The Proprotein Convertases in Hypercholesterolemia and Cardiovascular Diseases: Emphasis on Proprotein Convertase Subtilisin/Kexin 9

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Abstract
The secretory proprotein convertase (PC) family comprises nine members, as follows: PC1/3, PC2, furin, PC4, PC5/6, paired basic amino acid cleaving enzyme 4, PC7, subtilisin kexin isozyme 1/site 1 protease (SKI-1/S1P), and PC subtilisin/kexin type 9 (PCSK9). The first seven PCs cleave their substrates at single/paired basic residues and exhibit specific and often essential functions during development and/or in adulthood. The essential SKI-1/S1P cleaves membrane-bound transcription factors at nonbasic residues. In contrast, PCSK9 cleaves itself once, and the secreted inactive protease drags the low-density lipoprotein receptors (LDLR) and very LDLR (VLDLR) to endosomal/lysosomal degradation. Inhibitory PCSK9 monoclonal antibodies are now prescribed to treat hypercholesterolemia. This review focuses on the implication of PCs in cardiovascular functions and diseases, with a major emphasis on PCSK9. We present a phylogeny of the PCs and the...
I. The Proprotein Convertases and Their Functions in Health and Disease

Post-translational modifications of secretory proteins fashion the final bioactive forms of primary protein products in various tissues and cells during development and in adulthood (Seidah and Guillemot, 2016). Of all post-translational modifications of proteins known, peptide bond cleavage represents one of the most drastic and irreversible reactions leading to two or more fragments of distinct, but sometimes complementary, biologic functions. From the time it was realized that secretory polypeptide hormones such as insulin (Steiner, 2011) and adrenocorticotropic hormone/β-endorphin (Chrétien, 2011) were synthesized from inactive precursors, pro-insulin and pro-opiomelanocortin, respectively, it took 23 years to identify the cognate proteases that generate various peptide hormones and protein products (Seidah and Chrétien, 1999; Seidah and Prat, 2012).

The mammalian genome encodes nine proprotein convertases (PCs). These serine proteases share sequence identity with bacterial subtilisin and yeast kexin (Julius et al., 1984; Mizuno et al., 1988), and their genes are referred as proprotein convertases subtilisin kexin types 1–9 (PCSK1 to PCSK9), with two exceptions for members 3 and 8, respectively, named FURIN and MBTPS1. The nine corresponding proteins are known as follows: PC1/3, PC2, furin, PC4, PC5/6, paired basic amino acid cleaving enzyme 4 (PACE4), PC7, subtilisin kexin isozyme 1/site 1 protease (SKI-1/S1P), and PCSK9. Except for PCSK9, they all cleave secretory proteins, such as polypeptide hormones, receptors, growth factors, adhesion molecules, membrane-bound transcription factors, and neuronal regulators, during embryonic development and in adulthood (Seidah and Prat, 2012). Such processing regulates essential cellular functions, and their dysregulation can lead to the metabolic syndrome, obesity, diabetes, hypertension, dyslipidemia, inflammation, cancer/metastasis, pain, memory deficits, anxiety/depression, neurodegeneration, iron dysregulation, and even viral and parasitic infections. Accordingly, a number of ongoing clinical trials are investigating the plausible roles of PCs in diseases.

Potentially the most advanced/promising clinical trials are for inhibitors of furin in cancer and viral infections, PACE4 in arthritis pain and prostate cancer, and PCSK9 in hypercholesterolemia and septic shock (Fig. 1).

The first seven basic amino acid–specific PCs were discovered between 1990 and 1996 (Seidah and Prat, 2012; Seidah, 2015). However, other precursors, such as brain-derived neurotrophic factor, cell surface glycoproteins of hemorrhagic fever viruses, and membrane-bound transcription factors [sterol regulatory element-binding proteins (SREBPs); endoplasmic reticulum (ER)–stress protein activating transcription factor 6; or cAMP-response element-binding proteins], are cleaved after nonbasic residues, predicting the implication of a distinct protease(s). This led to the simultaneous discovery and cDNA cloning of site 1 protease (S1P) (Cheng et al., 1999; Espenshade et al., 1999), also known as subtilisin-kexin isozyme 1 (SKI-1) (Seidah et al., 1999).

The transcription factors SREBP-1 and SREBP-2 are major regulators of cholesterol/low-density lipoprotein (LDL) receptor (LDLR) and fatty acid synthesis, respectively. They are anchored in the secretory pathway via a luminal loop separating two transmembrane–cytosolic domains. The initial cleavage by SKI-1/S1P in the luminal loop within the cis/medial Golgi is followed by an intramembrane cleavage in the first transmembrane domain by the metalloclopeptide site-2 protease in the medial Golgi (Rawson et al., 1997). The released cytosolic N-terminal basic helix-loop-helix leucine zipper-containing domains of both factors are then translocated to the nucleus, where they activate their respective target genes. SKI-1/S1P was the first PC implicated in the regulation of lipid metabolism, as its inactivation in the liver decreased by 75% lipid synthesis (Yang et al., 2001). However, the complete Mbtbs1 gene inactivation in mouse resulted in an early embryonic death with no epiblast formation (Yang et al., 2001), thus hampering clinical applications of SKI-1/S1P inhibitors. Only short treatments with the SKI-1/S1P small-molecule inhibitor PF-429242 (Hawkins et al., 2008) were suggested for persistent arenavirus

ABBREVIATIONS: αα, amino acid; ADH, autosomal dominant hypercholesterolemia; apoB, apolipoprotein B; ApoER2, apolipoprotein E receptor 2; ARH, autosomal recessive hypercholesterolemia; CVD, cardiovascular disease; eeKO, endothelial cell–specific KO; ER, endoplasmic reticulum; FH, familial hypercholesterolemia; Gdf11, growth differentiation factor 11; GOF, gain-of-function; GWAS, genome-wide association studies; HDL, high-density lipoprotein; HepKO, hepatocyte-specific KO; KO, knockout; LDL, low-density lipoprotein; LDLr, LDL receptor; LOP, loss-of-function; Lp(a), lipoprotein (a); LPS, lipopolysaccharide; LRPs, LDLR-related protein-1; mAb, monoclonal antibody; PACE4, paired basic amino acid cleaving enzyme 4; PC, protease convertase; PCSK, proprotein convertase and kexin; S1P, site 1 protease; S1P1, subtilisin kexin isozyme 1; SNP, single-nucleotide polymorphism; SREBP, sterol regulatory element-binding protein; TGF, triglyceride; VLDLR, very LDLR.
(Pasquato et al., 2012; Pasquato and Kunz, 2016), hepatitis C (Blanchet et al., 2015), and Dengue viral infections (Uchida et al., 2016). Mbtps1 inactivation in osteoblasts further showed that SKI-1/S1P is critical for extracellular matrix signaling and axial elongation during somitogenesis and vertebral development, and possibly linked to the caudal regression syndrome (Achilleos et al., 2015). In addition, Mbtps1 inactivation in osteocytes stimulates soleus muscle regeneration and increased muscle size and contractile force with age (Gorski et al., 2016). Understanding bone–muscle cross-talk may provide a novel approach to prevention and treatment of age-related muscle loss.

Although the first seven PCs cleave their substrates after basic residues often organized in pairs within the motif K/R-2Xn-K/R↓[where Xn = 0, 1, 2, or 3 amino acids (aa)], SKI-1/S1P recognizes the motif R-X-(aliphatic residue)-X↓. In contrast, the last convertase, PCSK9 (Seidah et al., 2003), which is abundantly synthesized by the liver and released into the circulation, is only found in some vertebrates. Interestingly, because the bovine PCSK9 genome exhibits a premature stop codon in exon 10 (Ding et al., 2007; Cameron et al., 2008), this revealed that fetal bovine serum, widely used in cell biology, does not contain a PCSK9 protein. Like all other PCs, PCSK9 undergoes in the ER an autocatalytic cleavage between its pro and catalytic domains at the site VAFAQ152↓ (Naureckiene et al., 2003; Benjannet et al., 2004), where Gln152 is critical (Benjannet et al., 2012). Note that, different from the eight other members of the family, it does not require the presence of any basic amino acid at the vicinity of the cleavage site.

