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CHAIR'S CORNER

Welcome to the summer issue of the LVDD newsletter. I hope all of you are enjoying the summer and have had time off to enjoy it with family and friends. The LVDD team is very excited to welcome you all to Philadelphia for our annual events during the 68th AACC Annual Scientific Meeting.

This year we have the pleasure of having a keynote lecture during the Monday night LVDD Dinner Meeting on August 1st from Professor Dan Rader, who will be updating us all with the latest research in lipoprotein research. During this session the LVDD team will also announce the Cooper award for this year. It will be presented to Professor Hitoshi Chiba for excellent contributions to service in the area of lipoproteins and vascular diseases. Dr. Chiba will follow up with a presentation on his key achievements.

Dr. Vincent Delatour from the National Metrology Institute in France will, during the Tuesday International Lipoprotein Standardization Forum, present data comparing multiple methods used for testing lipoprotein particle numbers. Dr. Delatour will present data from the BioSITrace study. We hope to follow his presentation with a panel discussion with representatives from companies involved in this study. During this symposium we will also have oral presentations from the top three abstracts submitted to AACC in the field of lipoproteins.

I would also like to encourage those of you interested in knowing more about LVDD activities and wish to be more involved to attend our Annual Executive Committee Meeting. This will be held August 2nd 4-5.30pm at the Marriott Downtown Room 304. Please note the venue and date change from what is listed in the program. The reason for the change was due to a conflict with other important program activities.

Finally, as I mentioned in the previous newsletter, the AACC's new governance framework has created a new structure featuring a Science & Practice Core Committee comprising all of the chairs of all AACC Divisions. The first Committee meetings will be held early October 2016. This new committee will serve as the focus for AACC activities in research, science and the translation of science into practice. I will keep you all updated on the progress and activities taking place at the meeting in October in the next newsletter.

In this newsletter Associate Professor Dayami Lopez from the North Carolina Central University presents a very comprehensive review on the implications of the PCSK9 protein as an indirect regulator of plasma LDL levels and as a regulator of several risk factors associated with increased metabolic risk. In this review, Dr. Lopez makes the case for using PCSK9 as a biomarker for predicting disease severity in humans.

I hope you enjoy reading this newsletter. Wish you all well until we meet in person in Philadelphia.

Best wishes,
Amar A. Sethi, MD. PhD.

PCSK9: A Biomarker of Disease Severity

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ABSTRACT

Heart disease causes more death and disability than any other disease in the US. High cholesterol, as measured by low density lipoprotein (LDL) levels, is a well-known risk factor for heart disease as LDL particles are involved in the formation of atherosclerotic plaques. Proprotein convertase subtilisin/kexin-9 (PCSK9) is an indirect regulator of plasma LDL levels since it controls the LDL receptor expression at the plasma membrane. In addition to controlling the LDL receptor expression, PCSK9 also appears to regulate the development of glucose intolerance, insulin resistance, abdominal obesity, inflammation, and hypertension. Interestingly, the magnitude of PCSK9's involvement in the onset of these metabolic abnormalities appears to be associated with age, sex, and ethnic background. Herein, we will review the possible role of PCSK9 as a biomarker of disease severity in humans.

Introduction

Familial hypercholesterolemia (FH), a major risk factor for coronary artery disease (CAD), is typically caused by defects in genes that code for proteins involved in the clearance of low density lipoprotein (LDL) [1-3]. Mutations in three main genes can cause FH in an autosomal dominant manner: the LDL receptor, apolipoprotein B (ApoB), and proprotein convertase subtilisin/kexin 9 (PCSK9) [4-17]. Heterozygous FH (HeFH) patients have reduced cellular uptake of LDL, especially by hepatocytes, increased plasma LDL concentrations, and premature CAD [4]. Homozygous FH (HoFH) is a rare, severe disorder, caused by extremely low or absent plasma clearance of LDL, substantially raised LDL concentration, and accelerated development of CAD [4]. About 15% of patients diagnosed with FH by genetic testing not always show a hypercholesterolemic phenotype [18]. Conversely, 60% of patients who are negative for known mutations in the three genes mentioned above, but have a clinical FH phenotype (non-FH), can be associated with an accumulation of polymorphisms in multiple genes [19]. Thus, it has been suggested that in patients without known mutations for LDL receptor, ApoB, and PCSK9, hypercholesterolemia may be polygenic rather than monogenic [19]. For PCSK9, it has been shown that high serum levels of this convertase, which positively associate with LDL concentration in FH patients,

contribute to the phenotypic severity of hypercholesterolemia [20, 21].

Statins and hypercholesterolemia

Hypercholesterolemia, in general, can improve with a proper diet and an exercise program and with the use of cholesterol-lowering treatments [22, 23]. Despite using an intensive treatment plan, many HeFH and non-FH hypercholesterolemic patients are unable to achieve the recommended target levels of LDL [4, 24]. Furthermore, first-line lipid-lowering therapies, such as statins, are only modestly effective for HoFH patients [4]. Statins effectively reduce (30-55%) LDL levels and CAD risk in hypercholesterolemic patients [25-30]. However, statin intolerance has been a major limitation in the widespread use of these drugs [29-34]. Many patients (10-25%) are not able to tolerate statins either at all or at a dose sufficiently high to lower their LDL to recommended goal levels [29-42].

Statin-induced myalgia or myopathy, which is confirmed by an increase in the patient's creatine kinase (CK) levels, is the most common side effect reported for these drugs [43-47]. Muscle pain due to a statin is highly responsible for the lack of patient's compliance with treatment, for a reduction in the patient's exercise routine, which asseverates his/her condition, and for 13% of the hospitalizations related to statin use [48-50]. In fact, athletes usually complain about having difficulties in their performance when taking statins [49]. Why this side effect appears is not clearly understood. However, it is suggested that it could be due to factors such as patient-specific (advanced age, small body size, female sex, ethnic background, genetic background, renal and hepatic dysfunction, hypothyroidism, diabetes, metabolic syndrome, and alcohol abuse), statin-specific (dose, lipophilicity, and type of metabolism), and whether the statin is taken alone or with other drugs [51-65]. In some rare cases, muscle damage due to statin treatment could be so intense that leads to rhabdomyolysis [51].

