

# Lipid Disorders

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**THE YEAR IN  
LIPID DISORDERS**

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**P. P. TOTH**

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# THE YEAR IN LIPID DISORDERS

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VOLUME 2

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EDITED BY  
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CLINICAL PUBLISHING  
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## **Clinical Publishing**

an imprint of Atlas Medical Publishing Ltd

Oxford Centre for Innovation

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Email: [info@clinicalpublishing.co.uk](mailto:info@clinicalpublishing.co.uk)

Web: [www.clinicalpublishing.co.uk](http://www.clinicalpublishing.co.uk)

## **Distributed in USA and Canada by:**

Clinical Publishing

30 Amberwood Parkway

Ashland OH 44805 USA

Tel: 800-247-6553 (toll free within U.S. and Canada)

Fax: 419-281-6883

Email: [order@bookmasters.com](mailto:order@bookmasters.com)

## **Distributed in UK and Rest of World by:**

Marston Book Services Ltd

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Tel: +44 1235 465500

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Email: [trade.orders@marston.co.uk](mailto:trade.orders@marston.co.uk)

© Atlas Medical Publishing Ltd 2010

First published 2010

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A catalogue record for this book is available from the British Library.

ISBN 13 978 1 84692 061 5

ISBN e-book 978 1 84692 622 8

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Project Manager: Prepress Projects Ltd, Perth, UK

Printed by Marston Book Services Ltd, Abingdon, Oxon., UK

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# Contents

*Editors and contributors* vii

---

## **Part 1**

### **Clinical aspects of atherosclerotic disease** 1

- 1.** Proprotein convertase subtilisin/kexin type 9 (PCSK9): molecular function and impact of its mutations and polymorphisms on cholesterol levels and related diseases 3  
**Marianne Abifadel, Catherine Boileau**
  - 2.** Metabolic syndrome, diabetes and cardiovascular risk 25  
**Mohamed. H. Ahmed, Rasaq Olufadi, Christopher D. Byrne**
  - 3.** Diet and exercise in the prevention and treatment of diabetes and coronary heart disease 47  
**Gang Hu, Jaakko Tuomilehto**
  - 4.** Imaging developments in vascular disease 61  
**Colin Berry**
- 

## **Part II**

### **Lipids, lipoproteins and atherosclerosis** 79

- 5.** Ezetimibe: mechanism of action update and recent controversies 81  
**Harry R. Davis Jr, Margaretann Halleck**
- 6.** Oxidative stress, lipoprotein oxidation and atherosclerosis 97  
**Richard W. James**
- 7.** Updating the metabolism of apolipoprotein B-100-containing lipoproteins in dyslipidaemia 119  
**Gerald F. Watts, Dick C. Chan, P. Hugh R. Barrett**
- 8.** Triglycerides and atherosclerosis 141  
**Thomas D. Dayspring, Gregory S. Pokrywka**

---

**Part III****Prevention and treatment strategies 167**

- 9.** JUPITER: the crossroads between statin therapy, inflammation and cardiovascular risk reduction 169  
**Peter P. Toth**
- 10.** New clinical trials of lipid-regulating agents 187  
**Anders G. Olsson**
- 11.** Statin myopathy 205  
**Charles Harper, Terry A. Jacobson**
- 12.** Nuclear receptors (PPARs, LXRs and TRs) and their role in regulating lipid metabolism 223  
**Sue-Anne Toh, John Millar**

---

**Part IV**

- 13.** Inflammation, inflammatory disease and coronary atherosclerosis 245  
**Robert S. Rosenson, Kyaw K. Soe**
- 14.** Mechanisms of insulin resistance 273  
**Vasudevan A. Raghavan**
- 15.** Haemostasis–dyslipidaemia interactions in coronary heart disease 291  
**Vellore J. Karthikeyan**

*Acronyms/abbreviations* 311

*Index of papers reviewed* 315

*General index* 323

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# Part 1

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Clinical aspects of  
atherosclerotic disease

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# 1

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## Proprotein convertase subtilisin/kexin type 9 (PCSK9): molecular function and impact of its mutations and polymorphisms on cholesterol levels and related diseases

MARIANNE ABIFADEL, CATHERINE BOILEAU

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### Introduction

Hypercholesterolaemia is one of the major causes of coronary heart disease (CHD). Mendelian forms of hypercholesterolaemia have been identified: first the autosomal dominant form (autosomal dominant hypercholesterolaemia [ADH]) and later the autosomal recessive form (ARH).

ADH (OMIM 144400) is characterized by a selective increase in the levels of low-density lipoprotein cholesterol (LDL-C) in plasma, giving rise to tendon and skin xanthomas, arcus corneae and cardiovascular deposits, leading to progressive and premature atherosclerosis, CHD and mortality. ADH is a heterogeneous genetic disorder. Its most frequent and archetypal form is familial hypercholesterolaemia (FH), the frequency of which is 1 in 500 in heterozygotes and 1 per million in homozygotes [1]. The disease is co-dominant, with homozygotes more severely affected than heterozygotes. FH is caused by mutations in the gene that encodes the LDL receptor (*LDLR* at 19p13.3; MIM 606945, 143890) [1]. The second form of ADH is familial defective apolipoprotein B (apoB)-100, caused by mutations in the apoB gene (*APOB* at 2p23–p24; MIM 107730, 144010) encoding the ligand of the LDL receptor [2].

Through the French Research Network for ADH, French families with hypercholesterolaemia and no mutations in the *LDLR* or *APOB* gene were recruited. Some of these families allowed us, using a positional cloning approach, to identify the third gene implicated in ADH: proprotein convertase subtilisin/kexin type 9 (*PCSK9*; MIM 607786) at 1p32.3 [3]. The discovery of PCSK9 and its suggested

role in cholesterol homeostasis shed light on a major player in the regulation of plasma LDL-C levels that has been extensively studied and constitutes one of the most interesting therapeutic targets for the reduction of LDL-C.