The most amazing and key feature that distinguishes PCSK9 from the other PCs is its inability to get rid of its prodomain, leading to a secreted inactive protease. Indeed, after autocatalytic cleavage of the prodomain, which acts as an intramolecular chaperone and potent but transient inhibitor in the other PCs (Seidah and Prat, 2012), in mature PCSK9 it remains noncovalently attached to its catalytic pocket (Seidah et al., 2003), thereby preventing any in trans catalytic activity of the enzyme (Cunningham et al., 2007). Thus, PCSK9 acts as a protease only once in its lifetime, whereas the other PCs cleave and activate/inactivate secretory precursor proteins either in the cis/medial (SKI-1/S1P) or trans Golgi network, cell surface or endosomes (furin, PC4, PC5/6, PACE4 and PC7), or immature secretory granules (PC1/3 and PC2) (Seidah and Prat, 2007, 2012; Creemers and Khatib, 2008; Seidah et al., 2008, 2013; Turpeinen et al., 2013). In contrast, PCSK9 binds various receptors and escorts them to endosomes/lysosomes for degradation (Seidah and Prat, 2012; Seidah et al., 2014). Interestingly, from 1990 to the present, a literature statistical analysis of the interest in PCs, and especially in PCSK9, revealed that, since its discovery in 2003, it has stimulated wide interest, as exemplified by the >1850 manuscripts published on PCSK9, compared with a total of >4100 manuscripts reported on all PCs (Fig. 2).

In this review, we will focus on the newly described aspects of the biology of PCSK9, its mechanism of action, as well as its validated and possibly new targets.
We will also discuss the consequences of PCSK9 natural mutations on the metabolism of LDL cholesterol (LDL-c), as well as the clinical applications of its inhibitors in pathologies. Finally, we will summarize the implication of other PCs in cardiovascular diseases (CVD) and how some of them can regulate PCSK9.

II. Phylogenetic and Haplotype Analyses of PCSK9 in Human Species

A phylogenetic tree based on the amino acid sequences of the PCs revealed that SKI-1/S1P is the most ancient member of the family (Seidah et al., 1999) and is found in parasites and the plant kingdom (Barale et al., 1999). Although the closest members form couples, that is, PC1/PC2; furin/PC4, PC5/PACE4; and SKI-1/PCSK9, the highly conserved PC7 (Seidah et al., 1996) is difficult to place on the phylogenetic tree (Fig. 3A).

Alignment of PCSK9 protein sequences of modern and archaic human species revealed only three variations between the modern human reference sequence and either the Denisovan individual, H449L, V474I, and G670E, or the Altai neandertal individual, A53V, V474I, and G670E. Interestingly, the benign polymorphic variants A53V and V474I were also detected in modern humans before. Also, G670E is a major common variant in modern humans, with only ~13% carrying a Gly670 in the Chinese Han population (He et al., 2016). We have consistently found a Glu670 in all of our DNA sequences of PCSK9 to date. The common His449 in modern humans and neandertal is replaced by Leu449 in the Denisova individual (H449L).

The functional consequence of this replacement in the hinge domain (aa 422–452) (Cunningham et al., 2007; Saavedra et al., 2012) is the loss of a positive charge at the acidic pHs of endosomes/lysosomes and begs for more detailed cellular analyses. In that context, a similar loss of positive charge, R434W, in the same domain resulted in a loss-of-function (LOF) associated with lower levels of circulating PCSK9 and LDL-c (Dubuc et al., 2010).

Given the possible LOF phenotype of the Denisovan PCSK9 protein (H449L variation), we investigated whether its genomic haplotype could have been adaptively introgressed into modern humans. Recent studies have demonstrated that several important genes show signs of adaptive introgression from neandertal and/or Denisovans to modern humans, such as the EPAS1 gene important for high altitude adaptation in Tibetans (introgressed from Denisovans to modern humans) (Huerta-Sánchez et al., 2014) or human Toll-like receptors important for our innate immunity (introgressed from both neandertals and Denisovans into modern humans) (Dannemann et al., 2016). However, PCSK9 does not show a pattern consistent with...
adaptive introgression for the denisovan haplotype (H449L variation, Fig. 3B). In case of adaptive introgression, we would have expected this haplotype to be present in high (or higher) frequency within certain populations in our data, as well as genetic distance to the archaic haplotype to increase with a sharp incline.

In conclusion, only minor changes in PCSK9 primary protein sequence occurred within the years that separate modern humans from either denisovans (estimated separation ~380,000–473,000 years ago) (Prüfer et al., 2014) or neandertals (estimated separation ~550,000–765,000 years ago) (Prüfer et al., 2014). However, we found one interesting amino acid change (H449L) in denisovans that may result in a LOF of PCSK9. In contrast to the conserved protein sequence of PCSK9 between hominids, their genomic sequences are quite variable in individuals outside of East Asia. Furthermore, it does not show any pattern consistent with adaptive introgression. More in-depth analyses (including all nine PCs and more extensive sampling) will be needed to get a more detailed picture of the evolutionary history of PCSK9 and the other eight PCs.

III. The Enzymatically Inactive Mature PCSK9 Regulates Circulating LDL Cholesterol Levels

Screening of nucleotide databases with conserved sequences encoding the active site residues of S1P/SKI-1 as baits led us to report in February 2003 (Seidah et al., 2003) the cDNA cloning and protein analysis of the ninth and last member of the PC-family, a putative convertase, originally called neural apoptosis-regulated convertase-1. Millennium Pharmaceuticals had released part of its cDNA in a patent database as belonging to a group of genes upregulated upon induction of apoptosis in primary cerebellar neurons by serum withdrawal. Neural apoptosis-regulated convertase-1, now known as PCSK9, belongs to the proteinase K family of subtilases, and its catalytic domain exhibits ~25% of sequence identity with that of its closest family member SKI-1/S1P (Seidah et al., 2003) (Fig. 3A). Human PCSK9 mRNA (NM_174936.3) spans 3710 bp over 12 exons encoding a 692 amino-acid protein (NP_777596.2).

Chromosomal mapping of the PCSK9 gene revealed it to be located on chromosome 1p32 (Seidah et al., 2003), close to a predicted third major gene locus [familial hypercholesterolemia (FH)] for autosomal dominant familial hypercholesterolemia (ADH) located at 1p34.1-p32 in a large French family in whom the two genes known to be implicated in this disease, the LDLR and APOB genes, had been excluded (Varret et al., 1999). This locus was previously associated with an increase in the hepatic secretion of LDL, but not high-density lipoprotein (HDL) cholesterol, or triglycerides (TG) (Varret et al., 1999; Hunt et al., 2000). Armed with this information and the major expression of PCSK9 in liver (Seidah et al.,
ing of several genes led to the identification of a common region on chromosome 1. This positional cloning approach allowed the identification of a new multiplex family that analyses in 23 French non-LDLR/non-APOB families had studying this region of chromosome 1. Intensive genetic

effect, we contacted a French team that was actively working on the genetics of hypercholesterolemia culminated in the first report that appeared in June 2003, revealing that human PCSK9 was the FH3 gene critical for LDL-c regulation (Abifadel et al., 2003).

This discovery was soon followed by the identification of the devastating PCSK9 D374Y mutation in a large Utah pedigree (Timms et al., 2004) and 1 month later in three Norwegian subjects (Leren, 2004). Other ADH-causing PCSK9 mutations were discovered in the following years (Abifadel et al., 2009, 2014), and, more recently, 16 different PCSK9 mutations were found in patients from eight countries (Hopkins et al., 2015).

It was evident from the analysis of the first identified mutants S127R and F216L that PCSK9 plays a major role in the regulation of LDL-c, but not TG (Abifadel et al., 2003). This was confirmed by the seminal experiments in mice done by Maxwell and Breslow (2004), who demonstrated that PCSK9 promotes the degradation of the LDLR. Adenoviral-mediated expression of PCSK9 led to high plasma LDL-c levels in a LDLR-dependent manner and resulted in the quasi-absence of the LDLR in the liver. As the mouse gene Pcsk9 was strongly downregulated by dietary cholesterol (Maxwell et al., 2003), they hypothesized that PCSK9 was coregulated with the LDLR to rapidly modulate lipoprotein clearance. Because the two first loci related to FH were that of the LDLR and its ligand apolipoprotein B (apoB), it was very gratifying that a strong functional link could be established between the LDLR and PCSK9. Thus, gain-of-function (GOF) mutations resulting in higher activity or levels of PCSK9 were expected to lower liver LDLR levels and hence raise circulating LDL-c, due to its reduced clearance by the remaining LDLR. The demonstration that, conversely, LOF mutations lead to higher LDLR concentrations and hence reduced LDL-c levels was first achieved by Cohen et al. (2005) 2 years later. By screening 32 subjects from the Dallas Heart study that had the lowest LDL-c levels, they discovered two heterozygote nonsense mutations present in ~2% of the African American population, Y142X and C679X. The latter are responsible for a ~40% drop in circulating LDL-c and associated with a ~88% reduction in the risk of CVD (Cariou et al., 2009; Stein and Raal, 2014). This result opened a new potential therapeutic application because Lp(a) has long been known to be an independent risk factor for atherosclerosis for which no known therapeutic lowering agent had been identified. The reduction in Lp(a) levels was recently rationalized by the fact that, under supraphysiologic levels of the LDLR resulting from PCSK9 mAb treatments, the LDLR becomes a functional receptor of Lp(a) both in cells (Romagnuolo et al., 2015) and in humans (Raal et al., 2016). It was also shown that PCSK9 binds the apoB component of Lp(a), but not to apo(a) (Romagnuolo et al., 2015). Interestingly, a recent report suggests that PCSK9 is associated with Lp(a) in the plasma of patients with very high circulating Lp(a) concentrations, and that the Lp(a)-associated PCSK9 levels seem to be directly correlated with plasma Lp(a) levels, but not with total plasma PCSK9 levels (Tavori et al., 2016), suggesting that polymorphic variations in Lp(a) and its levels may affect its affinity to PCSK9. Whether the reported association of Lp(a) with PCSK9 in plasma samples is direct or requires a bridging protein is not clear, because we have shown in vitro that PCSK9 does not directly bind Lp(a), whereas it binds LDL with a Kd of ~130 nM (Romagnuolo et al., 2015).

