PCSK9 as a Biomarker of Cardiovascular Disease

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Abstract

PCSK9 is a secreted protein which in circulation promotes lysosomal degradation of the low-density lipoprotein receptor (LDLR). Hence, it is informative to measure its concentration in blood. Circulating PCSK9 exists in several forms – its parent form and a furin-cleaved form and a low-density lipoprotein (LDL)-bound form and a free form. The furin-cleaved and LDL-bound forms are much less active. Most studies have not clearly distinguished among these forms. In a different context, in individuals receiving anti-PCSK9 monoclonal antibody (mAb) therapy, there is an Ab-bound form and a mAb-free form. Future measurements of circulating PCSK9 will need to account for the heterogeneity of PCSK9 in circulation.

Circulating PCSK9 concentration shows a diurnal rhythm and it decreases beyond ~16 h of fasting. It has a wide 50- to 100-fold range within a population and is rightward skewed, higher in women than men, and higher after menopause. In male adolescents, it decreases with age, while in female adolescents, it increases with age. It is elevated in pregnancy at term, and umbilical cord blood has lower serum PCSK9 levels than maternal blood. Ideally, measurement of plasma PCSK9 should be standardized to time of day and feeding status.

Plasma PCSK9 levels associate with LDL cholesterol (LDL-C) levels in most states of health and disease. The positive correlation between serum PCSK9 and LDL-C is relatively weak in contrast to the much greater effect of genetic variation in PCSK9 activity on serum LDL-C levels. GOF and LOF mutations and polymorphisms of PCSK9 are often reflected in higher and lower plasma PCSK9 levels, respectively, but this is not always the case.

Plasma PCSK9 measurement in various states of health and disease has enhanced our knowledge of lipoprotein metabolism in general and in abnormal states associated with CVD. It is currently not seen as a clinical marker of CVD risk or disease progression. There are early reports indicating an association between plasma PCSK9 levels and vascular disease, independent of other traditional risk factors, but underlying pathophysiologic mechanisms are still unclear. Plasma PCSK9 may serve in the future as a biomarker for the selection of patients for anti-PCSK9 mAb therapy.

It has also been measured in response to various forms of lipid-altering drug therapy. Statin therapy increases plasma PCSK9 levels. These data will need further development in order to serve as an aid to the establishment of the pharmacokinetic and pharmacodynamic properties of various forms of therapy. In clinical practice, it could be helpful in determining statin dosage, but its measurement is not essential as it is possible to simply titrate the dose of statin upward or downward as guided by LDL-C response. In clinical research, plasma PCSK9 levels provide hypothesis-generating information which could help the design of basic science experiments that explore mechanisms of regulation, metabolism, and role of PCSK9.

Keywords

PCSK9 • Circulating forms • Biomarker • Cardiovascular disease • Health and disease • Statins

Abbreviations

ApoB100	Apolipoprotein B100
BMI	Body mass index
CAD	Coronary artery disease
cIMT	Carotid intima-media thickness
CKD	Chronic kidney disease
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Cardiovascular
CVD	Cardiovascular disease
EGF-A	Epidermal growth factor-like repeat A
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FH	Familial hypercholesterolemia
FIELD	Fenofibrate intervention and event lowering in diabetes
GOF	Gain of function
HeFH	Heterozygous familial hypercholesterolemia
HoFH	Homozygous familial hypercholesterolemia
LDL	Low-density lipoprotein
LDL-A	Low-density lipoprotein apheresis
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LOF	Loss of function
mAb	Monoclonal antibody
MS	Mass spectrometry
NARC-1	Neural apoptosis-regulated convertase 1
Non-FH	Nonfamilial hypercholesterolemia
PCSK9	Proprotein convertase subtilisin-kexin 9
SNP	Single-nucleotide polymorphism
SREBP2	Sterol-responsive element-binding protein 2
T1DM	Type 1 diabetes mellitus
TC	Total cholesterol
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
TRL	Triglyceride-rich lipoproteins
TSH	Thyroid-stimulating hormone
VLDLR	Very low-density lipoprotein receptor

Key Facts of PCSK9

- PCSK9 was first discovered in 2003 as an important regulator of blood cholesterol levels through the identification of individuals with PCSK9 mutations that led to more active forms of PCSK9 and very high blood cholesterol levels.
- Conversely, mutations of the PCSK9 gene that lead to less active forms of PCSK9 are associated with lower blood cholesterol levels and lower risk for coronary artery disease.
- Drugs that inhibit PCSK9 action are already under development to treat high blood cholesterol.
- Since PCSK9 is active mostly while it is in blood circulation, it is informative to measure its level in blood.
- Measurement of blood levels of PCSK9 has provided added information on how cholesterol and other blood fats are regulated in the body in health and in various disease conditions.
- Blood PCSK9 levels are also directly linked to disorders that result from blockages in blood vessels, independent of other risk factors such as blood cholesterol levels.
- Treatment of high blood cholesterol with statins, a very effective and widely used class of cholesterol-lowering drugs, increases plasma PCSK9 levels, thereby blunting their full cholesterol-lowering effect.
- The addition of anti-PCSK9 therapy has the ability to further lower blood cholesterol levels in those who are already on statins.

Definitions

Carotid intima-media thickness A measurement of the inner two layers of the main artery in the neck to evaluate the presence, progression, or regression of atherosclerosis

Gain-of-function mutation A mutation that results in an enhanced activity of the protein encoded by the gene

Heterozygote An individual with a gene variation that affects a single allele of a specific gene

Homozygote An individual with a gene variation that affects both alleles of a specific gene

Loss-of-function mutation A mutation that results in reduced or no activity of the protein encoded by the gene

Mutation A change in the nucleotide sequence of a gene that is rare (usually <1 % of the population) and may be associated with a clear manifestation of disease

Parabiosis An intracellular enzyme that converts a variety of inactive precursor proteins within cells into active products

Single-nucleotide polymorphism A variation of a single nucleotide in the nucleotide sequence of a gene. This is often seen in a significant proportion of the population and is more common than a mutation. Its effect on biologic function is usually less than that of a mutation

Sterol-responsive element-binding protein 2 A protein that upregulates synthesis of enzymes involved in intracellular sterol production

Variant/variation Any change in the nucleotide sequence of a gene. This term includes mutations and polymorphisms

Introduction

Proprotein convertase subtilisin-kexin 9 (PCSK9) was discovered in 2003 as the ninth member of the secretory subtilase family of proprotein convertases (PCs) that are responsible for proteolytic activation of precursor proteins in the secretory pathway. PCSK9 is expressed primarily in the liver, small intestine, kidneys, and brain (Seidah et al. 2003). It was initially named neural apoptosis-regulated convertase 1 (NARC-1) for its ability to enhance recruitment of undifferentiated neural progenitor cells into the neuronal lineage (Seidah et al. 2003). The importance of PCSK9 in lipoprotein homeostasis was recognized in the same year, when two single-nucleotide polymorphisms (SNPs) in the *PCSK9* gene resulting in the amino acid conversion of S127R within the prodomain and F216L within the catalytic domain of PCSK9 were shown to associate with a form of autosomal dominant familial hypercholesterolemia (FH) (Abifadel et al. 2003). Conversely, loss-of-function (LOF) variants of PCSK9 were subsequently shown to be associated with lower low-density lipoprotein cholesterol (LDL-C) and a significant reduction in risk of coronary artery disease (CAD) (88 % and 47 % for C679X and R46L heterozygotes, respectively) (Cohen et al. 2006).

