cause insulin resistance. For example, the accumulation of diacylglycerol in tissues due to overwhelming of lipid oxidation or conversion into triacylglycerol may lead to the activation of PKCε which interferes with insulin signaling (Samuel et al., 2010). Moreover, CD36 is associated with insulin resistance, obesity and T2DM due to its function in metabolism by participating in the uptake of free fatty acids, TG storage and secretion (Koonen et al., 2007). Demers *et al.* demonstrated that PCSK9 KO mice had a ~4-fold increase in hepatic triglyceride (Demers et al., 2015). Lebeau *et al.* demonstrated that along with increased LD under both normal control diet and high fat diet, PCSK9 KO mice also showed decreased lipogenesis through the downregulation of *Pgc1α* mRNA expression, a coactivator of liver X receptor (LXR) transcription factor which activates fatty acid synthase (FAS) (Lebeau et al., 2019). Indeed, lipogenic genes are coordinately regulated at the transcription level due to common features in the promoters. In particular, the glycolytic and lipogenic transcription factors SREBP-1c and carbohydrate responsive element binding protein (ChREBP) are required for the induction lipogenic genes and may also include USF and LXR (Linden et al., 2018). The process of *de novo* lipogenesis is tightly controlled by hormones and nutritional status or substrate influx, therefore the activities and expression of the genes involved in this process are regulated during the fasting and refeeding cycle. As such, analyzing the expression of these genes may provide an insight into the signaling pathways that are upregulated. Therefore, we assessed *de novo* lipogenesis by measuring the expression of key genes by Q-PCR during a metabolic challenge to better investigate how this process is regulated in PCSK9 WT and KO mice. We analyzed the expression of *Srebf1, Chrebp, Fasn,* and *Acc* (Acetyl-CoA carboxylase) in 4-months old male PCSK9 WT and KO mice. Our results clearly demonstrate regulation by nutritional status of these genes for both genotypes. We expected

PCSK9 KO mice to demonstrate decreased expression of these genes under basal conditions, however there was no significant difference between both groups for the level of expression for any of the genes analyzed (Figure III.5.3.A-D). Although preliminary results (not shown) demonstrate an upregulation of *Scd1* (stearoyl-CoA desaturase-1) in PCSK9 KO mice *vs.* WT mice (2-2.5-fold increase) under steady state conditions. Our results are in accordance with previous studies demonstrating that fatty acid synthesis is downregulated during fasting, instead the product of ACC, malonyl-coA, is decreased allowing for β-oxidation of fatty acids (Heijboer et al., 2005). Furthermore, staining of neutral lipids from livers of these mice demonstrated that there were no significant differences in lipid droplets content between PCSK9 WT and KO mice under any of conditions of the fasting and refeeding challenge, in contrast to what had been previously published (Demers et al., 2015; Lebeau et al., 2019) (Figure III.5.3.E). Although they correspond well with the observation that the lipogenic genes from both genotypes are similarly regulated. Therefore, lipid synthesis and accumulation into lipid droplets was very similar between both genotypes suggesting that the absence of PCSK9 does not lead to excess substrate influx through previously published increased CD36 mediated uptake of FFAs from adipose tissue during fasting (Demers et al., 2015; Lebeau et al., 2019).

