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REVIEW

Molecular and cellular biology of PCSK9: impact on glucose homeostasis

Sègbédé E. R. Tchéoubi^{a,b} , Casimir D. Akpovi^b , Frédérique Coppée^c , Anne-Emilie Declèves^c ,
Sophie Laurent^a , Clément Agbangla^d  and Carmen Burtea^a 

^aGeneral, Organic and Biomedical Chemistry Unit, Faculty of Medicine and Pharmacy, Research Institute for Health Sciences and Technology, University of Mons – UMONS, Mons, Belgium; ^bNon-Communicable Diseases and Cancer Research Unit, Laboratory of Applied Biology Research, University of Abomey-Calavi – UAC, Abomey-Calavi, Benin; ^cLaboratory of Metabolic and Molecular Biochemistry, Faculty of Medicine and Pharmacy, Research Institute for Health Sciences and Technology, University of Mons – UMONS, Mons, Belgium; ^dLaboratory of Molecular Genetics and Genome Analyzes, Faculty of Sciences and Technics, University of Abomey-Calavi – UAC, Abomey-Calavi, Benin

ABSTRACT

Proprotein convertase subtilisin/kexin 9 (PCSK9) inhibitors (PCSK9i) revolutionised the lipid-lowering therapy. However, a risk of type 2 diabetes mellitus (T2DM) is evoked under PCSK9i therapy. In this review, we summarise the current knowledge on the link of PCSK9 with T2DM. A significant correlation was found between PCSK9 and insulin, homeostasis model assessment (HOMA) of insulin resistance and glycated haemoglobin. PCSK9 is also involved in inflammation. PCSK9 loss-of-function variants increased T2DM risk by altering insulin secretion. Local pancreatic low PCSK9 regulates β -cell LDLR expression which in turn promotes intracellular cholesterol accumulation and hampers insulin secretion. Nevertheless, the association of PCSK9 loss-of-function variants and T2DM is inconsistent. InsLeu and R46L polymorphisms were associated with T2DM, low HOMA for β -cell function and impaired fasting glucose, while the C679X polymorphism was associated with low fasting glucose in Black South African people. Hence, we assume that the impact of these variants on glucose homeostasis may vary depending on the genetic background of the studied populations and the type of effect caused by those genetic variants on the PCSK9 protein. Accordingly, these factors should be considered when choosing a genetic variant of PCSK9 to assess the impact of long-term use of PCSK9i on glucose homeostasis.

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Introduction

Diabetes mellitus (DM) is a metabolic disorder characterised by a prolonged hyperglycaemia. DM is an important public health problem because of its direct cost, its economic burden and its disabling consequences such as blindness, heart attacks and stroke, kidney failure, lower limbs amputation [1, 2]. According to WHO, type 2 diabetes mellitus (T2DM) represents >90% of DM cases worldwide. Its prevalence is growing, especially in low- and middle-income countries such as sub-Saharan Africa, where particular features like ketosis-prone T2DM and a high frequency of acute and chronic T2DM complications exist [3, 4]. Although changes in habit and lifestyle contribute to the increase of DM incidence in these countries, genetic and epigenetic factors remain important players [1, 2, 5]. T2DM is in fact a complex polygenic disease that shows many threatening complications, including dyslipidemia [6] and its related cardiovascular [7] and nephrological disorders [8]. The management of the cardiovascular complications often requires the use of lipid-lowering drugs.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protein [9, 10] involved in cholesterol/lipoprotein metabolism through low density lipoprotein (LDL) receptor (LDLR) regulation. PCSK9

binds to LDLR and drives it into lysosomes for degradation, preventing LDLR recycling to the cell surface. *PCSK9* gene can be the subject of loss-of-function (LOF) mutations that are associated with low LDL-cholesterol (LDL-c) level in plasma, and gain-of-function (GOF) mutations characterised by LDLR depletion with ensuing elevated plasmatic LDL-c level. LOF mutations are frequent in the *PCSK9* gene. Taking advantage of this knowledge, many pharmaceutical companies are still racing for the development of cholesterol-lowering drugs, the so-called PCSK9 inhibitors (PCSK9i). While PCSK9i demonstrate high efficiency [11, 12], there are issues about the risk of T2DM incidence as seen with statins [11]. *PCSK9* has been indeed associated to many parameters related to glucose homeostasis [13–15] and the study of the link between DM and *PCSK9* is still of great interest and continues to mobilise the scientific community efforts, especially regarding the genetic aspects where many controversies exist. Since clinical use of PCSK9i is too recent, PCSK9 cholesterol-lowering genetic variants are often used as proxy to evaluate the long-term impact of PCSK9i [16, 17].

In this review, we will summarise current knowledge on the association of PCSK9 and T2DM with an emphasis on the genetic aspect and explore if all PCSK9 cholesterol-lowering variants can reflect the effects of PCSK9i on glucose homeostasis.

Pcsk9 biology

The gene and its mutations

PCSK9 is a serine protease discovered in 2003 separately by a Canadian and a French research teams [9, 10] as a negative regulator of LDLR. Formerly known as neural apoptosis regulated convertase 1 (NARC-1), PCSK9 is a member of the proprotein convertases (PC) family that includes PC1, PC2, PC4, PC5, PC7, the furin, the paired basic amino acid-cleaving enzyme 4 (PACE4) and the subtilisin kexine isoenzyme (SKI)-1 [18]. The *PCSK9* gene is located at chromosome 1p32.3. The canonical *PCSK9* gene encompasses 12 exons and 11 introns. Since the first mutations described by Abifadel and colleagues [9], many common and rare variants of *PCSK9* have been identified including dbSNP: rs1874776, rs4926675, rs45448095, rs373507733, rs2479409 and rs11591147 [19–21]. To date, several hundred variant alleles have been described. The *PCSK9* variants can be classified according to several characteristics. The most used classification is based on the impact of the mutation on the protein activity and distinguishes LOF and GOF mutations (Tables 1 and 2). LOF mutations are associated with a reduction of the PCSK9 protein abundance and/or activity, which results in increased number of cell surface LDLR and low plasma LDL-c level. The C679X, Y142X and R46L are examples of LOF mutations [22–24]. In contrast, GOF mutations are characterised by an elevation of the PCSK9 protein abundance or/and activity, leading to an increase of LDLR turnover from the cell surface and high plasma LDL-c level. S127R, F216L and D374Y are examples of GOF mutations [9, 18].

However, based on the expression of recombinant proteins in cell culture, Horton *et al.* [25] proposed another classification that focuses on the functional defects observed in the synthesis, trafficking or secretion of the variant protein (Tables 1 and 2): (1) null allele with no detectable protein (Y142X); (2) variants that interfere with the autocatalytic cleavage prodomain and where the protein is not secreted but can interact with the LDLR in the secretory pathway between the cell membrane and the endoplasmic reticulum (ER) (S127R, Δ97R, G106R, L253F); (3) mutations where the transport of the normally cleaved protein from the ER to cell membrane is delayed (C679X) and retained in the ER; (4) LOF variants that affect the protein stability because of a furin-mediated post-translational cleavage (e.g.: A443T) and (5) variants that modify PCSK9 affinity to LDLR (GOF D374Y, F216L, F379A). Noteworthy, this classification may not distinguish some GOF from LOF as they show similar post-translational processing, e.g. both S127R (GOF) and L253F (LOF) impair autocatalytic cleavage and secretion of PCSK9. Therefore, the circulating level of PCSK9 may not reflect the type of mutation [26, 27].

