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RELATIONSHIP BETWEEN PLASMA LEVELS OF PCSK9, VASCULAR EVENTS AND MARKERS OF SUBCLINICAL ATHEROSCLEROSIS AND INFLAMMATION

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ABSTRACT

Contesto e scopo: La proproteina convertasi subtilisina/kexina di tipo 9 (PCSK9), uno dei principali regolatori del metabolismo del recettore delle LDL, è stata associata allo sviluppo di aterosclerosi. Diversi studi hanno confermato tale associazione attraverso vie lipidiche e non lipidiche. Tuttavia, le relazioni dirette tra PCSK9 circolante e marcatori di aterosclerosi subclinica e clinica sono ancora da chiarire. Pertanto, abbiamo valutato le relazioni tra i livelli plasmatici di PCSK9 ed alcuni indici di aterosclerosi subclinica (marcatori di imaging) e clinica (eventi vascolari; EV). Un altro obiettivo è stato l'identificazione dei determinanti indipendenti di PCSK9, con particolare attenzione ai lipidi e ai biomarcatori infiammatori. Infine, abbiamo anche valutato la relazione tra alcuni marcatori di imaging e quattro SNPs del gene *PCSK9*, noti per essere associati alla presenza di bassi livelli di colesterolo LDL. Per validare i risultati ottenuti in quest'ultima parte, le analisi genetiche sono state replicate in una coorte indipendente reclutata nel Regno Unito (UK).
Metodi: Lo studio è stato realizzato sfruttando le banche dati, biobanche e la banca di immagini dello studio IMPROVE. 3,703 soggetti europei (54-79 anni; 48% uomini), privi di EV al basale e definiti ad alto rischio per la presenza di almeno tre fattori di rischio vascolare, sono stati reclutati e seguiti per 36 mesi. PCSK9 è stata misurata tramite ELISA e trasformata in logaritmo prima delle analisi. I marcatori di imaging convenzionali [spessore medio-intimale carotideo (cIMT, dall'inglese intima-media thickness) e dimensione della placca carotidea] ed emergenti [cambiamento di cIMT nel tempo, ecolucenza dello spessore del complesso medio intimale della carotide comune misurato in zone libere da placca (PF CC-IMT$_{mean}$), ecolucenza della placca più grande rilevata in tutto l'albero carotideo e punteggio di calcio carotideo (cCS, dall'inglese carotid calcium score)] sono stati misurati su scansioni ultrasonografiche conservate nella banca di immagini. In particolare, l'ecolucenza è stata misurata in termini di mediana della scala dei grigi (GSM, dall'inglese grey scale median) della distribuzione dei pixel di una specifica regione d'interesse, mentre il cCS è stato calcolato come somma delle lunghezze dei coni d'ombra acustici generati dal calcio all'interno delle placche carotidee. I lipidi sono stati misurati con metodi enzimatici (ad eccezione del colesterolo LDL che è stato calcolato con la formula di Friedewald). Tra i marcatori

infiammatori, la proteina C reattiva ad alta sensibilità (hs-PCR) è stata misurata con la turbidimetria, mentre il conteggio dei globuli bianchi (WBC, dall'inglese white blood cells) e la formula leucocitaria sono stati misurati localmente. Tutti i soggetti dello studio IMPROVE e della coorte UK (n=22,179; 48 % uomini) sono stati genotipizzati.

Risultati: Nell'analisi univariata, PCSK9 correlava positivamente con colesterolo totale, LDL e HDL e con trigliceridi e basofili (tutte le p <0.0001), mentre correlava negativamente con neutrofili ed eosinofili (entrambe le p=0.04). Le correlazioni positive osservate con hs-PCR e con il conteggio dei WBC erano solo vicine alla significatività statistica (p=0.060 e 0.064, rispettivamente). Le terapie con fibrati o statine (positivamente; entrambe le p <0.0001), così come sesso maschile e storia familiare di diabete (negativamente; entrambe le p <0.05) erano i predittori indipendenti più forti dei livelli plasmatici di PCSK9. Nell'analisi non aggiustata, si osservava una correlazione negativa tra PCSK9 e variabili basali di cIMT (IMT_{mean}, IMT_{max}, $IMT_{mean-max}$, e PF CC-IMT_{mean}), una correlazione negativa tra PCSK9 e la variazione di cIMT nel tempo (Fastest-$IMT_{max-progr}$) e cCS (tutte le p ≤0.01), mentre si osservava un trend positivo tra PCSK9 e GSM sia del PF CC-IMT_{mean} che della placca carotidea (entrambe le p ≤0.0001). Il cCS (positivamente) e il GSM del PF CC-IMT_{mean} (positivamente) erano associati significativamente (o vicini alla significatività) a PCSK9 in diversi modelli multivariati (tutte le p ≤0.064). Tutte le correlazioni osservate all'analisi univariata tra PCSK9 e le variabili basali di cIMT, Fastest-$IMT_{max-progr}$ e GSM della placca carotidea perdevano la significatività statistica dopo aggiustamento delle stesse per età, sesso, latitudine ed altri potenziali confondenti. Durante il follow-up [mediana (intervallo interquartile): 3.01 (2.98; 3.12) anni], sono stati registrati 215 EV: 125 coronarici, 73 cerebrali e 17 EV periferici. Tra questi, 37 erano eventi hard (infarto miocardico, morte improvvisa ed ictus). Nell'analisi non aggiustata, PCSK9 era associata positivamente ad eventi combinati e coronarici (entrambe le p <0.01), ma non ad eventi cerebrovascolari. Anche in questo caso, tuttavia, tutte le associazioni osservate perdevano la significatività statistica dopo aggiustamento delle analisi per età, sesso e stratificazione per latitudine. La mancanza di associazione con EV era confermata anche nel modello aggiustato per tutti i fattori confondenti considerati e nelle analisi focalizzate sugli eventi hard. Per quanto riguarda il ruolo delle varianti genetiche, nessuno dei quattro SNPs considerati

correlava con cIMT (IMT_{mean}, IMT_{max}, $IMT_{mean-max}$) quando l'analisi era effettuata nei soggetti reclutati nello studio IMPROVE. La variante rs11591147, invece, correlava negativamente con l'IMT_{max} misurato nella popolazione UK (p=0.002). Combinando le quattro varianti genetiche in uno score, la relazione con cIMT era non significativa nello studio IMPROVE, mentre era negativa e significativa nella popolazione UK (tutte le p <0.01).

Conclusioni: I livelli plasmatici di PCSK9 non sono associati a EV. Per quanto riguarda i marcatori dell'aterosclerosi subclinica, i livelli plasmatici di PCSK9 non sono associati né alla dimensione della lesione, né all'ecolucenza della placca carotidea, ma sono associati all'ecolucenza dello spessore della parete carotidea e al carotid calcium score. Ulteriori studi sono pertanto necessari per comprendere meglio il ruolo di tale proproteina nell'ecolucenza dello spessore della parete carotidea e nel carotid calcium score. La terapia con fibrati o statine, così come il sesso maschile e la storia familiare di diabete sono i predittori indipendenti più forti di PCSK9 circolante. È stata inoltre confermata l'associazione, precedentemente osservata, tra PCSK9 circolante e alcuni marcatori lipidici ed infiammatori. La relazione tra i livelli plasmatici di PCSK9 ed altri marcatori infiammatori (neutrofili, basofili ed eosinofili) merita ulteriori indagini, così come merita ulteriori indagini l'associazione tra le quattro varianti genetiche di *PCSK9* selezionate e il cIMT nella coorte britannica, in quanto lascia intravvedere un possibile ruolo di SNPs o polimorfismi genici di *PCSK9* nell'aterosclerosi e nelle strategie della sua prevenzione.