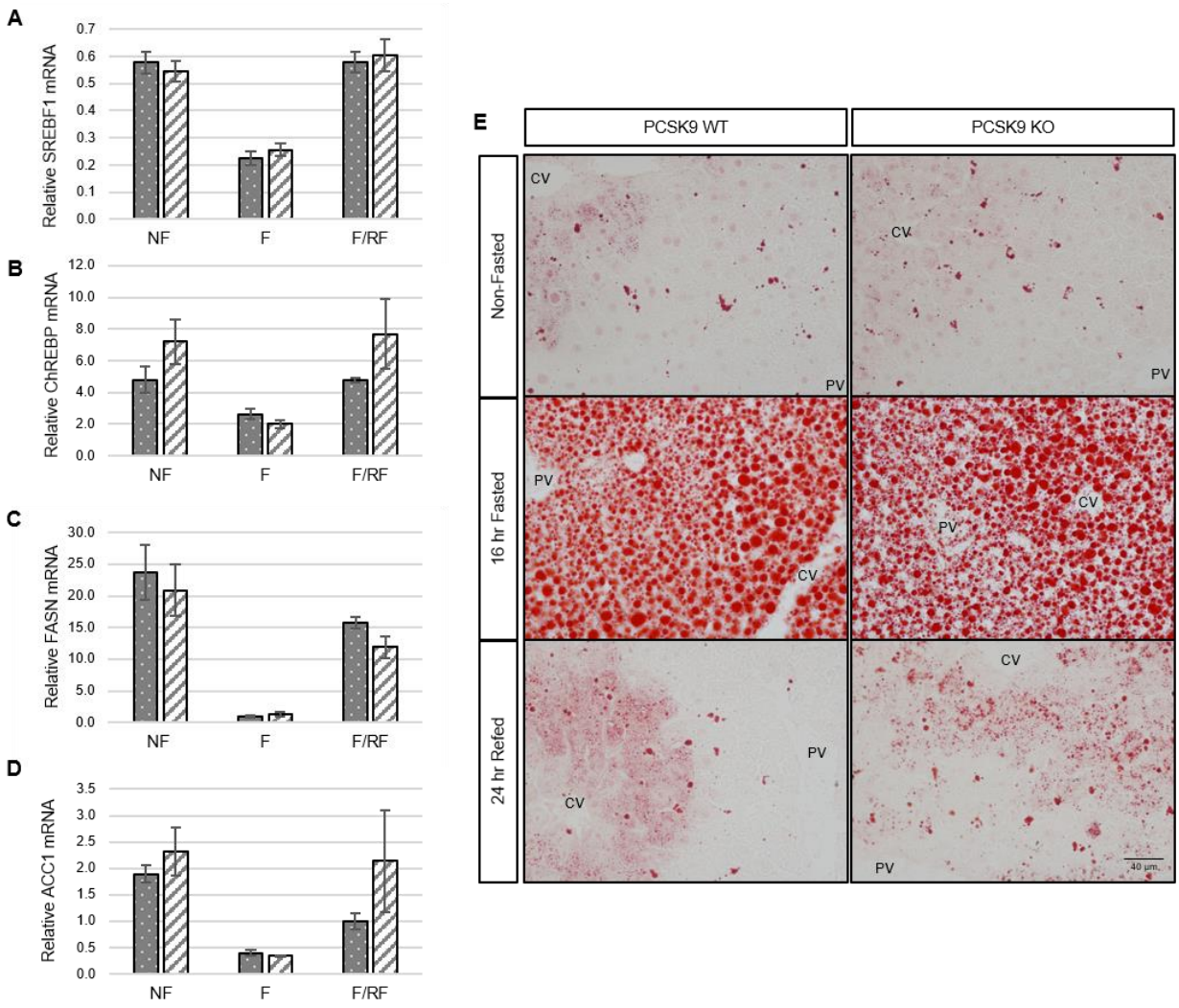

Figure III.5. 3 PCSK9 deficiency does not modulate liver fatty acid synthesis in response to O/N fasting and refeeding.

PCSK9 WT and KO mice were fasted for 16 hrs and refed for 24 hrs. ***A-D,*** the expression of genes implicated in *de novo* lipogenesis was measured by Q-PCR from liver RNA samples; n = 4-6 mice/condition for each genotype. ***E,*** Liver neutral lipid droplet staining (oil red O) was performed for both groups. Representative images from 3 independent experiments show lipid droplet size and concentration decreasing gradient from the central vein (CV) towards the portal vein (PV); n = 7-9 mice/genotype, scale bar: 40 µm. Data represented by mean ± SEM.

III.5.4. An O/N fasting and refeeding challenge does not modulate LDLR expression in contrast to InsR

Previous studies had demonstrated that PCSK9 is regulated by nutritional status (Costet et al., 2006; Persson et al., 2010) and it was suggested that post-translational regulation of LDLR by PCSK9 was important to maintain stable plasma LDLc during fasting conditions (Persson et al., 2010). Therefore, we firstly confirmed that overnight fasting leads to almost undetectable plasma and liver concentrations of PCSK9 and that refeeding induces its expression (Figure III.5.4.A). Figure III.5.4.B shows that refeeding is a potent inducer of PCSK9 expression as post-16hr of fasting, refeeding increased PCSK9 linearly; at 24 hrs post refeeding, PCSK9 levels increased by ~21-fold. Then we investigated whether LDLR protein expression is altered by fasting and refeeding. Our results demonstrate that in PCSK9 WT mice the LDLR is stable under all conditions, while in PCSK9 KO mice, a significant increase in LDLR is observed under refeeding conditions, likely due to the combined effect of increased SREBP-1c activity and the absence of circulating PCSK9 (Figure III.5.4.C). Therefore, PCSK9 is required of the stable protein expression of LDLR in hepatocytes, which likely participates in maintaining stable LDLc plasma concentrations even under strenuous fasting conditions (Persson et al., 2010).

It is known that insulin is the major regulator of InsR levels, where prolonged elevated insulin plasma levels lead to downregulation of InsR, exemplified in obese and insulin resistant patients or mice which show decreased InsR levels (Bar et al., 1976; Grunberger et al., 1989; Soll et al., 1975). Previous reports suggested that InsR is upregulated during fasting (Contreras et al., 1990; Rouiller et al., 1988; Tozzo and Desbuquois, 1992). Gorden *et al.* also demonstrated that corticosteroids increase InsR (Fantus et al., 1981). Since fasting conditions lead to increased corticosteroids in both mice and humans (Bergendahl et al., 1996; Jensen et al., 2013), corticosteroids could be at least in part responsible for the upregulation of InsR under fasting conditions. In our study, we confirm that total protein and cell surface hepatocyte InsR levels significantly increase after an overnight fast (Fig. 5A, B). Moreover, PCSK9 WT and KO mice show similar regulation of this receptor in contrast to the differentially regulated LDLR (Figure III.5.5.A, B). Our results suggest that PCSK9 does not participate in the post-translational regulation of InsR.