Another side effect associated with statins is liver toxicity, which is confirmed when the levels of alanine (ALT) and aspartate amino transaminases (AST) increase as a result of using the drug [43-69]. Like for myopathy, these elevations are dose-dependent and reverted once the statin treatment is removed or modified [66, 67].

Severe liver toxicity occurs only in about one per million patients per year, and there is no indication that patients with alcoholic steatosis or hepatitis C are at a higher risk of developing liver toxicity when using statins [67, 68].

Based on these side effects, specific recommendations are given to monitor patients on statin therapy [70]. Measurements of CK, AST, and ALT are mandatory before starting the statin treatment, at 1-3 months after starting the drug, and yearly after that [70]. Statin therapy should stop whenever CK levels reach five times and AST and ALT three times over the normal limit [70]. After waiting for three months without any treatment, the same drug at a lower dose or a different statin may be tried [70]. If the patient is recognized as statin intolerant, other drugs, which come with their list of side effects, could be introduced either alone or in combination with a smaller dose of statin [70-75]. Since the treatment with statins has been extended to children as young as eight years [76-78], the number of patients that will show symptoms of adverse effects is expected to continue to increase as time progresses [79-81]. According to the FDA, pravastatin should be used in children over eight years, whereas lovastatin, atorvastatin, and simvastatin should be used when the child is above ten years [82].

Many times, patients tolerate statins, but once their LDL falls below a certain level the clinical benefits of reducing LDL diminish causing a reduction in the patients' protection against CAD [83-86]. Patients that fail to achieve adequate reduction of LDL levels upon statin treatment are considered statin resistant [83-86]. Resistance to statin treatment appears to result from polymorphisms in multiple genes involved in cholesterol homeostasis [34]. Also, patients with inflammatory states and human immunodeficiency virus infection have diminished LDL lowering in response to statin treatment [34].

Upregulation of PCSK9 as a side-effect of statin treatment

When cholesterol biosynthesis inhibitors such as statins are administered, an initial depletion in hepatic cholesterol levels occurs. As a result, sterol regulatory element binding protein-2 (SREBP-2) is activated, which in turn upregulates the expression of the LDL receptor and PCSK9 via the sterol regulatory elements (SREs) found in the promoters of these genes [87-93]. The degradation rate of the LDL receptor protein is also increased due to the upregulation of PCSK9 [87-93]. In

fact, it is well established in humans and animal models that statin treatment increases plasma PCSK9 levels and conversely partially attenuates the effects of statins on the LDL receptor [94-99]. For example, daily treatment with 40 mg of atorvastatin increases circulating PCSK9 levels by 34% [95]. Increasing the dose of atorvastatin to 80 mg per day enhances PCSK9 levels up to 47% [100, 101]. Furthermore, treating daily with 20 mg of rosuvastatin increases plasma levels of PCSK9 by 28% in men and by 35% in women [102]. Similar results are reported in other studies [99, 103]. These data supported the development of PCSK9 inhibitors for the treatment of hypercholesterolemia, especially when combined with statins [94, 95].

The principle of the PCSK9 inhibitor was also confirmed using PCSK9 knockout (KO) mice [94]. Inactivation of PCSK9 in mice enhances sensitivity to lovastatin treatment as compared to wild-type mice [94]. Accordingly, several missense and loss-of-function (LOF) mutations in the PCSK9 gene have been linked to increased statin response and hypocholesterolemia, pointing again to the benefit of using PCSK9 inhibitors [96, 104, 105]. In fact, PCSK9 inhibitors are currently a reality [106, 107].

The discovery of PCSK9

PCSK9 was discovered in 2003 when gain-of-function (GOF) mutations in this gene were identified as causative of FH in an autosomal dominant manner and associated with a higher risk of CAD [108-126]. In fact, serum PCSK9 levels have been identified as a significant predictor of carotid atherosclerosis, independently of other risk factors [126]. LOF mutations of PCSK9 have also been discovered and are associated with hypocholesterolemia and a significant protection against CAD [120-142]. Many studies support a positive correlation between wild-type PCSK9 levels and atherogenic lipoproteins [143-150]. PCSK9 increases serum triglyceride levels by reducing the hepatic degradation of ApoB-containing lipoproteins and by enhancing production and secretion of chylomicrons in the intestine [149-154]. Interestingly, it has been proposed that the ratio of ApoB to PCSK9 should be considered a good indicator of metabolic risk in humans [155]. PCSK9 is expressed and secreted by many tissues but primarily by the liver, small intestines, and kidneys [156-158]. Recent reports suggest that PCSK9 could also be found in cerebrospinal fluid [159] and at the sites of atherosclerotic plaques [160]. Normal levels of PCSK9 in human plasma vary from 30 ng/mL up to 4 µg/mL [146,

161-163].

PCSK9 is synthesized as a 74 kDa soluble zymogen (proPCSK9) that undergoes autocatalytic processing in the endoplasmic reticulum to release the prodomain (14 kDa) from the N-terminal, resulting in a processed enzyme of about 60 kDa [164-166]. This autocleavage is necessary both for activation of the convertase and to allow its departure from the endoplasmic reticulum [164, 165]. Mature PCSK9 has three distinct domains with the prodomain noncovalently bound to the catalytic domain and the cysteine-rich, histidine-rich, C-terminal domain (CHRD), resulting in a triangular pyramid shape [122]. The presence of the prodomain in the catalytic channel must obstruct the access of other proteins and peptides to PCSK9 [122].