Background and purpose: Proprotein convertase subtilisin/kexin type 9 (PCSK9), one of the main regulators of LDL receptor metabolism, has been associated with atherosclerosis development. Several studies have confirmed such association through both lipid and non-lipid pathways. However, the direct relationships between circulating PCSK9 and markers of subclinical and clinical atherosclerosis are still matter of debate. Therefore, we investigated the relationships between plasma PCSK9 levels and some indexes of subclinical (imaging markers) and clinical (vascular events; VEs) atherosclerosis. Another objective was the identification of the independent determinants of PCSK9, with particular attention to lipids and inflammatory biomarkers. Finally, we also assessed the relationship between some imaging markers and four SNPs of the *PCSK9* gene, known to be associated with the presence of low levels of LDL-cholesterol. In order to validate the results obtained in this last part, the genetic analyses were replicated in an independent cohort recruited in the United Kingdom (UK).

Methods: The study was carried out taking advantage of databases, biobanks and imaging-bank of the IMPROVE study. 3,703 European subjects (54-79 years; 48% men), free of VEs at baseline and defined at high risk for the presence of at least three vascular risk factors, were recruited and followed-up for 36 months. PCSK9 was measured by ELISA and log-transformed prior to analyses. Conventional imaging markers [carotid intima-media thickness (cIMT) and carotid plaque-size], and emerging imaging markers [cIMT change over time, echolucency of the intima-media thickess of common carotid measured in plaque free areas (PF CC-IMT$_{mean}$), echolucency of the biggest plaque detected in the whole carotid tree, and carotid calcium score (cCS)] were measured on ultrasonographic scans stored in the imaging-bank. In particular, echolucency was measured in terms of grey scale median (GSM) of pixels distribution of a specific region of interest, whereas cCS was calculated as sum of lengths of acoustic shadow cones generated by calcium within carotid plaques. Lipids were measured with enzymatic methods (except for LDL-cholesterol, which was calculated by Friedewald's formula). Among inflammatory markers, high-sensitivity C-reactive protein (hs-CRP) was measured by turbidimetry, whereas white blood cells (WBC) count and the leukocyte formula had already been measured locally. All the IMPROVE study and UK (n=22,179; 48% men) subjects have been genotyped.

Results: In the univariate analysis, PCSK9 was positively correlated with total, LDL-, and HDL-cholesterol, and with triglycerides and basophils (all p <0.0001), whereas was negatively correlated with neutrophils and eosinophils (both p=0.04). The positive correlations observed with hs-CRP and WBC count were just close to the statistical significance (p=0.060 and 0.064, respectively). Fibrates or statins therapies (positively; both p <0.0001), as well as male sex and family history of diabetes (negatively; both p <0.05) were the strongest independent predictors of plasma PCSK9 levels. In the unadjusted analysis, a negative correlation was observed between PCSK9 levels and basal cIMT variables (i.e. carotid IMT_{mean}, IMT_{max}, $IMT_{mean-max}$, and PF CC-IMT_{mean}), a negative correlation between PCSK9 and cIMT change over time (Fastest-$IMT_{max-progr}$) and cCS (all p ≤0.01), whereas a positive trend was observed between PCSK9 and GSM of both PF CC-IMT_{mean} and carotid plaque (both p ≤0.0001). The cCS (positively) and the GSM of PF CC-IMT_{mean} (positively) were significantly (or almost significantly) associated with PCSK9 in several multivariate models (all p ≤0.064). All correlations observed in the univariate analysis between PCSK9 and basal cIMT variables, Fastest-$IMT_{max-progr}$ and GSM of carotid plaque lost the statistical significance after adjustment for age, sex, latitude, and other potential confounders. During the follow-up [median (interquartile range): 3.01 (2.98; 3.12) years], 215 VEs were recorded: 125 coronary, 73 cerebral and 17 peripheral VEs. Among these, 37 were hard events (i.e. myocardial infarction, sudden death and stroke). In the unadjusted analysis, PCSK9 was positively associated with combined and coronary events (both p <0.01), but not with cerebrovascular events. Also in this case, however, all the associations observed lost the statistical significance after adjustment of the analyses for age, sex, and stratification for latitude. The lack of association with VEs was confirmed also in the model adjusted for all confounding factors considered, and in the analyses focused on hard events. With regard to the role of genetic variants, none of the four SNPs considered was correlated with cIMT (i.e. IMT_{mean}, IMT_{max}, $IMT_{mean-max}$) when the analysis was performed in the subjects recruited in the IMPROVE study. The rs11591147 variant, by contrast, was negatively correlated with IMT_{max} measured in the UK population (p=0.002). By combining the four genetic variants in a score, the relationship with cIMT was not significant in the IMPROVE study, whereas was negative and significant in the UK population (all p <0.01).

Conclusions: Plasma PCSK9 levels are not associated with VEs. Regarding markers of subclinical atherosclerosis, PCSK9 levels are associated neither with lesion size, nor with carotid plaque echolucency, but are associated with echolucency of carotid wall thickness and with carotid calcium score. Therefore, further studies are needed to better understand the role of such circulating proprotein in carotid wall thickness echolucency and in carotid calcium score. Fibrates or statins therapies, as well as male sex and family history of diabetes are the strongest independent predictors of PCSK9 levels. The associations, previously observed, between circulating PCSK9 and some lipid and inflammatory markers have been confirmed. The relationship between plasma levels of PCSK9 and other inflammatory markers (neutrophils, basophils and eosinophils) deserves further investigation, as does the association between the four selected *PCSK9* variants and cIMT in the UK cohort, as it suggests a possible role of *PCSK9* SNPs or gene polymorphisms in atherosclerosis and in its preventive strategies.

INTRODUCTION

Circulating proprotein convertase subtilisin/kexin type 9 (PCSK9), a relatively new identified protein synthesized mainly in the liver, plays an important role in lipid metabolism.[1, 2] PCSK9 enhances endosomal and lysosomal degradation of the hepatic low-density lipoproteins (LDL) receptors present on the cell surface,[3] thus indirectly increasing LDL-cholesterol levels. PCSK9 plays a key role in the development of atherosclerosis.[1, 2] Supporting this thesis, since 2003 human studies showed that dominant "gain-of-function" mutations of *PCSK9* gene are associated with a familial form of hypercholesterolemia and with an increased risk of coronary artery disease (CAD);[4] by contrast, "loss-of-function" mutations cause hypocholesterolemia, and protection from CAD.[5] Experimental studies in animal models corroborated these observations.[6-9] On these bases, the inhibition of circulating PCSK9 is now recognized as a target both to treat hypercholesterolemia and to prevent atherosclerosis.[10, 11]

Besides the lipid-pathway, several studies consistently demonstrated that PCSK9 is related with atherosclerosis also through inflammation.[12] Moreover, circulating PCSK9 has been associated with traditional markers of subclinical atherosclerosis, such as carotid intima-media thickness (cIMT), in some[13, 14] but not all[15] studies. Circulating PCSK9 has been also associated with plaques,[13, 14, 16] with 10-years atherosclerosis progression,[17] with coronary calcium score,[18] and with the fraction and absolute volume of necrotic core within coronary plaques.[19] No studies, instead, have evaluated the association with new markers of subclinical atherosclerosis, such as carotid plaques echolucency or carotid plaques content of calcium. A significant association has been also observed between circulating PCSK9 levels and the incidence of vascular events (VEs),[20] but also such association was not consistent across all the studies.[21] So, even if the aforementioned studies suggest that the involvement of PCSK9 in atherosclerosis development acts through both lipid and non-lipid pathways, the direct relationship between circulating PCSK9 and subclinical and/or clinical atherosclerosis is still matter of debate.

AIMS OF THE STUDY

In order to add further insights on this issue, in the present project we have investigated the relationships between plasma PCSK9 levels and some indexes of subclinical atherosclerosis (imaging biomarkers) and VEs. Regarding the subclinical atherosclerosis, we have considered both conventional biomarkers (cIMT and carotid plaque-size), and emerging biomarkers (cIMT change over time, echolucency of cIMT and plaques, and the calcium content of carotid plaques). Another objective of the study was the identification of the independent determinants of PCSK9, with particular attention to lipids and inflammatory biomarkers. Finally, we also assessed the relationship between some imaging markers and four single nucleotide polymorphisms (SNPs) of the *PCSK9* gene, known to be associated with the presence of low levels of LDL-cholesterol.[22] In order to validate the results obtained in this last part, the genetic analyses were replicated in an independent cohort recruited in the United Kingdom (UK).

PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9

Proprotein convertase subtilisin/kexin type 9 (PCSK9), a member of the family of secretory serine endo-proteases, is the ninth and last member of mammalian proprotein convertases (PCs) discovered in 2003.[4] It is primarily synthesized and secreted by hepatocytes with a lower contribution of other tissues, such as gut, kidney, and nervous system.[23, 24] During 1970s, PCs were identified as a special group of proteases able to convert an inactive secretory precursor into a mature protein (or peptide) or into several bioactive fragments; therefore, according to their action site and activities, all PCs are involved in activation/inactivation mechanisms in several physiological and clinically relevant processes.[25-27] Some of these processes are known to affect the cardiovascular health.[28]

Because PCSK9 is highly expressed in the liver and *PCSK9* gene is localized on human chromosome 1p32, a region associated with familial hypercholesterolemia (FH),[29] *PCSK9* gene was suspected and early confirmed as the third FH locus, whereas the low-density lipoproteins receptor (*LDLR*) and apolipoprotein B (*ApoB*) genes were the first and the second loci, respectively.[4] Such pioneering work identified an important new player in cholesterol homeostasis and represented the first step in research involving PCSK9 as a promising therapeutic target in lowering the concentrations of LDL-cholesterol in hypercholesterolemic patients and, therefore, in reducing the risk of atherosclerosis, a major determinant of cardiovascular diseases (CVDs).

BIOSYNTHESIS AND STRUCTURE

In humans, *PCSK9* gene is situated on the small arm of chromosome 1p32.3, includes 12 exons and 11 introns, and encodes for a glycoprotein of 692 aminoacid, i.e. PCSK9.[28, 30] PCSK9 is synthesized as a soluble zymogen of about 75 kDa, denoted as proPCSK9. The proPCSK9 undergoes a specific autocatalytic cleavage at the sequence Val-Phe-Ala-Gln152↓Ser-Ile-Pro (VFAQ152↓SIP) within cell endoplasmic reticulum, thus releasing the propeptide (~15 kDa) from the N-terminus, and finally resulting in a mature and active PCSK9 (aa 153–692) of almost 60 kDa

(Figure 1).[30] This observation highlights that the autocatalytic cleavage of the pro-domain is strictly necessary for maturation and activation of PCSK9.

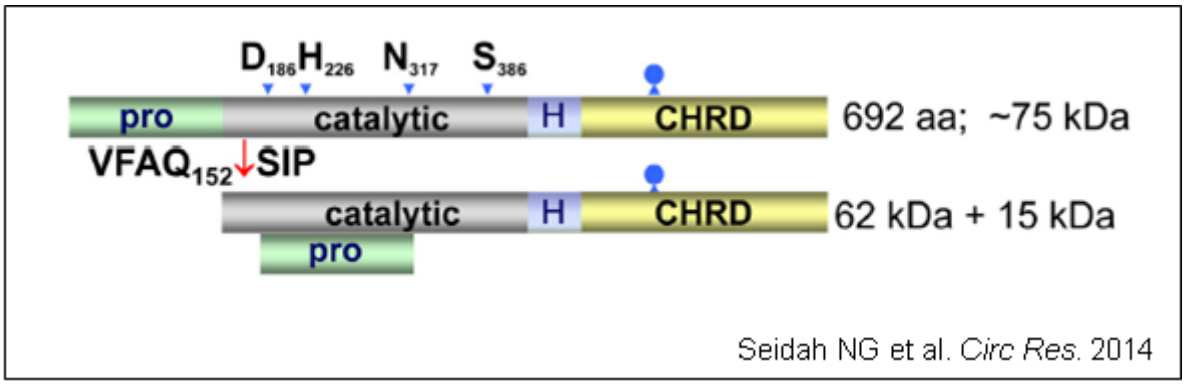

Figure 1. Schematic representation of the processing of proprotein convertase subtilisin/kexin type 9 (PCSK9) zymogen. VFAQ152↓SIP, aminoacid sequence Val-Phe-Ala-Gln152↓Ser-Ile-Pro; D186, Asp186; H226, His226; N317, oxyanion hole Asn317; S386, Ser386; H, hinge region; CHRD, cys-his–rich domain.

The mature PCSK9 catalytic subunit (aa 153–421) includes the active sites Asp186, His226, and Ser386 and the oxyanion hole Asn317, all typical of subtilisin-like serine proteases;[31] a small hinge region (H; aa 422–439) is localized between the catalytic subunit and the C-terminal cys-his–rich domain (CHRD; aa 440–692) (Figure 1).

Unlikely other PCs, mature PCSK9 remains not-covalently bonded to its inhibitory prosegment (aa 32–152) and, therefore, is secreted as a PCSK9–prosegment complex.[30, 32] Such complex is enzymatically inactive, because the prosegment is located in the active site cleft of PCSK9 and inhibits the interaction with other substrates.[33] Thus, unlikely other PCs that have substrates such as hormones, receptors, metalloproteases, growth factors, membrane-bound transcription factors, and surface glycoproteins, PCSK9 has itself as the only substrate, and its activity is linked to its binding to specific target proteins and to the transport of the resulting complexes towards degradation compartments within the cell.

MECHANISMS OF ACTION

The first and main target of circulating PCSK9 that has been identified is the low-density lipoproteins receptor (LDLR) at the surface of hepatocytes plasma membrane.[6, 7, 30]

Normally, at low to normal PCSK9 levels, the LDLR binds the circulating LDL-cholesterol particles to regulate the cholesterol homeostasis (Figure 2). Once the LDLR–LDL-cholesterol complex has formed, it is internalized into the cells via endosomal pathway (i.e. clathrin heavy chain–coated vesicles), and the acidic pH of such endosomes causes the dissociation between LDLR and LDL-cholesterol particle. At this point, the LDLR is recycled to the cell surface, whereas LDL-cholesterol is directed to lysosomes to be degraded. In such manner, cholesterol is now available to be recovered and distributed within the cell.[34]

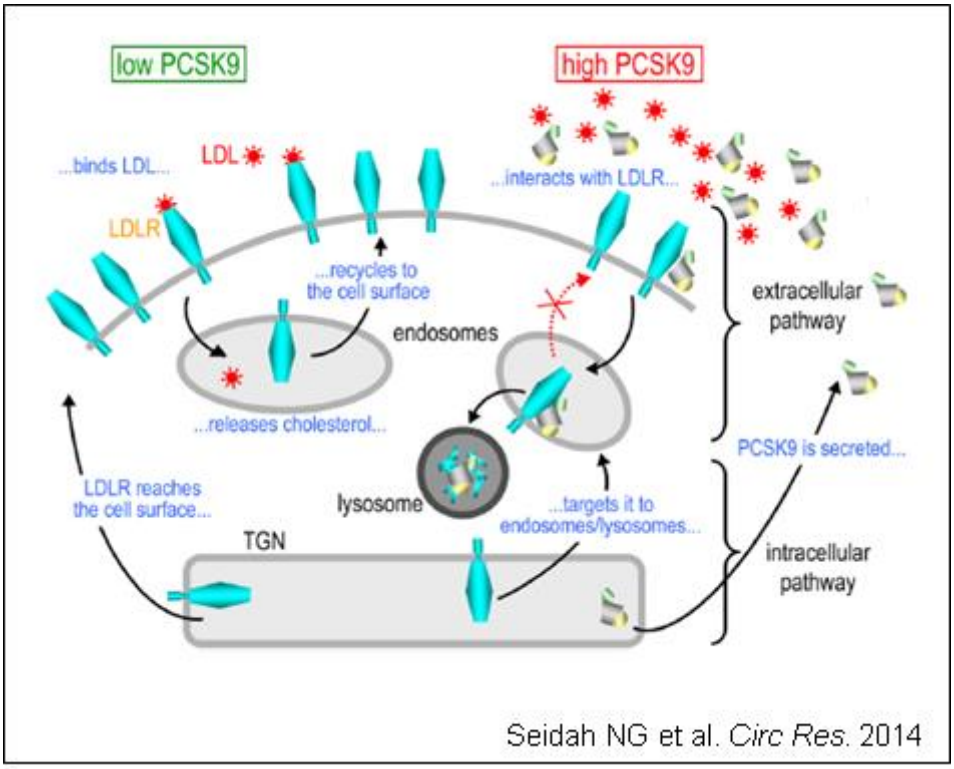

Figure 2. Function and recycling of the low-density lipoprotein receptor (LDLR) on the cell surface or its degradation by proprotein convertase subtilisin/kexin type 9 (PCSK9) through extracellular or intracellular pathways. TGN, *trans* Golgi network.