These clinical observations were accompanied by overexpression studies of PCSK9 in mice which showed a reduction in LDLR protein but not mRNA, suggesting that PCSK9 accelerated turnover of the LDLR, resulting in hypercholesterolemia (Benjannet et al. 2004; Maxwell and Breslow 2004; Park et al. 2004). On the other hand, knockout of *PCSK9* in mice resulted in a 2.8-fold increase in LDLR protein (compared to wild-type littermates) leading to increased clearance of LDL and a decrease in plasma cholesterol levels of about 40 % (Rashid et al. 2005). Again, mRNA was not affected. PCSK9 mRNA levels are responsive to cellular cholesterol levels through the transcription factor, sterol-responsive element-binding protein 2 (SREBP2) (Horton et al. 2003; Maxwell et al. 2003).

Subsequent studies showed that secreted PCSK9 binds to the epidermal growth factor-like repeat A (EGF-A) domain of the LDLR (Zhang et al. 2007) and is

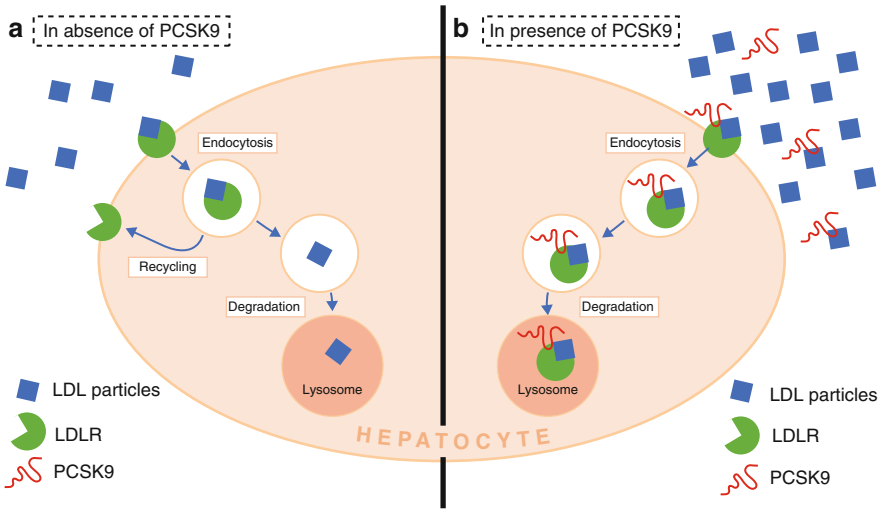

Fig. 1 Mechanism of action of PCSK9. (**a**) In the absence of PCSK9, the LDL particle in the endosome is directed to the lysosomal compartment for degradation, while the LDLR is recycled back to the cell surface through the endosomal recycling pathway. (**b**) In the presence of PCSK9, PCSK9, bound to the EGF-A domain of the LDLR, is endocytosed with the LDL-LDLR complex. PCSK9 prevents recycling of LDLR to the cell surface and directs LDL-LDLR to the lysosomal compartment for degradation

endocytosed with the LDLR (Lagace et al. 2006). PCSK9 redirects the LDLR from the endosomal recycling pathway to the lysosomal compartment for degradation, thereby modifying circulating LDL-C levels (Fig. 1) (Nassoury et al. 2007; Zhang et al. 2007).

There is significant evidence that PCSK9 is secreted before it interacts with cell surface LDLR. The introduction of PCSK9 into mice, directly or through parabiosis, reduced hepatic LDLR levels (Lagace et al. 2006). Addition of recombinant PCSK9 to medium of cultured cells results in LDLR degradation (Lagace et al. 2006; Fisher et al. 2007). This need for secretion draws attention to the potential value of measuring the concentration of PCSK9 in circulation. There is also evidence that PCSK9 may interact with the LDLR without prior secretion (Poirier et al. 2009).

Although it is identified as a member of a family of proteolytic enzymes, PCSK9's natural substrate is unknown and so is its physiological function, although its promotion of LDLR degradation indicates a role in cholesterol homeostasis. It is interesting to note that this role does not require its enzymatic property (McNutt et al. 2007).

Detection of PCSK9 in Human Circulation

The initial experiments on NARC-1 clearly showed that CHO and HK293 cells, stably or transiently transfected with human or mouse *NARC-1*, secreted NARC-1/PCSK9 into the culture medium (Seidah et al. 2003). This observation suggested

that PCSK9 could be detected in human plasma. Confirmation of this was soon published (Alborn et al. 2007; Mayne et al. 2007, 2008; Lambert et al. 2008; Dubuc et al. 2010). In studies involving total and liver-specific *pcsk9* knockout mice, it has been demonstrated that the main source of circulating PCSK9 is the liver, despite significant expression in the intestine and kidneys (Rashid et al. 2005; Zaid et al. 2008).

PCSK9 has since been measured in plasma or serum in normal and abnormal conditions, and this chapter reviews the potential role of circulating PCSK9 measurement as a biomarker in cardiovascular disease (CVD). A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). This chapter examines PCSK9's role as an indicator of "normal biologic processes," "pathogenic processes," and "pharmacologic responses to a therapeutic intervention," but it will be restricted to the context of CVD. Before doing this, we discuss the various forms of circulating PCSK9 and address the question of whether plasma PCSK9 levels reflect genetic variance of the *PCSK9* gene.

Circulating Forms of PCSK9

PCSK9 is synthesized as a ~74 kDa proprotein from which a prodomain of ~14 kDa is autocatalytically cleaved within the secretory pathway. The prodomain remains attached to PCSK9 by non-covalent bonds. This autocatalytic cleavage is essential for the exit of PCSK9 from the endoplasmic reticulum (ER) as demonstrated in the Q152H variant in which this cleavage fails to occur resulting in markedly lowered plasma PCSK9 levels (Mayne et al. 2011). There is also evidence that in the absence of the N-terminal sequence of the prodomain (aa 31–58), the PCSK9 complexed with the prodomain is severalfold more active in degrading LDLR, suggesting that the prodomain is a negative regulator of PCSK9's degradation activity on the LDLR (Benjannet et al. 2010). It is likely that most reported assays of PCSK9 measure the prodomain-associated PCSK9 molecule.