In the present chapter, we will summarize the molecular functions of PCSK9, the impact of its mutations and polymorphisms, and the role that this enzyme plays in LDL metabolism and risk for CHD, focusing on recently published studies.

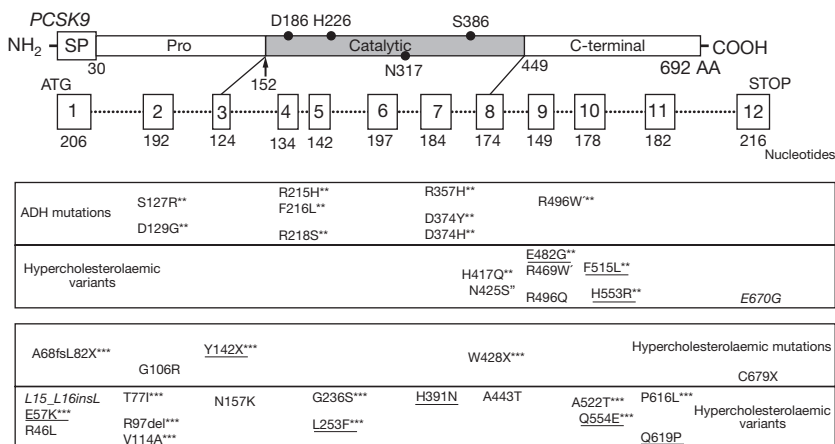
*PCSK9* mutations inducing ADH are relatively rare but well documented (familial segregation analysis, *in vitro* mutagenesis, etc.) [4]. Most enzymopathies are recessively inherited; thus, the dominance of the ADH trait associated with *PCSK9* was in favour of a gain-of-function mechanism [3]. This was confirmed by cellular and animal models showing that these gain-of-function mutations decreased the number of LDL receptors at the cell surface, leading to hypercholesterolaemia [5]. Two years after the first report of gain-of-function mutations leading to hypercholesterolaemia, loss-of-function mutations were described by Cohen *et al.* [6] and were found to be associated with lower cholesterol levels and a reduction in CHD. Thus, as for *APOB*, some *PCSK9* gene variants might lead to hypercholesterolaemia whereas others lead to hypocholesterolaemia. However, the frequency of *PCSK9* functional single nucleotide polymorphisms (SNPs), their elevated number and their impact on cholesterol levels in several populations mean that *PCSK9* variants are more important than variants of the *LDLR* or *APOB* gene.

The gain-of-function and loss-of-function mutations and polymorphisms are distributed in all *PCSK9* domains (Fig. 1.1), with no structural clue to predict their functional relevance. Besides the rare mutations implicated in ADH, some variants are functionally relevant in cholesterol regulation and their distribution and impact vary in different populations, whereas other SNPs have no clinical impact and are generally more frequent in all populations [4].

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## PCSK9: from gene to protein

*PCSK9* cDNA (NM\_174936.2) spans 3617 base pairs over 12 exons that encode the 692 amino acid protein PCSK9 (NP\_777596.2). This enzyme is a member of the mammalian serine proprotein convertase (PC) family that is responsible for the proteolytic maturation of secretory proteins including neuropeptides, prohormones, cytokines, growth factors, receptors and serum and cell surface proteins [7]. *PCSK9* protein was formerly named neural apoptosis-regulated convertase 1 (NARC-1) as it was first identified by the cloning of cDNAs upregulated following apoptosis induced by serum deprivation in primary cerebellar neurons. NARC-1 was more precisely characterized by Seidah *et al.* [7] as the ninth member of the subtilisin family of kexin-like proconvertases. *PCSK9* is particularly expressed in the liver, gut, kidney and nervous system [7]. The structure of this enzyme, its autocatalytic cleavage in the endoplasmic reticulum (ER), which is essential for transport from this compartment and for secretion [8,9], and the cleavage of the secreted form by other proprotein convertases, particularly furin and/or PC5/6A [8], are shown in

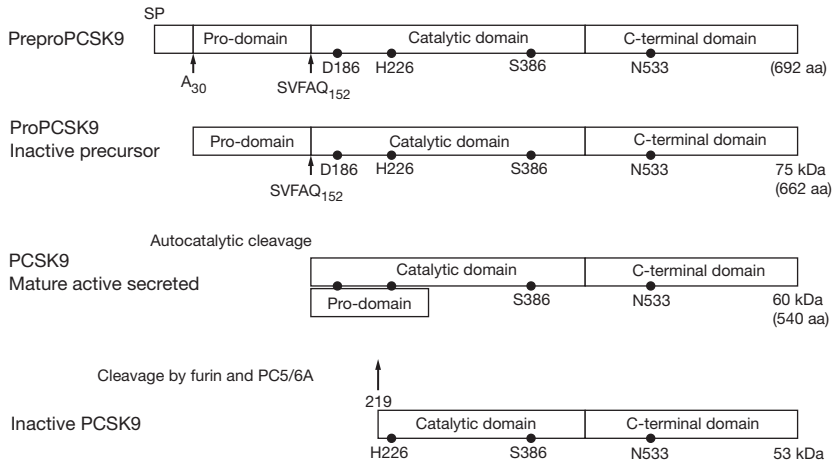


**Fig. 1.1** Genetic localization of PCSK9 natural variants present in the coding region and their association with cholesterol levels [4]. The active site D, H and S residues and the oxanion hole N residue in the catalytic domain are represented. Variants of PCSK9 associated with a mutation in the LDLR gene are depicted by \*; variants found only in patients with high cholesterol levels are depicted by \*\*; variants found only in patients with low cholesterol levels are depicted by \*\*\*; variants found only in black populations are underlined; and variants found with a high frequency in both black and white populations are in italic. SP, signal peptide; pro, pro-segment.