The PCSK9 protein: structure and function

PCSK9 protein is expressed mainly in hepatocytes, and also at a lower level in intestines, lung, renal mesenchymal cells, pancreas, smooth muscle cells, endothelial cells, cardiomyocytes and macrophages [28–32]. It is synthesised as a 120KDa preproPCSK9 molecule, composed of 692 amino acids and comprising several domains (Figure 1). The N-terminal domain starts with a signal sequence (Met1-30Ala) that is cleaved in the ER. The prodomain of PCSK9 (Gln31-Gln152) that undergoes autocatalytic cleavage at a Val-Phe-Ala-Gln↓Ser-Ile-Pro site [33] in the ER but remains bound to the catalytic domain through the secretory pathway. This heterodimeric complex is required for exit from ER and secretion. The catalytic domain (Ser153-454Gln) where the proteolytic active site (so-called catalytic triad amino acids Asp186, His226 and Ser386) is inactivated by the non-covalently associated prodomain. The catalytic domain ends with a hinge region covering amino acids Glu405 to Gly452. This region contains the amino acid residues critical for PCSK9 secretion [34]. The cysteine- and histidine-rich C-terminal domain (CTD, Leu455-Gln692) which consists of 3 similar modules (M1, M2 and M3). Even if this CTD is not critical for LDLR binding, a body of evidences indicated that PCSK9 CTD is essential for LDLR-mediated endocytosis, its trans-Golgi network localisation, the affinity of its auto-catalytic domain and an effective degradation of PCSK9-LDLR complex in late endosomes/lysosomes [35].

After its synthesis, the PCSK9 binding to GRP94 (Glucose-regulated protein 94) in the ER prevents premature LDLR degradation *in situ* [35, 36]. PCSK9 transport from ER requires SEC24A subunit to be incorporated into coat protein complex II-coated vesicles [37, 38]. Moreover, there are conflicting data regarding the implication of the sorting receptors surfeit 4 (SURF4) [39, 40], sortilin and Amyloid precursor protein-like protein 2 (APLP2) in the PCSK9 secretion [41, 42].

PCSK9-induced LDLR degradation occurs both *via* intracellular (Golgi to lysosome) and extracellular endosomal routes (Figure 2). The intracellular pathway (where PCSK9 binds and shuttles newly formed LDLR directly from the trans-Golgi network to the lysosomal compartment) involves clathrin light chains a and b [43], whereas the extracellular one would be mediated by clathrin heavy chain [44] and requires LDLRAP1/ARH (Autosomal recessive hypercholesterolaemia protein) adaptors [45]. Gustafen *et al.* [46] proposed that heparan sulphate proteoglycans present at hepatocytes surface interact with the heparan sulphate-binding site contained in the PCSK9 prodomain and facilitate subsequent PCSK9:LDLR complex formation. Recently, in HepG2 cells and in mice liver tissue, the Src homology 3 binding domain (SH3BD) of adenyl cyclase-associated protein 1 (CAP-1) was demonstrated to bind directly to the CTD of PCSK9 (Figure 3). Then, CAP-1

Table 1. Classification of *PCSK9* natural LOF mutations based on functional defects and associated LDL-c level in plasma.

Variants	Exon number location	Class of mutation and functional defect					LDL-c level (mg/dL)	References
		Class1 Null	Class 2 Processing	Class 3 Transport from ER	Class 4 Stability	Class 5 Affinity for LDLR		
R46L	1	–	–	(higher retention in TGN)	?	?	86 ± 20	[24,158–160]
Δ97R	1	–	x	x	–	–	58	[160]
G106R	2	–	x	x	–	–	89	[24]
Y142X	3	x	–	–	–	–	53 ± 40	[22,159]
Q152H	3	–	x	? (blocks secretion)	–	–	77.34	[152,161,]
C679X	12	–	–	x	–	–	15–68 ± 27	[22,75,160]
L253F	5	x	x	–	–	–		[46,160,161]
S386A	7	–	x	–	–	–		[103]
A443T	8	–	–	–	?	?		[75,160]
R434W	8	–	–	? (reduced secretion) No localisation in TGN	–	–		[35]

–: no defect observed; x: defective; ?: not defined; ER: endoplasmic reticulum; TGN: Trans Golgi network.

Table 2. Classification of PCSK9 natural GOF mutations based on functional defects and associated cholesterol level.

Mutations	Chromosomal location	Class of mutation and functional defect					LDL-c level (mg/dL)	References
		Class1 Null	Class 2 Processing	Class 3 Transport from ER	Class 4 Stability	Class 5 Affinity for LDLR		
D374Y	7	-	-	-	-	x	350 ± 97	[18,161]
F216L	4	-	-	-	-	?	227 ± 112	[9,162]
R218S	4					?	216 ± 48	[163]
D129G	2			(reduced secretion); increased TGN accumulation		?		[52,53]
S127R	2	-	x	x	?	?	287 ± 72	[9,162,164]
L108R	2					?	266 ± 53	[52,53]
D35Y	1					?	249 ± 19	[53]
R357C	6					?	112.14	[165]
S636R	12					?	255.2	[166]

-: no defect observed; x: defective; ?: not defined; ER: endoplasmic reticulum; TGN: Trans Golgi network.

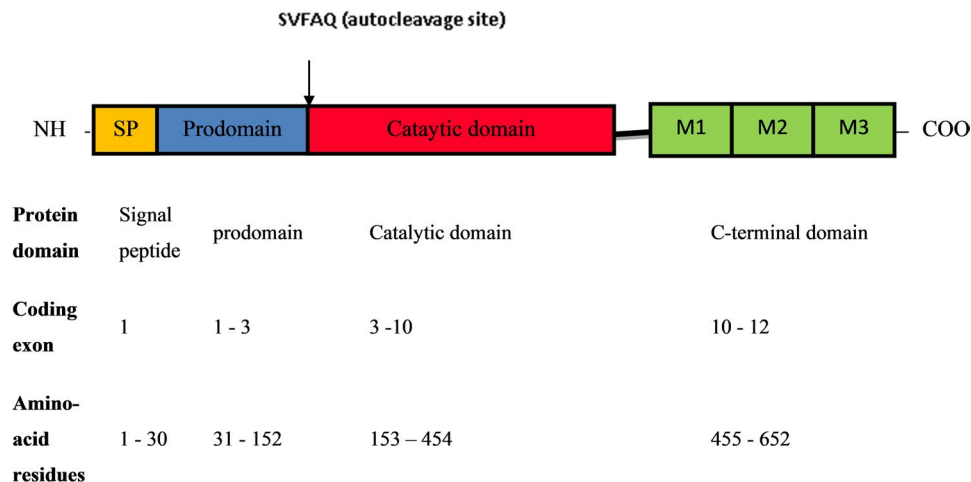


Figure 1. The domains of the PCSK9 protein. The protein comprises a signal peptide, a prodomain, a catalytic domain which ends with a hinge region (aa 405-452) and a C-terminal cysteine-rich, histidine-rich domain composed of 3 modules M1 (aa 457-528), M2 (aa 534-601) and M3 (aa 604-692).