There are three other issues about circulating PCSK9 to address. The various forms of circulating PCSK9 are illustrated in Fig. 2. PCSK9 circulates as its parent form as well as a furin-cleaved form. Membrane-bound furin, another PC, cleaves the parent PCSK9 at the Arg^{218}-Gln^{219} peptide bond (Benjannet et al. 2006; Essalmani et al. 2011) to result in a furin-cleaved form that lacks a ~7 kDa segment, Ser^{153}-$Arg^{218,}$ within the catalytic domain. The ~53 kDa furin-cleaved PCSK9 has been reported to have no LDLR degradation activity likely because of the loss of a region of PCSK9 required for LDLR binding as well as the concomitant loss of the ~14 kDa prodomain (Benjannet et al. 2006; Essalmani et al. 2011). Another study, however, has shown that furin cleavage did not result in dissociation of the prodomain (Han et al. 2014). With regard to LDLR degradation activity, yet another study has shown that furin-cleaved PCSK9 does have LDLR degradation activity, but it is less efficient than the intact parent PCSK9 (Lipari et al. 2012).

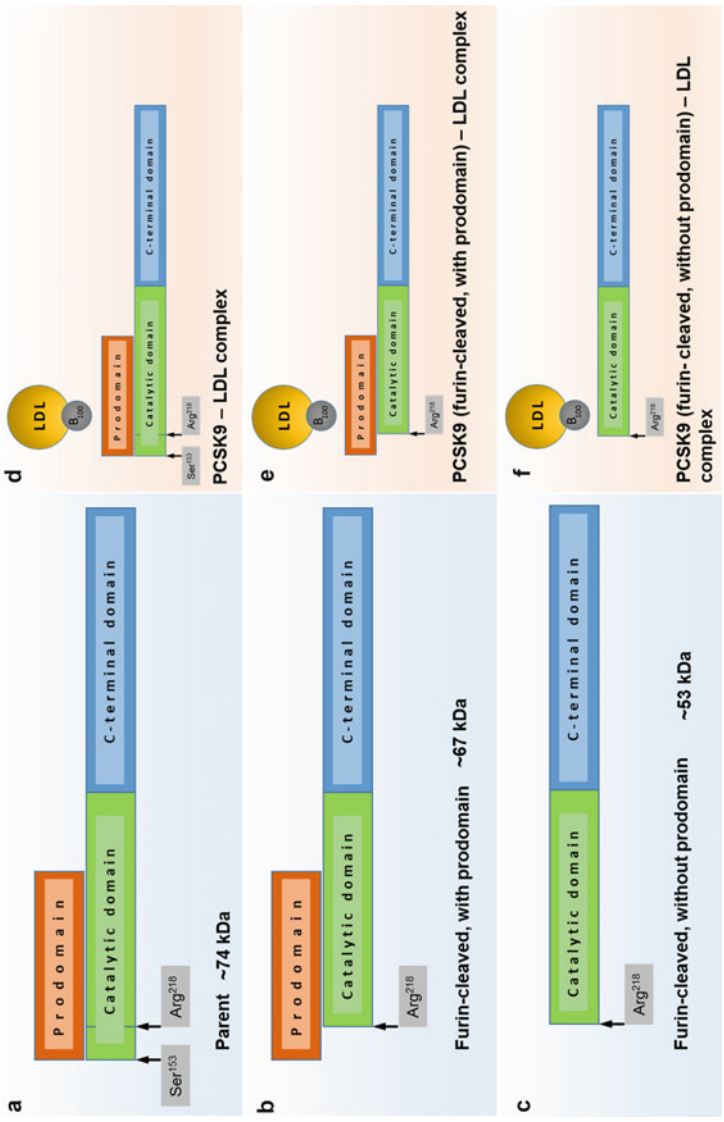

Fig. 2 (continued)

Until recently, most reports on circulating PCSK9 have not distinguished between the parent PCSK9 and furin-cleaved PCSK9. However, two recent reports have distinguished between these two forms of PCSK9 (Han et al. 2014; Hori et al. 2015). A method to isolate the truncated form from the parent PCSK9 was developed using differential binding of monoclonal antibodies (mAbs) to the two forms; the C-terminal domain PCSK9 mAb binds both forms and a catalytic domain mAb binds only the parent PCSK9 (Han et al. 2014). Mass spectrometry (MS) and ELISA methods to detect PCSK9 indicated that ~30 % of total PCSK9 was in the truncated furin-cleaved form. This was demonstrated in serum samples from only 20 human subjects. A recombinant form of this truncated PCSK9 was shown to be inactive on LDLR. They further showed that the weak correlation demonstrated between total PCSK9 (parent plus furin-cleaved PCSK9) and LDL-C was not improved by considering only the active parent form of PCSK9 (Han et al. 2014). Confirmation of these findings in a larger population is needed.

The other group that looked into this also used differential binding of mAb to measure the two forms of circulating PCSK9 (Hori et al. 2015). They found that the furin-cleaved form constituted 15 % of total PCSK9, unlike the study of Han et al. in which the figure was 30 %. They also demonstrated that both forms of PCSK9 was removed by LDL apheresis (LDL-A) by about 46–56 %.

Apart from these two studies, most other studies reporting on serum or plasma PCSK9 levels have not distinguished between the parent and the furin-cleaved forms of PCSK9. Since the furin-cleaved form has been shown to be inactive or less active, it is theoretically necessary to measure the parent form alone for proper interpretation of the role of PCSK9 activity in any situation. However, whether it is essential to do so is still unclear. A superior method that could distinguish the various forms of circulating PCSK9 is MS (Dewpura and Mayne 2011), but this is expensive and not widely available.

The next aspect of circulating PCSK9 is that ~30–40 % is associated with LDL particles via an interaction with apolipoprotein B100 (apoB100) (Sun et al. 2012; Kosenko et al. 2013; Tavori et al. 2013), likely at a single apoB100 molecular site (Kosenko et al. 2013). In one study, PCSK9 associated with LDL is a monomer, while the rest of unassociated PCSK9 is found in larger complexes. It also showed that PCSK9 existing as complexes is the active form of PCSK9 (Fan et al. 2008; Kosenko et al. 2013). However, there is also evidence that monomeric PCSK9 is active (Lagace et al. 2006). In cell culture studies, LDL inhibited PCSK9 binding to

Fig. 2 Multiple forms of circulating PCSK9. The parent form of PCSK9 has a prodomain that is attached to it by non-covalent bonds. (**a**) About 15–30 % of PCSK9 has a ~7 kDa segment, Ser153–Arg218, within the catalytic domain cleaved off by furin. The prodomain has been variably reported as either remaining attached (**b**) or concomitantly lost from the parent molecule upon furin cleavage (**c**). About 30–40 % of these intact (**d**) and furin-cleaved PCSK9, in turn, are bound to LDL particles via an interaction with apoB100 (**e**, **f**). The binding sites on PCSK9 and apoB100 have not been clearly determined. Not shown in the figure are PCSK9 molecules that are bound to monoclonal antibodies (mAbs) in patients receiving anti-PCSK9 mAb therapy for treatment of hypercholesterolemia

LDLR, and this was independent of the LDL-LDLR interaction (Kosenko et al. 2013). Specifically in FH patients, gel filtration chromatography analysis has shown that 20 % of total plasma PCSK9 exists in the apoB-containing fraction, and both the parent and the furin-cleaved forms of PCSK9 in this fraction were reduced by 92–97 % by LDL-A.