**Fig. 1.2.** The 60-kDa mature form and the furin cleaved form of PCSK9 are present in the circulation [8,9].

## Impact of PCSK9 on the LDL receptor

The ability of PCSK9 to degrade the LDL receptor is independent of its catalytic activity [9]. The role of PCSK9 serine protease activity seems limited to catalysing the self-cleavage of pro-PCSK9, which is essential for PCSK9 secretion from liver cells and for the reduction in the number of LDL receptors [10]. Although the mechanism by which PCSK9 regulates LDL receptor degradation is not fully resolved, it seems to involve both intracellular and extracellular pathways [8]. PCSK9 may work in a post-ER compartment, where it may target the LDL receptor for degradation in lysosomes. But secreted PCSK9 may also bind to the first epidermal growth factor-like repeat (EGF-A) of the LDL receptor at the cell surface, and the PCSK9/LDL receptor complex could be internalized into endosomal/lysosomal compartments [10]. PCSK9-LDL receptor affinity is increased in the endosome because of its higher acidity [10]. Failure to release PCSK9 may hinder receptor recycling and reduce the cell surface abundance of LDL receptor [11]. The LDL receptor would



**Fig. 1.2** Structure, maturation and post-translational modifications of PCSK9. Prepro-PCSK9 is synthesized with a signal peptide (SP; amino acids 1–30), a pro-domain (amino acids 31–152) and a catalytic domain, followed by a 243-amino-acid cysteine-rich and histidine-rich C-terminal region [8,11]. PCSK9 is synthesized as an inactive proenzyme and contains a triad of residues (D186, H226 and S386) that are required for catalytic activity [7]. The 74-kDa precursor form of PCSK9 undergoes intramolecular autocatalytic cleavage in the endoplasmic reticulum (ER), which produces a 60-kDa catalytic fragment. Autocatalytic cleavage of the zymogen in the ER is essential for transport from this compartment and for secretion. The cleaved pro-domain of 14 kDa remains associated with the catalytic domain, permitting the mature protein to move from the ER into the secretory pathway and assisting in the proper folding and the regulation of catalytic activity of the enzyme. Also, the 60-kDa mature and secreted form is cleaved at the motif RFHR↓218 into an 53-kDa inactivated fragment by other proprotein convertases, particularly furin and/or PC5/6A [8,11].

then be rerouted from the endosome to the lysosome, where it is degraded [10]. Thus, PCSK9 functions as a chaperone to prevent LDL receptor recycling and/or target LDL receptors for lysosomal degradation [9].

## Major hypercholesterolaemic mutations and gain-of-function mechanisms

The first two mutations of *PCSK9* we identified in families with hypercholesterolaemia were the p.S127R mutation, which was found in two French families in exon 2 in a highly conserved region between species, and the p.F216L mutation in exon 4, identified in a French family in which the proband died from myocardial infarction at age of 49 years with a total cholesterol level of 441 mg/dl and an

LDL-C level of 356 mg/dl [3,4]. The p.D374Y mutation was reported in 2004 in a hypercholesterolaemic Utah kindred [12], in three Norwegian families and in three English families including 12 affected individuals suffering from severe hypercholesterolaemia and with a family history of premature CHD [13]. Another mutation, p.D374H, which concerns the same amino acid, was identified in two unrelated Portuguese patients and one relative who presented a very severe phenotype with extremely high total cholesterol levels and a familial history of premature CHD [14]. Two mutations adjacent to the p.F216L mutation are also linked to hypercholesterolaemia: the p.R218S mutation, which was found in a French proband ascertained at age 45 years with 402 mg/dl total cholesterol and 293 mg/dl LDL-C and presenting with tendinous xanthoma and arcus corneae [15], and the p.R215H mutation in two families from Norway [16]. Furthermore, the p.D129G mutation was identified in a family originating from New Zealand [17]; it also segregates with the ADH phenotype and concerns highly conserved residues.

These missense gain-of-function mutations of *PCSK9* reported in families with ADH cause a clinical phenotype resembling FH caused by *LDLR* gene mutations: tendon xanthomas, CHD, premature myocardial infarction and stroke. To characterize the mechanisms underlying hypercholesterolaemia, *in vivo* and *in vitro* studies as well as animal models were analysed. *In vivo* kinetic studies of apoB-100-containing lipoproteins conducted in two French subjects carrying the p.S127R mutation in *PCSK9* showed that this mutant dramatically increased the production rate of apoB-100 (threefold) compared with control subjects or *LDLR*-mutated patients and led to a higher direct overproduction of very low-density lipoprotein (VLDL) (threefold), intermediate-density lipoprotein (IDL) (threefold), and LDL (fivefold) [18]. A lower conversion rate of VLDL and IDL compared with control subjects and heterozygous FH patients was also observed. Finally, the LDL fractional catabolic rate was slightly decreased (by 30%) compared with control subjects but was still higher than in *LDLR*-mutated subjects [18]. The p.D374Y gain-of-function mutation was also associated with increased apoB-100 secretion [13,19].

In animal models, human mutant *PCSK9* (p.S127R and p.F216L) overexpressed in the liver of mice leads to hypercholesterolaemia owing to a dramatic decrease in hepatic LDL receptor levels through a post-transcriptional mechanism [5]. This was also seen with overexpression of wild-type *PCSK9* in mice, which caused a twofold increase in plasma total cholesterol and a fivefold increase in non-high-density lipoprotein (HDL) cholesterol levels with no effect on *LDLR* mRNA level [5].