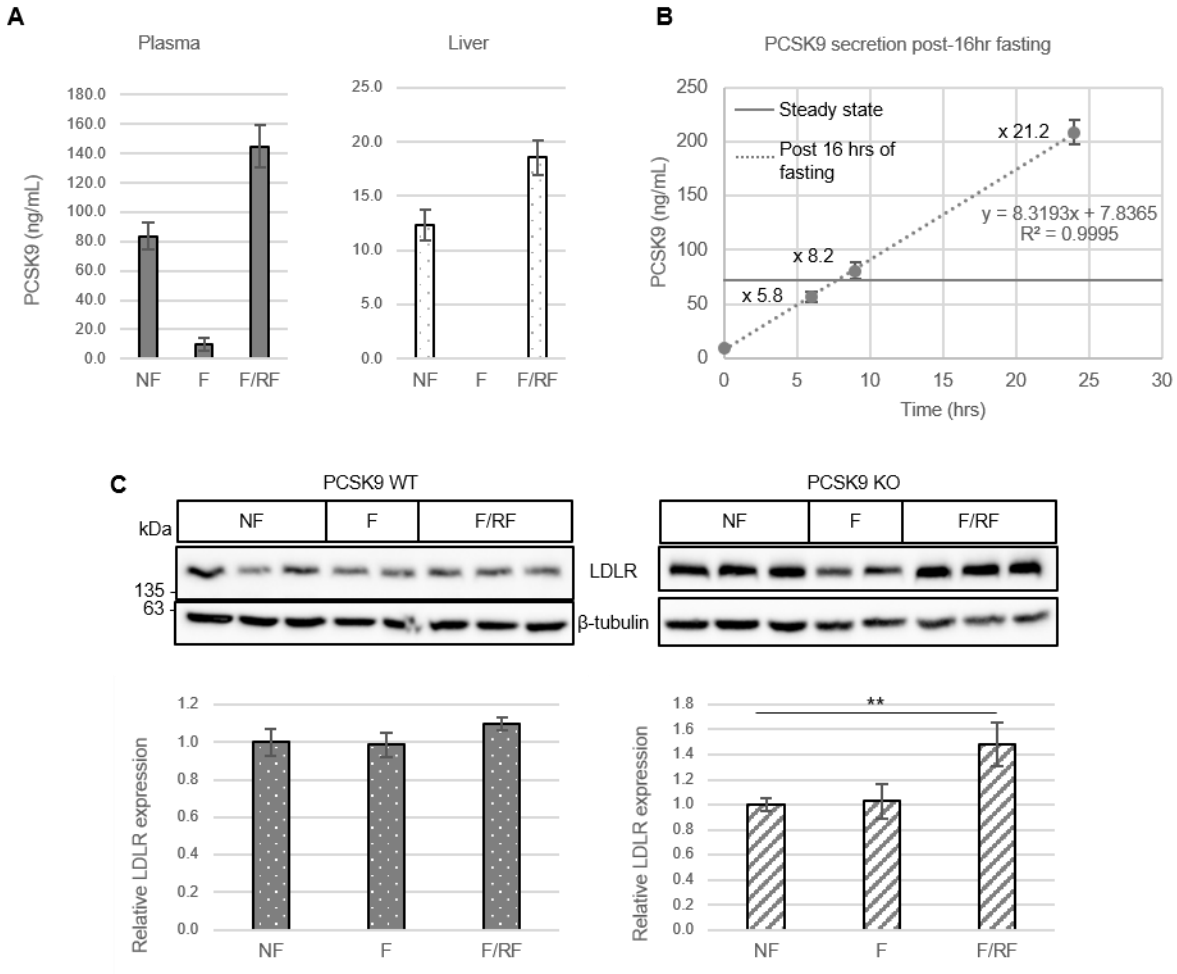

Figure III.5. 4 PCSK9 maintains stable and hepatic LDLR expression after a strenuous O/N fasting challenge.

A, plasma and liver PCSK9 concentrations during fasting and refeeding challenge measured by ELISA; *n* = 5-6 mice/condition. ***B,*** plasma PCSK9 concentrations observe a linear increase upon refeeding post 16-hr fast. PCSK9 levels were measured by ELISA. Mean steady state PCSK9 concentration is represented by a full-line, whereas the refeeding linear curve is represented by a dotted-line; *n* = 5 mice/condition. ***C,*** immunoblot analysis of LDLR from livers of PCSK9 WT and KO groups; *n* = 4-6 mice/condition for each genotype. Data represented by mean ± SEM. *p<0.05, **p<0.01.

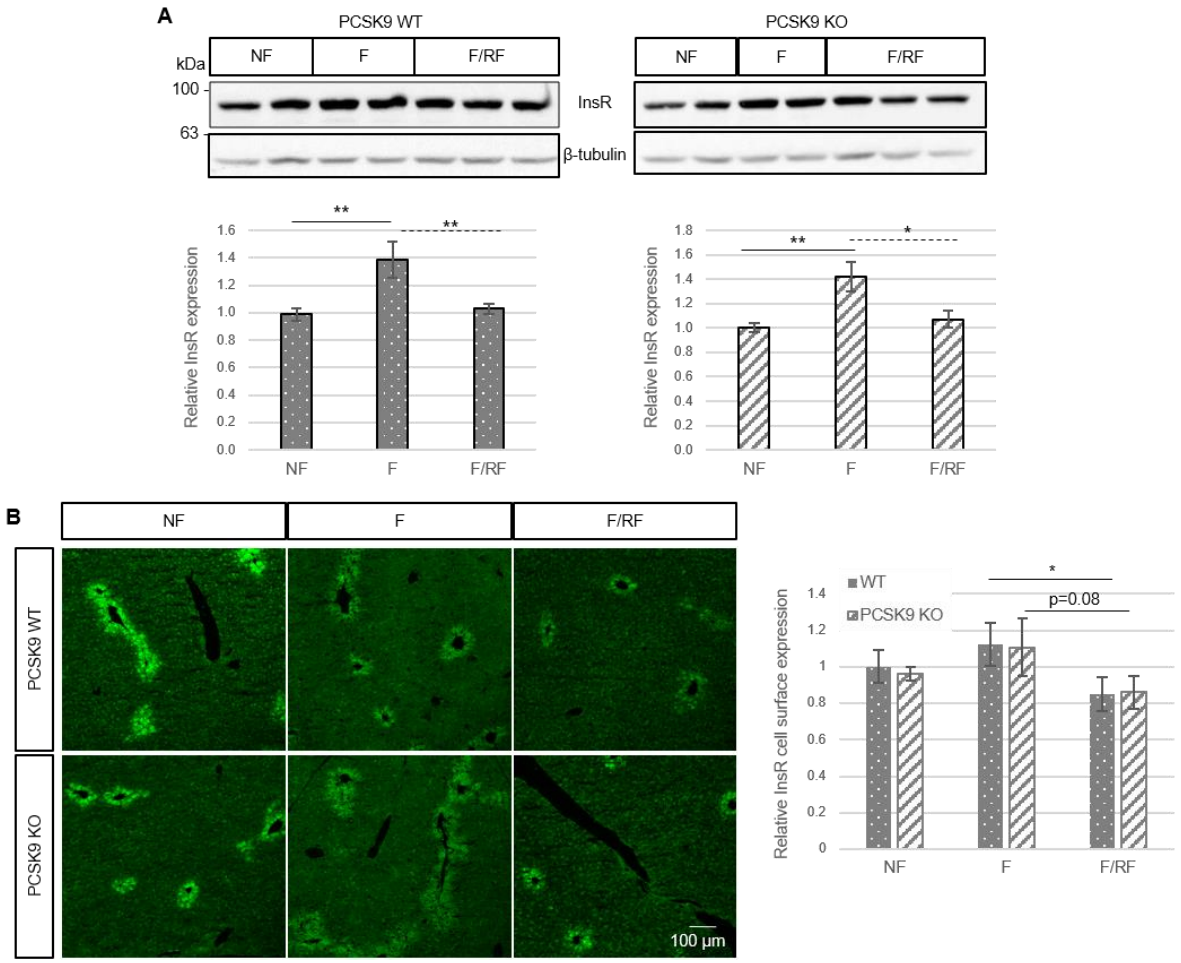

Figure III.5. 5 Hepatocyte InsR levels are regulated by nutritional status in the presence and absence of PCSK9.