IV. The Absence of PCSK9 Leads to a Sex- and Tissue-Specific Subcellular Distribution of the LDLR

In mice, PCSK9 is highly expressed in liver, pancreatic islets, intestine, and kidney (Seidah et al., 2003; Zaid et al., 2008; Langhi et al., 2009). PCSK9 knockout (KO) mice, as well as mice lacking specifically PCSK9 in
the liver [hepatocyte-specific KO (HepKO)], were generated (Rashid et al., 2005; Zaid et al., 2008; Parker et al., 2013). In these mice, the lack of PCSK9 leads to a two- to threefold increase of the LDLR content in the liver, pancreas, or intestine, as estimated by Western blotting, with no change in LDLR mRNA levels (Rashid et al., 2005; Zaid et al., 2008; Roubtsova et al., 2015).

GOF mutations in human PCSK9 or PCSK9 overexpression in mice stimulate apoB-containing lipoprotein secretion from the liver (Ouguerram et al., 2004; Park et al., 2004; Sun et al., 2005; Lambert et al., 2006). In contrast, PCSK9 deficiency in mice leads to lower expression in mice stimulate apoB-containing lipoprotein secretion from the liver (Ouguerram et al., 2004; Park et al., 2004; Sun et al., 2005; Lambert et al., 2006). In contrast, PCSK9 deficiency in mice leads to lower expression of SREBP-2 and 3-hydroxy-3-methylglutaryl-CoA reductase mRNA levels in their regenerating liver, suggesting the sensing of a lack of intracellular cholesterol. How elevated levels of LDLR do not lead to cholesterol accumulation in the liver or pancreatic islets (Langhi et al., 2009) remains an enigma. A possibility is that more substantial amounts of cholesterol are excreted via transintestinal cholesterol excretion (Le May et al., 2013). Whether transintestinal cholesterol excretion increases in individuals lacking PCSK9 or treated with mAbs remains to be verified.

PCSK9 is undetectable in the plasma of HepKO mice, demonstrating that the circulating protein originates exclusively from hepatocytes (Zaid et al., 2008). Circulating PCSK9 can also regulate peripheral receptors. For example, in HepKO or complete KO mice, very LDLR (VLDLR) immunolabeling increases at the cell surface of visceral adipocytes that do not express PCSK9 locally. Intriguingly, this increase was ~10-fold higher in female than in male KO mice (Roubtsova et al., 2011), providing the first evidence for a sex-dependent cell surface accumulation of a PCSK9-targeted receptor in PCSK9 KO mice. In liver, pancreas, and small intestine of KO male and female mice, the absence of PCSK9-triggered LDLR degradation led to similar two- to threefold increases in the total protein levels of LDLR. However, the subcellular distribution of the receptor differed in a sex- and tissue-dependent manner. LDLR cell surface immunolabeling was dramatically increased in the liver and pancreatic islets of KO males, but not females. Conversely, LDLR cell surface labeling was higher in the small intestine of females, similar to the VLDLR in perigonadal fat depots (Roubtsova et al., 2015). Moreover, the accumulation of the LDLR at the cell surface of male but not female hepatocytes was evidenced by Western blot analysis of plasma membrane preparations (Roubtsova et al., 2015). This sex-specific distribution is dictated by estrogens, as illustrated by the accumulation of the LDLR at the hepatocyte surface in ovariectomized female mice supplemented with placebo, whereas those supplemented with 17β-estradiol exhibited low surface levels of the LDLR (Roubtsova et al., 2015).

The physiologic relevance of the above regulation by 17β-estradiol of the LDLR subcellular distribution is of the highest interest if it has a counterpart in humans. The sex-dependent efficacy of the PCSK9 mAbs has not yet been extensively examined. The long-term study LTS11717 conducted on 1438 men and 872 women (predominantly postmenopausal) resulted in 65.5% and 53.4% drops in LDL-c in men and women, respectively, thus showing a 22% higher efficacy in men (see Supplemental Fig. S4 in Robinson et al., 2015). However, a subgroup analysis revealed that postmenopausal women responded with a 16% higher efficacy than premenopausal women (54.8% versus 47.3%). Thus, men (65.5% drop) responded with 38.5% and 19.5% higher efficacies than premenopausal (47.3% drop) and postmenopausal (54.8% drop) women, respectively (http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/EndocrinologicandMetabolicDrugsAdvisoryCommittee/UCM449865.pdf). Clearance experiments as well as extensive analyses of phase 3 clinical trials will be needed to confirm the existence in women of a lower response to PCSK9 inhibition.

V. Role of PCSK9 in Inflammation, Sepsis, and Viral Infections

Inflammation and infection induce marked changes in lipid and lipoprotein metabolism, and administration of bacteria endotoxins induces hyperlipidemia in animals (Khovidhunkit et al., 2004). Lipopoly saccharide (LPS) administration, a model for Gram-negative bacterial infection, increases LDL and VLDL levels, most likely via decreased LDLR and VLDLR protein levels in the liver (Liao et al., 1996), possibly caused by LPS-stimulated expression of PCSK9 in liver (Feingold et al., 2008). Sepsis is a complex disease characterized by systemic activation of inflammation and coagulation, most commonly in response to bacterial infection. Severe sepsis, accompanied by dysfunction of at least one organ, affects 0.3–1% of individuals/yr in the United States with mortality rates of ~30% (Gaieski et al., 2013). Therapies include antibiotic treatments and hemoperfusion filtration that specifically remove LPS from circulation (Cruz et al., 2009).

Mice lacking LDLR exhibit increased mortality in a cecal ligation and puncture (CLP) model (Lanza-Jacoby et al., 2003), suggesting that LDLR plays a notable role in sepsis. In that context, Walley et al. (2014) recently showed that exogenous human PCSK9 reduces LPS
uptake in vitro by HepG2 cells, and that inhibitory PCSK9 antibodies reduced plasma endotoxin levels in mice subjected to CLP, blunted the cytokine response, and doubled survival rates. In humans, PCSK9 LOF (R46L) polymorphisms were associated with better survival of septic patients and reduced cytokine levels, whereas GOF (E670G) polymorphisms had the opposite effect. Furthermore, i.v. injection of endotoxin to healthy volunteers generated lower cytokine levels in R46L individuals (Walley et al., 2014). Finally, it was recently shown that LPS binds plasma lipids to be internalized in human HepG2 cells and mouse primary hepatocytes by the LDLR, but also other receptors, including the VLDLR (Topchiy et al., 2016). It is important to note that, in septic patients, the median levels of PCSK9 were positively correlated with the extent of the respiratory and cardiovascular failure (Boyd et al., 2016), leading to the hypothesis that reducing PCSK9 levels, and hence increasing LDLR-mediated hepatic clearance of pathogen lipids, may be protective (Walley et al., 2014). Indeed, lower circulating levels of endotoxins may reduce their binding to Toll-like receptors that leads to nuclear factor κB activation and subsequent proinflammatory cytokine secretion by leukocytes (Savva and Roger, 2013). In agreement, we have recently shown that, in a mouse CLP model of sepsis, PCSK9 deficiency confers protection against systemic bacterial dissemination, organ pathology, and tissue inflammation, particularly in the lungs and liver, whereas PCSK9 overexpression exacerbates early hypercoagulable and proinflammatory cytokine secretion by leukocytes (Savva and Roger, 2013). In situ hybridization analyses at embryonic day 17 (Fig. 4) and lower signals in the telencephalon (Fig. 4B). At this developmental stage, the mouse placenta did not show PCSK9 expression. In embryonic membranes (Fig. 4, C–F), PCSK9 mRNA was present within the smooth muscle cell layer, but not in endothelial or embryonic blood cells. The role of PCSK9 in these tissues is not known and maybe vary during development. Interestingly, PCSK9 mRNA and protein are expressed in human placenta at full-term in patients with gestational diabetes mellitus, a common complication of pregnancy aggravated by obesity (Dubé et al., 2013). In situ hybridization analyses at embryonic day 17 (Fig. 5, upper panel) and postpartum day 10 (Fig. 5, lower panels) in mouse revealed that PCSK9 is mostly expressed in liver, small intestine, colon, cerebellum, and kidney. At postpartum day 10, PCSK9 and LDLR colocalize especially in the liver and intestine, but also in thymus (Seidah et al., 2003; Zaid et al., 2008). The function of PCSK9 in extrahepatic tissues is still poorly understood (Seidah, 2016).