*In vitro* studies showed that the two gain-of-function mutations p.S127R and p.D374Y resulted in a 23% decreased level of cell surface LDL receptors and a 38% decreased level of internalization of LDL compared with wild-type *PCSK9* [20]. The p.S127R variant interferes with autocatalytic cleavage, which is crucial for secretion from the cell, but its exact mechanism in hypercholesterolaemia has not yet been elucidated. The role of the p.D374Y mutation is better understood; it affects the affinity of *PCSK9* for the LDL receptor and enhances receptor degradation. In fact, the gain-of-function mutation p.D374Y binds the LDL receptor 25 times more tightly than wild-type *PCSK9* at neutral pH, remains in a high-affinity complex at

acidic pH and is approximately 10-fold more active in reducing LDL receptor levels than the wild-type protein [11,21].

The natural gain-of-function mutations p.R218S, p.F216L and p.D374Y result in total (p.R218S) or partial loss of the furin/PC5/6A processing of PCSK9 at the motif RFHR↓218, which leads to an inactive form (of 53 kDa). Thus, these gain-of-function mutations act by increasing the stability of PCSK9 [8].

Other rare mutations (p.R357H for example) that were found in hypercholesterolaemic patients [15], but absent in normolipidaemic control subjects, are evolutionarily conserved between species. Some other rare variants were found only in patients with high LDL-C from specific populations (African American: p.F515L for example) but their association with the disease is less evident [4]. The most important *PCSK9* mutations or polymorphisms are reported in Fig. 1.1.

---

## PCSK9 and hypocholesterolaemia

Two years after our first report of the role of PCSK9 in cholesterol metabolism and disease, Cohen *et al.* [6] searched for mutations in *PCSK9* in subjects with low plasma levels of LDL-C (LDL-C < 58 mg/dl) from the Dallas Heart Study, a multiethnic population of Dallas County, Texas (52% African American, self-identified as ‘Blacks’, 29% European American, self-identified as ‘Whites’, 17% Hispanic and 2% other ethnicities, in accordance with US census categories [22]). They identified two nonsense mutations: p.Y142X in exon 3, found in 0.4% of African Americans, and p.C679X in exon 12, found in 1.4% of African Americans but very rarely in European Americans (<0.1%). Subjects with nonsense mutations had significantly (28%) lower plasma levels of total cholesterol and LDL-C, but not all of them were hypocholesterolaemic. Their LDL-C values ranged from the first to the fiftieth percentiles adjusted for age and sex when compared with the entire sample of African Americans in the Dallas Heart Study [6]. In the United States, 1 of every 50 African Americans has a nonsense mutation in *PCSK9*. This mutation was also found at this same high frequency in a Nigerian population [23] and in 3.7% of African women from Zimbabwe [24], in whom it was associated with a 27% reduction in LDL-C.

Other variants of *PCSK9* were also associated with lower plasma levels of LDL-C (reductions ranging from 3.5% to 30%), particularly the p.L253F and the p.A443T variants found in black populations and the p.R46L mutation found in white populations [23,25,26]; however, these polymorphisms might be found also in normo- or hypercholesterolaemic individuals because of other genetic or environmental factors influencing cholesterol levels [3,15,23].

Hallman *et al.* [27] studied the relationship between *PCSK9* mutations and serum LDL-C levels in childhood and adulthood in the Bogalusa Heart Study. They analysed associations of one missense (p.R46L) and two nonsense (p.Y142X and p.C679X) *PCSK9* mutations with serum LDL-C levels in 478 African Americans

and 1086 white people, from 4 to 38 years of age. Longitudinal LDL-C profiles were significantly lower in white people with the L46 allele and in African Americans with the X142 or X679 allele. These results showed that these *PCSK9* variants are associated with significantly lower LDL-C levels starting in childhood [27].

The *in vitro* studies of these variants showed that no protein was detected with the p.Y142X mutation, probably due to nonsense-mediated mRNA decay, whereas the p.C679X mutant was cleaved normally but was misfolded and retained in the ER [28]. Nassoury *et al.* [29] showed that some of the mutants associated with hypocholesterolaemia either remain in the ER (this is the case for the p.C679X and the p.G106R mutations, the latter segregating with low LDL-C levels in a Norwegian family [25]) or do not sort to endosomes (p.L253F and p.Q554E), resulting in loss of function. Thus, sorting of *PCSK9* to the cell surface and endosomes is required for *PCSK9* to fully promote LDL receptor degradation, and retention in the ER prevents this activity [29]. Some variants may have several functional defects at the same time. In animal studies, livers of knockout mice lacking *PCSK9* (*Pcsk9*<sup>-/-</sup>) display increased LDL receptor levels (but not mRNA), which leads to a decrease in plasma cholesterol levels of 48% compared with wild-type littermates [30].