Conversely, when PCSK9 levels are higher than normal, circulating PCSK9 interacts with the LDLR 1:1 by binding the epidermal growth factor-A domain in the LDLR extracellular portion

(Figure 2).[35, 36] The PCSK9–LDLR complex, although also entering the cells via endosomal pathway,[37, 38] does not dissociate at a low pH, but is even more strongly associated.[33] This increased affinity of PCSK9 for the LDLR prevents LDLR to be recycled and, through some unclear mechanisms, the complex is directed to lysosomes where the LDLR is degraded.[30, 37]

Actually, it has been demonstrated that PCSK9 targets the LDLR not only extracellularly, but also through an intracellular pathway (Figure 2).[3, 39, 40] In particular, clathrin light chains, that are unnecessary for clathrin-mediated endocytosis, are essential for the clathrin-mediated trafficking between the *trans* Golgi network (TGN) and endosomes/lysosomes within the cell.[41] This intracellular route does not affect the ability of exogenous PCSK9 to degrade LDLR, and, therefore, the two pathways seem to be independent. Both the extracellular and intracellular pathways require the presence of the PCSK9 CHRD to degrade the LDLR; indeed, without this domain, the PCSK9–LDLR complex is internalized into the endosomes but is not able to reach lysosomes, whereas is recycled to the cell membrane.[42, 43]

Studies have also demonstrated an increase in plasma cholesterol in an LDLR-independent way.[44] Regardless of expression of LDLR, almost the 30% of plasma PCSK9 has been associated with LDL-cholesterol probably by enhancing the hepatic and intestinal production of LDL structural protein, i.e. apoB.[44] Investigations on cells, animals, and humans have proposed that PCSK9 is involved in the regulation of apoB production and secretion; moreover, they have convincingly demonstrated a direct interaction between PCSK9 and apoB.[45] The PCSK9–apoB interaction prevents the degradation of apoB within the cell, leading to an augmented secretion of both apoB and apoB-containing lipoproteins [e.g. very low-density lipoproteins (VLDL) and LDL].

Whatever the mechanism of action, PCSK9 indirectly increases circulating LDL-cholesterol levels, the main atherosclerotic risk factor. For this reason, since its discovery in 2003 PCSK9 has been identified as a new player in the development of atherosclerosis.

ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease of the arterial wall (mainly elastic and muscular arteries of medium to large caliber, such as coronary arteries, descending thoracic aorta, abdominal aorta, lower limb arteries, and carotid arteries), leading to the progressive formation of atherosclerotic plaques.[46] Such disease is characterized by a slow and asymptomatic development, and may manifest itself with sudden, often disabling or even fatal, events, such as myocardial infarction, stroke, and lower limb arteriopathies. The underlying mechanism is extremely complex and involves both a disequilibrium of lipid metabolism and an altered inflammatory response from the arterial wall.[47]

PATHOGENESIS OF ATHEROSCLEROSIS AND FORMATION OF ATHEROCLEROTIC PLAQUES

The atherosclerotic process starts with an early phenomenon known as endothelium dysfunction, that is a damage to the endothelial cells of tunica intima, the inner layer of arterial wall directly in contact with the lumen.[48] Hemodynamic forces and vascular risk factors (VRFs), such as hyperlipidemia, hyperglycemia, hypertension, smoking, as well as infections and immunological injuries cause abnormal attachment between one endothelial cell and another one, or between one endothelial cell and connective tissue.[49] In this condition of lack of anatomical continuity of the endothelium cellular monolayer,[50, 51] the normal endothelial function is altered and is followed by adhesion and aggregation of both platelets and monocytes, as well as by release of pro-inflammatory chemokines at the injury sites. Plasma constituents (e.g. monocytes, lipoproteins, and/or metabolites) start to infiltrate in the subendothelium, followed by lipid deposition and modification (e.g. oxidation of LDL-cholesterol), macrophages rich in lipid droplets (macrophage foam cells) formation, new connective tissue matrix proteins synthesis, vascular smooth muscle cells (VSMCs) proliferation, apoptosis and necrosis, calcification, angiogenesis, arterial remodeling, thrombosis and more.[52] All these processes lead to a progressive morphological alteration of the arterial wall and, finally, to the formation of the atherosclerotic plaque.[53, 54]

Early lesions, or fatty streaks, are silent and do not cause obstruction of the arterial lumen. They are primarily characterized by the accumulation of macrophage foam cells (Figure 3, top panel), that play a key role in plaques development by accumulating and transforming LDL particles in the pro-atherogenic oxidized-LDL within the arterial wall. T lymphocytes and mast cells are also present, but they are fewer than macrophages.[55, 56] The successive accumulation of extracellular lipids and cholesterol crystals, covered by a thin layer of collagen, constitute the so called “lipid core”, identifiable only in advanced-stage lesions (atheroma). Between the lipid core and the endothelium, several cellular types can be identified, including lymphocytes, mast cells, macrophages, and VSMCs. Calcium particles may be found in dispersed VSMCs, as well as in lipid cores. Capillaries surround the lipid core, particularly at the lateral margins of these lesions.

Fibroatheromas differ from atheromas because of the presence of a consistent fibrous coating (the “fibrous cap”) rich in collagen (Figure 3, middle panel). The fibrous tissue may invade the most part of the lesion thickness than does the underlying lipid core. In other situations, fibrous plaques include a calcified lipid core (Figure 3, bottom panel). Lesions with a predominant mineralization are called “calcific plaques”, and such mineral deposits represent the remnants of dead cells, extracellular lipid, and, therefore, lipid cores. Also in this case, capillaries are present at the edges of the lipid core.

Both atheromas and fibroatheromas can be clinically relevant, because may evolve in fissurated plaques, as well as in lesions with hematoma, and/or thrombus; all associated with the onset of ischemic VEs.[57]

Each of these lesions represents a different stage of chronic inflammation of the arterial wall.