A, immunoblot analysis of InsR from liver during fasting and refeeding challenge from both PCSK9 WT and KO groups; *n* = 7-9 mice/condition for each genotype. ***B,*** Representative images from 3 independent experiments of immunofluorescent labelling and confocal imaging of livers from both groups during fasting and refeeding challenge (InsR in green), scale bar: 100 µm; *n* = 7-9 mice/condition for each genotype. Data represented by mean ± SEM. *p<0.05, **p<0.01.

III.6. Discussion

From Mendelian randomization studies demonstrating the association of LDL decreasing variants with increased risk for type 2 diabetes came about the hypothesis that life-long exposure of tissues to increased LDLc would lead to lipotoxicity and impaired glucose and insulin homeostasis (Ference et al., 2016; Lotta et al., 2016). In particular, LOF variants in *PCSK9* were associated with parameters suggestive of either liver or muscle insulin resistance or impaired β-cell secretion (Lotta et al., 2016). In order to investigate these associations, we studied 6 months-old PCSK9 WT and KO mice, which have had a life-long exposure to decreased circulating LDLc and therefore more efficient clearance of this lipotoxic particle. Previous studies had reported controversial results, where the absence of PCSK9 would lead to impaired glucose tolerance, affected pancreatic islet integrity, liver lipid droplet accumulation and increased visceral fat mass in some studies but not others (Da Dalt et al., 2019; Demers et al., 2015; Langhi et al., 2009; Lebeau et al., 2019; Mbikay et al., 2015; Mbikay et al., 2010). In the present report, we could not find any evidence for glucose intolerance, impaired β-cell function, lipid droplet accumulation in hepatocytes, increased perigonadal fat mass or liver insulin resistance. As such, the absence of PCSK9 did not lead to lipotoxicity in these mice. Therefore, it is likely that genetic composition within the C57BL/6J strain may have strong influence over the PCSK9 KO phenotype.

An example where genetic background could highly influence intra-islet cholesterol content is the composition and abundance of HDL. Indeed, heritability estimates in humans range from 40-70%, suggesting strong influence of genetic background on HDL levels (Peacock et al., 2001). ~40 different loci in mice and humans have been identified to alter HDL levels, with almost all loci being shared by equivalence between mice and humans suggesting common underlying genes (Rollins et al., 2006). Moreover, these genes are not yet known to contribute to HDL metabolism. This is important for our studies because in mice plasma cholesterol is mainly comprised in HDL particles, HDL is protective against ectopic lipid accumulation and induced lipotoxicity due to its intrinsic properties. *Ex vivo* incubation of HDL with LDL abolishes the LDL induced lipotoxic effects on pancreatic islets (Cnop et al., 2002) likely due to its antioxidant and anti-inflammatory properties (Kruit et al., 2010a); thereby preventing conversion of LDL to oxidized LDL which leads to impaired β-cell function (Brites et al., 2017). Moreover, HDL particles enhance reverse cholesterol transport, clearing and preventing ectopic cholesterol accumulation (Farbstein and

Levy, 2012). Therefore, it would be interesting to evaluate whether HDL composition, levels and antioxidant, anti-inflammatory properties might affect PCSK9 KO glucose and insulin homeostasis.

In the present study we did not find any evidence of decreased *de novo* lipogenesis in the liver that would result from excessive lipoprotein internalization, nor did we observe any differences in lipid droplet accumulation. Demers *et al.* had found increased visceral fat mass in PCSK9 KO mice confirming data our group previously published (Demers et al., 2015; Roubtsova et al., 2011). This group also found increased liver LD content. This could be a result of increased adipose tissue release of free fatty acids and uptake in the liver, particularly since visceral fat is more susceptible to insulin resistance than subcutaneous fat. This increased substrate influx is thought to be responsible for ~60% of the triglycerides stored in hepatocytes in patients with NAFLD (Donnelly et al., 2005).

Overall, these studies in mice are limited by the clear distinction that mice have elevated HDLc/LDLc ratios in contrast to humans, where the majority of cholesterol is in apoB-containing lipoproteins, in particular in LDL fractions. Therefore, the impact of decreasing PCSK9 in hypercholesterolemic patients will likely lead to important internalization of LDLc into insulin sensitive tissues possibly without the protective effects of HDL described above. Therefore, even though recent clinical trials demonstrated that decreasing PCSK9 in high risk ASCVD patients did not increase new onset diabetes or alter glycemic indices (Ray et al., 2019a; Sabatine et al., 2017), it is still too early to conclude that there will not be any effects in the longer term. Of important note, however, is that diabetic patients demonstrate greater absolute benefit in reducing cardiovascular events from PCSK9 inhibitors than normoglycemic patients (Ray et al., 2019a). The latter strongly encourages the use and continued development of these inhibitors even with the possibility that they may, like statins, result in type 2 diabetes.

III.7. Additional Information

III.7.1. Funding

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III.7.3. Conflict of interest

The authors declare no conflict of interest with the contents of this article.

III.7.4. Author contributions

JC conducted all the experiments, analyzed the results and wrote the article. AR assisted in some animal experimentations. JM contributed to histological preparations. AP generated the PCSK9 KO mouse lineage and maintained the mouse colonies. NGS contributed to the conception of the experiments, analysis of the data and writing of the article.

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Chapter IV: Discussion and Perspectives

In this thesis we discussed a relatively new protein, the 9th member of the mammalian family of proprotein convertases, PCSK9, with relation to metabolic disease especially. Briefly, this family of enzymes are synthesized as zymogens and require autocatalysis for protein maturation and proper folding. PCSK9 autocatalytic cleavage in the ER allows for ER exit, however, no other enzymatic substrate than itself have been identified to date. Accordingly, PCSK9 is secreted with its prodomain tightly bound in its catalytic cleft. Instead, PCSK9 regulates target proteins non-enzymatically, although the underlying mechanisms are not yet understood. The interacting proteins linking PCSK9 to the endosomal system allowing for the rerouting of the LDLR for lysosomal degradation rather than recycling to the cell surface have not yet been identified. *PCSK9* is a polymorphic gene and the identification of variants in either hyper- or hypocholesterolemic patients have helped characterize the activity towards the LDLR in relation to structure as well as to interacting proteins, such as annexin A2, apoB and furin, for example.