Missense and nonsense mutations of *PCSK9* causing mild hypocholesterolaemia are actually quite common and have a marked effect on plasma LDL-C levels and on protection against CHD. However, *PCSK9* mutations could be responsible for severe hypocholesterolaemia in homozygous or compound heterozygous carriers. Hooper *et al.* [24] reported a woman originating from Zimbabwe, homozygous for p.C679X, with very low LDL-C levels (15 mg/dl). Furthermore, Zhao *et al.* [28] reported a compound heterozygote for the p.Y142X mutation and an in-frame 3-base pair deletion (c.290\_292delGCC) that deletes an arginine at codon 97. This 32-year-old African American woman had no immunodetectable circulating *PCSK9*. She had an LDL-C level of only 14 mg/dl and was apparently healthy, fertile and normotensive, with grossly normal hepatic, neuronal and renal function tests [28]. In one Sicilian hypobetalipoproteinaemia kindred, a deletion in exon 1 (c.202delG) was found, which causes a frameshift in mRNA leading to a premature stop codon (p.A68fsL82X) [31]. The proband and his father were overweight and had fatty liver. The mutation was found in two unrelated hypocholesterolaemic blood donors living in the same area. These subjects were healthy and had no clinical or laboratory signs of liver disease; the other routine laboratory tests were normal [31]. In the Dallas Heart Study, no significant difference in the median hepatic triglyceride content or in the prevalence of hepatic steatosis between the subjects with and those without an LDL-lowering mutation in *PCSK9* was observed in either ethnic group [23]. Hypocholesterolaemia due to a deficiency of *PCSK9* appears to be benign, in contrast to other Mendelian forms of severe hypocholesterolaemia such as abetalipoproteinaemia (MIM 200100) and homozygous hypobetalipoproteinaemia (MIM 107730), which are both associated with malnutrition, hepatic steatosis, steatorrhoea and manifestation of fat-soluble vitamin deficiency [28]. Until now, *PCSK9* variants have been associated only with altered cholesterol levels in man,

as compound heterozygotes for loss-of-functions mutations in *PCSK9* seem to be healthy and have a normal lifespan [5,28].

---

## Impact of *PCSK9* variants on coronary heart disease

The impact of *PCSK9* variants on cholesterol levels and CHD was analysed in several populations (African [24], American [32], European [4,25]) and studies (ARIC [32], PROSPER [33], LCAS [34], TEXGEN [34], PLIC [35]) by evaluating the protection of the loss-of-functions variants or the severity of coronary atherosclerosis associated with gain-of-function polymorphisms.

### *The ARIC study*

The impact of *PCSK9* variants in the clinic was ascertained by the protection against CHD found to be associated with three cholesterol-lowering *PCSK9* variants (p.C679X, p.Y142X and p.R46L) in the Atherosclerosis Risk in Communities (ARIC) study [32]. The comparison of the incidence of CHD (myocardial infarction, fatal CHD, coronary revascularization) over a 15-year period, according to the presence or absence of these *PCSK9* variants, was established in 3363 black and 9523 white participants, from 45 to 64 years of age, constituting four American communities [32]. It is noteworthy that the exclusion criteria in this study included the use of lipid-lowering drugs and the presence of symptomatic cardiovascular disease at baseline. The nonsense mutations occurred in 2.6% of the black subjects examined and were associated with a 28% reduction in mean LDL-C level and an 88% reduction in the risk of CHD. p.R46L, found in 3.2% of the white subjects examined, was associated with a 15% reduction in LDL-C and a 47% reduction in the risk of CHD [32]. These results confirmed the importance of the duration of cholesterol reduction in preventing CHD. In fact, individuals with loss-of-function *PCSK9* mutants have low LDL-C concentrations (40 mg/dl lower) throughout their lives, which reduces CHD incidence by 88%, whereas using a statin to reduce cholesterol level by 40 mg/dl for 5 years reduces CHD by only 23% on average. Thus, lowering LDL-C well before atherosclerosis has become advanced is crucial to attain an important reduction in the incidence of CHD [36]. These *PCSK9* alleles were also associated with a reduced risk of carotid atherosclerosis with a mean intima-media thickness slightly but significantly lower among carriers than among non-carriers in both groups of subjects [32].

In a recent study, Folsom *et al.* [37] hypothesized that *PCSK9* variants that lower LDL-C levels are associated with reduced prevalence and incidence of peripheral artery disease (PAD). However, their analysis in the same population-based cohort as in the ARIC study provided mixed evidence that *PCSK9* variants associated with low LDL-C are associated with reduced occurrence of PAD. Their cross-sectional analysis suggested a halving of PAD prevalence for carriers of these *PCSK9* variants

[37], which is consistent with findings in this same cohort for CHD [32]. In contrast, the low-cholesterol *PCSK9* variants were not associated with incident PAD. LDL-C level itself was a comparable risk factor for PAD prevalence and incidence; thus, the lack of association between *PCSK9* and incident PAD is surprising and might be due to the fact that *PCSK9* variants were relatively uncommon. Therefore, although this study was large, it had statistical power to detect only moderately large associations [37].

### *The PROSPER study*

In the PROSPER study (Prospective Study of Pravastatin in the Elderly at Risk [33]), 2804 men and 3000 women, aged 70–82 years, with pre-existing vascular disease or at least one of three major vascular risk factors (diabetes, smoking or hypertension) were randomized to pravastatin 40 mg/day ( $n=2891$ ) or placebo ( $n=2913$ ). The mean LDL-C reduction in this study in the active group was 32%, and the risk of developing CHD was decreased by 19% over 3.2 years, which was statistically significant. The study of the *PCSK9* p.R46L variant in the 5783 participants (with mean age 75.3 years in PROSPER, of whom 43% had a history of vascular disease at baseline) showed that 3.5% were carriers of the p.R46L variant. These subjects had significantly ( $P<0.001$ ) lower levels of LDL-C (mean reduction 10%), no difference in LDL-C-lowering response to pravastatin, and a non-significant (19% unadjusted and 9% adjusted) decreased risk of vascular disease at baseline. These data support the concept that the rare allele of the p.R46L SNP at the *PCSK9* locus significantly lowers LDL-C but does not greatly reduce CHD risk in an elderly population with a high prevalence of cardiovascular disease [33].