The noncovalently bound PCSK9/prodomain complex departs from the endoplasmic reticulum and migrates through the secretory pathway until it is secreted into the bloodstream [167, 168]. In other convertases, there is a second maturation step that releases the prodomain resulting from further proteolytic processing or a change in environmental stimuli, such as pH change or increase in calcium concentration [169]. This second maturation phase is absent in PCSK9 [169]. One possible explanation is that PCSK9 does not contain a target loop typical of other convertases that serves as the site for the second cleavage that destabilizes the prodomain [122]. No physiological event has been identified that causes the dissociation of the inhibitory prodomain from PCSK9 [169].

Protein modifications in PCSK9 include glycosylation at N533 [165], sulfation at Y38 [123], and phosphorylation at S47 and S688 [170]. Glycosylation and sulfation do not appear to be essential for PCSK9 activity [123], but phosphorylation protects PCSK9's prodomain against proteolysis and may affect its activity [170, 171]. PCSK9 also has an alternative spliced variant [172]. The alternative spliced variant results from an in-frame deletion of the eighth exon which encodes for 58 amino acids [172]. The spliced variant of PCSK9 is expressed in multiple tissues, including the liver, small intestines, prostate, uterus, brain, and adipose tissue [172]. Unlike wild-type PCSK9, the spliced form is not autoprocessed or secreted [172]. The physiological function of the spliced form is currently unknown.

PCSK9 is also cleaved to a 53 kDa-truncated protein by the action of furin [173, 174]. Both full-length and the truncated forms of PCSK9 could be detected in

human and mouse plasma associated by more than 80% with ApoB containing lipoproteins [174-178]. Indeed, both forms of PCSK9 can be significantly decreased (46-56%) in FH patients after a single LDL-apheresis treatment [177-179].

The primary function of PCSK9

PCSK9 controls LDL levels mainly by influencing the number of LDL receptor molecules expressed at the cell surface, especially in the liver [128, 129, 135]. The LDL receptor removes lipoproteins from the circulation through a process that involves endocytosis of the lipoprotein/receptor complex within clathrin-coated regions [180-183]. At the cell surface, the LDL receptor's extracellular domain is extended, exposing the ligand-binding domain (open position), which allows the binding of lipoproteins [184]. The cytosolic tail of the LDL receptor contains an ⁸²³FDNPVY sequence that is necessary and sufficient for rapid clathrin-mediated endocytosis [185, 186]. Internalization of the lipoprotein/receptor complex into hepatic cells is facilitated by the LDL receptor adaptor protein 1 (LDLRAP1) that interacts with the FDNPVY sequence of the LDL receptor [187, 188].

After endocytosis, the LDL receptor-ligand complex is delivered to the endosome [184]. Acidification of the endosome is carried out by the activity of V-type ATPases [182]. The low pH facilitates folding of the LDL receptor back upon itself, bringing the β -propeller region closer to the ligand-binding domain (closed position), which results in displacement of the lipoprotein particle [189, 190]. After being released from the receptor, the lipoprotein moves to the lysosome [182]. At that point, cholesteryl esters contained within the particle are hydrolyzed to form cholesterol and free fatty acids, and proteins are degraded into free amino acids [182]. Most of the receptor molecules are recycled back to the cell surface, where they can once again bind and internalize lipoproteins [184, 189]. Only a small percentage of LDL receptor molecules are degraded during each cycle [191]. It has been estimated that each LDL receptor molecule completes about 150 cycles before its final degradation [191].

GOF mutations of PCSK9 cause a 23% decrease in cell surface expression of LDL receptors and a 38% decrease in internalization of LDL [128, 129, 135]. The LOF mutations of this convertase lead to a 16-28% increase in cell surface LDL receptors and a 35% increased internalization of LDL [128, 129, 135]. After its

secretion, the catalytic domain of PCSK9 interacts with the EGF-A domain of the LDL receptor at neutral pH of the plasma membrane with a 1:1 stoichiometry and a K_d of 170-750 nM [192-199]. Once they interact, the PCSK9/receptor complex enters the endosomal pathway [162, 200]. In contrast to the interaction between lipoprotein and receptor, the affinity of PCSK9 for the LDL receptor at the acidic pH of the endosome is increased 150-170-fold (K_d of 1-8 nM) [196-201]. Thus, PCSK9 locks the LDL receptor in the open position, which prevents regular recycling sending the receptor to the lysosome for degradation [196-198, 202, 203].

Another form of PCSK9 has a K_d of 42 nM [122], which appears to correspond to the 53 kDa truncated PCSK9 made by furin [195]. Thus, both wild-type and truncated PCSK9 can bind to and trigger degradation of LDL receptors and elevate serum cholesterol levels [174, 204]. The catalytic activity of PCSK9 does not participate in the degradation of LDL receptor within the lysosome, but it is essential for the activation, folding and secretion of PCSK9 as explained above [205-207]. Once the PCSK9/receptor complex reaches the lysosome, the LDL receptor is degraded forming a 17 kDa C-terminal LDL receptor fragment that is further degraded by γ -secretase [208]. The complementary 143 kDa ectodomain of the LDL receptor is degraded by endosomal cysteine cathepsin [208, 209]. The LDL receptor is degraded earlier than PCSK9 [208, 209].

Deletion of the LDL receptor ligand binding domain does not significantly affect PCSK9 binding to the receptor, suggesting that PCSK9 can bind the LDL receptor independently of lipoproteins [210]. However, it is currently unknown whether the LDL receptor can bind lipoproteins and PCSK9 simultaneously or whether PCSK9 binding to the EGF-A affects lipoprotein binding to the receptor [210]. LDL and VLDL do appear to affect the LDL receptor binding affinity for PCSK9 suggesting that the contribution of secreted PCSK9 to LDL receptor lowering may be reduced by plasma LDL and VLDL levels [211].