VI. Other Physiologic Roles of PCSK9

We investigated by in situ hybridization the expression of mouse PCSK9 at embryonic day 14.5 (Fig. 4). High signals were observed in the hepatic primordium, embryonic membranes, midgut and umbilical artery, and lower signals in the telencephalon (Fig. 4B). At this developmental stage, the mouse placenta did not show PCSK9 expression. In embryonic membranes (Fig. 4, C–F), PCSK9 mRNA was present within the smooth muscle cell layer, but not in endothelial or embryonic blood cells. The role of PCSK9 in these tissues is not known and maybe vary during development. Interestingly, PCSK9 mRNA and protein are expressed in human placenta at full-term in patients with gestational diabetes mellitus, a common complication of pregnancy aggravated by obesity (Dubé et al., 2013). In situ hybridization analyses at embryonic day 17 (Fig. 5, upper panel) and postpartum day 10 (Fig. 5, lower panels) in mouse revealed that PCSK9 is mostly expressed in liver, small intestine, colon, cerebellum, and kidney. At postpartum day 10, PCSK9 and LDLR colocalize especially in the liver and intestine, but also in thymus (Seidah et al., 2003; Zaid et al., 2008). The function of PCSK9 in extrahepatic tissues is still poorly understood (Seidah, 2016).

Multiple studies revealed that PCSK9 can escort not only the LDLR to lysosomal degradation compartments, but also its family members VLDLR, apolipoprotein E receptor 2 (ApoER2), and LDLR-related protein-1 (LRP1) (Poirier et al., 2008; Roubitsos et al., 2011; Canuel et al., 2013), best seen in livers lacking LDLR and PCSK9 (unpublished data). This suggests that, in FH patients, especially those with very low levels of functional LDLR, PCSK9 may also regulate LRP1 levels. In addition, the fatty acid transporter CD36 seems to be another target of PCSK9 (Demers et al., 2015), although the exact binding site has not yet been determined. This is in line with the observed hypertrophy of visceral adipocytes in PCSK9 KO mice...
(Roubtsova et al., 2011) that indicates higher binding of TG-rich lipoproteins and fatty acid uptake via increased VLDLR and CD36. Elucidation of the role(s) of PCSK9 as an intracellular and/or paracrine regulator of LDLR and/or VLDLR protein levels in gut, kidney, and pancreas will require tissue-specific KOs, as was done in hepatocytes (Zaid et al., 2008). Although expressed in kidney, PCSK9 is not secreted into the urine (Dubuc et al., 2004), and it does not seem to play a role in controlling blood pressure even under induced hypertensive conditions (Berger et al., 2015). However, patients with chronic kidney disease exhibit higher liver expression and circulating levels of PCSK9 (Konarzewski et al., 2014), most likely due to enhanced expression of HNF1α in hepatocytes (Sucajtys-Szulc et al., 2016). Finally, although PCSK9 does not cross the blood brain barrier, little is known about the function of PCSK9 in the cerebellum, or other neuronal cells (Seidah et al., 2003). PCSK9 has been shown to reduce LDLR levels, and possibly those of VLDLR and ApoER2, during brain development and after ischemic stroke in adult mice, although poststroke behavior and lesion volume were not affected by the lack of PCSK9 (Rousselet et al., 2011). The possible role of PCSK9 in some neurodegenerative diseases is yet to be conclusively shown and validated in humans.

**VII. Cellular Studies: Mechanism of Action of PCSK9**

Surprisingly, 13 years after the discovery of PCSK9, no sorting molecular mechanism(s) can yet explain its ability to induce the degradation of the LDLR (Seidah et al., 2014) in late endosomes/lysosomes (Nassoury et al., 2007) (Fig. 6). Our data revealed that the C-terminal Cys-His-rich domain (CHRD) of PCSK9, which contains three repeat structures called M1, M2, and M3 (Cunningham et al., 2007), is needed for the PCSK9–LDLR complex (Nassoury et al., 2007), including the repeat domain M2 (Saavedra et al., 2012), which has 14 His (Cunningham et al., 2007) and a number of natural mutations associated with it (Seidah et al., 2014). This critical importance of the CHRD in regulating the ability of PCSK9 to induce the degradation of the LDLR was confirmed in two independent studies (Zhang et al., 2008; Poirier et al., 2016) and was shown to be inhibited by mAbs (Ni et al., 2010; Schiele et al., 2014) or single-domain antibodies that target the CHRD, without inhibiting the PCSK9–LDLR complex formation (Weider et al., 2016).

We suggested that a putative protein Xp binds the CHRD of extracellular PCSK9 in the PCSK9–LDLR complex and escorts it to lysosomes for degradation by...
unknown resident hydrolases (Canuel et al., 2013; Seidah et al., 2014; Butkinaree et al., 2015). In view of the importance of the cytosolic adaptor protein, autosomal recessive hypercholesterolemia (ARH), which contains a critical NP-X-Y motif for entry into clathrin-coated endosomes (Lagace et al., 2006), it was thought that the cytosolic tail (CT) of the LDLR (containing an NP-X-Y motif) controls this ARH-dependent internalization. However, we and others realized that PCSK9 can enhance the degradation of LDLR lacking this CT (LDLR-ΔCT) or replaced with another one lacking an NP-X-Y motif (Holla et al., 2010; Canuel et al., 2013). Thus, we proposed that the putative Xp may be a membrane-bound protein with an NP-X-Y motif in its CT. Proteomic analyses of livers of mice lacking or not PCSK9 suggested that the LDLR family member LRP1 is also upregulated in the absence of PCSK9 in liver (Canuel et al., 2013). However, the implication of the PCSK9 target LRP1 as a putative Xp was soon eliminated (Canuel et al., 2013). We also recently discarded two other Xp candidates proposed in the literature, that is, sortilin (Gustafsen et al., 2014) and amyloid precursor-like protein 2 (DeVay et al., 2013), as neither protein is critical for the in vivo activity of PCSK9 on LDLR in hepatocytes (Butkinaree et al., 2015), and mice lacking both proteins do not exhibit significantly different LDLR levels in the liver (Fig. 7). Thus, the identity and necessity of Xp are yet to be defined and validated in cells and in vivo, for the ability of PCSK9 to enhance the degradation of the LDLR and possibly other targets.

A genetic approach showed that the cytosolic adaptor protein Sec24A implicated in the ER–Golgi trafficking of the coat protein complex COPII vesicles was critical for the secretion of PCSK9 from the ER into the medium (Chen et al., 2013). This facilitation is thought to be mediated by a membrane-bound protein that binds PCSK9 in the ER lumen and Sec24A in the cytosol via its CT. It was also recently reported that the LDLR does not bind efficiently PCSK9 in the ER because of the presence of a competitive chaperone like-protein GRP94 that prevents such ER interaction (Poirier et al., 2015). Finally, Leren (2014) hypothesized that the membrane-bound PCSK9–LDLR complex is first shed into a soluble form upon entry into acidic endosomes via a cathepsin-like cysteine protease inhibited by E64, which may facilitate its efficacious degradation in lysosomes.

Although it was reported that in mice the cytosolic adaptor protein ARH is essential for the ability of extracellular PCSK9 to enhance the degradation of the LDLR in liver hepatocytes (Lagace et al., 2006), recent clinical trials using an inhibitory PCSK9 mAb Alirocumab revealed that absence of ARH in some autosomal recessive hypercholesterolemic patients, although reducing the activity of PCSK9, still left room for an ARH-independent pathway, at least as ascertained in lymphocytes (Thedrez et al., 2016). Similar results were also observed in ARH-negative lymphocytes (Pasano et al., 2009), revealing the presence of an ARH-independent pathway for PCSK9 activity on LDLR. The presumed relatively lower contribution of this pathway in human liver (Thedrez et al., 2016) is yet to be unambiguously validated.