### *The LCAS, TexGen and other studies*

Concerning gain-of-function variants, a common *PCSK9* haplotype encompassing the p.E670G variant was found to be an independent determinant of plasma LDL-C levels and of the severity of coronary atherosclerosis in the Lipoprotein Coronary Atherosclerosis Study (LCAS) and the TexGen populations from Houston (Texas) [34]. The LCAS population comprised 372 subjects who had plasma LDL-C levels of 1.15–1.90 g/dl and one or more coronary lesions causing 30–75% diameter stenosis, whereas the TexGen population comprised 319 subjects who had normal plasma LDL-C levels of <1.30 g/dl. The p.E670G (c.2009A>G) common SNP accounts for 3.5% of LDL-C level variability, with higher levels with the GG genotype, intermediate with the AG genotype, and lowest with the AA genotype. Plasma total cholesterol, apoB and lipoprotein a (Lp[a]) levels were also associated with the p.E670G SNP in these populations [34]. These associations were not found in the Dallas Heart Study [23] nor in the PROSPER study, in which 6.0% of participants were carriers of the G allele at p.E670G, with no significant relationships with baseline LDL-C, response to pravastatin or vascular disease risk [33].

In a European study [38] of 506 patients attending the lipid clinic of the University Hospital in Hamburg, Germany, it was established that the p.E670G polymorphism

in the *PCSK9* gene is associated with increased LDL-C in men but not in women. This observation explains the discrepancy in the results between the Dallas Heart Study and the LCAS [34] as the majority of the probands in the LCAS were men, but does not explain the lack of association in healthy men in the UK [26]. Another study has suggested gender as a factor in *PCSK9* physiology, with plasma *PCSK9* levels correlated with total cholesterol and LDL-C in men but not in women [39]. The explanation for these gender discrepancies is still unknown.

Nevertheless, a study of a potential link between the *PCSK9* gene and the risk of ischaemic stroke or intracranial atherosclerosis has recently shown that the p.E670G polymorphism also seems to be associated with the risk of the large vessel atherosclerosis stroke subtype in a Belgian population [40]. In an independent Finnish autopsy study comprising a total of 604 Caucasian subjects (64.3% men and 35.7% women) who died suddenly out of hospital, p.E670G tended to be associated with increased atherosclerosis of the large intracerebral arteries [40].

### *The PLIC study*

In a recent study, Norata *et al.* [35] investigated the effects of two common polymorphisms of the *PCSK9* gene (p.E670G and p.I474V) on the intima-media thickness of the common carotid artery and the possible relation with polymorphisms of apoE in 1541 middle-aged subjects selected from the general population enrolled in the PLIC study and confirmed the major findings in a second free-living population. The PLIC study is a prospective population-based study designed to investigate the presence and progression of atherosclerotic lesions and intima-media thickness in the common carotid artery in a local cohort of men and women. They showed that the 670G carriers were associated with significantly increased plasma total cholesterol, LDL-C and apoB levels, whereas no significant differences were observed in carriers of the p.I474V SNP. The intima-media thickness was significantly increased in p.E670G carriers compared with individuals homozygous for the E allele ( $0.640 \pm 0.102$  mm vs.  $0.652 \pm 0.092$  mm,  $P < 0.05$ ). The presence of the p.E670G allele was also significantly associated with a greater progression of intima-media thickness compared with 670EE subjects. Plasma total cholesterol, LDL-C, apoB and intima-media thickness significantly increased from apoE-2-*PCSK9*-670EE carriers to apoE-4-*PCSK9*-670G carriers [35].

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## Genotype-phenotype correlations

Even if the effect of some variants of *PCSK9* is small, their cumulative effect might significantly impact cholesterol variability and CHD susceptibility. Furthermore, the elevation of LDL-C might not be the only atherogenic role of *PCSK9* gain-of-function mutations [36]. Further investigation is needed to determine if *PCSK9* also exerts a direct toxicity on the arterial wall which is reduced with the loss-of-

function variants that protect against atherosclerosis [36]. Moreover, recent results have shown that *PCSK9* polymorphisms might modify the FH phenotype and that some of them could be associated with other dyslipidaemia disorders.

### *PCSK9 as a modifier gene of FH*

Recently, we showed that *PCSK9* is a major gene accounting for cholesterol variability not only in normolipaeamic subjects but also among FH patients sharing the same *LDLR* mutation [41]. We reported for the first time that *PCSK9* might constitute a modifier gene in FH [41].

More than 1000 *LDLR* mutations have been reported worldwide having different impacts on the severity of the disease. Nevertheless, the spectrum of FH mutations is much reduced in some populations, such as the Lebanese population, facilitating the investigation of genes that might influence the FH phenotype. We showed in Lebanese FH patients sharing the same *LDLR* mutation (the p.C681X mutation, which accounts for more than 80% of FH patients in Lebanon) that the polymorphism c.61\_63dupCTG (p.Leu21dup, also denoted as L10), an in-frame insertion of one leucine in the stretch of nine leucines in exon 1 of *PCSK9*, known to be associated with lower LDL-C levels in general populations, is also associated with a reduction in LDL-C levels in FH [41]. In these FH patients, the L10 allele in exon 1 was associated with a reduction in LDL-C levels of 49.3 mg/dl (16%) and an increase in HDL-C of 12.1 mg/dl (28%), with significant *P*-values of 0.02 and 0.006, respectively, after adjusting for age and sex [41]. This polymorphism, c.61\_63dupCTG, is common in both African American (allele frequency 0.27) and Caucasian (allele frequency 0.143) populations [34,42]. The variant was studied in a Caucasian population of 403 unrelated volunteers with total cholesterol levels under 150 mg/dl and was associated with lower LDL-C levels, the L9/L10 heterozygous carriers having 10–15 mg/dl lower LDL-C than L9/L9 carriers [42].