There is evidence that PCSK9 can act on the LDL receptor after biosynthesis, but before it reaches the basolateral surface of the hepatocyte (endogenous pathway), and after PCSK9 secretion via internalization of PCSK9-LDL receptor complexes (exogenous pathway) [209, 212-214]. The LDL receptor also plays a critical role in facilitating the trafficking of PCSK9 from the endoplasmic reticulum to downstream sites in the secretory and endocytic pathways [206, 215]. In fact, it has been shown that in cells lacking the LDL receptor,

PCSK9 is mostly found in the endoplasmic reticulum, whereas when both proteins are present, PCSK9 is always found colocalizing with the LDL receptor [215]. Furthermore, the endosomal immunoreactivity of these proteins could be enhanced in the presence of NH₄Cl [208, 215], which has also been shown to prevent the PCSK9-dependent degradation of the LDL receptor [164].

Regulation and other potential roles of PCSK9

In addition to controlling the LDL receptor protein expression, PCSK9 appears to play conflicting roles in atherosclerosis development, inflammation, thrombosis, apoptosis, glucose intolerance, insulin resistance, abdominal obesity, and hypertension [163, 216-259]. Interestingly, the magnitude of PCSK9's involvement in the onset of these metabolic abnormalities appears to be associated with age, sex, and ethnic background [163, 216-259].

An increase in estrogen levels reduces plasma PCSK9 levels in female rats and in women undergoing *in vitro* fertilization [223, 260]. In females, PCSK9 is higher in the follicular phase than in the ovulatory or the luteal phases (30 and 21% higher, respectively) [261]. Postmenopausal females have 22% more PCSK9 than premenopausal females [163, 261, 262]. Another study found that PCSK9 levels significantly increase in pregnant women at the time of parturition when estrogen levels are the lowest [263]. In general, estrogen levels are inversely correlated to circulating PCSK9 in premenopausal females [261, 264]. These results would identify estrogen as a negative regulator of PCSK9 levels [163, 223, 260-264]. However, several studies have reported that women have 10% more circulating PCSK9 than men suggesting that this reverse relationship between estrogen and PCSK9 levels may only apply to females [163, 260-262, 264-266]. Changes in testosterone levels do not affect the amount of circulating PCSK9, so it does not appear to be responsible for the differences between men and women [264]. Nonetheless, testosterone deficiency in pigs has been related to an increase in PCSK9 expression and a corresponding increase in LDL levels [267].

Interestingly, giving estrogen replacement therapy to postmenopausal women does not correct PCSK9 levels suggesting that the loss of endogenous estrogen after menopause is not the only cause for the increase in circulating PCSK9 [262, 264]. Changes in thyroid hormone levels also appear to be responsible for the

increase in PCSK9 levels in post-menopausal women [163, 266, 268-270]. A study showed that there is a direct relationship between the ratio of secreted phosphoPCSK9 relative to total secreted PCSK9 with increasing concentrations of β -estradiol, suggesting a change in the functional rate of PCSK9 in the presence of β -estradiol [171]. However, there is no confirmation that phosphorylation alters the activity of PCSK9. Interestingly, high levels of phosphoPCSK9 have also been detected in correlation with insulin resistance [271].

It is important to mention that the differences in PCSK9 levels between males and females start as early as puberty [146, 163, 262]. Plasma PCSK9 levels have been shown to increase in girls while decrease in boys, during their teenage years, paralleling changes in their LDL cholesterol and TC levels [146, 163, 262]. Independently of the potential roles that estrogen and/or testosterone might have in this process, hormonal regulation underlying these gender differences in PCSK9 levels at this age have been associated with growth hormone (GH) [272]. GH reduces serum PCSK9 levels in humans [272], but in rodent, GH stimulation tends to increase PCSK9 expression [273]. One effect that has been confirmed for GH is that it is responsible for the diurnal variation of PCSK9 levels that mimics the diurnal variation of cholesterol synthesis [159, 260, 272]. Circulating PCSK9 levels are at the lowest between 3–9 pm and peak between midnight and 4:30 am in healthy subjects [159, 272]. The baseline levels for PCSK9 is reached by 8:00 am [159, 272]. Thus, to correctly monitor PCSK9 levels, it will be needed to check them at a specify time of the day.

In mice, systemic inflammation induced by the administration of different compounds leads to increased expression of PCSK9 and decreased hepatic levels of the LDL receptor, in association with a significant increase in circulating LDL levels [237, 242]. PCSK9 also reduces the LDL receptor expression and LDL uptake in human and murine macrophages, which could potentially prevent vascular lipid accumulation and oxidation in these cells [160, 243]. However, the fact that PCSK9 reduces the expression of the ApoE receptor-2 [238], which is anti-inflammatory and anti-apoptotic in macrophages, the reduction in this receptor enhances inflammation [239, 240, 243, 244]. In mice, the lack of PCSK9 significantly reduces aortic cholesteryl esters whereas overexpression of PCSK9 results in an accelerated development of

atherosclerotic plaques [245]. These effects are dependent on the expression of the LDL receptor [245]. Minipigs overexpressing a human PCSK9 GOF mutant have a significant increase in aortic atherosclerosis compared with wild-type minipigs [246]. Interestingly, HepG2 cells expressing the naturally occurring GOF mutation p.D374Y have reduced expression of stress-response genes and specific inflammatory pathways as compared to cells expressing the wild-type PCSK9 [241]. Thus, it is unclear whether the activity, rather than the overall levels of PCSK9, is what is responsible for the PCSK9-dependent inflammatory effects.