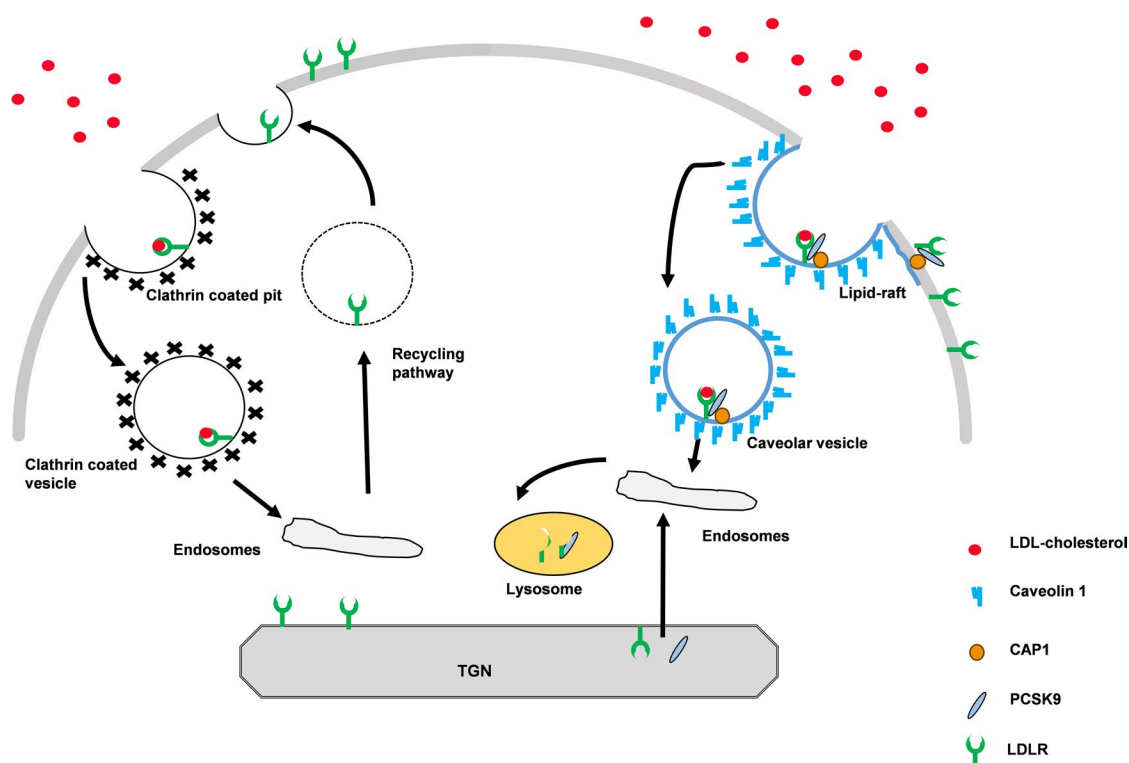


Figure 2. Schematic representation of extracellular and intracellular pathway of PCSK9 induced degradation of LDLR. LDLR: Low density lipoprotein receptor; TGN: Trans Golgi network; CAP1: Cyclase-associated protein 1; PCSK9: Proprotein convertase subtilisin kexine 9

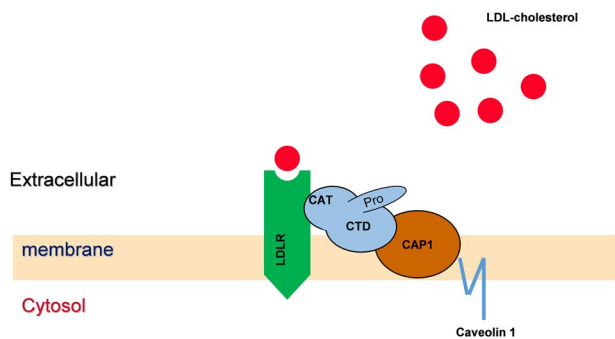


Figure 3. Schematic representation of PCSK9 interaction with the LDLR involving CAP1. LDLR: Low density lipoprotein receptor; CAP1: Cyclase-associated protein 1; PCSK9: Proprotein convertase subtilisin kexine 9; CAT: catalytic domain; CTD: C-terminal domain; Pro: prodomain

guides PCSK9:LDLR complex to undergo caveolae-dependent endocytosis in the lipid rafts and promotes LDLR degradation in lysosomes [47] (Figure 2) rather than the clathrin-coated pits hypothesis previously evoked in the HuH7 cells (hepatocytes). Noteworthy, PCSK9 and LDLR interaction was shown independent of CAP-1.

The catalytic activity of PCSK9 is not required for the degradation [48] of LDLR and its closest family members, such as the very low-density lipoprotein receptor (VLDLR), the apolipoprotein E receptor (LRP1/APOER) and the apolipoprotein E receptor 2 (ApoER2) [49]. PCSK9 also degrades the epithelial Na channel (ENaC), the β -secretase 1 β -site APP-cleaving enzyme 1 (BACE1), the cluster of differentiation 36 and 81 (CD36 and CD81) [50]. Precisely, PCSK9 amino acids 367 to 380 in the catalytic domain bind to the epidermal growth factor-like A domain (EGF-A) of LDLR in a pH-dependant manner, the affinity of the interaction increasing at acidic pH (pH values 6.5-5.5) [51]. The later interaction of the CTD with the LDLR ligand binding domain promotes the failure of the LDLR to adopt a closed conformation resulting in its shedding by γ -secretase [34]. Additional to the salt bonds, interactions such as van der Waals interactions between L108 of PCSK9 and the LDLR β -propeller domain [52, 53] potentiate the N-terminal domain (prodomain) binding to LDLR [54]. Consequently, PCSK9 remaining bound to LDLR in the early endosome, it allows LDLR ectodomain cleavage by a cysteine cathepsin. The cleaved ectodomain then goes for further degradation in lysosomes [55]. Another mechanism proposed for PCSK9-induced LDLR degradation involves a putative transmembrane protein that would connect PCSK9 CTD to cytosolic adaptors and divert the PCSK9:LDLR complex to lysosomes [56].

Transcriptional regulation of PCSK9

Many transcription factors and cofactors were found to bind the PCSK9 promoter [57–59]. The most known transcription factors are sterol-response element (SRE) binding proteins (SREBPs). SREBPs are known to activate enzymatic cascades involved in cholesterol, phospholipid, fatty acid and triglyceride biosynthesis [60]. In transgenic mice, SREBP-1a or SREBP-2 overexpression was associated with a significant increase in PCSK9 expression [61] whereas the knock-out of SREBP-2 resulted in decrease of its expression [62]. Of note, SREBP-2 upregulates LDLR expression as well [63], which could somewhat mitigate the effect of sole PCSK9 upregulation.