This was the first report of an impact of a *PCSK9* polymorphism as a modifier of LDL-C levels in FH subjects [41]. Furthermore, additive effects of mutations in the *LDLR* and *PCSK9* genes on the phenotype of FH have been reported in several studies [15,43]. Three probands heterozygous for mutations in both the *LDLR* and *PCSK9* genes have been reported with plasma levels of LDL-C higher than those in their relatives with either mutation alone [15,43]. These patients displayed a digenic form of hypercholesterolaemia, suggesting that *PCSK9* and *LDLR* mutations have additive effects on LDL metabolism.

### *PCSK9 variant and familial combined hypercholesterolaemia*

Interestingly, the leucine stretch in exon 1 of *PCSK9* is more heterogeneous, and an insertion of two leucines (L11, p.L21tri also designated p.L15\_L16ins2L) in the leucine stretch of the signal peptide instead of one (L10) seems to be associated with a different phenotype. We have recently identified this L11 allele in two French Canadian families with familial combined hypercholesterolaemia (FCHL) and in one French Canadian woman and her father with hypercholesterolaemia [44]. Like

other *PCSK9* variants that are associated with cholesterol variability, the L11 allele was associated with hypercholesterolaemia in the FCHL families studied according to family segregation and the statistical analysis carried out [44].

The L11 allele was reported by Chen *et al.* [34] with a very low frequency. They found the L11 allele in 7 out of 372 (1.8%) individuals in the LCAS population, which comprised American subjects who had plasma LDL-C levels of 115–190 mg/dl (2.96–4.90 mmol/l) despite diet, and one or more coronary lesions causing 30–75% diameter stenosis [34].

Thus, L11 is a very rare *PCSK9* allele that seems to increase total and LDL-C levels in a moderate way and might also be observed in FCHL, where it contributes with other genetic and environmental factors to the appearance of the phenotype of this common, multigenic and highly atherogenic disease characterized by elevated levels of either plasma cholesterol or triglyceride or both in members of the same family. It is noteworthy that the p.R496Q variant of *PCSK9* had been identified by Cameron *et al.* [20] in a subject homozygous for apoE-2 who presented with type III hyperlipoproteinaemia.

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## Ethnicity variation and human evolution

Some *PCSK9* variations are confined to specific populations with the same ethnic origin. This difference could simply result from genetic drift. However, the high frequency of nonsense mutations in individuals of African ancestry suggests that positive selective pressure has maintained these alleles in the population [6]. We can only speculate on the nature of this selective pressure. Thus, it is possible that nonsense *PCSK9* mutants confer a selective advantage because they reduce susceptibility to severe parasitic or viral infections. Increased LDL receptor activity might also reduce the exposure of peripheral tissues to organisms or bioactive cytokines that circulate in association with lipoprotein particles [6].

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## Regulation of *PCSK9* transcription

*PCSK9* is regulated as a typical cholesterologenic gene and constitutes a target of the sterol regulatory element-binding protein (SREBP) transcription factors [45]. A twofold decrease in *PCSK9* mRNA levels was observed in livers of mice fed a cholesterol-rich diet. In contrast, *PCSK9* was highly upregulated in SREBP-2 transgenic mice [45]. This is due to the presence in the *PCSK9* gene promoter of two conserved sites for transcription factor binding: Sp1 and SRE (sterol-responsive element) [46]. Furthermore, a highly conserved hepatocyte nuclear factor 1 (HNF1) binding site residing 28 base pairs upstream from SRE is a critical sequence motif for *PCSK9* transcription and its regulation by berberine [47], a natural plant extract [16]. Moreover, hepatic *PCSK9* expression seems to be regulated by nutritional and hormonal status, as summarized in Table 1.1.

**Table 1.1** Hormonal, dietary and pharmacological regulation of PCSK9

Downregulation/decrease	Upregulation/increase	Study/remarks/references
Suppression of SREBP-2	Overexpression of SREBP-2	Animal models  45,46 ; results from presence of Sp1 and SRE in the promoter
Cholesterol feeding	Cholesterol depletion	Animal models  45,48
Glucagon	Insulin	Animal models  48 ; PCSK9 levels in human plasma positively associated with insulin  49
Ethinylestradiol		Animal models  48
Chenodeoxycholic acid		Immortalized human hepatocytes  49
Farnesoid X receptor agonist		Immortalized human hepatocytes  49
	Inflammation	Animal models  50
	Statin	Cellular and animal models  46 ; PCSK9 levels in human plasma increased by statin  55
PPAR agonist (fibrate) decreased PCSK9 mRNA and protein <i>in vitro</i>  51 ; fenofibrate decreased circulating PCSK9 in the FIELD study  57	Fenofibrate increased circulating PCSK9  56	Discrepancies due to the type of patient population studied and differences in analytical technique used  56
Berberine		Immortalized human hepatocytes  52 ; results from (HNF1) binding site upstream of SRE  47

HNF1, hepatocyte nuclear factor 1; PCSK9, proprotein convertase subtilisin/kexin type 9; SRE, sterol-responsive element; SREBP-2, sterol regulatory element-binding protein-2.

## Circulating PCSK9

PCSK9 is present in human plasma, but the factors that contribute to differences in plasma concentrations have not been well characterized. Several teams have developed an enzyme-linked immunosorbent assay (ELISA) to measure PCSK9 in plasma. Serum PCSK9 measured by ELISA seems to be directly correlated with serum LDL-C and total cholesterol |53| and positively associated with fasting glucose

and insulin in several studies [54]. In hypercholesterolaemic subjects, PCSK9 levels were higher than in control subjects, and increased in proportion to the statin dose, combined or not with ezetimibe [46]. Atorvastatin (40 mg/day) increases human serum levels of PCSK9 by 34% compared with baseline and placebo [55]. These results could explain why statins do not achieve as much LDL-C lowering as might otherwise be expected. Thus, the addition of a PCSK9 inhibitor to statin therapy may result in additional beneficial LDL-C lowering [56].