Mouse genetic studies have been critical to further the understanding of *Pcsk9* gene and tissue expression. Interestingly, PCSK9 is expressed in several tissues, including the liver, intestine, kidney, brain (cerebellum), and more (Zaid et al., 2008) but the only tissue contributing to circulating PCSK9 is the liver (Zaid et al., 2008). The latter allowed for the demonstration that circulating PCSK9 accounts for 2/3 of the regulation of LDLc in mice such that whole body KO mice demonstrate ~40% drop in total cholesterol and ~ 80% drop in LDLc *vs.* liver specific PCSK9 KO results in ~60% drop in LDLc and ~27% drop in total cholesterol (Zaid et al., 2008), further suggesting partial regulation by the locally expressed PCSK9 in peripheral tissues through a paracrine or intracellular activity. On the other hand, the regulation of TGs by PCSK9 is much less well understood. Several epidemiologic studies have demonstrated association of PCSK9 with fasting plasma TGs, large buoyant VLDL and IDL (Guardiola et al., 2015; Kwakernaak et al., 2014). Variants in PCSK9 and mechanistic studies from mouse and cell models together demonstrate both intracellular and extracellular pathways in the regulation of apoB-containing TG-rich lipoproteins. Indeed, PCSK9 would protect apoB-lipoproteins from presecretory degradation which is mediated by LDLR. PCSK9 would also increase apoB secretion through an LDLR independent mechanism. Lastly, through the regulation of the LDLR, PCSK9 modulates

the fractional catabolic rate of these lipoprotein particles (for extensive review view Dijk *et al.* (Dijk et al., 2018)).

There is no evidence yet for the direct implication of PCSK9 in the regulation of plasma insulin or plasma glucose, however epidemiological studies suggest association of PCSK9 plasma levels with markers of hepatic insulin resistance (HOMA-IR, fasting plasma glucose, fasting plasma insulin); moreover, Mendelian randomization studies suggest an implication for the inhibition of PCSK9 activity towards the development of T2DM. Thus, in this thesis we were interested in investigating: (i) the direct modulation of InsR signaling; and (ii) the effect of the absence of PCSK9 on glucose and insulin homeostasis.

In summary of the first study, our results have shown that, *in vivo* in mice, and *in vitro* in immortalized human hepatocytes, PCSK9 mediated the downregulation of InsR downstream signaling, an effect that was counter-acted *in vitro* by mAb inhibitors but not by sdAb which target the catalytic and CHRD domains, respectively. Although the CHRD does not appear to be involved in this process it is nevertheless interesting to note that the CHRD domain is homologous to resistin (Hampton et al., 2007), an adipokine associated with insulin resistance and obesity. Therefore, this is the first report demonstrating that PCSK9, *via* its catalytic domain, may antagonize insulin function.

As a second part to this study, we show that proPCSK9 and proInsR interact in the ER, decreasing total protein and mRNA levels of the InsR, reminiscent of proPCSK9 exerting a dominant negative effect on the WT allele counterpart in patient studies (Cariou et al., 2009; Mayne et al., 2011). We demonstrate that the co-expression of these proteins results in acute ER stress, by upregulated XBP1 splicing and upregulated IRE1α exonuclease activity. The latter is known to alleviate the ER by cleaving ER targeted mRNA through the RIDD process (Hollien and Weissman, 2006). Since we also observed decreased InsR mRNA, we propose that IRE1α leads to reduced InsR mRNA under these cellular conditions. Therefore, oligomerization and misfolding of proPCSK9, especially by uncleaved, ER retained PCSK9 variants, may trigger the downregulation of the InsR. These results are in line with previous studies since the InsR is a known target of UPR, where downregulated expression, decreased signaling, and impaired transport from the ER to the plasma membrane have been reported (Brown et al., 2020; Ozcan et al., 2004; Xu et al., 2010).

The second study investigated glucose and insulin homeostasis in older, PCSK9 WT and KO mice to verify the effect of long-term absence of PCSK9 on pancreatic islet and liver function. A handful of other studies have reported controversial results of glucose and insulin homeostasis in these PCSK9 WT and KO mice some of which were suggestive of impaired islet function and integrity and liver steatosis (Da Dalt et al., 2019; Demers et al., 2015; Langhi et al., 2009; Lebeau et al., 2019; Mbikay et al., 2015; Mbikay et al., 2010; Zaid et al., 2008). In our study, WT and KO mice presented similar healthy β-cell and liver phenotypes even though PCSK9 KO mice express 2-3-fold more LDLR in liver and pancreatic islets. Da Dalt *et al.* show that PCSK9 KO islets accumulate lipids and cholesterol (~3-fold increase in intracellular cholesterol), the authors further suggest that cholesterol is responsible for impaired β-cell function (Da Dalt et al., 2019). Whereas, Langhi *et al.* observed no differences in intra-islet cholesterol, they also show that incubating WT and KO islets with LDLc for 24 hrs does not lead to greater internalization of cholesterol or affect KO islet glucose stimulated insulin secretion more significantly than WT islets (Langhi et al., 2009). It should be noted however that once in culture islets start losing the regulatory effect of PCSK9 on LDLR (Langhi et al., 2009). There have been two PCSK9 KO mice that were developed, the first published by Rashid *et al*. (Rashid et al., 2005) and the second published by Zaid *et al* (Zaid et al., 2008). Importantly they were generated by targeting different sections of the genes for deletion, the first targeted part of exon 2 to exon 4 included, whereas the latter targeted the proximal promoter and exon 1 (Rashid et al., 2005; Zaid et al., 2008). These may have important consequences since the editing presented in first study according to the design may be permissible to transcription of the signal peptide, part of the catalytic domain and the CHRD domain, allowing for this hypothetical product to enter the secretory pathway. Whereas the second study presents a strategy that even if a product was transcribed through a second transcription start site (that would only have resulted randomly from the gene edition) in contrast to the earlier design it would not be allowed to enter the secretory pathway. Moreover, if the design by Rashid *et al*. did indeed result in a protein product it would be almost entirely constituted of the CHRD domain, which we have reviewed in section I.1.3.3 as highly homologous to resistin, an adipokine implicated in diabetes. Altogether, these differences in the approaches used to generate the KO strains could, at least in part, explain the heterogeneity in the reported glucose and insulin related phenotypes of the PCSK9 KO mice.