Another aspect to consider about measurement of circulating PCSK9 is that during anti-PCSK9 mAb therapy, papers have reported on measurement of plasma-free or unbound PCSK9 levels. In this setting, free PCSK9 refers to PCSK9 that is unbound to the mAb. It is unclear whether the unbound PCSK9 is partially bound to LDL particles and whether it is total or only mature or only furin-cleaved PCSK9 that is measured. The method for measurement of free PCSK9 in evolocumab trials has been well characterized (Colbert et al. 2014). Plasma-free PCSK9 levels were decreased by as much as ~90 %, the magnitude and duration of PCSK9 reductions being related to the extent and duration of LDL-C reductions (Blom et al. 2014). In trials on alirocumab, both total and free PCSK9 levels have been measured. Total PCSK9 was obtained by dissociating soluble PCSK9 from PCSK9/alirocumab complexes by means of an acid-wash step. In these trials, plasma total PCSK9 levels were markedly increased, likely the result of slower clearance of the bound complex from the circulation. In contrast, there was a marked reduction in free PCSK9 concentration (Roth et al. 2012). These are not unexpected findings. Plasma-free PCSK9 levels have the potential of serving as a biomarker of pharmacological response. However, the full value of plasma total and free PCSK9 measurement as an aid to the establishment of the pharmacokinetic and pharmacodynamic properties of this form of therapy has not been fully developed (Colbert et al. 2014).

PCSK9 Is a Polymorphic Gene: Do Plasma PCSK9 Levels Reflect Gene Variation?

PCSK9 is located on human chromosome 1(1p32) and has 12 exons. Many studies have demonstrated the presence of a large number of variations in the *PCSK9* gene (Abifadel et al. 2009). Among these variants, some are gain-of-function (GOF) mutations that are associated with a clear phenotype such as autosomal dominant hypercholesterolemia, while others are LOF mutations associated with hypocholesterolemia. In addition to these important but rare mutations, polymorphisms that have smaller effects (some elevating, others lowering) or no effects on LDL-C levels are much more common.

The key question here is whether the GOF or LOF is mediated through an increase or a decrease in circulating plasma PCSK9 levels, respectively. This would be dependent on the functional defects associated with the mutation/polymorphism. For those that are associated with decreased secretion of PCSK9 due to various secretory defects, LOF would be expected to be mediated by decreased circulating PCSK9 levels. There is a report of a young female with compound heterozygosity for LOF PCSK9 mutations who had both a very low LDL-C and

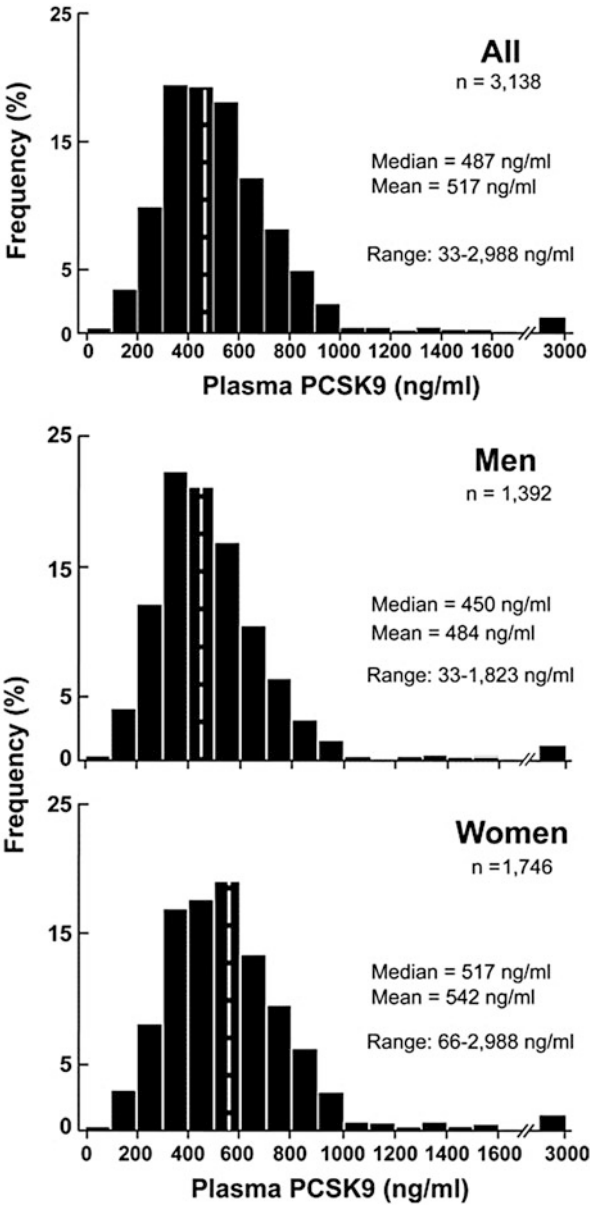

Fig. 3 Distribution of fasting plasma concentrations of PCSK9 in the Dallas Heart Study. The distribution of fasting plasma concentrations of PCSK9 in the Dallas Heart Study ($n = 3138$) excluding individuals on statins (97 women and 117 men) in all subjects (**a**), in men only (**b**; $n = 1392$), and in women only (**c**; $n = 1746$). Significant differences were observed between men and women after adjustment for age, ethnicity, BMI, diabetes, and plasma levels of LDL-C, HDL-C, and triglycerides ($P < 0.0001$) (Reprinted with permission from Lakoski et al. 2009)

an undetectable level of plasma PCSK9 (Zhao et al. 2006). However, the functional defects associated with mutations/polymorphisms are not all known.