Hepatocyte nuclear factor 1 (HNF1)-binding site is also present in the PCSK9 promoter. HNF1- α cooperate with SREBP to regulate PCSK9 at transcriptional level [64, 65]. In a recent study, Shende *et al.* [66] showed that the liver specific knock-down of HNF1- α in mice

is associated with up-regulation of liver LDLR protein and decreased serum level of PCSK9 and LDL-c. The mammalian target of rapamycin complex 1 (mTORC1)/PK δ pathway, whose activation by insulin inhibits HNF1- α , reduces PCSK9 expression in hepatocytes [67].

Another factor is also found in association with the PCSK9 promoter: the histone H4 transcription factor (HiNF-P), which bind a sequence between the SRE and HNF1 binding sites. Activation of HiNF-P enhanced PCSK9 transcription through histone H4 acetylation [59]. In addition, histone deacetylase Sirtuin (Sirt) 6 recruitment to the PCSK9 promoter by forkhead-box protein O3 (FoxO3) [68] as well as bile acid receptor resulted in HNF1 activity suppression, thereby repressing PCSK9 transcription [69].

Peroxisome proliferator-activated receptor (PPAR) isoforms are involved in lipid homeostasis and decreased SREBP-2 [70]. It has been shown that PPAR regulates PCSK9 in various manners. PPAR- α attenuated PCSK9 promoter activity, which resulted in PCSK9 transcription down-regulation and in addition increased plasma PCSK9 furin/PC5/PC6 cleavage in hepatocytes [71]. On the contrary, PPAR- γ induced PCSK9 mRNA and protein expression in HepG2 cells by inhibiting extracellular-regulated kinases (ERK) 1/2 activity; whereas, PCSK9 expression is decreased subsequent to ERK1/2 activation by epidermal growth factor (EGF) [72, 73]. Finally, Liver X receptor (LXR) increases PCSK9 expression [69, 74]. So PCSK9 regulation appears very complex, suggesting it implication in many pathways.

Circulating PCSK9 regulation and elimination

PCSK9 circulates in an intact heterodimeric form of apparent molecular weight of 75 kDa and a truncated form of 52-55 kDa that can reach 30-40% of the circulating PCSK9 [75]. The truncated form was thought to be a product of cleavage by furin [75–77] or hepsin [78]. The circulating amount of PCSK9 ranged from 33 ng/mL to 2988 ng/mL, with a median value of 517 ng/mL in women and 450 ng/mL in men [79]. In fact, the plasma level of PCSK9 level is regulated by many physiological factors as described hereafter.

Nychthemeron

Circulating PCSK9 follows a diurnal rhythm synchronous with cholesterol synthesis. The lowest plasma concentration of PCSK9 has been detected between 3:00 and 9:00 p.m. while a peak is observed around 4:30 a.m. These variations are approximately $\pm 15\%$ around the mean level and seem to be an effect of the growth hormone [80]. This finding leads Cariou and colleagues to suggest that plasma PCSK9 dosage should be scheduled early in the morning [26].

Age and gender

Several studies pointed out that circulating PCSK9 concentration is higher in women than men. These sex differences start early in childhood till menopause, even post-menopause. A study revealed that in neonates, female exhibit higher serum PCSK9 level than male [81]. This observation remains true in childhood, adolescence [13] and adulthood as well [82]. Furthermore, circulating PCSK9 level is shown to be 22% higher in postmenopausal than premenopausal women [83], and in pregnant at parturition than non-pregnant women [84], suggesting that sex hormones may play a role in PCSK9 levels regulation. The observed sex variations in PCSK9 level persists even in patients with acute myocardial infarction [85].

Plasma level of PCSK9 varies according to age, especially in childhood and adolescence, in a sex dependant manner. While plasma PCSK9 concentration decreases in men, it increases in females from 9 to 13-16 years of age [13], in parallel with

cholesterol level at puberty [86]. Older men had also shown significantly higher levels of PCSK9 than younger men [87].

To address the effects of sex hormones on circulating PCSK9, the clinical trial NCT00848276 was conducted. It found that in men, testosterone did not affect PCSK9 level, except for a possible effect during testosterone ablation, whereas oestradiol was inversely related to plasma PCSK9 in women [88]. Surprisingly, oestradiol treatment showed no effect on plasma PCSK9 level, indicating that the oestradiol-related differences in PCSK9 levels may be the result of differences in receptor-mediated PCSK9 clearance through oestradiol-induced changes rather than production of PCSK9 and the lack of oestrogen after menopause may not be the only cause for the increase in circulating PCSK9 in ageing females [79, 88]. This confirms that gender difference in lipid and lipoprotein metabolism is not just a matter of sexual hormones [89]. Nevertheless, induction of endogenous oestrogen in women undergoing *in vitro* fertilisation reduced the circulating PCSK9 levels [89]. In addition, Ghosh's work demonstrated that during ovarian cycle, women exhibit lower PCSK9 levels in the ovulatory and the luteal phases than in the follicular phase [83]. Altogether, it appears clear that there are gender differences regarding the circulating PCSK9 concentrations. Moreover, in female gender, the modulation of its circulating levels might even be more complex. Further investigations are certainly needed to better understand these observations.

Diet

There is also a link between PCSK9 level and diet. For example, it has been showed that fasting decreased plasma PCSK9 level in healthy volunteers in a range of 20-35% after 18h, or more if fasting was prolonged [80, 90]. Conversely, circulating PCSK9 increased with refeeding [80]. Similar variations were observed in animal liver (at the PCSK9 protein and mRNA levels) [86]. It is suggested that this post-prandial PCSK9 elevation is due to insulin effect through the upregulation of SREBP1 [58]. Wu *et al.* [91] thought that low dietary cholesterol concentrations suppresses SREBP2 expression and decreased PCSK9 protein level in the course of fasting. Upon sterol depletion, PCSK9 expression increased for 24h in HepG2 cells [91]. Similarly, Mediterranean diet [92], oleic acid enriched diet [93] or polyunsaturated fatty acid (PUFA) diet [94] consumption reduced PCSK9 levels. However, plasma PCSK9 level increased upon a short-term high fructose diet in humans [95].

Physical activity

It was reported that physical exercise reduced hepatic expression and plasma levels of PCSK9 in rodents [96, 97]. Several studies have addressed in various ways the effects of physical activity on plasma PCSK9 level in human as well. While some of them found no significant correlation between PCSK9 levels and physical activity in abdominally obese and sedentary subjects [14] or subjects with high risk of T2DM [98], other studies observed decrease [99] in healthy subjects or increase [100] in men with cardiometabolic risk factors. These discrepancies may reside in the difference in the patients' conditions and physical activity exposures, a lack of standardised physical activity measurements methods or the design of the studies.

Moreover, PCSK9 levels can be regulated by many other factors like lipid lowering agents (statins, fibrates, glytazones), nutraceuticals and diseases (nephrotic syndrome, myocardial infarction) [86].