Fenofibrate (200 mg per day) significantly increased circulating PCSK9 levels by 25% compared with baseline in some studies [56], but a decrease of 8.5% after 6 weeks of treatment was reported by Lambert *et al.* [57] in diabetic patients from the FIELD study (Fenofibrate Intervention and Event Lowering in Diabetes). One possibility for the discrepancies between these results may be the type of patient population studied but differences in the analytical technique used may also have contributed [56]. A standardized method, with specific antibodies, is still needed to compare the levels of PCSK9 measured in different studies.

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## PCSK9 as a new drug target

Statins are among the most widely prescribed drugs in primary and secondary prevention of CHD. This class of drugs lowers plasma cholesterol through inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase, a key enzyme in cholesterol synthesis, triggering the SREBP-2 response and subsequent activation of LDL receptor synthesis. However, it is now established that statins induce an upregulation of PCSK9 [46] that might attenuate their cholesterol-lowering effect by reducing LDL receptor abundance at the cell surface. Thus, it was suggested that a combined statin–anti-PCSK9 therapeutic regimen could overcome this effect and enhance cholesterol reduction. Initial proof of concept was provided by statin administration to *Pcsk9*<sup>(-/-)</sup> mice, which produced an exaggerated increase in LDL receptors in liver and enhanced LDL clearance from plasma [30]. These experiments confirmed that inhibition of PCSK9 activity should enhance statin hypocholesterolaemic effects by increasing LDL receptor number [30,55].

Finally, the low plasma LDL-C levels and the reduction in CHD found in *PCSK9* loss-of-function mutation carriers who appear healthy suggest that the inhibition of PCSK9 could be an effective tool in the management of hypercholesterolaemia with no obvious adverse side-effects. In fact, several strategies to inhibit or lower PCSK9 have been investigated.

Administration of a second-generation antisense oligonucleotide inhibitor targeting murine PCSK9 in mice fed a high-fat diet for 6 weeks reduced total cholesterol and LDL-C levels by 53% and 38%, respectively, and resulted in a twofold increase in hepatic LDLR protein levels [58]. Liver-specific small interfering RNA (siRNA) silencing of *PCSK9* in mice and rats reduced *PCSK9* mRNA levels by 50–70% and was associated with up to a 60% reduction in plasma cholesterol

concentrations. In cynomolgus monkeys, siRNA-mediated reduction in *PCSK9* mRNA and protein resulted in lowering of LDL-C by approximately 50–60% within 48 h after administration; this reduction lasted for nearly 3 weeks [59].

The identification of small pharmacological molecules or antibodies that might block the interaction between PCSK9 and the LDLR constitute promising therapeutic strategies. Anti-PCSK9 antibodies seem effective therapeutics for treating hypercholesterolaemia. A monoclonal antibody, mAb1, that binds to an epitope on PCSK9 adjacent to the region required for LDLR interaction inhibited *in vitro* PCSK9 binding to the LDLR [60]. In wild-type mice, mAb1 increased hepatic LDLR protein levels approximately twofold and lowered total serum cholesterol by up to 36%. In cynomolgus monkeys, a single injection of mAb1 reduced serum LDL-C by 80%, and a significant decrease was maintained for 10 days [60]. Furthermore, a combination of mAb1 with a statin increases LDLR levels in HepG2 cells more than either treatment alone [60].

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## Safety of PCSK9 targeting

Nevertheless, future longer-term repeated dose studies are necessary to investigate the pharmacodynamic and toxicological consequences of these approaches. The only phenotype of PCSK9 deficiency detected in humans is lower plasma cholesterol levels, but it remains possible that ageing or exposure to environmental challenges, such as infectious agents or dietary toxins, may reveal other phenotypes associated with the absence of PCSK9.

It is noteworthy that conditional knockout mice lacking PCSK9 specifically in hepatocytes have impaired liver regeneration after a partial hepatectomy [61]. Furthermore, it has recently been shown that circulating liver PCSK9 has an antiviral effect on hepatitis C virus (HCV) and reduces the surface expression of CD81, a major HCV receptor [62]. PCSK9 has been reported to modulate levels of  $\beta$ -site amyloid precursor protein-cleaving enzyme 1 (BACE), and mice lacking PCSK9 show increased levels of total amyloid  $\beta$ -peptide in the brain [63]. It is noteworthy that no genetic association was found between *PCSK9* polymorphisms and Alzheimer's disease and plasma cholesterol level in Japanese patients studied by Shibata *et al.* [64]. Finally, it has also been shown that PCSK9 interacts with the VLDL receptor, apoE receptor 2 (apoER2) and annexin A2 [65]. The significance of the interaction between PCSK9 and these proteins is not presently known but these interactions should be kept in mind when considering PCSK9 as a therapeutic target. Possible other unknown functions of PCSK9 and unidentified binding partners might exist; thus, it is important for the safety of new cholesterol-lowering therapy to target specifically PCSK9 action on the LDL receptor.

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## Conclusion

The rapid progress achieved in the study of PCSK9 highlights the role of classical genetic approaches in identifying new genes implicated in lipid disorders. Other genes implicated in FH are still to be discovered. Their proteins might constitute new therapeutic targets in hypercholesterolaemia, as was the case for PCSK9.

The discovery of PCSK9's role in cholesterol homeostasis is crucial for the diagnosis of ADH, particularly when the implication of the *LDLR* and *APOB* genes is excluded. It has opened the way for a better understanding of the physiopathology of ADH and CHD and should improve the prevention and treatment of these diseases. This new therapeutic target might constitute a new approach to reducing cholesterol levels and CHD and enhancing the effectiveness of other lipid-lowering drugs.

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