PCSK9 expression in vascular smooth muscle and endothelial cells reach their highest levels at low shear stress (3–6 dynes/cm²) suggesting a link between PCSK9 and atherosclerosis development [242]. Furthermore, it was determined that under the low shear stress conditions, there is an NADPH oxidase-dependent reactive oxygen species (ROS) generation that follows the same pattern as the increase in PCSK9 expression [242]. In THP-1-derived macrophages, oxidized LDL (oxLDL) increases PCSK9 expression and alters the secretion of inflammatory chemokines in a dose-dependent manner [247]. Interestingly, suppressing the expression of PCSK9 expression prevents the oxLDL-induced proinflammatory chemokine synthesis and secretion by inhibiting the activation and translocation of the nuclear factor kappa B [247]. In human endothelial cells, ox-LDL treatment induces apoptosis in correlation with an increase in PCSK9 expression [248]. Again, if the expression of PCSK9 is abolished, there is a significant reduction in the ox-LDL-dependent induction of apoptosis by altering the Bcl-2/Bax ratio and preventing the activation of caspases-9 and -3 [248]. The lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) seems to be implicated in these pathways [242, 249]. Furthermore, induction of the pro-inflammatory factor suppressor of cytokine signaling-3 (SOCS-3) has been shown to increase expression of PCSK9 [250]. Likewise, periodontitis increases circulating PCSK9 levels in humans further confirming the link between PCSK9 and inflammation [85]. Thus, PCSK9 levels increase every time there is inflammation in the body. Interestingly, any factor that improves health and possibly reduces inflammation also decreases the levels of PCSK9 in plasma [251, 252, 274-277].

PCSK9 also appears to be involved in thrombosis [253-255]. It has been reported that the levels of PCSK9 in the plasma are positively and strongly correlated with

platelet (PLT) and white blood cell count in CAD patients [253, 254]. Patients with a high PCSK9 concentration also tend to have higher fibrinogen levels [255]. Additionally, the amount of circulating PCSK9 directly correlates with high sensitivity C-reactive protein (hs-CRP) levels [255, 256]. Even the ABO blood group, involved in cholesterol metabolism, has been identified as a determinant factor for plasma PCSK9 level [259].

Related to blood pressure, PCSK9 directly regulates the levels of epithelial sodium channel (ENaC) protein expression [278], and some rare variants in PCSK9 appear to influence blood pressure among African Americans [279]. Furthermore, recent reports on several ethnic populations have revealed that blood pressure is positively correlated with circulating PCSK9 levels [146, 163, 262]. These ethnic differences related to PCSK9's role in disease development could explain why African Americans are more susceptible to statin intolerance limiting the treatment options for this group.

In addition to these adverse effects, PCSK9 has positive functions that cannot be ignored. These positive functions include liver regeneration, protection against viral infections such as hepatitis C virus, promotion of brain development, especially the cerebellum, and possible protection against Alzheimer's disease [156, 165, 280-293]. One has to consider that PCSK9 may be second messenger generated as a response to a stress signal and has the intention of solving the problem. As a second messenger, positive and negative signals are activated, that could improve or worsen the situation. In fact, high plasma PCSK9 levels have been reported after a cerebral ischemia [240, 257], following an acute myocardial infarction [294, 295], in nephritic syndrome patients [296], in animal models of nephritic syndrome or chronic kidney disease [297, 298], and in patients with hepatocellular carcinoma [299]. For this reason, it has been proposed that circulating PCSK9 should be used as a biomarker of severity in patients with multiple trauma or CAD [300, 301].

The main question is what would happen if this second messenger is inhibited for an extended period by using the recently approved PCSK9 inhibitors. One might think that inhibiting the signal would be sufficient to solve the problem as in the case of protecting against melanoma invasion in mouse liver using PCSK9's inhibition [302]. Interesting, a case report showed that inhibition of PCSK9 using a locked nucleic acid antisense oligonucleotide resulted in a toxic acute tubular injury

in a healthy 56-year-old woman volunteer [303]. Fortunately, 44 days after the last oligonucleotide dose was administered, the patient recovered fully, and her kidney function returned to normal [303]. The underlying mechanisms that led to kidney damage in response to PCSK9 inhibition in this patient are currently unknown, but an effect of PCSK9 in stress and tissue damage responses may be involved.

Similar stress-related damage could be induced in the liver of hypercholesterolemic patients upon statin treatment resulting in the observed increase in plasma PCSK9 levels. The higher the dose or the potency of the statin, the greater the tissue stress that is induced. Therefore, caution should be taken when using PCSK9 inhibitors, and a careful evaluation of the risk/benefit ratio is needed before broadening the utilization of these biologicals. Thus, knowing how much PCSK9 is required for normal body's function, before commencing any treatment, is critical now that the era of PCSK9 inhibitors started.

Diabetes is one metabolic disturbance that is conditioned on the levels of PCSK9. Circulating PCSK9 levels are positively correlated with fasting blood glucose levels and the homeostasis model assessment of insulin resistance (HOMA-IR) in several cohorts of nondiabetic subjects [146, 163, 217, 225, 262]. Moreover, PCSK9 levels are directly correlated with the levels of glycated hemoglobin (HbA1c) in type 2 diabetic patients [232-234]. Interestingly, having low levels of PCSK9 can also lead to diabetes. Treatment with PCSK9 inhibitors, in addition to statins, results in a cholesterol accumulation in pancreatic islets that causes type-2 diabetes [304, 305]. In fact, diabetes has been reported in 1.8% of patients that had no diabetes before starting treatment with PCSK9 inhibitors [306-309]. It is critical to mention that a recently identified PCSK9 genetic variant, p.InsLEU, which causes FH, gives patients a higher risk of developing diabetes [231]. Other side-effects that have been seen in response to treatment with PCSK9 inhibitors are neurocognitive events, gastrointestinal disturbances, infections, and ophthalmologic events, all related to the additional roles attributed to PCSK9 [310, 311].