VIII. What Have We Learned from Key Natural Mutations on the Functions of PCSK9 and Its Partners?

Since the discovery of PCSK9 and its relationship to LDLc, >100 PCSK9 SNPs were reported (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=255738) with varied consequences (http://www.ucl.ac.uk/ldlr/LOVDv.1.1.0/search.php?select_db=PCSK9&srch=all). Many of these are silent, others are suspected to be damaging, and some have been validated for their functional effects. The structure of the 692-aa human PCSK9 protein (Fig. 6A) contains a prodomain (aa 31–152) that is noncovalently associated with the mature protease chain (aa 153–692),
keeping it in an enzymatically inactive state. The catalytic subunit (aa 153–404) is followed by a short hinge domain (Hi; aa 405–452) and the C-terminal CHRD (aa 453–692) (Seidah et al., 2003, 2014; Seidah and Prat, 2012) (Fig. 6A), composed of three repeats called M1, M2, and M3 (Cunningham et al., 2007). Mutations can be found in all four structural components of PCSK9. The catalytic subunit of PCSK9 directly binds the epidermal growth factor-like

![Fig. 6.](image1)

Fig. 6. A. Schematic representation of proPCSK9 (75 kDa, 692 aa) and its processed form PCSK9 (62 kDa) that is noncovalently bound to the autocatalytically processed prodomain (pro; 15 kDa) at VFAQ152-SIP. Notice the active site residues Asp, His, and Ser and the oxyanion hole Asn, as well as the single N-glycosylation site (in blue). B. Schematic of the extracellular pathway of PCSK9-induced degradation of the LDLR. High PCSK9 levels (left part of the graph) or GOF forms enhance the degradation of the LDLR following the endocytosis of the PCSK9–LDLR complex in clathrin-coated vesicles, together with the LDL, leading to the degradation of the PCSK9–LDLR complex in lysosomes. This results in low levels of the LDLR at the cell surface and increased levels of circulating LDL-c. Low levels of PCSK9 or LOF forms lead to increased levels of cell surface LDLR because the latter can be recycled back to the surface after delivery of LDL particles to acidic endosomes/lysosomes. PCSK9 mAbs target the extracellular pathway by sequestering extracellular PCSK9 and hence preventing its binding to the LDLR.

![Fig. 7.](image2)

Fig. 7. The lack of amyloid precursor-like protein 2 and/or sortilin has no effect on total cholesterol, PCSK9, and LDLR levels. Mice deficient for amyloid precursor-like protein 2 (Appl2/--) and/or sortilin (Sort1+/+ or Sort1/--) were analyzed for their level of circulating cholesterol and PCSK9 and those of LDLR protein in liver extracts. No changes were observed. Note that, individually, amyloid precursor-like protein 2 or sortilin deficiency does not affect these parameters (Butkinaree et al., 2015).
domain-A domain of the LDLR with nM potency (Zhang et al., 2007) and is critical for the ability of PCSK9 to enhance the degradation of the LDLR (Zhang et al., 2007; Saavedra et al., 2012).

Interestingly, the prodomain of PCSK9 also makes contact with the β-barrel domain of the LDLR (Lo Surdo et al., 2011), as also evidenced by specific mutations therein, for example, L108R, that enhance the functional activity of PCSK9 in enhancing the degradation of the LDLR in endosomes/lysosomes (Abifadel et al., 2012). However, the various crystal structures of PCSK9 reported at both neural and acidic pHs with or without the ectodomain of the LDLR could not detect a PCSK9 reported at both neural and acidic pHs with or without the ectodomain of the LDLR could not detect a specific structure for N-terminal aa 31–59 of the prodomain (Cunningham et al., 2007; Lo Surdo et al., 2011). This suggests that alone this 29-aa sequence of the prodomain may be quite mobile, most likely predicting that a partner may be needed to stabilize in space this highly acidic segment, or that it may bind, albeit weakly, to another segment of PCSK9. It has been suggested that the prodomain may interact with apoB100 on LDL (Kosenko et al., 2013), but not VLDL (Lagace, 2014) particles, although the importance of the aa 31–59 segment of the prodomain in this interaction has yet to be defined. Interestingly, deletion of the acidic aa 31–51 sequence of the prodomain enhances the affinity of PCSK9-Δ31–51 for the LDLR by >sevenfold (Kwon et al., 2008), suggesting that, if apoB binds this region, it results in a dampening of PCSK9-binding affinity for the LDLR. The physiologic importance of the 122–aa prodomain (aa 31–152) is exemplified by mutations that result in LOF (e.g., R46L, Δ97, G106R, Q152H) or GOF (e.g., E32K, D35Y, L108R, S127R) of PCSK9. Japanese patients that are E32K homozygotes have very high LDL-c, but still lower than FH patients that completely lack functional LDLR (Mabuchi et al., 2014).

Although many natural mutations within the PCSK9’s catalytic domain (aa 153–452) result in either GOF (Table 1) or LOF (Table 2) (Abifadel et al., 2009), one of the most damaging GOF mutations is the Anglo-Saxon D374Y mutation (Timms et al., 2004), which enhances the binding affinity of PCSK9 to the LDLR by >10-fold (Cunningham et al., 2007). A corresponding mutation H306Y in LDLR has a similar enhancing effect on the PCSK9–LDLR complex formation (McNutt et al., 2009). Interestingly, the GOF F216L and R218S mutations reduce or eliminate the ability of furin to cleave and inactivate PCSK9 (Benjannet et al., 2006). That furin processing of PCSK9 at Arg218↓ in vivo (Essalmani et al., 2011) does inactivate PCSK9 was recently confirmed by functional analysis of human plasma PCSK9 (Han et al., 2014). Furthermore, such processing was shown to extend the half-life of a distinct anti-PCS9 mAb that recognizes the N-terminal sequence aa 168–181, which, different from Evolocumab or Alirocumab, does not stericly impede the inactivating furin cleavage at Arg218↓ (Schroeder et al., 2015). Mutations within the hinge domain and the CHRD domain also resulted in either GOF or LOF of PCSK9 (Tables 1 and 2) (Abifadel et al., 2009). The function of these mutations relates to their regulation of the ability of the PCSK9–LDLR complex to be directed to late endosomes/lysosomes for degradation (Nassoury et al., 2007; Zhang et al., 2008; Poirier et al., 2016) (Fig. 6B, left part). However, the underlying mechanism and the importance of post-translational modifications, for example, Asn533 glycosylation (Seidah et al., 2003) or Ser688 phosphorylation (Gauthier et al., 2015; Tagliabracci et al., 2015) in this process are still unknown. In vitro biochemical experiments suggested that the CHRD may interact with the ligand binding domain of the LDLR at acidic pHs (Yamamoto et al., 2011), but this is not evident from the available PCSK9–LDLR crystal structures (Lo Surdo et al., 2011). We suggested that the CHRD may bind an as yet unknown protein X that would then direct the PCSK9–LDLR complex toward late endosomes/lysosomes (Seidah et al., 2014; Butkinaree et al., 2015). Alternatively, the CHRD may displace a protein required for the default recycling of the LDLR to the cell surface found in most tissues (Fig. 6B, right part), thereby allowing the PCSK9–LDLR complex to reach the degradation compartments. It is thus conceivable that, in the future, specific PCSK9 and/or LDLR mutations may be identified that would disrupt or enhance the interaction with such putative regulator and hence favor the recycling or degradation pathways of the LDLR.

IX. Ongoing Outcome Clinical Trials

Several pharmaceutical strategies were and are being developed to reduce PCSK9 levels/activity on LDLR degradation and were reviewed (Seidah et al., 2014; Elbitar et al., 2016; Stein, 2016). In essence, an encapsulated in nanoparticles antisense RNA interference oligonucleotide when i.v. injected once at 0.4 mg/kg resulted in ~70% and ~40% drops in PCSK9 and LDL-c levels, respectively (Fitzgerald et al., 2014). It is now being tested in phase 2 clinical trials. However, the present, most prevalent approach consists in blocking mAbs that inhibit the binding of extracellular or circulating PCSK9 to the LDLR (Stein and Raal, 2014). Three human mAbs are now in multiple phase 3 clinical trials. Two of them, Evolocumab (Repatha; Amgen; Thousand Oaks, CA) and Alirocumab (Praluent; Sanofi; Bridgewater, NJ), are already prescribed to FH patients worldwide, whereas the Bococizumab (Pfizer; Groton, CT) is not yet available commercially (Ito and Santos, 2016). However, very recently Pfizer discontinued the global development of Bococizumab, as the efficacy of this noncompletely humanized mAb decreased with time (http://www.pfizer.com/news/press-release/press-release-detail/pfizer_discontinues_global_development_of_bococizumab_its_investigational_pcsk9_inhibitor). In all cases, the s.c. injection of either 150 mg
Every 2 weeks or 420 mg every 4 weeks results in a consistent and sustained ~50–60% reduction in LDL-c. The safety and tolerability profile is very good, especially for hypercholesterolemia patients who fail to obtain an optimal clinical response to statin therapy, those who are statin-intolerant or have contraindications to statin therapy. The combination of these mAbs with other lipid-lowering agents such as statins or the nonstatin ezetimibe/ fenofibrate resulted in a modest 50% reduction in major adverse cardiovascular events following more than 1 year of treatment.