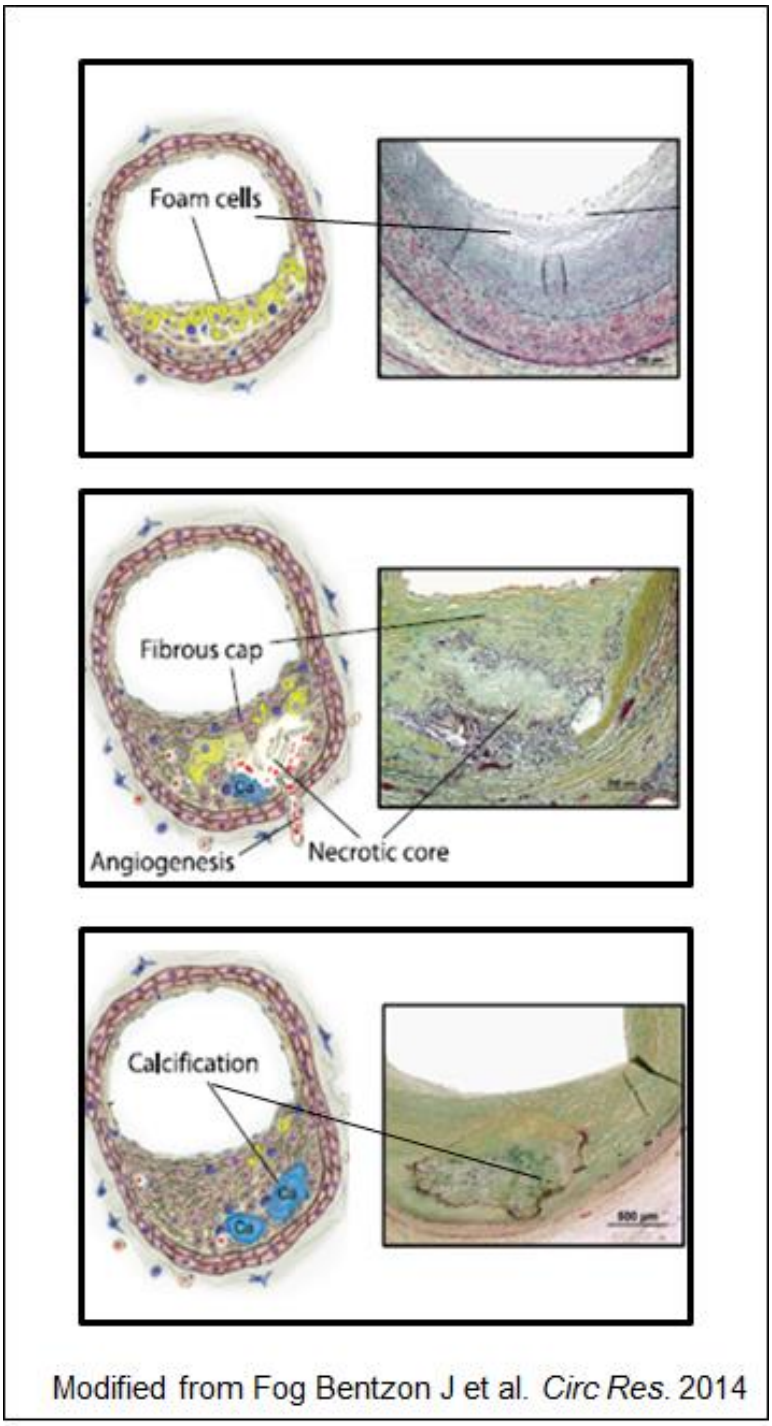

Figure 3. Morphological alterations of the arterial wall and formation of the plaque. Top panel, fatty streak characterized by macrophage foam cells; middle panel, fibroatheroma characterized by a consistent fibrous cap; bottom panel, fibroatheroma characterized by deposits of calcium (i.e. calcific plaque).

IMAGING MARKERS OF SUBCLINICAL ATHEROSCLEROSIS

High-resolution B-Mode ultrasound is a non-invasive diagnostic technique used to evaluate subclinical atherosclerosis in superficial arteries not shielded by bone surfaces, such as extra cranial carotid arteries (Figure 4).

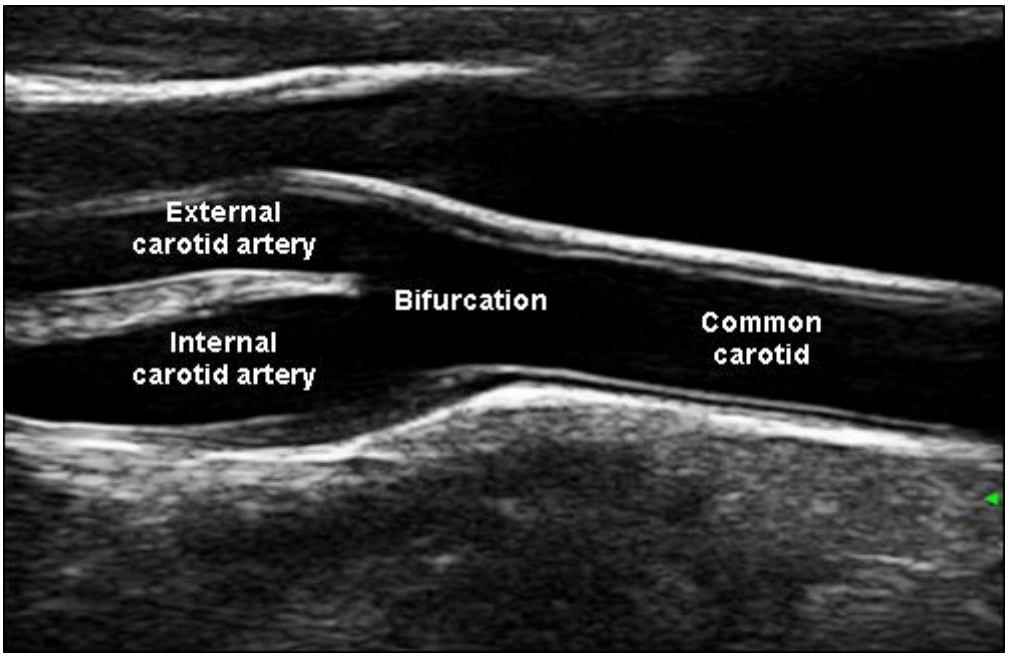

Figure 4. Ultrasonographic anatomy of common carotid, bifurcation, internal and external carotid arteries.

Providing information about not only the arterial lumen but also the vessel walls,[58-60] this technique allows to measure several imaging biomarkers related to both size and composition of early asymptomatic lesions. Among the imaging biomarkers, the most studied have been: the carotid intima-media thickness (cIMT) and its change over time, the carotid plaque, and the echolucency of both cIMT and carotid plaques. The content of calcium in carotid plaque indexed in terms of length of acoustic shadow (LAS), which is usually generated by plaque calcium (Figure 5), is a variable never measured before and measured just in this study.

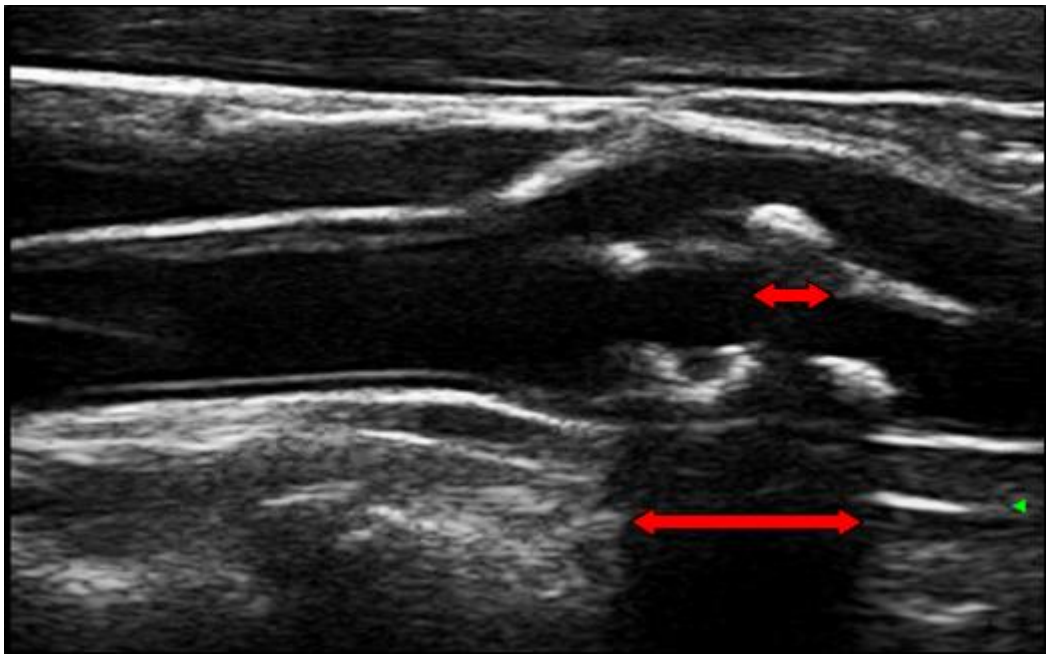

Figure 5. Ultrasonographic image representing the length of an acoustic shadow (LAS) generated by calcified carotid plaques.