Critical studies have demonstrated that cholesterol accumulation in mouse islets *in vivo* is dependent on ABCA1 cholesterol efflux activity, even under conditions of hypercholesterolemia, suggesting that the cell maintains balanced intracellular cholesterol by upregulating efflux to HDL cholesterol acceptor (Brunham et al., 2007; Kruit et al., 2010b). Future studies looking at the effect of PCSK9 inhibition on β-cell function could include a characterization of β-cell cholesterol efflux, HDL lipoprotein particle antioxidant and inflammatory content, as well as expression of ABCA1.

Lipid accumulation in liver lipid droplets in PCSK9 KO *vs.* WT mice could be due to several mechanisms such as increased TG hydrolysis in adipose tissue resulting in increased substrate influx and increased expression of fatty acid transporter CD36, increased *de novo* lipogenesis, and/or impaired VLDL assembly and secretion, for example. Interestingly, Lebeau *et al.* showed that their KO mice have liver steatosis and that it is likely not due to increased *de novo* lipogenesis but to CD36 mediated lipid internalization, as demonstrated by significant decrease in lipid accumulation upon CD36 inhibition (Lebeau et al., 2019).

Altogether, there have not yet been enough studies to conclude that PCSK9 regulation of the LDLR may or may not lead to lipotoxicity in insulin responsive tissues. All these studies were conducted with the C57BL/6J mouse strain, however, these strains, through breeding in the vendor houses, acquire genetic drift. Indeed, several variants, including the well described deletion in the nicotinamide nucleotide transferase (*Nnt*) gene, that may influence physiology have been identified in these substrains (Zurita et al., 2011). Additionally, environmental confounders, such as gut microbiota will significantly influence the metabolic profile. Increased ratio of Firmicutes to Bacteroidetes in addition to decreased bacterial diversity are strong indicators for predisposition to metabolic syndrome (Agus et al., 2016; Marcher et al., 2019; Stephenson et al., 2018). Hence, the large heterogeneity between the described analyses could be explained by the inherent differences in mouse substrain susceptibility, accounted for genetic variability and gut microbiome, to insulin resistance, fatty liver and weight gain (Kern et al., 2012; Vercalsteren et al., 2019).

In summary, the work presented in this thesis requires further investigation as the underlying mechanisms linking PCSK9 to TG metabolism and insulin signaling are not yet well defined. Nevertheless, these hypotheses are highly relevant today because PCSK9 is an extremely promising target to decrease cardiovascular events in CVD patients, even though longer studies are required to demonstrate efficiency in lowering mortality. Among early concerns in PCSK9

inhibition therapy were the effect of decreasing LDLc to extremely low levels (Robinson et al., 2015) and the risk in developing adverse effects, similarly to statins. Clinical trials have shown modest but significant changes in TG and HDL levels, ~15% decrease and ~5-7% increase, respectively (Robinson et al., 2015; Sabatine et al., 2015) no affect on diabetes or glucose homeostasis (Ray et al., 2019a; Sabatine et al., 2017). Our studies are the first to support a direct implication for PCSK9 in insulin signaling, therefore suggesting that increased PCSK9 may contribute to hepatic insulin resistance, which in addition to its previously described effects on TG-rich lipoprotein secretion and decreased fractional catabolic rate, suggest that PCSK9 may drive dyslipidemia (view Figure IV. 1.A). As such, decreasing PCSK9 would be beneficial in ASCVD patients (view Figure IV. 1.B). With regard to concerns over affecting β-cell toxicity, it is interesting to note that in contrast to the whole body knockout models, and the PCSK9 LOF Mendelian randomization studies, in CVD patients treated with PCSK9 inhibitors, whether mAb or inclisiran siRNA, only circulating PCSK9 will be targeted, allowing PCSK9 expressed from β-cells to regulate LDLR levels and the cell to adjust intracellular cholesterol accordingly (studies from our lab and from Da Dalt *et al.* show that locally expressed PCSK9 strongly regulates LDLR *vs.* weak regulation by circulating PCSK9 (Da Dalt et al., 2019). Therefore, β-cells would be protected through this mechanism from cholesterol induced lipotoxicity in these patients. Lastly, targeting PCSK9 through inclisiran could lead to previously unexpected advantages that may be proposed from the results of the first study. Indeed, in addition to well-characterized extracellular functions of PCSK9, our study proposes that PCSK9 may also regulate other proteins, perhaps in a less specific manner, through proPCSK9 oligomerization in the ER leading to interacting proteins' downregulation as for the InsR and TFRC. As such, preventing PCSK9 translation through mRNA silencing could rescue these effects.

PCSK9 is known to have several targets, some of which were presented in the literature review of this thesis; hence it is likely that all targets have not yet been identified. In this thesis we demonstrate that ER-retained PCSK9 variants may lead to the activation of acute ER stress and the downregulation of ER-targeted mRNA. A recent publication from our group demonstrates that ER-retained PCSK9 does not cause ER stress *in vivo* but does lead to the increase in the expression of ER chaperones (Lebeau et al., 2020). Under conditions where acute ER stress is not resolved, chronic ER stress and the activation of the UPR will lead to further adaptation of the ER, although prolonged activation of the UPR will then lead to apoptosis. These conditions may be detrimental

and lead to a large heterogeneity of disease types, from metabolic diseases to developmental and neurological diseases, depending on which tissue the protein is normally expressed such that impaired function of this tissue will determine the disease phenotype (Schroder and Kaufman, 2005). Therefore, effects of ER-retained, uncleaved PCSK9 may become apparent when accompanied by metabolic stress or other genetic predispositions, careful screening of liver function should probably be done in carrier of these specific PCSK9 mutations, particularly when accompanied by the metabolic syndrome or other predisposing genetic diseases. For example, a patient carrying an ER-retaining, uncleaved PCSK9, G316C in addition to polymorphisms in two genes associated with NAFLD, developed steatosis (Di Filippo et al., 2017).