In the Dallas Heart Study where plasma PCSK9 levels were measured in individuals with known mutations/polymorphisms, it was shown that LOF mutations/polymorphisms were associated with lower plasma PCSK9 levels (Fig. 3) (Lakoski et al. 2009). However, there are exceptions to an association between

LDL-C effect and circulating PCSK9 levels. Subjects with the GOF variant, D374Y, had markedly elevated LDL-C, but their plasma PCSK9 levels were low (Humphries et al. 2009). In subjects with the minor A53V variant which is associated with a reduction in LDL-C by ~15 %, there was no difference in their plasma PCSK9 levels from normal controls with no *PCSK9* variant (Mayne et al. 2013).

Given the large body of information available on PCSK9's role in LDL clearance, variants are currently designated as having GOF or LOF properties based on their effect on serum LDL-C levels. As knowledge of other possible functions of PCSK9 evolve, it is possible that the definition of GOF and LOF pertaining to PCSK9 may change. This concept is particularly plausible in the area of triglyceride-rich lipoprotein metabolism (Tavori et al. 2015).

Physiological Status of Circulating PCSK9

Circulating PCSK9 shows a diurnal rhythm synchronous with cholesterol synthesis and growth hormone secretion. It also shows a significant fall of ~35 % after 18 h of fasting (Persson et al. 2010). In another study, blood was collected every 4 h from the start of fasting and there was no change in serum PCSK9 level after 12 h of fasting, but a significant fall of ~14 % was seen at 16 h (Browning and Horton 2010). Ideally, measurement of plasma PCSK9 should be standardized to time of day and feeding status.

Diet also seems to have an effect on circulating plasma PCSK9 levels. A Mediterranean diet that reduces LDL-C is associated with a reduction in PCSK9 levels even in the absence of weight loss (Richard et al. 2012).

PCSK9 concentrations vary very widely among studies, likely the result of different binding characteristics of various polyclonal and monoclonal antibodies and PCSK9 standards used in the assays. Thus, inter-study comparisons of absolute PCSK9 levels should not be made until standardized assays are used. Plasma PCSK9 concentrations also show a wide range within a population using a single assay. It was measured by ELISA in the large multiethnic population of the Dallas Heart Study ($n = 3138$) (Lakoski et al. 2009). The distribution of fasting plasma PCSK9 levels in this population is shown in Fig. 4. They showed a rightward skew in distribution of PCSK9 levels in the population. There was a 100-fold range in plasma PCSK9 levels. Females had higher levels than males, and this difference persisted after adjusting for age, ethnicity, BMI, systolic BP, menopausal status, and fasting levels of glucose, LDL-C, HDL-C, TG, and CRP. Postmenopausal women had higher levels than premenopausal women. Curiously, in postmenopausal women, there was no difference in PCSK9 levels between those receiving and those not receiving estrogen replacement therapy, suggesting that the higher PCSK9 levels in postmenopausal women may be the result of diminished LDLR-mediated clearance of PCSK9 since hypoestrogenemia in menopause is associated with a decrease in LDLR (Walsh et al. 1991).

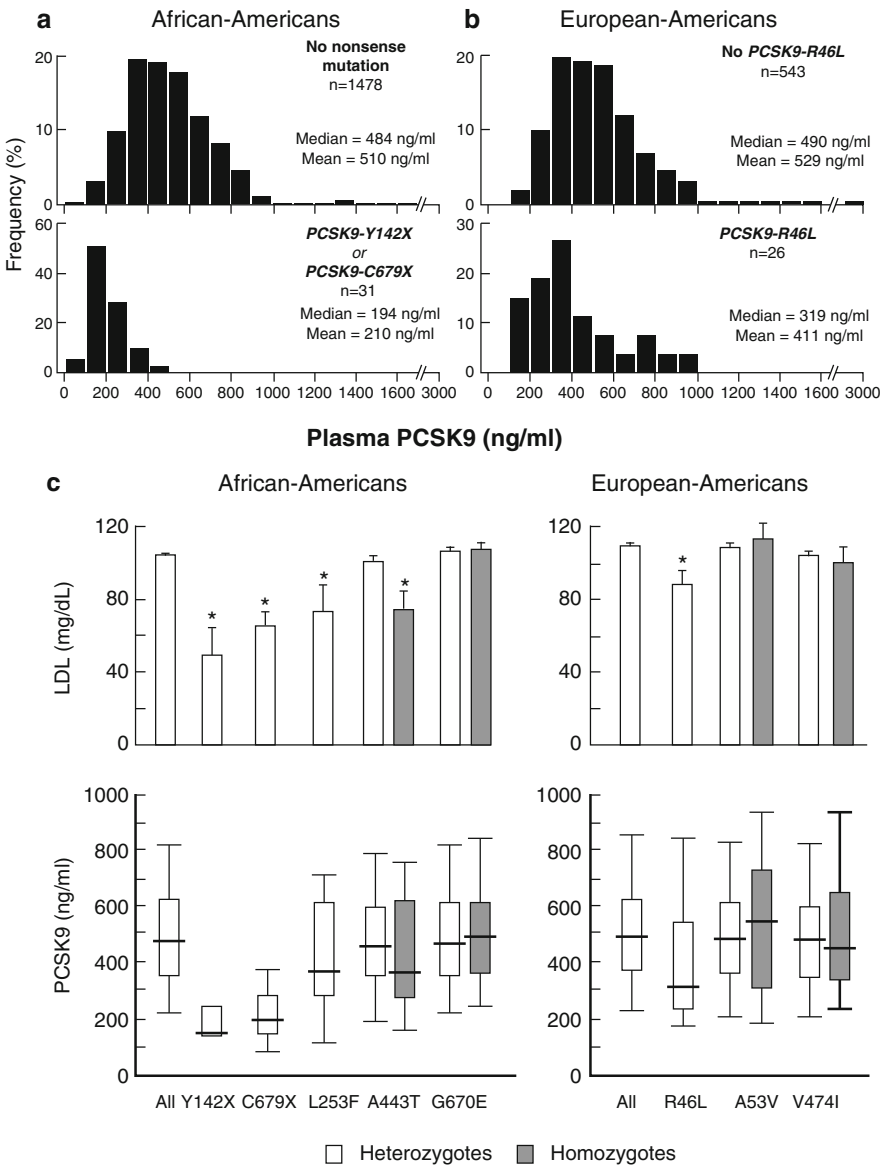

Fig. 4 Distribution and median plasma levels of plasma PCSK9 levels in African-Americans and European-Americans with various sequence variations in PCSK9. The distribution of plasma PCSK9 levels in African-Americans who were heterozygous for a nonsense mutation in PCSK9 (Y142X or C679X) (**a**), European-Americans heterozygous or homozygous for PCSK9:R46L (**b**), and median plasma levels of PCSK9 in African-Americans and European-Americans with various sequence variations in PCSK9 (**c**). (**a**) African-American individuals heterozygous for a nonsense mutation had significantly lower median PCSK9 concentrations compared with those without the mutation after adjusting for age and sex ($P < 0.0001$) and after adjusting for LDL-C ($P < 0.0001$). (**b**) In European-Americans, individuals heterozygous for R46L had significantly lower plasma

In a population of 1739 French-Canadian children and adolescents aged 9, 13, and 16 years, plasma PCSK9 measured by ELISA decreased with age in boys, but it was the opposite in girls (Baass et al. 2009).