Below a quick summary of these variables.

Carotid intima-media thickness (cIMT)

The intima-media thickness of extra cranial carotid arteries has been the first imaging variable identified as a marker of subclinical atherosclerosis. The method to determine this variable was developed and validated about thirty-five years ago[58] thanks to a study showing that, by measuring the distance between the ultrasonographic "blood-intima" and "media-adventitia" interfaces, it was possible to obtain a real estimate of the intima-media complex.

Two parallel echogenic (i.e. bright) lines form a typical ultrasonographic image of cIMT: the most luminal line is generated by the blood-intima interface, whereas the most external line is generated by the media-adventitia interface (Figure 6).[58] These two lines are separated by an anechoic (i.e. dark) space (Figure 6).[58]

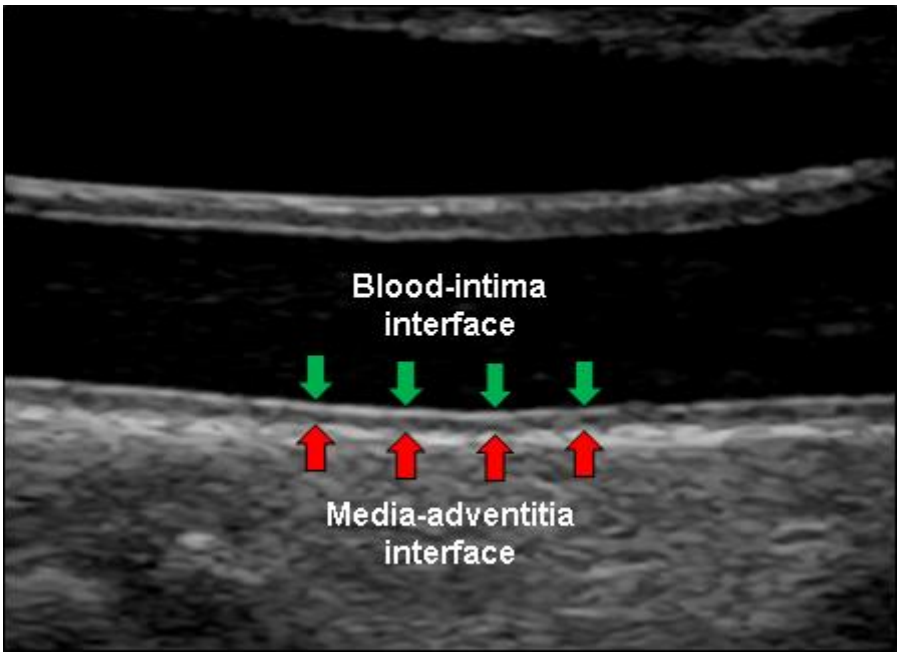

Figure 6. A typical ultrasonographic image of carotid intima-media thickness (cIMT). The most luminal echogenic (i.e. bright) line is generated by the blood-intima interface, whereas the most external echogenic line is generated by the media-adventitia interface. The two lines are separated by an anechoic (i.e. dark) space.

Carotid IMT measurement has been used in numerous clinical and epidemiological studies, both cross-sectional and longitudinal, to refine the cardiovascular risk. Several studies have shown that cIMT is associated not only with traditional VRFs,[61-65] but also with non-traditional VRFs, such as postprandial lipemia,[66] saturated dietary fat,[67] hyperhomocysteinemia,[68] E-selectin[69] and high-sensitivity C-reactive protein (hs-CRP) levels,[70] asymmetric dimethylarginine levels,[71] metabolic syndrome,[72] insulin hypersensitivity,[73] bacterial infections,[74] and psychosocial factors.[75] In addition, the higher the number of VRFs, the higher the cIMT, independently from age and sex (Figure 7).[76]

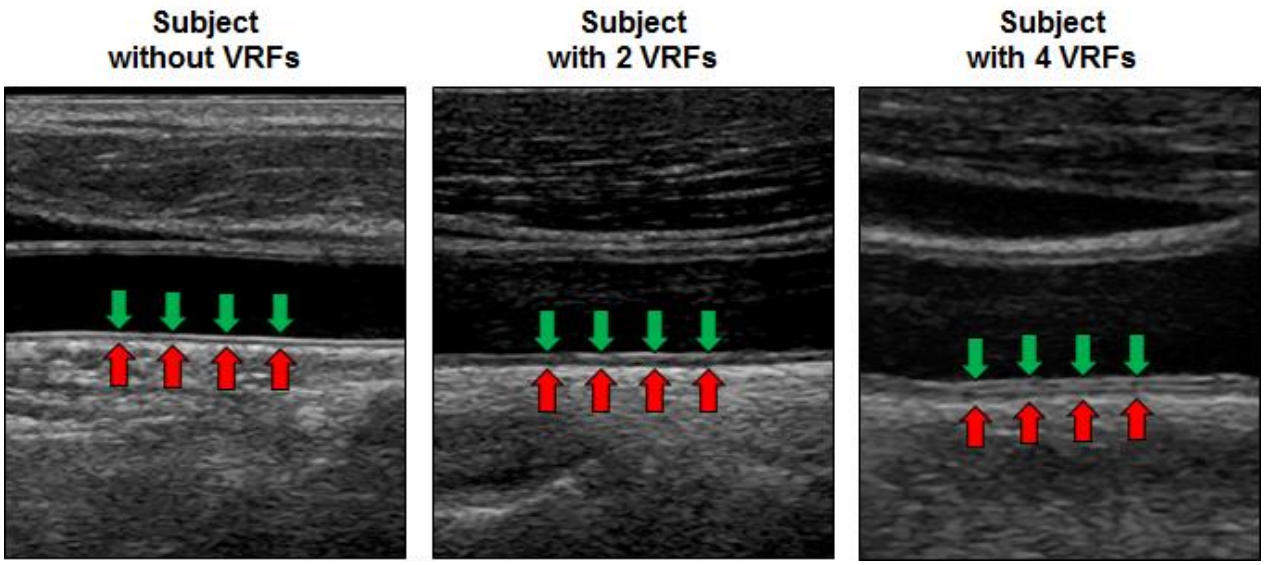

Figure 7. Carotid intima-media thickness (cIMT) increases with the increasing number of VRFs, independently from age and sex. VRFs, vascular risk factors.

A thicker cIMT has been also associated with the presence of plaques in carotids[77] and coronary arteries,[78-80] as well as with the incidence of cardiac and cerebral VEs.[63, 81-84]

On these bases, cIMT is now considered a surrogate marker of atherosclerosis not only in carotid arteries, but also in other vascular districts, such as coronary arteries.[85] This suggests that this variable can be used to assess atherosclerosis in the carotid district instead of coronary district, and, most importantly, together with plaques[86] it can be used as a predictive index of incident VEs.[63, 78, 84, 87]

Carotid IMT changes over time

Carotid IMT changes over time have been associated with traditional VRFs,[88] with coronary atherosclerosis,[89] and used as main end-point in interventional trials evaluating the anti-atherosclerotic effect of hypolipidemic, hypoglycemic or antihypertensive treatments.[90, 91]

Despite used in several studies, the clinical relevance of cIMT changes over time is still matter of debate, because studies failed in identifying a direct association between cIMT progression and incidence of VEs. In 2013, a Milan group (the lab where I performed my thesis) succeeded in identifying a novel approach to measure cIMT progression: the Fastest-IMT$_{max\text{-}progr}$.[92] Such variable, described in details in Materials and Methods and reflecting the fastest focal increase of

cIMT that occurred in the whole carotid arteries regardless of the specific location, was an independent predictor of cardiovascular events.