### X. Other Genes and Loci Implicated in LDL-c Regulation

About 50 years after the pioneering work of Khachadurian (1964) on the genetics of FH in Lebanese families, and ~40 years after the discovery by Brown and Goldstein (1974) of the LDL receptor that paved the way to statin therapy, our discovery of PCSK9 and its implication in FH opened the way to intensive studies by researchers and pharmaceutical companies, leading to a promising new class of lipid-lowering drugs: the PCSK9 mAbs. The race to discover new genes implicated in FH and its cardiovascular complications is still very vigorous. In 2001, the LDLRAP1 gene encoding ARH was identified in families suffering from autosomal recessive hypercholesterolemia (Garcia et al., 2001). PCSK9 was the third gene implicated in ADH after the LDLR and APOB genes (Innerarity et al., 1987). Familial mutations in the APOE gene were recently linked to ADH (Awan et al., 2013; Marduel et al., 2013). Other mutations have been reported in genes encoding proteins implicated in cholesterol metabolism or regulation, notably SREBP2 (Muller and Miserez, 2002) and CYP7A1, but they are limited to rare cases (Pullinger et al., 2002).

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**TABLE 1**

<table>
<thead>
<tr>
<th>Protein Position</th>
<th>Nucleotide Position</th>
<th>Exon</th>
<th>Geographical Origin of Patients</th>
<th>Clinical Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Val141Le</td>
<td>c.10G &gt; A</td>
<td>1</td>
<td>Japan</td>
<td>CAD, PVD, arcus, xanthomas</td>
<td>Hopkins et al., 2015</td>
</tr>
<tr>
<td>p.Glu22Lys</td>
<td>c.94G &gt; A</td>
<td>1</td>
<td>Japan</td>
<td>CAD, stroke, arcus, xanthoma</td>
<td>Miyake et al., 2008</td>
</tr>
<tr>
<td>p.Asp35Tyr</td>
<td>183G &gt; T</td>
<td>1</td>
<td>France</td>
<td>Mild dyslipidemia</td>
<td>Abifadel et al., 2012</td>
</tr>
<tr>
<td>p.Glu48Lys</td>
<td>142G &gt; A</td>
<td>1</td>
<td>Netherlands</td>
<td>Mild dyslipidemia</td>
<td>Hopkins et al., 2015</td>
</tr>
<tr>
<td>p.Pro71Leu</td>
<td>212C &gt; T</td>
<td>2</td>
<td>Netherlands</td>
<td>CAD, stroke</td>
<td>Hopkins et al., 2015</td>
</tr>
<tr>
<td>p.Arg96Cys</td>
<td>286C &gt; T</td>
<td>2</td>
<td>Netherlands</td>
<td>CAD</td>
<td>Hopkins et al., 2015</td>
</tr>
<tr>
<td>p.Leu105Arg</td>
<td>323G &gt; C</td>
<td>2</td>
<td>France: Republic of Mauritius</td>
<td>CAD</td>
<td>Abifadel et al., 2012</td>
</tr>
<tr>
<td>p.Ser127Arg</td>
<td>c.381T &gt; A</td>
<td>2</td>
<td>2 French families</td>
<td>Tendon xanthomas</td>
<td>Abifadel et al., 2003; Cameron et al., 2006; Nassoury et al., 2007</td>
</tr>
<tr>
<td>p.Asp129Gly</td>
<td>c.388A &gt; G</td>
<td>2</td>
<td>1 South African patient</td>
<td>CAD, early MI</td>
<td></td>
</tr>
<tr>
<td>p.Asp129Asn</td>
<td>c.390G &gt; A</td>
<td>2</td>
<td>Norwegian patient</td>
<td>Stroke, arcus</td>
<td></td>
</tr>
<tr>
<td>p.Arg215His</td>
<td>c.644G &gt; A</td>
<td>4</td>
<td>2 Norwegian families</td>
<td>CAD</td>
<td>Cameron et al., 2006</td>
</tr>
<tr>
<td>p.Phe216Leu</td>
<td>c.645T &gt; C</td>
<td>4</td>
<td>1 French family</td>
<td>Early MI</td>
<td>Abifadel et al., 2003; Benjannet et al., 2004</td>
</tr>
<tr>
<td>p.Arg218Ser</td>
<td>c.654A &gt; T</td>
<td>4</td>
<td>1 French family</td>
<td>Tendinous xanthomas, arcus cornea</td>
<td>Benjannet et al., 2004; Allard et al., 2005</td>
</tr>
<tr>
<td>p.Arg357His</td>
<td>c.107G0 &gt; A</td>
<td>7</td>
<td>1 French proband</td>
<td>Family history of CVD</td>
<td></td>
</tr>
<tr>
<td>p.Asp374Tyr</td>
<td>c.110G0 &gt; T</td>
<td>7</td>
<td>1 Utah family</td>
<td>Achilles tendon xanthomas, arcus</td>
<td>Benjannet et al., 2004; Leren, 2004; Timms et al., 2004; Naoumova et al., 2005</td>
</tr>
<tr>
<td>p.Asp374His</td>
<td>c.1110G0 &gt; C</td>
<td>7</td>
<td>2 Portuguese probands + 1 relative</td>
<td>Severe phenotype. Premature CAD, arcus, xanthomas</td>
<td>Bourbon et al., 2008</td>
</tr>
<tr>
<td>p.Ser465Leu</td>
<td>c.1394 C &gt; T</td>
<td>9</td>
<td>Netherlands</td>
<td>CAD</td>
<td>Hopkins et al., 2015</td>
</tr>
<tr>
<td>p.Arg496Tyr</td>
<td>c.1486G &gt; T</td>
<td>9</td>
<td>Italy, Netherlands</td>
<td>Higher total cholesterol</td>
<td>Piscotta et al., 2006; Hopkins et al., 2015</td>
</tr>
</tbody>
</table>

CAD, coronary artery disease; CVD, cardiovascular disease; MI, myocardial infarction; PVD, peripheral vascular disease.
Using classic approaches based on positional cloning and linkage analysis, presumptive new loci for ADH were mapped to 8q24.22 (Cenarro et al., 2011) and to 16q22.1 (Marques-Pinheiro et al., 2010). In the next-generation sequencing era, parametric linkage analysis combined with exome sequencing in an ADH family resulted in the identification of the variant p.Glu97Asp in signal-transducing adaptor family member 1 (Fouchier et al., 2015) or genome-wide association studies (GWAS) (Stitziel et al., 2014). Other approaches using exome sequencing (Cariou et al., 2009) or resequencing (Patel et al., 2016), however, did not identify any major new gene. This may be explained by the difficulty to diagnose polygenic cases in ADH, estimated to \( \sim 13\% \) (Talmud et al., 2013), a value close to the percentage of PC1/3 alone or in combination with PC2 can generate diseases such as obesity or diabetes (Fig. 1). PC1/3 and PC2 sequentially cleave proinsulin into insulin and PC2 activates by cleavage most of the polypeptide hormone precursors in endocrine and neural tissues. The lack of either convertase results in \( \sim 20\% \) of normal hormone in corticotrophs of the pituitary gland or tissue-specific manner to generate adrenocorticotropin also differentially cleave pro-opiomelanocortin in a \( \alpha \)-melanotrophic hormone and \( \beta \)-endorphin in the hypothalamus and pituitary melanotrophs, respectively (Benjannet et al., 1991; Seidah et al., 2013). In fact, PC1/3 alone or in combination with PC2 can generate previously (Taylor et al., 2003; Seidah and Prat, 2007, 2012; Seidah et al., 2008, 2013; Turpeinen et al., 2013; Ramos-Molina et al., 2016). In this work, we will only briefly summarize our present understanding of their physiologic and pathologic roles in both health and in CVD.

### XI. Implication of the Enzymatically Active PCs in CVD

PCSK9 is not the only PC to be implicated in CVD, and relevant studies on other PCs were reviewed previously (Taylor et al., 2003; Seidah and Prat, 2007, 2012; Seidah et al., 2008, 2013; Turpeinen et al., 2013; Ramos-Molina et al., 2016). In this work, we will only briefly summarize our present understanding of their physiologic and pathologic roles in both health and in CVD.