Human plasma PCSK9 has been shown to be elevated in pregnancy at term when compared to nonpregnant, age-matched female controls, and umbilical cord blood had lower serum PCSK9 levels than maternal blood (Peticca et al. 2013). In another neonatal study, circulating PCSK9 levels showed gender-based differences and were significantly correlated with LDL-C. Their results suggest that PCSK9 could play an important role in regulating LDL-C levels during the fetal period (Araki et al. 2014).

In most populations, plasma PCSK9 levels correlate with multiple demographic and lipid and carbohydrate metabolism variables. The highest correlation is with LDL-C in nondiabetic (Alborn et al. 2007; Mayne et al. 2007; Lakoski et al. 2009) and in diabetic patients (Lambert et al. 2008). PCSK9 associates with LDL-C levels in most states of health and disease. This is consistent with the joint regulation of LDLR and PCSK9 by SREBP2 and our current understanding of PCSK9's role in LDLR degradation. Both are cleared from circulation by the same pathway. The positive correlation between serum PCSK9 and LDL-C is, however, relatively weak in contrast to the much greater effect of genetic variation in PCSK9 activity on serum LDL-C levels (Lakoski et al. 2009). Thus, serum PCSK9 levels provide a limited indication of functional PCSK9 activity. This may in future be explained in part by consideration of which of the different forms of circulating PCSK9 are measured.

The next highest correlation is with triglycerides (Lakoski et al. 2009) suggesting a possible link to the metabolism of triglyceride-rich lipoproteins as well. However, an association with TG is not consistently found, with some reports indicating no correlation (Lambert et al. 2008; Mayne et al. 2008). The correlation with serum glucose, insulin, and HOMA raises the possibility of a causative role in the metabolic syndrome.

Circulating PCSK9 in Abnormal States Associated with CVD Dyslipidemias (Familial Hypercholesterolemia, Familial Combined Hyperlipidemia)

The majority of patients with familial hypercholesterolemia (FH) have an LDLR gene mutation that results in absent or defective LDLR (FH1) or a mutation in the apolipoprotein B100 (apoB100) gene that results in a defective apoB100 (FH2). FH

Fig. 4 (continued) PCSK9 in age and sex-adjusted models ($P = 0.0004$) and after adjusting for plasma LDL-C levels ($P = 0.004$). (**c**) Relationship between nonsynonymous variants in PCSK9 and plasma levels of LDL-C in African-Americans (all, $n = 1607$) (*left*) and European-Americans (all, $n = 909$) (*right*) in the Dallas Heart Study. Individuals heterozygous for nonsynonymous variants not associated with changes in LDL-C were included in the analysis (PCSK9: G670E, A53V, V474I). Subjects taking statins were excluded.*, $P < 0.05$ (Reprinted with permission from Lakoski et al. 2009)

resulting from a GOF PCSK9 mutation (FH3) is considerably less common. Thus, serum PCSK9 levels reported in FH patients where genetic defect is not specified can be assumed to mostly reflect levels in FH1 (and to a lesser extent, FH2) and not FH3. High serum PCSK9 levels have been reported in patients with FH. Patients with homozygous FH (HoFH) have higher PCSK9 levels than patients with heterozygous FH (HeFH) who in turn have higher levels than non-FH controls. Their levels are further increased with statin therapy, more so in HeFH than in HoFH (see below) (Raal et al. 2013). As with other populations, serum PCSK9 levels correlate with serum LDL-C levels, indicating that PCSK9 plays a role in the regulation of LDL-C in FH, as in the general population. A recent report has demonstrated that elevated PCSK9 levels were equally detrimental for patients with HeFH or non-FH; a 100-ng/ml increase in PCSK9 led to an increase in LDL-C of 0.20–0.25 mmol/l in controls and HeFH alike, irrespective of their LDLR mutation (Lambert et al. 2014).

The mechanism for elevation in plasma PCSK9 in FH is likely a decrease in LDLR-mediated elimination of PCSK9. This phenomenon is supported by studies in transgenic mice overexpressing human PCSK9 showing that the clearance of serum PCSK9 is due predominantly to LDLR-mediated uptake PCSK9 (Tavori et al. 2013). However, there is also evidence for LDLR-independent pathways for clearance of PCSK9, but these remain to be more clearly demonstrated (Cameron et al. 2012; Tavori et al. 2013).

It is interesting to note that in FH, LDL-C elevation is proportionately greater than PCSK9 elevation, thereby giving rise to higher LDL-C/PCSK9 ratios. The explanation for this is not clear, but a plausible one might be that the higher PCSK9 level from diminished LDLR-related PCSK9 elimination might promote degradation of LDLR from the normal LDLR allele in HeFH, thus setting off a vicious circle of even higher circulating LDL-C (Tavori et al. 2013). Yet another explanation is that the elevation in circulating PCSK9 may not be as much as one might expect from the LDLR deficiency. Thus, the correlation between LDLR function and serum PCSK9 levels is poor, and there is overlap of PCSK9 levels among FH and non-FH patients. This may stem from the ability of dysfunctional LDLR to clear PCSK9 and from clearance of PCSK9 through non-LDLR pathways (Tavori et al. 2013).

Plasma PCSK9 levels are high in patients with familial combined hyperlipidemia (FCH), and they are correlated with plasma lathosterol levels but not markers of cholesterol absorption, suggesting that SREBP2 activation partly accounts for the high PCSK9 in FCH (Brouwers et al. 2013).

Diabetes, Glucose Homeostasis, and PCSK9

There are conflicting reports on the relationship between glucose and insulin metabolism and circulating PCSK9. In the Dallas Heart Study cohort, there was a correlation between plasma PCSK9 and plasma levels of insulin and glucose (Lakoski et al. 2009). Also, in a cohort of children and adolescents aged 9–16, there were positive associations between PCSK9 and fasting glucose, insulin, and homeostasis

model assessment of insulin resistance (HOMA-IR) (Baass et al. 2009; Dubuc et al. 2010). There is also evidence from rodent studies that *PCSK9* expression is induced by insulin and suppressed by fasting via a pathway involving SREBP1c and liver x receptor (Costet et al. 2006).