Carotid plaque

Studies have shown that the presence of carotid plaques (Figure 8) is able to predict future cardiovascular events independently from cIMT and even in a better way,[93-95] probably because carotid plaques represent a later stage of atherosclerosis.

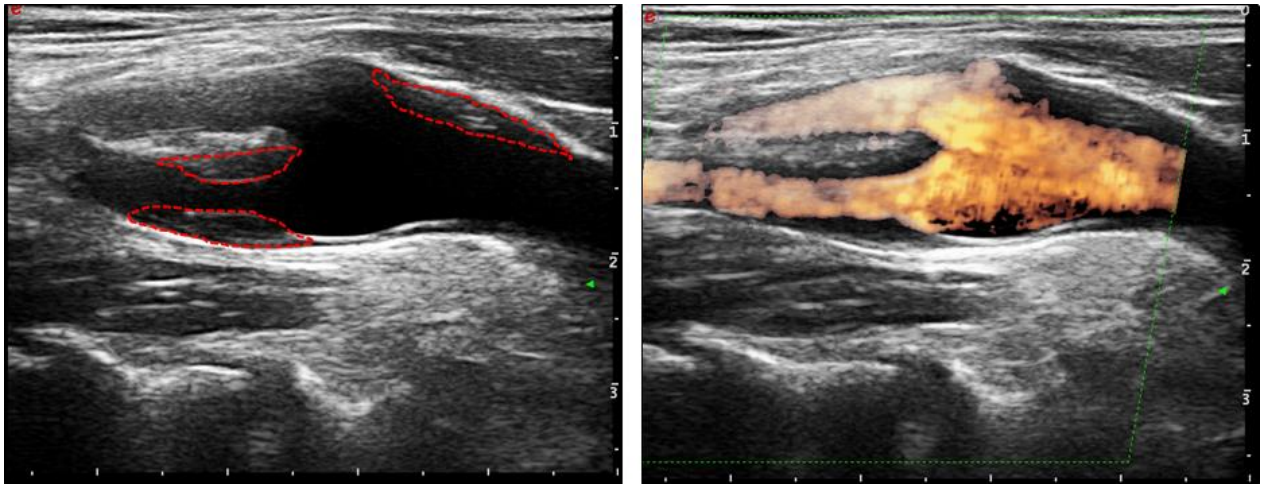

Figure 8. Ultrasonographic images of carotid plaques without (left panel) and with (right panel) color-doppler technique.

However, the relationship between atherosclerotic plaques and VEs is not merely due to plaques dimension, but also to their stability and/or vulnerability to rupture. Indeed, it is well known that stenotic plaques might be stable and asymptomatic, while other plaques, even if smaller, might have a composition that makes them more prone to rupture,[96, 97] and, therefore, more dangerous.

Echolucency of cIMT and echolucency of carotid plaques

Echolucency of a specific region of interest (ROI) on high-resolution B-Mode ultrasonographic images is another marker of subclinical atherosclerosis that allows to describe arterial wall features in terms of texture and composition.[98, 99] For example, echolucent plaques appear darker (Figure 9, left panel), suggesting the presence of a higher content of lipids and macrophages[100, 101] and,

sometimes, of intra plaque haemorrhage.[100, 102] By contrast, echorich plaques appear clear or even white (Figure 9, right panel), thus outlining the presence of fibrous tissue and, possibly, calcifications.[100] On these bases, echolucency also allows to discriminate echolucent-unstable plaques from echorich-stable plaques.[103-105]

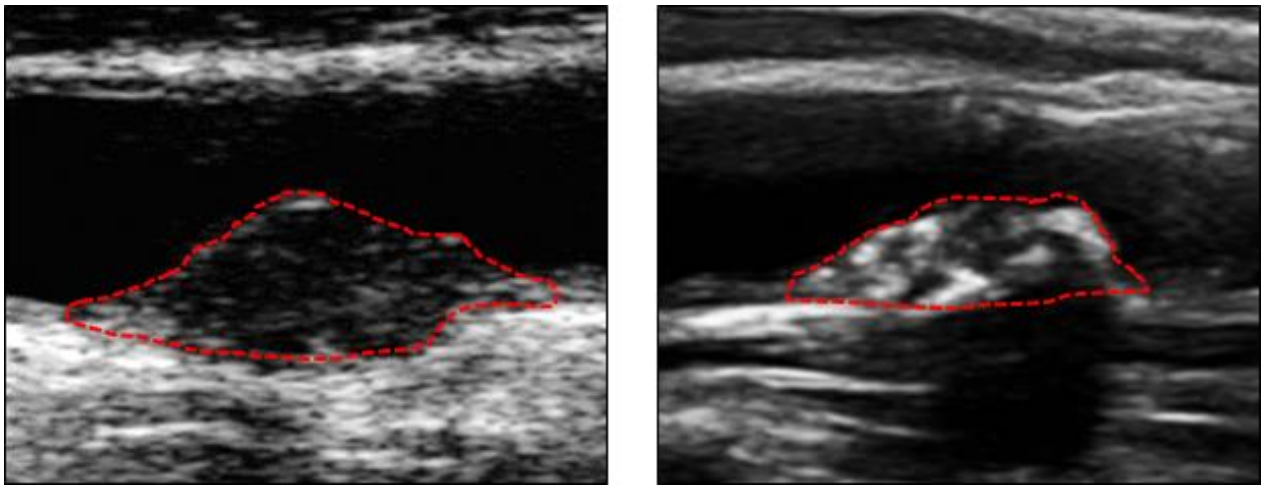

Figure 9. Echolucent carotid plaque (left panel) rich in lipids and macrophages; echorich carotid plaque (right panel) rich in calcium and fibrotic tissue.

Differences in plaque echolucency also allows to assume differences in the inflammatory activity.[100, 106, 107] Plaque echolucency has also been associated with low HDL-cholesterol levels,[108] with high levels of fasting and postprandial triglyceride-rich lipoproteins,[109] with oxidized-LDL and with hs-CRP,[110] thus suggesting that lipid profile, oxidative stress and inflammation play a role in determining the plaque echolucency itself.

Finally, both echolucency of cIMT and echolucency of carotid plaques have been related with several VRFs[107, 111, 112] and with the incidence of VEs,[83, 113-115] thus suggesting that even echolucency of the arterial wall in plaque free areas may be important from a prognostic point of view. This is of particular interest because the cIMT echolucency is measurable even in the earliest phases of the disease, i.e. in those situations (e.g. in children or young adults) where the plaques are very rare or completely absent.

Carotid calcium score (cCS)

The calcium contained in an advanced atherosclerotic plaque reflects the ultrasonographic waves. Consequently, in an ultrasonographic image an acoustic shadow is formed below a calcified plaque (see Figure 5, page 21). We have hypothesized that the greater the length of the acoustic shadow (LAS), the greater the amount of calcium into the plaque. Previous studies assessed the carotid calcium score (cCS) by computed tomography (CT). This technique allows to measure the amount of calcium in different vascular districts, e.g. carotid and coronary arteries.[116, 117] Coronary artery calcium (CAC) score has been widely accepted as a marker of coronary atherosclerosis[118] and as a reliable predictor of VEs.[119] These findings justify our decision to implement a simple ultrasonographic method to easily measure the carotid plaque calcium and to investigate whether the presence of calcium, measured in terms of LAS, could be an additional independent index of atherosclerosis and CVDs.

INFLAMMATORY BIOMARKERS

Based on the relationship between their plasma levels and the occurrence/extent of atherosclerosis, many soluble inflammatory markers have been studied as promoters of atherosclerosis progression, as predictors of VEs, and as determinants of therapy effectiveness.[120, 121] In our study, we considered two markers of inflammation: the high-sensitivity C-reactive protein (hs-CRP) and the white blood cells (WBC) count.