Future studies related to the work presented in this thesis could include an investigation on the mechanism for PCSK9 mediated decreased P-Akt. For example, our preliminary data shows direct binding of PCSK9 to the insulin receptor *in vitro*; and/or an analysis of mTORC2, which phosphorylates Akt, proper recruitment to PIP3 at the plasma membrane and endomembranes. Additionally, further investigations on the role of PCSK9 on insulin and glucose homeostasis could be done on mouse models which have similar lipid profiles to humans. For example, the apoE*3-Leiden mice on a Western type diet show humanized lipoprotein cholesterol distribution (van Vlijmen et al., 1994). These mice can also be expressing CETP, if a model for atherosclerosis is required (Westerterp et al., 2006). Under these conditions, inhibiting PCSK9 in the circulation would lead to a greater absolute internalization of LDLc into insulin responsive tissues; furthermore, the decrease in HDL relative to WT mice, would remove the protective effect that benefits mice in favoring reverse cholesterol transport and preventing ectopic lipid accumulation.

Ongoing and recent studies on PCSK9 have shown that this protein plays a role in inflammation (Ricci et al., 2018), viral infection (Khademi et al., 2018; Labonte et al., 2009), neointimal formation and smooth muscle cell proliferation (Ferri et al., 2016). Accordingly, inhibiting PCSK9 has demonstrated decreased psoriatic lesions through reduced inflammatory reactions (Luan et al., 2019) and decreased macrophage induced smooth muscle cell proliferation in the atherosclerotic plaque (Ding et al., 2015). However, the expression of PCSK9 appears to be protective in septic patients (Paciullo et al., 2017). Lastly, PCSK9 was recently shown to target MHC class I presented on tumor cells for degradation such that PCSK9 inhibition resulted in reduced tumor growth (Liu et al., 2020). Altogether these data suggest that in the future PCSK9 may be targeted for further applications than just hypercholesterolemia.

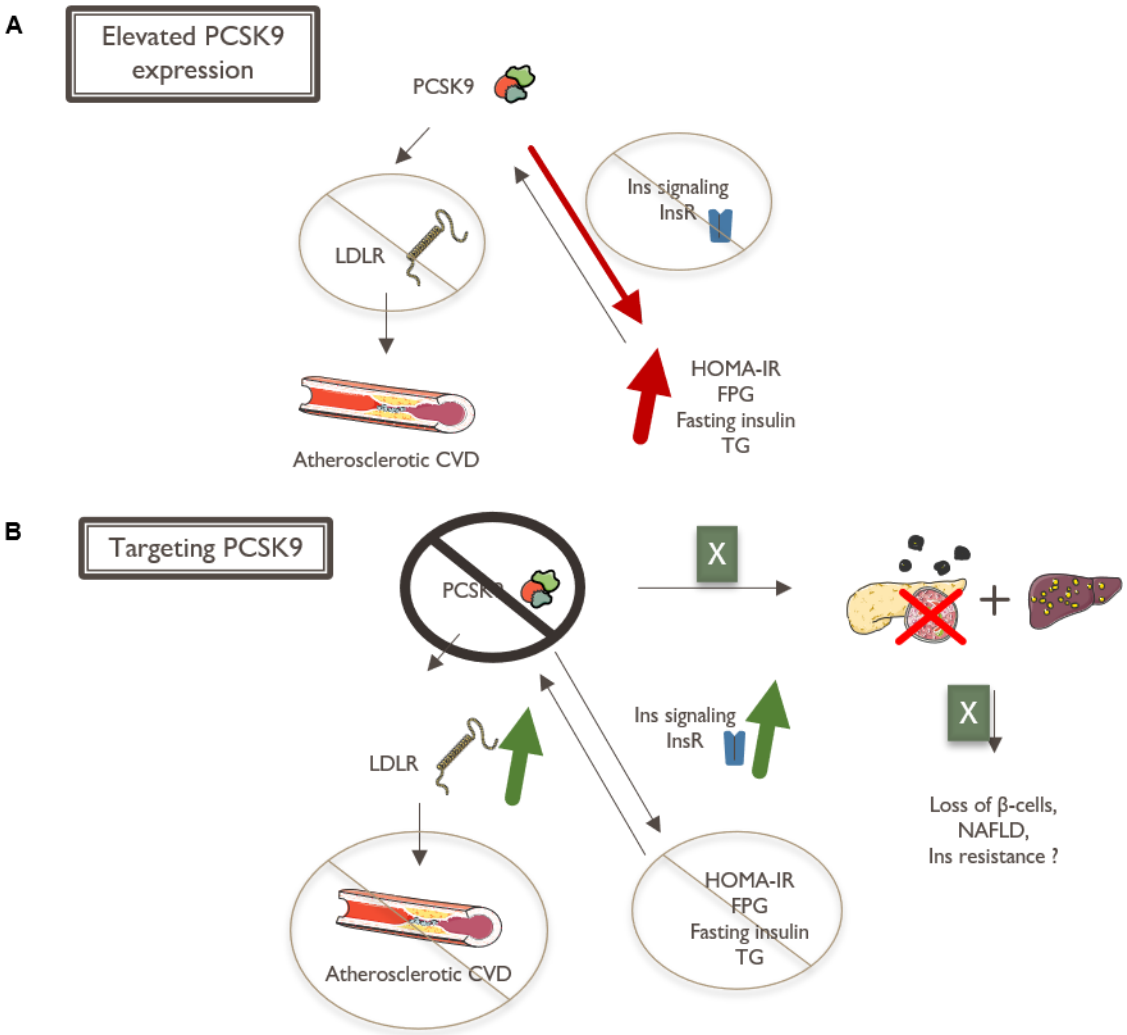

Figure IV. 1 Summary diagram of main thesis findings

A, Elevated levels of PCSK9 downregulate the LDLR increasing the risk for cardiovascular disease. Elevated levels of PCSK9 also regulate insulin signaling and insulin receptor levels through an extracellular and an intracellular pathway, respectively, which in turn may account for the association with markers of insulin resistance. ***B,*** Targeting either extracellular or intracellular PCSK9 is beneficial for the prevention of cardiovascular disease and could hypothetically reverse or prevent insulin resistance known to be associated with increased PCSK9. This thesis also shows that in mice, the prolonged absence of PCSK9 does not lead to impaired β-cell function or fatty liver. The graphic illustrations used in this figure are from Servier Medical art (https://smart.servier.com).