PCSK9 elimination

Many mechanisms are proposed to explain the elimination of PCSK9. It was observed that iodinated PCSK9 accumulated mainly

in liver and kidney [101]. Based on the observation that plasma PCSK9 half-life increases from 5 to 15min in wildtype as compared with *LDLR*^{-/-} mice [102], it was suggested that LDLR:PCSK9 complex degradation in lysosomes is a principal way of PCSK9 elimination [30]. Scientific observations revealed that there is a delay between the initial interaction of PCSK9 with LDLR and the final LDLR degradation [45]. For instance, when PCSK9 encounters LDLR, the newly formed LDLR:PCSK9 complexes take 2-3h to reach lysosomes [45]. Then, LDLR:PCSK9 complex degradation *in vitro* is effectively observed 12 to 24h after the addition of PCSK9 to the medium. Besides, humans treated with PCSK9i therapy showed significant LDL-c lowering only after 2-3 days of treatment [45].

Nevertheless, in the absence of LDLR effect, PCSK9 removal rate remains high indicating there is another important pathway of PCSK9 clearance [103]. Other proposed mechanisms are the proteolytic degradation and the pathway *via* APLP2 [41].

PCSK9 and diabetes-related conditions

T2DM and insulin resistance are two closely linked conditions. In addition to metabolic disorders, pro-inflammatory factors are the most important causative factors of T2DM and insulin resistance. Hence, we review hereby the association of PCSK9 with low grade systemic inflammation and insulin resistance, both common in T2DM patients.

PCSK9 and low-grade systemic inflammation

Besides lipid metabolism regulation, PCSK9 plays a critical role in inflammation. Early in 2013, Li and his colleagues found a positive correlation between plasma PCSK9 and white blood cells count and its subsets [104], suggesting a PCSK9 association with chronic inflammation. This corroborated the positive correlation observed between serum PCSK9 levels and C-reactive protein (CRP) in the whole population Dallas Heart Study [79]. This finding was confirmed among type 1 diabetic patients and in accelerated atherosclerosis of systemic lupus erythematosus patients [105]. In the meantime, it has been proven that CRP increased PCSK9 expression by activating p38 mitogen activated protein kinase (p38MAPK)-HNF1- α pathway *in vitro* in HepG2 cell [106].

PCSK9 and pattern recognition receptors of innate immune cells

Pattern recognition receptors (PRR) recognise pathogen-associated motif pattern (PAMPs) like pathogen lipids (lipopolysaccharide (LPS), lipoteichoic acid and phospholipomannan), which eventually results in inflammatory response [106]. The well described PRR involved in this interaction are Toll-like receptors (TLR). In fact, LPS binds to TLR4 and activates nuclear factor (NF)- κ B pathway, thus inducing pro-inflammatory cytokines and chemokines [106]. Lipoteichoic acid and glycolipids of fungi induce inflammation in the same way, respectively *via* TLR2, TLR4 and TLR6 [107,108].

Pathogen lipids in the host, firstly sequestered in HDL, are then transferred to LDL, VLDL and chylomicrons [109]. However, there is an equilibrium between lipoprotein-associated and non-lipoprotein-associated pathogen lipids [110]. While incorporation of pathogen lipids into LDL particles hampers their binding to TLR, it fortunately enables their hepatic clearance through LDLR and bile secretion [108]. PCSK9 may enhance inflammatory response in subjects with sepsis. Accordingly, in many cohorts of PCSK9 LOF patients with sepsis shock, a lower inflammatory response was described as compared to GOF mutations carriers [111]. The evidence of LDLR (at least partly) mediation in that PCSK9 effect was brought by the observation that PCSK9 resistant

LDLR gene mutations improved survival of patients with septic shock [108]. Moreover, *LDLR*^{-/-} mice did not benefit from the effect of transcriptional reduction of PCSK9 expression conversely to wild type. In line, PCSK9 inhibited LPS uptake by human liver cells and increased inflammation in mice [111, 112].

Furthermore, overexpression of PCSK9 in apolipoprotein E (APOE) KO mice was accompanied by increased accumulation of macrophages in atherosclerotic lesions and elevated expression of TLR4 and NF-κB compared to mice with *PCSK9* RNA silencing, which showed opposite effects [113]. In addition, increased levels of phosphorylated IκBα, of IκBα degradation, and of nuclear translocation of NF-κB was seen in macrophages exposed *in vitro* to high PCSK9 levels, either overexpressed or added in the culture medium. These data indicated that PCSK9 triggers inflammation involving TLR4/NF-κB pathway. Obviously, such modifications in TLR4 and NF-κB, key regulators of inflammatory response, are followed by variations in cytokine levels [113].

In the gut epithelium, the binding of absorbed LPS to TLR-2 and TLR-4 also activated JNK and IKKβ/IκBα/NF-κB pathways. [114].

In addition, PCSK9 was shown to regulate oxidised-LDL (oxLDL) uptake in macrophages [32, 115]. Elevation of PCSK9 level can induce up to five-fold upregulation of oxLDL uptake *via* overexpression of scavenger receptors (LOX-1, SRA and CD36), which in turn triggers subsequent ox-LDL induced inflammatory cytokines expression.

Thus, PCSK9 is involved in inflammation both at systemic and local level. At a systemic level, PCSK9 exerts its pro-inflammatory effects *via* LDLR. Locally, elevated PCSK9 is associated with macrophage recruitment, increased expression of cell receptors (TLR, SRA, CD36, LOX-1) and activation of TLR, NF-κB and JNK pathways. The subsequent secretion of pro-inflammatory cytokines may lead to the inactivation of IRS-1 and 2 and therefore, to the impairment of insulin sensitivity.

PCSK9, chemokines and cytokines

The above-mentioned activation of TLR4/NF-κB pathway by PCSK9 increased the expression of inflammatory markers like TNF-α, IL-1β and MCP-1 both at mRNA and protein levels [113], IL-6, CXCL2 and CCL2 [116]. Experiments in macrophages cell lines (THP-1 and RAW264.7) and in murine and human primary macrophages revealed induction of inflammatory biomarkers by PCSK9. In fact, Ricci and colleagues [116] showed that THP-1 and human primary macrophages exposed to 2.5 μg/mL of PCSK9 for 24 h presented an induction of ≈2-9 fold of TNF-α, IL-1β, CXCL2 and MCP-1 mRNA expression. These increments were lesser in murine *LDLR*^{-/-} than *LDLR*^{+/+} bone marrow macrophages, suggesting once more that LDLR is involved in pro-inflammatory effects of PCSK9 on macrophages. However, recombinant PCSK9 treatment results in similar increase of TNF-α in *LDLR*^{-/-} mice and wild type, thereby indicating that another pathway may be involved in PCSK9-induced inflammatory response. On the other hand, PCSK9 is regulated by many cytokines, including adipokines [73, 117–119].

First, *PCSK9*^{-/-} mice present higher visceral adiposity and their adipocytes are hypertrophied [120]. Adipocyte hyperplasia and hypertrophy are characteristics of adipose tissue inflammation. Indeed, in an obesity context, there is an accumulation of M1 pro-inflammatory macrophages in adipose tissues [121]. Increased infiltration of M1-polarized macrophages [121] results in higher secretion of pro-inflammatory cytokines and chemokines. These cytokines are described to upregulate the *PCSK9*, *ApoB*, *SREBP1* and other *de novo* lipogenesis genes expression in HepG2 cell line [117]. For instance, TNF-α, leptin and resistin increased *PCSK9* mRNA level *via* JAK/STAT pathway. The observed *PCSK9* induction

by TNF-α is totally blocked by siRNA targeting *SOCS3* (Suppressor of cytokine signalling 3) that inhibits the JAK/STAT pathway. Similarly, siRNA targeting *STAT3* also blocked the effect of TNF-α on *PCSK9* expression [118]. It was also reported [119] that resistin increased *PCSK9* mRNA and protein expression possibly *via* *SREBP2* and post-translational stabilisation of the *PCSK9* protein.