Future considerations

The design of more efficient drugs/pharmaceuticals against PCSK9, with a minimum of adverse effects, still needs further investigation to identify conditions and/or factors that may influence complex formation between PCSK9 and the LDL receptor, while allowing PCSK9 to

perform its positive actions. **Figure 1** summarizes the known regulators of PCSK9 expression and function discussed above, as well as proposes the possible roles of PCSK9 levels in the response of hypercholesterolemic patients to statin treatment. Having high levels or overactive PCSK9 would lead to statin resistance since any additional increase in PCSK9 levels due to the statin would only reduce the LDL receptor even more. Thus, a further attenuation in LDL uptake would be seen (statin resistance). On the other hand, having low levels or underactive PCSK9 would result in diminished liver regeneration due to tissue stress from the drug. Hence, these patients would show symptoms of liver toxicity (high AST and/or ALT levels) triggering the discontinuation of the statin. Eventually, the patients would be classified as statin intolerants. It is currently unknown whether an underactive PCSK9 does not perform the positive roles of this convertase. The hypothetical model illustrated in **Figure 1** also considers the potential risk of developing diabetes related to PCSK9 levels. In the case of diabetes, high levels of PCSK9 alone have been shown to cause hyperglycemia and insulin resistance in humans [146, 163, 217, 225, 262]. Low levels of PCSK9 are known to cause dysmorphism, inflammation, and apoptosis of the pancreas islet in animal studies [227, 312]. Therefore, diabetes will develop if PCSK9 levels are either too high or too low.

Imperative is to contemplate that not only the overall levels of circulating PCSK9 should be considered but also its activity. In fact, it has previously reported that high levels of PCSK9 protein are not sufficient to determine the number of PCSK9/LDL receptor protein complexes that forms within a cell [313]. Under the conditions examined in the study, most of the PCSK9 produced as a result of incubating hepatic cells with a hormone-rich medium was inhibited by a secreted factor [313]. Thus, the identification of this endogenous inhibitor could provide additional light into the roles of PCSK9 in hypercholesterolemia and the severity of other diseases.

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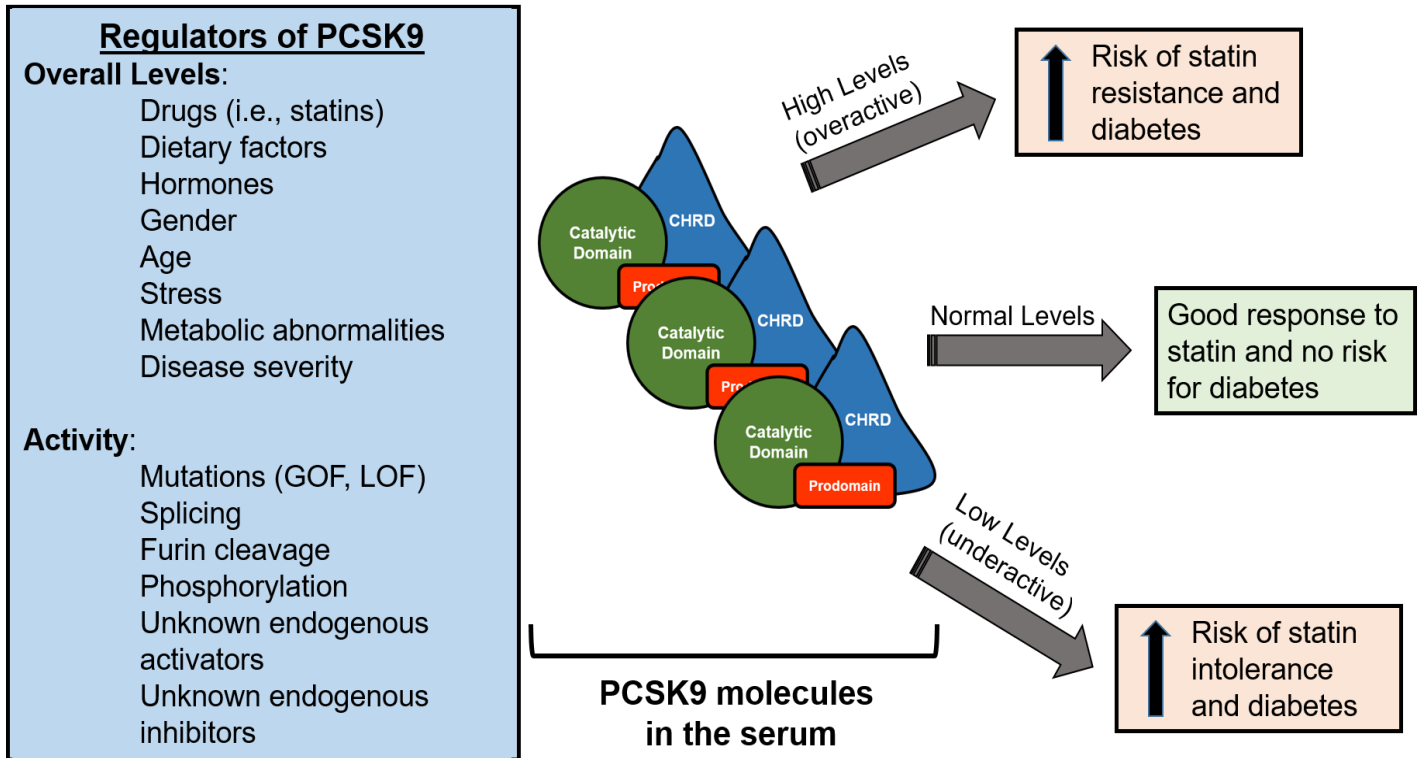
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Figure 1. Hypothetical model of the effects of PCSK9 levels on the response to statin and the risk of developing diabetes of hypercholesterolemic patients. The known regulators of PCSK9 expression (overall levels) and function (activity) are summarized.