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Appendices

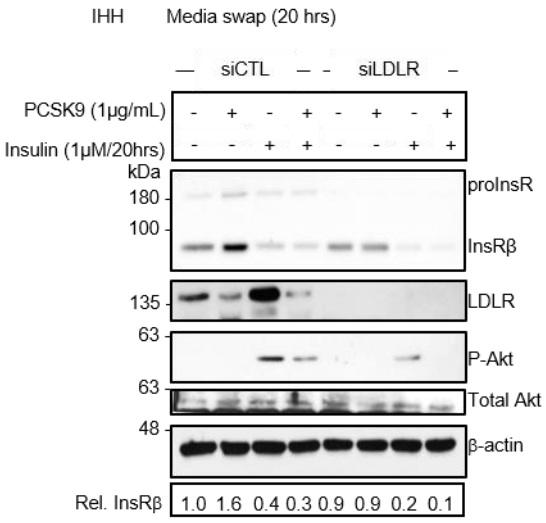

Figure II.5.S. 1 Exogenous PCSK9 decreases insulin signaling *in vitro* independently from the LDLR.

IHH cells were transfected with either control scrambled siRNA or three different siRNAs targeting the LDLR. They were then incubated O/N with serum-free conditioned media lacking or containing 1 µg/mL of PCSK9-V5 and in the presence or absence of 1 µM insulin. Cell lysates were analyzed for the activation of Akt (pS473) by WB. The data are representative of at least three independent experiments.

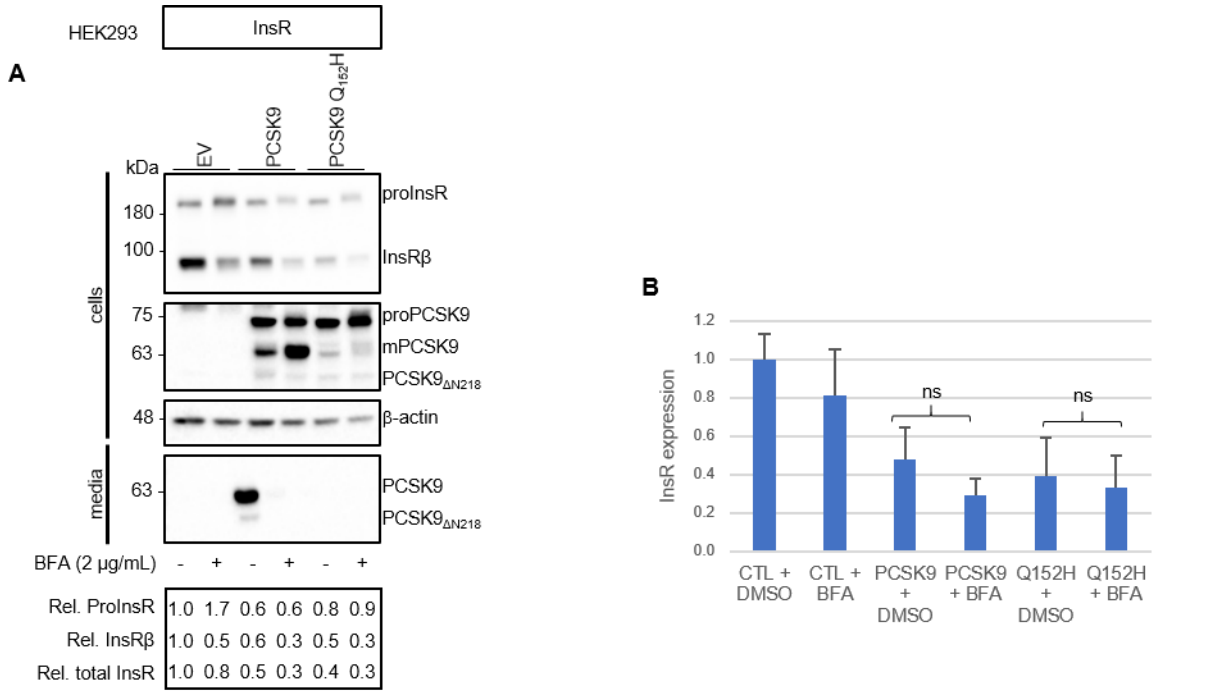

Figure II.5.S. 2 Retaining PCSK9 in the ER does not increase the downregulatory effect on InsR.

A and ***B***, HEK293 cells transiently co-expressing InsR with the indicated plasmids were treated with BFA (2 µg/mL, O/N) and analyzed by WB. The data are representative of at least three independent experiments and are presented with mean ± standard deviation. Statistical comparisons with controls were performed by an unpaired two-tailed Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

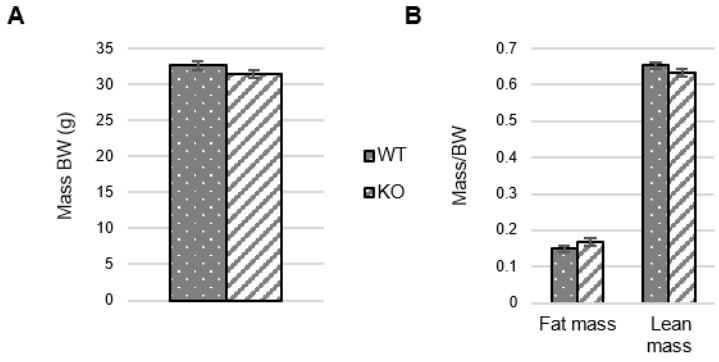

Figure III.5.S. 1 PCSK9 WT and KO mice showed equivalent body weight, lean and fat mass.

A, Mice from both genetic groups were weighed. ***B,*** fat and lean mass were measured by magnetic resonance imaging; *n* = 9-10 mice/genotype. Data represented by mean ± SEM.