Another major adipokine, adiponectin is well-known for its anti-inflammatory, insulin sensitising and thus antidiabetic and cardioprotective effects [122]. Adiponectin exerts its effects by binding to its receptors. Two main adiponectin receptors (AdipoR) were described, AdipoR1 and AdipoR2 [123], either of them being involved in the regulation of inflammation, oxidative stress, glucose and lipid metabolism [123, 124]. While AdipoR1 activity involves AMP-activated protein kinase (AMPK) activity, the lack of AdipoR2 reduces PPAR-α activity [124]. In an elegant study, AdipoR activation has been shown to induce *PCSK9* expression through increasing PPAR-γ mRNA and protein levels and activation of PPAR-responsive element that is present in the *PCSK9* promoter [73]. Adiponectin effects on *PCSK9* may appear complex, since AdipoRs activation induced both PPAR-α and PPAR-γ that have opposite effects on *PCSK9*. ADP355, an AdipoR agonist that mainly activates AdipoR1, increases *PCSK9* expression in mice by the activation of AMPKα and subsequent PPAR-γ upregulation. However, AdipoR2 activation slightly affected *PCSK9* expression as shown in AdipoRs silencing experiments probably *via* PPAR-α [73].

PCSK9, insulin, glucagon and insulin resistance

Several studies showed that insulin *via* its receptors upregulate *PCSK9* gene transcription and protein level. Epidemiological data found significant correlation between *PCSK9* and insulin, *homeostasis model assessment of insulin resistance* (HOMA-IR) [13, 79] and glycated haemoglobin [125]. However, there is an apparent discrepancy in studies regarding the influence of insulin on *PCSK9*, suggesting a complexity of their relationship. While hyperinsulinemic glucose clamp in healthy and T2DM subjects did not affect plasma *PCSK9* level [125], it decreased *PCSK9* concentrations in post-menopausal obese patients [126] and hyperinsulinemia increased *PCSK9* expression in mice [58]. In small intestine of obese patients with insulin resistant, *PCSK9* transcription was downregulated [50]. Regarding T2DM, some studies revealed no difference in plasma *PCSK9* levels of T2DM, pre-diabetic and individuals with normal glucose metabolism [125, 127], whereas other studies found elevated *PCSK9* concentration in T2DM patients [127]. Mechanistically, chronic hyperinsulinemia led to inactivation of the IRS pathway [67], and consequently inhibited HNF-1α, a positive regulator of *PCSK9*. It is then suggested that (1) the overall effect of insulin on *PCSK9* level is neutral [86] and (2) different pathways with opposite effects (at least inhibition of HNF-1α *via* mTORC versus SREBPs) must be involved in *PCSK9* regulation in simultaneous hyperglycaemia and hyperinsulinemia conditions [50].

Glucagon is another important hormone involved in glucose homeostasis, T2DM pathogenesis [128, 129] and lipid regulation [130]. Its relationship with *PCSK9* was pointed out by Spolitu *et al.* when investigating the role of glucagon receptor (Gcgr) in plasma cholesterol regulation [128]. The authors demonstrated that mice injected with glucagon exhibited decreased plasma *PCSK9* and LDL-c levels without change in VLDL-c, HDL-c or body weight compared to controls. As expected, Gcgr silencing or blocking lowered blood glucose level and increased plasma *PCSK9* level. However, no change was observed in hepatic *PCSK9*, LDLR and their transcription factors HNF-1α and SREBP2 at the mRNA

level. They reported that Gcgr signalling modulates PCSK9 through its lysosomal degradation and the exchange protein directly activated by the cAMP-2 (Epac2) and Ras-related protein-1 (Rap1) pathway.

Taken together, available data suggest that insulin upregulates, whereas glucagon down-regulates PCSK9 expression.

PCSK9 and type 2 diabetes mellitus: genetic aspects

Evidence from animal models

In murine models, four main studies - with opposite results - have caught our attention. The first one suggested that PCSK9 deficiency does not alter insulin secretion [131]. These authors detected the PCSK9 expression in δ -cell, but not in β -cells of human pancreatic islets. In 2-3 months-old male PCSK9^{-/-} mice, they showed that PCSK9 was not involved in β -cell apoptosis nor impaired insulin secretion. According to their findings, PCSK9 deficiency led to a 200% increase of LDLR protein in β -cells but did not alter cholesterol content nor glucose-stimulated insulin secretion. Moreover, *in vivo* glucose tolerance test was found similar in PCSK9^{+/+} and PCSK9^{-/-} mice.

One year later, another team published a totally contradictory result in older C57BL/6 mice of both sexes. These authors detected PCSK9 mRNA and protein expression in β -cells and gave evidence that lack of PCSK9 led to cholesterol accumulation in β -cells, insufficient insulin secretion and elevated plasma glucose level in mice of both sexes aged of more than four months [132]. Especially, glucose intolerance was observed in male PCSK9^{-/-} mice, not in female. Besides, their islets displayed inflammation and apoptotic features. Nevertheless, their findings were similar to those of Langhi *et al.* in mice before 4 months, suggesting that plasma levels of insulin and glucose impairment is not only gender- but also age-dependent.

To confirm this hypothesis, Mbikay's team assessed effects of gender and diet on lipid and glucose homeostasis by comparing 8 months old PCSK9^{-/-} mice with gender and aged-matched wild type mice [133]. On a normal or a western diet, PCSK9^{-/-} mice of both sexes exhibited low lipid level and pancreatic islet dysmorphism, with large possibly immature secretory granules. Female PCSK9^{-/-} mice on either diet showed higher plasma glucose and impaired glucose-stimulated insulin secretion. In addition to this phenotype, male mice exhibited glucose intolerance.

Later, low serum insulin level and glucose intolerance, but not insulin intolerance, increased insulin content and size of Langerhans islets were confirmed in PCSK9^{-/-} mice [134]. These results were reverted in PCSK9^{-/-}/LDLR^{-/-} mice. Then, authors concluded that the observed insulinopenia was in relation with the effect of PCSK9 on pancreatic LDLR.