JOURNAL WATCH

Makover ME, Schloss M. The very high residual degree of death and disease from atherosclerosis needs new approaches. J Clin Lipidol 2016; 10: 466-468.

This editorial addresses an important concern of many of us: that guidelines have become practice mandates rather than a tool to guide diagnosis and treatment. Drs. Makover and Scloss remind us that atherosclerosis is too complex a disease to manage with “simple algorithmic guidelines”, and randomized controlled trial (RCTs) used almost exclusively to create these guidelines have limitations as well, including the fact that hard outcomes such as myocardial infarction and stroke do not address preclinical disease which may be undertreated.

Rather than rely on general risk calculators and guidelines that address the “average” patient, physicians need to apply knowledge gathered from a variety of sources and address the “unique needs of each patient”.

Joshi PH, Khokhar AA, Massaro JM, et al. Remnant lipoprotein cholesterol and incident coronary heart disease: The Jackson Heart and Framingham Offspring Cohort Studies. J Am Heart Assoc 2016; 5: e002765 doi:10.1161/JAHA.115.002765.

This study included 4,991 participants of the Jackson Heart Study and a random subset of 818 Framingham Offspring Study subjects. Lipoprotein subfraction cholesterol concentrations were measured after separation by vertical spin density gradient ultracentrifugation (VAP) at Atherotech (Birmingham, AL). Remnant lipoprotein cholesterol (RLP-C) was defined as the sum of VLDL₃ and IDL cholesterol fractions. A total of 146 CHD events were ascertained within eight years of follow up. Hazard ratios (HR) for a 1 SD increase in RLP-C was 1.23 (1.06-1.42; 95% confidence interval) after adjustment for age, sex, BMI, smoking, blood pressure, diabetes and lipid lowering medication use. RLP-C association with CHD was attenuated by additional adjustment for LDL cholesterol and HDL cholesterol (HR: 1.12 (0.95-1.33, p=0.16).

Afshar M, Pilote L, Dufresne L, Engert JC, Thanassoulis G. Lipoprotein(a) interactions with low-density lipoprotein cholesterol and other cardiovascular risk factors in premature acute coronary syndrome. J Am Heart Assoc 2016; 5:e003012 doi: 10.1161/JAHA.115.003012.

The aim of this study was to identify interactions between Lp(a) with LDL cholesterol and other risk factors in 939 participants from the prospective GENESIS-PRAXY study of premature acute coronary syndromes. Mean age was 49 years and 31.0% had baseline Lp(a) concentrations >50 mg/dL. Serum Lp(a) concentration was associated with LDL cholesterol concentrations and individuals with elevated LDL-C levels were likely to have elevated Lp(a), as well. The odds ratio (OR) for Lp(a) concentration > 50 mg/dL was 1.0, 1.60 (1.08-2.38), 1.54 (0.93-2.55), and 4.04 (1.96-8.34), for LDL- cholesterol levels of ≤2.5, 2.5 to ≤3.5, 3.5 to ≤4.5, and >4.5 mmol/L, respectively.

This study confirms previous reports of possible synergy between Lp(a) and LDL cholesterol as cardiovascular disease risk factors, and highlights the frequent elevation of Lp(a) concentration in patients with premature coronary events.

Leander K, Mälarstig A, van't Hooft FM, et al. Circulating PCSK9 predicts future risk of cardiovascular events independently of established risk factors. Circulation 2016; doi: 0.1161/ CirculationAHA. 115.018531.

This is likely the largest prospective study to date looking at circulating PCSK9 concentrations and cardiovascular disease risk. This prospective study identified 485 cardiovascular disease cases after 590,430 person-years of follow up, 304 men and 181 women. Serum PCSK9 concentration was only modestly associated with LDL cholesterol (r=0.18) and triglycerides (r=0.12). Univariate analysis gave a hazard ratio (HR) of 1.22 (1.11-1.34; 95% confidence interval) for cardiovascular disease associated with a 1 SD increase in PCSK9. After adjustment for sex, LDL-cholesterol, HDL cholesterol, lipoprotein(a), triglycerides, hypertension, diabetes, obesity and overweight, physical activity, smoking, and statin use HR remained statistically significant: 1.15 (1.05-1.26).

It is very interesting that adjustment for LDL cholesterol did not alter the significance of an elevated PCSK9 concentration, suggesting that PCSK9 may have a pathophysiologic role beyond regulation of LDL receptors.

Zanoni P, Khetarpal SA, Larach DB, et al. Rare variant in scavenger receptor B1 raises HDL cholesterol and increases risk of coronary heart disease. *Science* 2016; 351: 1166-1171.

It is increasingly recognized that the paradigm associating high HDL cholesterol with atheroprotection is flawed. Intervention trials with niacin and CETP inhibitors resulting in sometimes dramatic increases in HDL cholesterol have failed to prevent coronary heart disease (CHD). Scavenger receptor B1 (SRB1) is recognized as a major receptor for HDL; therefore, one would expect a role for SRB1 in HDL clearance and modulation of risk for CHD. In this report, investigators studied individuals with very high concentrations of HDL cholesterol and identified a proband with a variant in the SRB1 gene that prevents binding of HDL to the receptor. Despite a very high concentration of HDL cholesterol (152 mg/dL), this individual had an increased mean carotid intima media thickness by ultrasound. Targeted sequencing in 328 additional individuals with elevated HDL cholesterol identified an additional 15 heterozygotes with the same SRB1 variant. Interestingly, these individuals were also at increased risk for CHD despite high HDL cholesterol concentrations.

Ruiz-Canela M, Toledo E, Clish CB, et al. Plasma branched chain amino acids and incident cardiovascular disease in the PREDIMED Trial. *Clin Chem* 2016; 62:582-592.