#### A. PC1/3 and PC2 in Diabetes and Obesity

PC1/3 and PC2 activate by cleavage most of the polypeptide hormone precursors in endocrine and neural tissues. The lack of either convertase results in diseases such as obesity or diabetes (Fig. 1). PC1/3 and PC2 sequentially cleave proinsulin into insulin (Smeekens et al., 1992; Steiner, 2011). The two enzymes also differentially cleave pro-opiomelanocortin in a tissue-specific manner to generate adrenocorticotropin hormone in corticotrophs of the pituitary gland or \( \alpha \)-melanotrophic hormone and \( \beta \)-endorphin in the hypothalamus and pituitary melanotrophs, respectively (Benjannet et al., 1991; Seidah et al., 2013). In fact, PC1/3 alone or in combination with PC2 can generate

### Table 2: PCSK9 gene LOF mutations and polymorphisms causing hypocholesterolemia

<table>
<thead>
<tr>
<th>Protein Position</th>
<th>Nucleotide Position</th>
<th>Exon</th>
<th>Geographical Origin of Patients</th>
<th>Clinical Features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine stretch: p.L21dup also designated p.L15_L16insL</td>
<td>c.dup61_63CTG also designated c.43_44insCTG</td>
<td>1</td>
<td>Frequently found in several populations</td>
<td>Decrease of LDL-c levels of 10–15 mg/dL</td>
<td>Abifadel et al., 2003; Chen et al., 2005; Yue et al., 2006</td>
</tr>
<tr>
<td>p.Arg46Leu</td>
<td>c.137 G &gt; T</td>
<td>1</td>
<td>Frequently found in several populations</td>
<td>Associated with a 15% reduction of LDL-c and 47% of CHD in whites; reduction of the risk of early-onset myocardial infarction</td>
<td>Abifadel et al., 2003; Cameron et al., 2005; Cohen et al., 2006; Kotowski et al., 2006; Scartezini et al., 2007; Dewpura et al., 2008; Kathiresan et al., 2008</td>
</tr>
<tr>
<td>p.A68fsL82X</td>
<td>c.202delG</td>
<td>1</td>
<td>Sicily: 1 kindred 2 hypocholesterolemic blood donors</td>
<td>Fatty liver in the proband and his father but not in blood donors from healthy carriers</td>
<td>Fasano et al., 2007</td>
</tr>
<tr>
<td>p.Thr77Ile</td>
<td>c.230C &gt; T</td>
<td>2</td>
<td>Sicily</td>
<td>Hypocholesterolemic blood donors R104C/V114A segregates with FHBL; dominant negative when associated with c.341T &gt; C (p.Val114Ala) on the same allele; impairs processing and secretion of wild-type PCSK9 in liver; low plasma PCSK9 concentration, LDL clearance rate &gt;200% of normal</td>
<td>Fasano et al., 2007; Cariou et al., 2009</td>
</tr>
<tr>
<td>p.Arg104Cys</td>
<td>c.310C &gt; T</td>
<td>2</td>
<td>French</td>
<td>Hypocholesterolemic blood donors R104C/V114A segregates with FHBL; dominant negative when associated with c.341T &gt; C (p.Val114Ala) on the same allele; impairs processing and secretion of wild-type PCSK9 in liver; low plasma PCSK9 concentration, LDL clearance rate &gt;200% of normal</td>
<td>Fasano et al., 2007; Cariou et al., 2009</td>
</tr>
<tr>
<td>p.Gly106Arg</td>
<td>c.316G &gt; A</td>
<td>2</td>
<td>1 Norwegian family</td>
<td>Hypocholesterolemic subjects; low autocrinecleavage</td>
<td>Berge et al., 2006; Nassoury et al., 2007</td>
</tr>
<tr>
<td>p.Val114Ala</td>
<td>c.341T &gt; C</td>
<td>2</td>
<td>Sicily</td>
<td>Hypocholesterolemic blood donors R104C/V114A segregates with FHBL; dominant negative when associated with c.341T &gt; C (p.Val114Ala) on the same allele; impairs processing and secretion of wild-type PCSK9 in liver; low plasma PCSK9 concentration, LDL clearance rate &gt;200% of normal</td>
<td>Fasano et al., 2007; Cohen et al., 2006; Kotowski et al., 2006</td>
</tr>
<tr>
<td>p.Tyr142X</td>
<td>c.426C &gt; G</td>
<td>3</td>
<td>French-Canadian</td>
<td>Reduction of PCSK9 levels by 79% and LDL-c by 48%</td>
<td>Hayemi et al., 2011</td>
</tr>
<tr>
<td>p.Q152H</td>
<td>c.436G &gt; C</td>
<td>2</td>
<td>French-Canadian</td>
<td>Hypocholesterolemic</td>
<td>Kotowski et al., 2006</td>
</tr>
<tr>
<td>p.Leu253Phc</td>
<td>c.757C &gt; T</td>
<td>5</td>
<td>Black American and Hispanic</td>
<td>Hypocholesterolemic: complete LOF due to early termination</td>
<td>Miyake et al., 2008</td>
</tr>
<tr>
<td>p.Trp428X</td>
<td>c.1284G &gt; A</td>
<td>5</td>
<td>1 Japanese subject</td>
<td>Hypocholesterolemic: complete LOF due to early termination</td>
<td>Miyake et al., 2008</td>
</tr>
<tr>
<td>p.Ala522Thr</td>
<td>c.1564G &gt; A</td>
<td>10</td>
<td>Sicily American, Hispanic</td>
<td>Mild reduction in LDL-c levels</td>
<td>Fasano et al., 2007; Kotowski et al., 2006</td>
</tr>
<tr>
<td>p.Glu574Glu</td>
<td>c.1660C &gt; G</td>
<td>10</td>
<td>Black American, Hispanic</td>
<td>Mild reduction in LDL-c levels</td>
<td>Fasano et al., 2007; Kotowski et al., 2006</td>
</tr>
<tr>
<td>p.Pro616Leu</td>
<td>c.1847C &gt; T</td>
<td>11</td>
<td>Sicily</td>
<td>Hypocholesterolemic blood donors R104C/V114A segregates with FHBL; dominant negative when associated with c.341T &gt; C (p.Val114Ala) on the same allele; impairs processing and secretion of wild-type PCSK9 in liver; low plasma PCSK9 concentration, LDL clearance rate &gt;200% of normal</td>
<td>Fasano et al., 2007; Cohen et al., 2006; Kotowski et al., 2006</td>
</tr>
<tr>
<td>p.Cys679X</td>
<td>c.2037C &gt; T</td>
<td>12</td>
<td>Black American, Hispanic</td>
<td>Reduction of LDL-c levels of 40% and CHD of 88% for heterozygous carriers</td>
<td>Fasano et al., 2007; Cohen et al., 2006; Kotowski et al., 2006</td>
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CHD, coronary heart disease; FHBL, familial hypobetalipoproteinemia; LDL-c, low-density lipoprotein cholesterol; LOF, loss-of-function.
both anorexigenic hormones (α-melanotrophic hormone, insulin, cholecystokinin, and glucagon-like peptide 1) and orexigenic peptides (neuropeptide Y, cocaine- and amphetamine-regulated transcript, and orexin/hypocretin). It was thus not a surprise that subjects lacking functional PC1/3 are obese (Jackson et al., 1997). PCSK1 LOF mutations can be found in ~1% of morbidly obese patients (Creemers et al., 2012), and PCSK1 represents the third most prevalent monogenic contributor to the risk of developing obesity (Choquet et al., 2013). To date, no human PC2 mutations associated with disease have been found. Interestingly, whereas PC1/3 and PC2 are critical for the generation of insulin, the absence of functional PC1/3 in humans has not been associated with insulin resistance or diabetes (Stijnen et al., 2016), suggesting the presence of compensatory pathways and/or modifier genes.

B. Furin and SKI-1/S1P in Cardiovascular Diseases

SKI-1/S1P is expressed in all cells, as furin. The importance of SKI-1/S1P in lipid metabolism has been discussed at the beginning of this review. It is encoded by the gene MBTFS1 for which no missense mutations were associated with disease, and a >90% reduction of SKI-1/S1P expression is required to decrease cholesterol and TG production in liver (Yang et al., 2001). The complete KO of this gene in mice leads to very early embryonic lethality (Mitchell et al., 2001).