In contrast, plasma PCSK9 levels were not increased by exposure to a 24-h infusion of insulin in a hyperinsulinemic glucose clamp experiment in eight healthy subjects and eight patients with T2DM (Kappelle et al. 2011). In another study, plasma PCSK9 levels were in fact decreased by 15.4 % during an acute 3-h euglycemic-hyperinsulinemic clamp study in 82 nondiabetic postmenopausal obese patients (Awan et al. 2014). In addition, plasma PCSK9 levels were demonstrated to be not different among patients with normal glucose metabolism, impaired glucose metabolism, and T2DM (Brouwers et al. 2011). The presence of T2DM, however, was associated with steeper regression slopes for the associations with non-HDL-C and apoB. Finally, no association was found between plasma PCSK9 levels and BMI, waist circumference, fat and fat-free mass, or visceral and subcutaneous adipose tissue measured by computed tomography in abdominally obese men. There was only a trend but insignificant decrease in plasma PCSK9 levels after weight loss associated with a lifestyle modification program in abdominally obese men (Arsenault et al. 2014).

The impact of PCSK9 deficiency on glucose metabolism also remains unclear with one study showing impaired glucose tolerance and pancreatic islet abnormalities in PCSK9-deficient mice (Mbikay et al. 2010), while another failed to detect any alteration in glucose homeostasis in PCSK9-deficient mice (Langhi et al. 2009).

With these conflicting data on the association of PCSK9 with insulin and glucose metabolism, PCSK9's role in the CVD associated with various forms of dyslipidemia, especially the metabolic syndrome, remains unclear.

Plasma PCSK9 levels have been reported in only one study in patients with type 1 diabetes mellitus (T1DM), and they have lower levels than patients with T2DM. However, data from this study need verification as ~60 % of their participants were on statin therapy, which affects plasma PCSK9 levels (Cariou et al. 2010).

Chronic Kidney Disease (CKD)

In chronic kidney disease, plasma PCSK9 levels seem to be dependent on the stage of CKD. In the earlier stages of CKD (2, 3, and 4) and in nephrotic syndrome, plasma PCSK9 levels are higher in association with higher LDL-C levels (Kwakernaak et al. 2013b). However, once patients have reached CKD stage 5 and are on hemodialysis, plasma PCSK9 levels are low in association with lower LDL-C levels (Abujrad et al. 2014; Jin et al. 2014). In contrast, patients with CKD stage 5 on peritoneal dialysis have high plasma PCSK9 levels as well as high levels of LDL-C (Jin et al. 2014). Thus, plasma PCSK9 levels tend to track with LDL-C levels in CKD. The underlying mechanisms responsible for changes in plasma PCSK9 levels in CKD are unknown.

Hypothyroidism

In a group of 64 nonobese subjects, plasma PCSK9 correlated positively with serum TSH (Kwakernaak et al. 2013a). However, an acute increase of TSH levels following administration of recombinant human TSH did not raise PCSK9 levels in patients who had previously undergone total thyroidectomy and radio-ablation for thyroid cancer (Gagnon et al. 2014). This might suggest that changes in plasma PCSK9 levels are due to the effect of thyroid hormone and not TSH. In a study of 20 hyperthyroid patients, studied before and after clinical normalization, hyperthyroidism was associated with reduced circulating PCSK9, which may contribute to lower plasma LDL cholesterol in hyperthyroidism (Bonde et al. 2014).

Circulating PCSK9 and Vascular Disease

The ultimate value of PCSK9 as a biomarker is when it serves as a useful marker not just of surrogate disease markers such as serum LDL-C but of morbidity and mortality from CVD. Evidence is just starting to emerge. In a cross-sectional study of 243 patients with angiographic CVD, plasma PCSK9 levels were positively associated with the extent of coronary stenosis expressed as Gensini score, independent of age, BMI, systolic BP, smoking, family history of CVD, glucose, LDL-C, HDL-C, and TC/HDL-C and LDL-C/HDL-C ratios. There was also an association of plasma PCSK9 with the number of diseased vessels. These patients were free of lipid-lowering drug therapy for at least 3 months before entry into the study (Li et al. 2014). Another study showed that elevated serum PCSK9 levels were associated with cardiovascular (CV) events in 504 patients with stable CVD on statin therapy during a 48-month follow-up period. This study provides an added perspective in that their patients were on statin therapy, which is known to elevate serum PCSK9 concentrations. Thus, with serum LDL-C well controlled by statin therapy, serum PCSK9 remained a good predictor of CV events (Werner et al. 2014). Finally, plasma PCSK9 levels were shown to be elevated with acute myocardial infarction in two independent retrospective angiographic populations. Their patients were not on statin therapy. In one population, plasma PCSK9 levels were not associated with CVD, but in the second population, they were (Almontashiri et al. 2014). Finally, there is also evidence of an association of serum PCSK9 levels with carotid intima-media thickness (cIMT) in hypertensive subjects, independent of age, sex, total cholesterol, triglycerides, and HDL-C (Lee et al. 2013), and in non-FH patients after adjustment for lipid levels and other traditional risk factors (Huijgen et al. 2012).

In mice, gene inactivation of PCSK9 was shown to reduce atherosclerosis. There was a direct relationship between PCSK9 expression and atherosclerosis. PCSK9 overexpression is proatherogenic, whereas its absence is protective (Denis et al. 2012).

Circulating PCSK9 in Response to Lipid-Altering Drug

Statins

Statins have been shown to elevate plasma PCSK9 levels by ~30–50 % in humans (Dubuc et al. 2004, 2010; Careskey et al. 2008; Mayne et al. 2008; Cariou et al. 2010). In some studies, the rate of PCSK9 increase was shown to depend on statin dose and duration of therapy. This is not surprising as statins reduce intra-hepatocyte sterol concentrations, triggering SREBP2 synthesis which results in upregulation not only of LDLR but also PCSK9 (Horton et al. 2003; Maxwell et al. 2003). In all these studies, the association between PCSK9 levels and serum LDL-C was disrupted by statin therapy. If not for the increase in serum PCSK9 and presumably its activity in promoting LDLR degradation, the LDL-C lowering effect of statins would be greater. This line of thinking has prompted the current vigorous efforts to find strategies to inhibit the action of PCSK9.

Fibrates

The response of plasma PCSK9 to fibrate therapy is not as consistent across reports as with statin therapy. Some studies have shown an increase in patients with combined hyperlipidemia (Mayne et al. 2008; Khera et al. 2015), in patients with high triglycerides and low HDL-C (Troutt et al. 2010), and in patients with impaired glucose tolerance or T2DM (Costet et al. 2010; Noguchi et al. 2011). Other studies have shown a decrease in plasma PCSK9. This was demonstrated in a group of 115 statin-naïve type 2 diabetes mellitus (T2DM) patients in the FIELD study where fenofibrate therapy reduced PCSK9 by 8.5 % (Lambert et al. 2008). In another study of T2DM individuals already on statin therapy, add-on fenofibrate therapy reduced serum PCSK9 by 13 % along with a decrease in the VLDL particle concentration (Kourimate et al. 2008; Chan et al. 2010). While statin therapy seems to increase PCSK9 expression at a transcriptional level with increased PCSK9 mRNA, the mechanism by which fibrates alter circulating PCSK9 is not clear.