The investigators measured plasma branched chain amino acids (BCAA; leucine, isoleucine, and valine) by LC/MS/MS to assess risk of cardiovascular disease among participants of the PREDIMED Trial. After a median 4.6 years of follow up 226 CVD case 744 random controls were compared. Univariate hazard ratio (HR) for a 1 SD increase in baseline BCAAs was 1.25 (1.07-1.45), 1.36 (1.17-1.58), and 1.18 (1.00-1.37) for leucine, isoleucine and valine, respectively. After adjustment for age, sex, intervention group, BMI, smoking, physical activity, and family history, HRs were attenuated. Interestingly HRs for stroke, rather than composite CVD were moderately higher.

Bartlett J, Predazzi IM, Williams SM, et al. Is isolated low high-density lipoprotein cholesterol a cardiovascular disease risk factor? New insights from the Framingham Offspring Study. *Circ Cardiovasc Qual Outcomes* 2016; 9:206-212.

The debate continues: Is low HDL cholesterol a cardiovascular disease risk factor? These new data from the Framingham Offspring Study cohort show that risk associated with HDL cholesterol is modified by concentrations of LDL cholesterol and triglycerides. The authors report that risk associated with low HDL cholesterol increased by 30% to 60% with elevated LDL cholesterol and/or triglycerides compared with isolated low HDL cholesterol alone. Interestingly, high HDL cholesterol was not protective of cardiovascular disease if accompanied by elevated LDL cholesterol or triglycerides.

Tavori H, Christian D, Minnier J, et al. PCSK9 association with lipoprotein(a). *Circ Res* 2016; 119:29-35.

The goal of this study was to characterize PCSK9 binding to Lp(a) particles. New monoclonal antibody therapy that blocks PCSK9 activity has been shown to reduce LDL cholesterol by as much as 65% and also decrease Lp(a) by about 35%. In part 1 of the study Lp(a) was isolated from plasma of two individuals with Lp(a) >100 mg/dL using iodixanol-based gradient ultracentrifugation. Following agarose gel electrophoresis and immunoblotting, intact (but not furin-cleaved) PCSK9 was found bound to the Lp(a) fraction. Immunoprecipitation studies confirmed PCSK9-Lp(a) binding when anti-PCSK9 pulled down apo(a), as well. Using a sandwich ELISA with anti-PCSK9 for capture and anti-apo(a) for detection the investigators found that on average there was 1.7-fold enrichment of Lp(a)-bound PCSK9 compared with LDL-bound. The functional significance of Lp(a)-PCSK9 binding remains to be determined.

Klempfner R, Erez A, Sagit B-Z, et al. Elevated triglyceride level is independently associated with increased all-cause mortality in patients with established coronary heart disease: Twenty-two-year follow up of the Bezafibrate Infarction Prevention Study and Registry. *Circ Cardiovasc Qual Outcomes* 2016; 9:100-108.

Like HDL cholesterol, there remains debate whether triglyceride is an independent risk factor for CHD. In this 22 year follow up of the BIP Study, 15,355 patients were stratified into five groups according to fasting serum triglyceride concentration: <100 mg/dL, 100-149 mg/dL, 150-199 mg/dL, 200-499 mg/dL, and ≥500 mg/dL. Age and sex adjusted survival was 41%, 37%, 36%, 35%, and 25% in the five progressively higher triglyceride groups, respectively. The hazard ratio (HR) for all-cause mortality for a 1 log triglyceride change was 1.15 (1.01-1.32) in a fully adjusted model that included LDL cholesterol and HDL cholesterol.

Despite data that showed a strong association of apo B concentration with increasing triglyceride concentration, apo B was not included in any of the regression models. I would have liked to see apo B or non-HDL cholesterol, which reflect LDL particle numbers, included in the analysis, as LDL particle number, but not necessarily LDL-cholesterol, may capture some of the risk associated with elevated triglycerides.

Romagnuolo I, Sticchi E, Attanasio M, et al. Searching for a common mechanism for placenta-mediated pregnancy complications and cardiovascular disease: role of lipoprotein(a). *Fertility and Sterility* 2016; 105:1287-1293.

As the authors point out, there is a common pathophysiology associated with endothelial dysfunction in both placental and vascular disease. Women with a history of pregnancy complications are at increased risk for cardiovascular disease. Therefore, they studied 360 women with a history of pregnancy complication related to vascular insufficiency, including preeclampsia (PE), small for gestational age neonates (SGA), and still birth (SB), and 270 women without pregnancy complications. For all patients with pregnancy complications the mean Lp(a) concentration was 28.6 mg/dL compared to 18.5 mg/dL in the control group ($p=0.03$). For the patient group 31.9% had Lp(a) >30 mg/dL compared with 17.1% of the control group ($p<0.0001$). Mean Lp(a) concentrations were 20.2, 28.3, and 32.4 mg/dL for SGA, PE, and SB groups, respectively. Odds ratio for pregnancy complications, stillbirth, and preeclampsia were 1.93 (1.20-3.09), 2.55 (1.29-5.05), and 2.43 (1.23-4.78), respectively.

This study corroborates a previous study showing a link between Lp(a) and preeclampsia and demonstrates for the first time a link between Lp(a) and still birth.

Stark KD, Van Elswyk ME, Higgins MR, Weatherford CA, Salem N. Global survey of the omega-3 fatty acids, docosahexaenoic acid, and eicosapentaenoic acid in the blood stream of healthy adults. *Prog Lipid Res* 2016; 63:132-152.

This is a comprehensive and systematic review of the literature to compare global differences in omega-3 polyunsaturated fatty acids in various blood fractions. The results from ~300 published studies show considerable variability in fatty acid profiles in various regions. Countries/regions such as Japan, Scandinavia, Alaska, Greenland had higher levels of EPA+DHA ($>8\%$ of total fatty acids in erythrocyte equivalents), while most of North and South America, India, and the Middle East had low EPA+DHA ($<4\%$).

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