Recently, an elegant study [135] investigated the molecular mechanisms that underline the association of PCSK9 LOF mutations with T2DM. The authors demonstrated that PCSK9 deficiency increased T2DM risk by altering insulin secretion but not peripheral insulin sensibility. Furthermore, they showed that liver-specific KO of PCSK9 in mice does not modify β -cell LDLR expression and interestingly preserve insulin secretion and glucose homeostasis. According to their results, reduction of local pancreatic PCSK9 level upregulates β -cell LDLR expression which in turn promotes intracellular cholesterol accumulation and hampered insulin secretion. To ascertain this hypothesis, a model of β -cell-specific KO of PCSK9 (β KO) (Pcsk9flox/flox; Tg(Ins1-cre/ERT)+/0) mice was generated [136]. Contrarily to whole-body KO of PCSK9 in mice, β KO mice showed normal level of plasma cholesterol, with a

downregulation of *Ldlr* (-32%) and *Hmgcr* (-29%) genes and an unchanged LDLR protein level in β KO cells. These data suggest that a regulation mechanism occurs in β KO islets to stabilise LDLR protein levels, an action that should prevent cholesterol induced β -cell toxicity and altered glucose homeostasis by keeping cholesterol uptake in a normal range. Noteworthy, the PCSK9 gene inactivation rate observed in β KO islets was estimated to be ~38%. Using both β KO and whole-body KO mice, authors demonstrated that loss of PCSK9 does not affect β -cell function and glucose homeostasis. Indeed, β KO mice exhibited normal glucose tolerance, glucose stimulated insulin secretion and insulin sensitivity. Islets also presented *ex vivo* glucose-stimulated insulin secretion in presence or absence of fatty acids similarly to the wild type. However, the increased secretion of insulin by β KO islets is likely inherent to the presence of Tg(Ins1-cre/ERT) [136]. Such findings, in agreement with those described by Langhi *et al.* in PCSK9 KO mice [131], support the view that loss-of function PCSK9 variants, like KO mice, are not expected to cause cholesterol accumulation and toxicity in β -cells. Indeed, the concomitant reduction of circulating LDLc seems to allow only a modest cholesterol intake by β -cells.

Overall, discrepancies persisted in animal models and may be at least attributed to the differences in the genetic background or the age of the mice. Moreover, pancreatic PCSK9 inhibition in the β KO mice islets was showed to be partial. In addition, the impact of circulating PCSK9 inhibition on the *Ldlr* gene regulation (as seen in β KO mice) remains to be investigated. Accordingly, conclusion from this study about the safety of PCSK9i should be considered carefully.

Evidence in human beings

In a Dutch cohort of 63,320 participants of a cross-sectional study, T2DM risk among familial hypercholesterolaemia patients (1.75%) was lower than unaffected subjects (2.93%) [137] and occurred through mechanisms reviewed elsewhere. Here, we focus on the effects of PCSK9 LOF mutations that are associated with reduced level of LDL-c.

It is now well established that statins have a diabetogenic effect due, at least partly, to cholesterol lowering [138]. This raised concerns at the new lipid lowering strategies with PCSK9i. Since PCSK9i are relatively new therapies, PCSK9 LOF mutations are proxies used to assess the link of PCSK9 mutations with T2DM. In this section, we summarise the published papers describing genetic associations of PCSK9 with T2DM or diabetic related parameters.

A typical case of HbA1c doubling after PCSK9i therapy was reported in 2019 in a 70years old man with history of cerebrovascular accident, hyperlipidaemia and T2DM [139]. After exhibiting intolerance to four different statins, ezetimibe and colesvelam, he was then shifted to PCSK9i namely Evolocumab, when an increase of HbA1c from 6.7% at baseline to 7.8% was observed 4 months later. Evolocumab treatment was replaced by Alirocumab and HbA1c level reached 13.7% after 5 months of treatment.

Similar to the above-mentioned effect of PCSK9i, a genetic study revealed an elevated risk of prediabetes/diabetes in familial hypercholesterolaemia patients carrying the InsLeu PCSK9 LOF variant [140]. InsLeu variant is a cholesterol-lowering variant affecting the signal peptide required for PCSK9 to enter the secretory pathway. It was suggested that the in-frame insertion of 1 or 2 Leu residue(s) in the signal peptide impairs PCSK9 cellular secretion, then the carriers of this polymorphism showed a lower PCSK9 plasma level [140, 141]. In a cohort of 764 patients, Saavedra and colleagues observed a proportion of 10% of prediabetic and

diabetic stages in the variant carriers versus 6% in non-carriers, in line with impairment of fasting glucose (31.3% versus 14.3%) [140].

Conversely, in a cohort of 1,745 healthy French Canadian infants of 9–16 years old [141], no alteration in glucose homeostasis/diabetes parameters (fasting plasma glucose, and insulin, HOMA-IR, adiponectin, leptin, and high-sensitivity C-reactive protein) was observed in *InsLeu* carriers compared with non-carriers. Another LOF variant, namely R46L, demonstrated a synergistic effect with ApoE genotype, translated by a 2-fold increase in insulin, HOMA-IR and leptin concentrations in ApoE3/E2 carriers compared with those without ApoE3/E2. However, the association of R46L variant to glucose homeostasis parameters are inconsistent. While a prospective study in French participants from the 'Data from an Epidemiological Study on the Insulin Resistance Syndrome' (DESIR) depicted no modification in fasting glucose, fasting insulin, HbA1c, HOMA- β , HOMA-IR, T2DM risk or incident T2DM in R46L variant carriers [142], another demonstrated that R46L lowered HOMA- β not HOMA-IR [135].

Surprisingly, by contrast to the idea that LOF PCSK9 variants may be associated with a risk of T2DM, recent studies in Black South African people revealed that the high frequency LOF C679X variant is associated with low fasting glucose but not glycated haemoglobin [143, 144]. In a 5 years prospective study [143] among 737 apparently healthy participants of both sexes, older than 35 years, authors examined the association of lipid and glucose related parameters with two LOF PCSK9 variants (C679X and A443T). They found that both C679X and A443T variant carriers were associated with reductions in LDL-c of -0.98 mmol/L [$(-1.29, -0.67)$; $p < 0.001$] and -0.39 mmol/L [$(-0.57, -0.20)$; $p < 0.001$] respectively. Nevertheless, only the nonsense C679X variant was associated with a -0.37 mmol/L [$(-0.61, -0.13)$; $p = 0.002$] reduction of fasting glucose level, not glycated haemoglobin. However, the mechanisms underlying this alteration remain unknown. This glucose lowering effect was found to start earlier in infancy. In adolescents of both genders aged of 18.0 ± 0.5 years from the Birth to Twenty (Bt20) Plus cohort, Chikowore *et al.* [144] confirmed a significantly lower fasting glucose of 0.30 mmol/L [$(-0.57, -0.02)$; $p = 0.035$] compared to non-carriers.

Several meta-analyses have also addressed the question at a larger scale. First of all, it is worthy to mention that in spite of the FOURNIER and ODISSEY studies [11, 12, 145] and the OLSER-1 extension study [146] that support the absence of link of PCSK9i with new onset T2DM, a meta-analysis of 20 phase 2/3 randomised clinical trials assessing PCSK9i versus placebo with a median follow-up of 78 weeks [147] demonstrated that PCSK9i induced increased fasting glucose [weighted mean difference 1.88 mg/dL (95% CI 0.91–2.68); $I^2 = 0\%$; $p < 0.001$] and HbA1c [0.032% (0.011–0.050); $I^2 = 15.5\%$; $p < 0.001$]. If this elevation is not sufficient to induce T2DM in short term, the intensity of the PCSK9i therapy ($p = 0.029$) and its long-term use ($p = 0.026$) may increase diabetes risk.