Ezetimibe

Another drug studied is the cholesterol absorption inhibitor, ezetimibe. There are reports that it further increases plasma PCSK9 levels in patients who were already on statin therapy (Davignon and Dubuc 2009; Dubuc et al. 2010), but there is another report that shows that it does not alter plasma PCSK9 levels (Berthold et al. 2013).

Niacin

The effect of niacin monotherapy on plasma PCSK9 levels has not been studied, but in studies where niacin was added to statin therapy or statin plus fibrate therapy, a decrease in plasma PCSK9 was observed. A positive association was noted between change in PCSK9 and low-density lipoprotein cholesterol levels with the addition of niacin suggesting that a portion of the LDL-C reduction seen with niacin therapy may be due to reduction in PCSK9 (Khera et al. 2015).

Bile Acid Resins

The effect of bile acid resin therapy on human plasma PCSK9 levels has not been studied, but it would be expected that it would elevate PCSK9 levels through upregulation of SREBP2. This was indirectly demonstrated in a human study in which preoperative cholestyramine treatment was associated with a 70 % increase in PCSK9 mRNA expression by liver tissue obtained at surgery (Nilsson et al. 2007).

Potential Applications to Prognosis, Other Diseases, or Conditions

Apart from PCSK9's role as a modulator of lipoprotein metabolism and CV health, there are indications that it may play an etiologic or prognostic role in other disease processes (Mbikay et al. 2013). Besides blood, PCSK9 has been demonstrated in human cerebrospinal fluid (CSF) (Chen et al. 2014). This is not surprising since PCSK9 was first recognized for its ability to enhance recruitment of undifferentiated neural progenitor cells into the neuronal lineage (Seidah et al. 2003). PCSK9 has also been shown to bind to and regulate other membrane-bound receptors such as the VLDLR (Poirier et al. 2008; Roubtsova et al. 2011) and the apolipoprotein E2 receptor (Poirier et al. 2008), both of which are highly expressed in the central nervous system (CNS). Unlike serum PCSK9 which shows a distinct diurnal pattern, CSF PCSK9 levels are constant throughout the day and are consistently lower than corresponding serum levels. The significance of PCSK9 in CSF remains to be explored.

Plasma PCSK9 has also been reported to be a late biomarker of severity in patients with severe trauma injury (Le Bras et al. 2013) and of periodontal infection (Miyazawa et al. 2012). Plasma PCSK9 levels may also serve a useful biomarker function in hepatitis C viral (HCV) infection. HCV is associated with VLDL and LDL particles in circulation, and these lipoviral particles enter hepatic cells through two receptors which are downregulated by PCSK9, namely, the LDLR and CD

81 (Labonte et al. 2009). Thus, PCSK9 may play a role in decreasing HCV uptake by the liver. However, it has not been demonstrated that plasma levels of PCSK9 serve as a useful prognostic biomarker of HCV infection.

Summary and Conclusions

PCSK9 is a secreted protein, and its role in promoting LDLR degradation occurs mostly after it has entered the circulation. Hence, it is informative to measure its concentration in blood. Circulating PCSK9 exists in its intact parent form as well as a furin-cleaved form, with loss of LDLR binding and degradation activity upon furin cleavage. Both forms may, in turn, be found free or bound to LDL particles, also with loss of LDLR binding and degradation activity upon binding to LDL particles. Thus, PCSK9 exists in several forms in circulation. Most studies reporting on serum or plasma PCSK9 levels have not distinguished among these various forms. It is theoretically important to do so, but there are currently no data to clearly indicate the need. In a different context, in individuals receiving anti-PCSK9 mAb therapy, there is yet another form of bound PCSK9, the mAb-bound form, leaving behind a lowered level of free PCSK9. Future measurements of circulating PCSK9 will need to account for the heterogeneity of PCSK9 in circulation.

GOF and LOF mutations and polymorphisms of PCSK9 are often reflected in higher and lower plasma PCSK9 levels, respectively, but this does not apply across the board.

Plasma PCSK9 has been measured in various states of health and disease, and results have enhanced our knowledge of lipoprotein metabolism in general as well as in abnormal states associated with CVD. It has also been measured in response to various forms of lipid-altering drug therapy.

It is currently not seen as a clinical marker of CVD risk or of disease progression. The few reports indicating an association between plasma PCSK9 levels and vascular disease, independent of other traditional risk factors including serum lipid levels, are the most direct evidence for a biomarker role for PCSK9. The underlying pathophysiologic mechanisms are, however, still unclear. Plasma PCSK9 may serve in future as a biomarker for the selection of patients for anti-PCSK9 therapy when drugs currently under development become available.

The alteration in plasma PCSK9 levels seen with drug therapy for dyslipidemia has also provided insight into pathophysiologic mechanisms. However, the data will need further development in order to serve as an aid to the establishment of the pharmacokinetic and pharmacodynamic properties of various forms of therapy.

In clinical practice, it is possible that it could be helpful in determining statin dosage, but its measurement is not essential as it is possible to simply titrate the dose of statin upward or downward as guided by LDL-C response.

In clinical research, plasma PCSK9 levels provide hypothesis-generating information which could help the design of basic science experiments that explore mechanisms of regulation, metabolism, and role of PCSK9.

Summary Points

- This chapter focuses on the role of proprotein convertase subtilisin-kexin 9 (PCSK9) as a key modulator of lipoprotein metabolism and cardiovascular health.
- PCSK9 is a secreted protein that promotes lysosomal degradation of low-density lipoprotein receptor (LDLR) resulting in an increase in serum low-density lipoprotein cholesterol (LDL-C).
- Circulating PCSK9 is heterogeneous as some of it undergoes furin-induced cleavage and/or binds to LDL particles, both resulting in a decrease in its LDLR degradation activity.
- Plasma PCSK9 shows a diurnal rhythm; decreases with fasting; is higher in women than men, after menopause, and in pregnancy at term; and is lower in umbilical cord blood.
- Plasma PCSK9 correlates with plasma LDL-C in most states of health and disease, indicating a role in the regulation of LDL-C levels.
- Gain-of-function and loss-of-function mutations and polymorphisms of PCSK9 are often reflected in higher and lower plasma PCSK9 levels, respectively, but this is not always the case.
- There is an association between plasma PCSK9 levels and vascular disease, independent of other traditional risk factors, but underlying pathophysiologic mechanisms are unclear.
- Statin therapy increases plasma PCSK9 levels, rendering statin therapy less effective.
- PCSK9 measurement could be helpful in determining statin dosage and in the future selection of patients for anti-PCSK9 therapy.

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