Furin is essential during development because deficient mouse embryos die at embryonic day 11 and exhibit multiple developmental abnormalities, particularly defects related to the function of endothelial cells (Roebroek et al., 1998). To define the role of furin in endothelial cells, a mouse endothelial cell-specific KO (ecKO) of the Furin gene was generated (Kim et al., 2012). Newborns die shortly after birth, and magnetic resonance imaging revealed ventricular septal defects and/or valve malformations. In addition, primary cultures of wild-type and ecKO lung endothelial cells revealed that ecKO cells are unable to grow, but are rescued by extracellular soluble furin. These cells are deficient for endothelin-1, transforming growth factor-β1, adrenomedullin, and bone morphogenetic protein 4 processing. The mature forms of the two last ones were reduced by ~90%. Thus, furin is a major regulator of cardiac functions, via the processing of multiple substrates. Recently, a GWAS revealed that a nucleotide A to C substitution in intron 1 of the Furin gene (rs17514846) was significantly associated with the development of the metabolic syndrome, with the protective minor A allele correlating with a 21% decreased serum TG (Ueyama et al., 2015). Interestingly, furin inactivates lipoprotein lipase, which is implicated in the hydrolysis of TG into fatty acids from TG-rich lipoproteins (Jin et al., 2005; Lei et al., 2011). The protective A allele in rs17514846 may thus be a furin LOF mutation leading to increased lipoprotein lipase activity, and hence to lower TG.

C. PACE4 in Blood Pressure Regulation

PACE4 is a cell surface enzyme bound to heparin sulfate proteoglycans that activates a number of receptors and growth factors (Seidah et al., 2008). In cardiomyocytes, PACE4 activates the serine protease corin that produces atrial natriuretic peptide/atrial natriuretic factor (Chen et al., 2015). Moreover, the authors identified in a hypertensive patient a dominant-negative D282N mutation in PACE4 that impedes the intracellular autoactivation of proPACE4 into PACE4. Mutated proPACE4 is retained in the ER in complex with normal PACE4, thereby inhibiting corin activation (Chen et al., 2015). Atrial natriuretic peptide is a hormone essential for sodium homeostasis (Flynn et al., 1983; Seidah et al., 1984) and reduces blood pressure (Yan et al., 2000). In agreement, PACE4 KO mice were found to be hypertensive on a normal salt (0.3% NaCl) diet, and this phenotype was exacerbated when the mice were placed on high-salt (4% and 8% NaCl) diets, indicating that corin activation by PACE4 is essential for maintaining normal blood pressure. Consistent with these findings, the PCSK6 gene was previously linked to hypertension in humans (Li et al., 2004).

D. PC5/6 in Heart Development and Extracellular Matrix Remodeling

Mice lacking PC5/6 die at birth and perfectly recapitulate the phenotypes of mice lacking the transforming growth factor-β–like growth differentiation factor 11 (Gdf11, also known as bone morphogenetic protein 11) (McPherren et al., 1999), including the absence of kidneys and major defects in the anteroposterior axis with extra-thoracic and -lumbar vertebrae, and a lack of tail (Essalmani et al., 2008; Szumska et al., 2008). In agreement, Gdf11 is exclusively activated by PC5/6 cleavage. In that context, high Gdf11 levels were associated with frailty, susceptibility to diabetes, or cardiac complications in old adults with severe aortic stenosis (Schafer et al., 2016). Thus, PC5/6 inhibition may reduce cardiac Gdf11 production. To circumvent lethality, studies were done in tissue-specific ecKO. Old ecKO mice lacking PC5/6 exhibit a cardiovascular hypotrophy associated with decreased extracellular matrix collagen deposition, decreased left ventricular diastolic function, and vascular stiffness, suggesting a trophic role of the enzyme PC5/6 most likely mediated by insulin-like growth factor 1/protein kinase B, also known as Akt/mammalian target of rapamycin signaling and control of autophagy (Marchesi et al., 2011). Among the endothelial cell surface precursors known to be cleaved by PC5/6 is the receptor protein tyrosine phosphatase receptor protein tyrosine phosphatase μ (Campan et al., 1996), of which KO in mice results in increased stiffness of mesenteric arteries (Koop et al., 2003), whereas our ecKO mice show the opposite phenotype (Marchesi et al., 2011).
et al., 2011). Finally, human intronic PCSK5 SNPs correlate with low levels of HDL cholesterol (Iatan et al., 2009), a correlation mediated by dietary polyunsaturated fatty acids (Jang et al., 2014).

E. PC7 in HDL, small dense LDL, and TG

Human GWAS studies suggest that PCSK7 mutations result in favorable CVD parameters, that is, high HDL, low TG (Guillemot et al., 2014; Peloso et al., 2014; Yao et al., 2015), low highly atherogenic small dense LDL (Hoogeveen et al., 2014), and reduced insulin resistance (Huang et al., 2015). Understanding the PC7 biology underlying these phenotypes should lead to powerful novel therapies for cardiometabolic disorders.

XII. Conclusions and Future Perspectives

The original hypothesis of the existence of secretory precursors, which upon limited proteolysis releases bioactive peptides or proteins, has been validated in multiple precursor proteins from various species (Chrétien, 2011; Steiner, 2011). The arduous process of hunting for the cognate proteinases responsible for such processing led to the identification of nine PCs with varied physiologic functions, which are sometimes associated with pathology (Seidah and Prat, 2012). The cumulative knowledge over the last 26 years led us to propose that inhibiting some of these convertases may in some cases be favorable to specific patients (Fig. 1). The most advanced target is PCSK9, as injectable inhibitory mAbs are now prescribed in clinics for the treatment of hypercholesterolemia, and will most likely be used for the prevention of septic shock. In the future, it is hoped that modulation of some of the other PCs for specific conditions may have new clinical applications, for example, PC7 in anxiety regulation (Wetsel et al., 2013), and possibly in hypertriglyceridemia (Peloso et al., 2014).

The present review exposed the many new facets of PCSK9 and its biology, concentrating only on its ability to enhance the degradation of the LDLR and VLDLR. However, PCSK9 has been shown to target for degradation other members of the LDLR family, including ApoER2 (Poirier et al., 2008), the fatty acid transporter CD36 (Demers et al., 2015), in liver and other tissues, such as small intestine, pancreas, brain, and adipocytes (Seidah et al., 2014). A lot more remains to be unraveled regarding the cellular trafficking of PCSK9 together with its targeted receptors, its complex web of interacting proteins, and its influence on the fate or levels of other proteins. Furthermore, PCSK9 has been shown to have a regulatory role of inflammatory processes independent from its effects on LDL-c (Leander et al., 2015). This is clearly a very exciting period in the field of dyslipidemia, where, thanks to new PCSK9-silencing therapies, LDL-c levels were lowered to unprecedented low levels, reaching 0.4 mM and lower. This is definitively good news for hypercholesterolemic patients who do not reach target levels of LDL-c with the available medications, cannot tolerate statins, or who experience painful side effects with statins, such as muscle pain, cramps, weakness to myopathy, and, rarely, rhabdomyolysis. Notably, homozygote FH patients who have minimal LDLR activity left can now be treated with PCSK9 mAbs, resulting in a ~30% decrease in circulating LDL-c (Raal et al., 2015). In these FH patients, switching from two to three times weekly LDL apheresis to a PCSK9 mAb combined or not with once per week LDL-c apheresis maintained the LDL-c-lowering effect, thereby giving a much better quality of life to these patients that is less dependent on the use of biweekly long sessions to clear LDL-c from their blood using special apheresis dialysis columns (Lappègård et al., 2016). Although the outcomes of the various ongoing phase 3 clinical trials using PCSK9 mAbs will not be known until 2017–2018 (Elbitor et al., 2016), already early signs revealed that this treatment results in a ~50% reduction in cumulative cardiovascular events within 1–2 years of treatment (Robinson et al., 2015; Sabatine et al., 2015). However, one should be cautious, as these results are only partial and not powered enough to unambiguously assess cardiovascular outcomes of prespecified endpoints in cardiometabolic diseases. Finally, the fact that PCSK9 is inactivated by some proteases such as furin (Benjannet et al., 2006; Essalmani et al., 2011) might open new strategies to enhance this inactivation mechanism, and thus lower the levels of active PCSK9. Indeed, recently a new mAb was reported that inhibits PCSK9 activity but still allows furin to inactivate it, resulting in a longer half-life of the antibody (Schroeder et al., 2015). The future will tell which strategies targeting PCSK9, including longer lasting mAbs, RNA interference (Fitzgerald et al., 2014), and small-molecule inhibitor approaches (Seidah, 2013), will find their way in dyslipidemia, cardiology, and sepsis in clinics worldwide, which would result in affordable and safe treatments and/or prevention of life-threatening conditions.

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Authorship Contributions

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