As far as genetic studies in human beings are concerned, many mendelian randomisation studies [17, 148–150] comprising hundreds of thousands of participants from various ethnic backgrounds indicated that some LDL-c lowering genetic variants of PCSK9 are linked with either higher fasting glucose, risk of T2DM, or other T2DM risk factors. A very interesting study [150] explored the association of T2DM and related biomarkers with four PCSK9 variants (rs11583680, rs11591147, rs2479409, and rs11206510) of which LDL-c-lowering effect ranged from -0.002 (-0.03 ; -0.02) mmol/L for rs11583680 to -0.34 (-0.036 , -0.032) mmol/L for rs1159147. The combined genetic score was associated with an increased bodyweight [1.023 kg (0.24–1.82)], waist-to-hip ratio [0.006 (0.003–0.010)], risk of T2DM [OR 1.29 (1.11–1.50)] and

fasting blood glucose [0.09 mmol/L (0.02–0.15)] per mmol/L lower LDL-c. However, no significant association was observed with HbA1c [0.03% (-0.01 – 0.08)], fasting insulin [0.00% (-0.06 – 0.07)] and body mass index [0.11 kg/m² (-0.09 – 0.30)]. These results were in accordance with former observations for other genetic polymorphisms affecting LDL-c levels, especially 3-hydroxy-3-methylglutaryl-co-enzyme A -reductase (*HMGCR*) variants [148]. Of note, the combined effects observed were largely driven by PCSK9 rs1159147 (R46L), probably due to its higher effect on LDL-c level. Many evidences, even conflicting, exist regarding pancreatic islets disruption in low LDL-c level conditions [131–135]. Nevertheless, an important heterogeneity was observed in T2DM genetic association elsewhere [149] when examining T2DM association with LDL-c-lowering alleles in or near Neemann-Pick type C1-like 1 (NPC1L1), *HMGCR*, PCSK9, ATP-Binding cassette transporter (ABC)G5/G8, and LDLR ($I^2 = 77.2\%$; $p = 0.002$).

To confirm these findings, Nelson and colleagues examined the link between the PCSK9 rs1159147 (T allele) variant with 80 diseases and traits [17]. The variant was confirmed to be significantly associated with many of studied conditions including, not limited to T2DM risk ($p < 6.25 \times 10^{-4}$), increased obesity biomarkers (body mass index, waist circumference, waist-to-hip ratio), diastolic blood pressure, type 1 diabetes, and insulin use. Noteworthy, the association of T2DM with the T allele of rs1159147 is largely independent of its association with obesity markers, suggesting that the link of rs1159147 with T2DM might involve, at least partly, other mechanisms than β -cell lipotoxicity and low-grade inflammation often seen in obese persons.

Together, genetic evidence in animals and human indicated that excessive plasma PCSK9 lowering variants that are associated with pancreatic PCSK9 deficiency (likely what happens in some LOF mutations) may cause disruption of β -cell morphology and function, thus lower insulin secretion, induce β -cell apoptosis and eventually lead to DM. This set a safer prospect for PCSK9i like Inclisiran, a small interfering RNA molecule that is directed for liver-specific PCSK9 gene silencing [151].

The opposite observations in some LOF variants (C679X associated with low blood glucose level versus *InsLeu* associated with high blood glucose level), could be attributed to the probable differences in genetic background of the subjects included in the studies. Although this hypothesis remains to be elucidated, the polygenic nature of T2DM suggests that PCSK9 LOF variants associated-T2DM may occur in presence of other additional genetic variations. Moreover, PCSK9 LOF variants associated-T2DM may involve other mechanisms apart from the above described pancreatic LDLR-mediated insulin lowering effect. One can assume for example that the accumulation of cholesterol in ER via LDLR upregulation could impair vesicles formation and maturation. Of note, contrarily to what could be expected, no ER stress was noticed in hepatocytes transfected with a PCSK9 LOF mutation (Q152H) affecting the catalytic domain in spite of the retention of misfold or uncleavable pro-PCSK9 in ER [152]. Indeed, PCSK9 remained bound mostly to GRP94 rather than GRP78, which protected cell from ER stress [153]. Nevertheless, to our knowledge, there is no such study reported in β -cells.

On the other hand, the cardioprotective effect of LOF variants may be responsible for a longer survival of the carriers who thereby could develop T2DM at later times.

PCSK9 and cardiovascular diseases

Cardiovascular diseases are the main macrovascular complications of T2DM, involving atherosclerosis. Atherosclerosis is now considered as a chronic inflammation disease of the artery wall.

Several studies showed that PCSK9 is an important player in atherosclerosis development. At a systemic level, serum PCSK9 promotes liver LDLR degradation and induces hypercholesterolaemia and elevated oxidised LDL (OxLDL), well-known atherosclerosis determinants. Serum PCSK9 level is thus considered as a cardiovascular disease biomarker [85, 154] and correlated positively with severity of artery lesions as well [155]. PCSK9 is involved in atherosclerosis by other non-canonical pathways as well [156]. At a local level, PCSK9 is secreted by vascular and blood cells involved in atherosclerotic plaque development: macrophages, endothelial cells and vascular smooth muscle cells. Intraplaque PCSK9 enhances oxLDL and foam cells production, and triggers local inflammation [32, 115, 156]. As far as PCSK9 genetic variants are concerned, LOF mutations or polymorphisms characterised by low level of LDL-c are associated with marked reduction of cardiovascular risk [157] whereas GOF variants are associated with high level of LDL-c and high risk of atherosclerotic cardiovascular diseases [9].

Conclusion

The discovery of PCSK9 as a key regulator of lipoproteins revolutionised the field of atherosclerotic cardiovascular therapy. However, many questions remain unanswered or partially elucidated, including T2DM risk associated with PCSK9i long term use. PCSK9i side effects are generally explored using PCSK9 LOF mutations as proxies. Scientific evidence about PCSK9 LOF mutations and T2DM association appear contradictory. While some variants are associated with significant elevation of glucose level and increased risk of T2DM, other variants are associated with no alteration of glucose metabolism or low plasma glucose level.

Then, the practice consisting in using PCSK9 LOF mutations as proxies to assess PCSK9i long term side effects should be more cautious. Regarding the conflicting reports about the PCSK9 LOF variants, we assume that their impact on glucose homeostasis and thus their association with T2DM may vary depending on the genetic background of the studied populations and the type of effect caused by those genetic variations on the PCSK9 protein. Accordingly, these factors should be considered when choosing a genetic variant of PCSK9 to assess the impact of long-term use of PCSK9i on glucose homeostasis.






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ORCID

Sègbédé E. R. Tchéoubi  <http://orcid.org/0000-0002-7473-5072>
 Casimir D. Akpovi  <http://orcid.org/0000-0002-1174-0560>
 Frédérique Coppée  <http://orcid.org/0000-0002-4791-8279>
 Anne-Emilie Declèves  <http://orcid.org/0000-0002-0838-2432>
 Sophie Laurent  <http://orcid.org/0000-0002-2589-3250>
 Clément Agbangla  <http://orcid.org/0000-0002-8783-0634>
 Carmen Burtea  <http://orcid.org/0000-0002-1117-1935>

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