High-sensitivity C-reactive protein (hs-CRP)

The CRP is the main marker of chronic low-grade inflammation.[122] According to this inflammatory status, CRP is produced in quantities much lower than that produced in acute inflammatory processes.[123] CRP is synthesized and secreted by hepatocytes,[123] VSMCs[124, 125] and by plaques foam cells in response to others pro-inflammatory molecules (e.g. interleukin-6).[123] CRP is also produced in response to acute inflammatory pathways involving cytokines (e.g. interleukin-6) and other pro-inflammatory molecules. In vascular tissues, CRP promotes a prothrombotic state

through the damage and the activation of local endothelium, thus playing a direct role in the atherosclerotic process.[126, 127]

CRP is also a marker of atherosclerosis; indeed, its plasma concentration (especially when measured with high-sensitivity methods, i.e. hs-CRP) is an independent determinant of both incidence of VEs and progression of atherosclerosis, even more powerful than other soluble markers as LDL-cholesterol.[128]

The usefulness of hs-CRP as a marker for the screening of subjects with CVDs was described, for the first time, in the JUPITER trial.[129] Such study demonstrated that the hs-CRP level achieved after statins treatment may be as important as the LDL-cholesterol concentration achieved. On this base, hs-CRP levels may be used to target high-risk individuals who may better benefit from early statins therapy.

The levels of hs-CRP are not affected by traditional VRFs and this makes this variable an independent determinant of clinical atherosclerosis. In fact, high levels of circulating hs-CRP have been independently associated with both VEs and cardiovascular and total mortality.[130, 131] Moreover, when combined with an algorithm for the assessment of global cardiovascular risk (e.g. Framingham Risk Score), hs-CRP improves the risk prediction of coronary events and the subjects' risk reclassification.[132] Finally, hs-CRP has also been consistently related with imaging markers of subclinical atherosclerosis, e.g. cIMT.[133]

White blood cells (WBC) count

Leukocytes, or white blood cells (WBC), are important cells belonging to the immune system. They originate in the bone marrow, but circulate into the bloodstream. There are five main types of WBC: neutrophils, monocytes, lymphocytes, eosinophils, and basophils.

The WBC count refers to the sum of WBC subtypes, and is another conventional and easy-to-measure marker of chronic low-grade inflammation. The relationship between WBC count and atherosclerosis was suggested, for the first time, many years ago.[134] Since then, several studies have confirmed a significant and positive association between WBC count and both CAD and carotid or femoral atherosclerosis in different populations with different degrees of baseline risk.[134,

[135] In particular, WBC count has been associated with traditional VRFs[134, 135] [e.g. age, body mass index (BMI), smoking, dyslipidaemia, diabetes mellitus and hypertension], with markers of subclinical atherosclerosis[136] (e.g. carotid and femoral IMT and plaques, even after adjustment for VRFs), and with future VEs (e.g. coronary[137-141] and cerebral[142-144] VEs).

CLINICAL MANIFESTATIONS OF ATHEROSCLEROSIS

Coronaropathies

Ischemic heart disease is commonly considered the most typical expression of coronary atherosclerosis, and angina pectoris, acute myocardial infarction or sudden death are the main clinical manifestations of this disease.[145-148]

The angina pectoris is characterized by recurrent precordial pain, generally of short duration, associated with myocardial ischemia. There are two different types of angina: unstable angina and stable angina. Unstable angina is generally due to an increased oxygen demand from the myocardium because of physical activity, or emotional states, or a transient thrombotic occlusion. Stable angina, instead, occurs also at rest. Patients with angina, especially if unstable, are considered at high risk of myocardial infarction.

During myocardial infarction, the lack of blood supply to the corresponding myocardial sector, due to a persistent thrombotic obstruction of a coronary artery, leads to the death of affected myocardial cells. Characteristic symptoms can be oppressive retrosternal pain – often involving upper limbs, neck or jaw – shoulders and arms numbness, and gastric pain. Other times, symptoms may be even completely absent (silent ischemia).

Coronaropathies are also the most frequent cause of sudden death.

Cerebrovascular diseases

A stroke[149] is the consequence of the lack of blood supply to a brain area due to the rupture of an artery (haemorrhagic stroke) or to an arterial occlusion induced by thrombotic material (thrombotic stroke). Without the oxygen supply, neurons die within few minutes and, consequently, all body

districts controlled by the hit region loose the chance to work. Haemorrhagic strokes are frequent in patients suffering from vascular diseases (aneurysms, arteriovenous malformations, arteritis) and hypertension. Thrombotic strokes are mostly related to embolic phenomena, and occur when a thrombus, formed usually in the heart, detaches, starts to circulate in the bloodstream and stops at the level of a cerebral artery, obstructing it. Cerebral thrombosis is the most frequent type of cerebral stroke. It occurs on arteries (e.g. carotid arteries) damaged by atherosclerotic plaques. Cerebral thrombosis is often preceded by transient ischemic attacks (TIAs),[149] which have to be considered important premonitory symptoms. TIAs occur when blood supply to the brain is temporarily reduced or stopped by thrombus formation or embolus passage. Symptoms generally last only a few minutes – although, occasionally, they can last up to several hours (but no more than 24 hours, otherwise the event has to be defined a stroke). TIAs symptoms are very similar to stroke symptoms, including: weakness and/or hypoesthesia of a limb or a body side, blurring or unilateral loss of vision, aphasia, and, more rarely, dizziness and diplopia. Symptoms duration and, above all, their complete reversibility make it possible to differentiate TIAs from stroke.

Peripheral vascular diseases

Atherosclerotic disease of abdominal or lower limbs arteries leads to acute or chronic ischemia of the extremities, which, as in coronary arteries, involves thrombotic and/or embolic processes due to atherosclerotic plaques.[150] Peripheral arteries occlusion can occur with acute pain at rest, or with intermittent pain that appears after physical activity (i.e. clauticatio intermittens). Acute pain at rest is typical of arterial embolism, while clauticatio intermittens is typical of atherosclerotic vasculopathy.

CIRCULATING PCSK9: ROLE IN ATHEROSCLEROSIS

BIOLOGICAL MECHANISMS

Circulating PCSK9 has been firstly related with atherosclerosis development through its involvement in lipid metabolism. However, several studies demonstrated that PCSK9 promotes atherosclerosis development also through a lipid-independent pathway, i.e. inflammation.[151-154]

PCSK9 and lipid metabolism

Many studies have described the effect of PCSK9 on LDLR and plasma LDL-cholesterol.[155-157] Two rare missense mutations (i.e. Ser127Arg or Phe216Leu substitutions) on *PCSK9* gene have been associated with autosomal-dominant hypercholesterolemia in 2003.[4] Since then, many *PCSK9* genetic variants with "gain-of-function"[158, 159] or "loss-of-function" [5, 159-161] effect have been identified. Human studies demonstrated that "gain-of-function" variants in *PCSK9* cause higher LDL-cholesterol concentrations, resulting in familial hypercholesterolemia and coronary heart disease,[4] whereas "loss-of function" variants were consistently associated with lower LDL-cholesterol concentrations and lower risk of VEs.[5, 162, 163]

Studies in experimental animals supported these findings. Animal models specifically designed to overexpress PCSK9 showed reduced hepatic LDLR expression, increased plasma LDL-cholesterol levels and anticipated severe hypercholesterolemia.[6, 7, 164] On the other hand, PCSK9 knockout mice presented an increased number of hepatic LDLR, reduced LDL-cholesterol up to 80% and reduced total-cholesterol up to 40%.[8, 164]

All these studies support the role of circulating PCSK9 in regulating LDL-cholesterol levels by modulating LDLR density on plasma cell membrane.

More recently, it has been demonstrated that PCSK9 effect on lipid metabolism is not only due to LDLR regulation, but also to the regulation of other receptors belonging to the LDLR superfamily [e.g. very low-density lipoproteins receptor (VLDLR),[165] apoE receptor 2 (ApoER2),[165] lipoprotein receptor-related protein 1 (LRP1),[166] and cluster of differentiation 36 (CD36)[167]]. All these receptors, indeed, are sensitive to circulating PCSK9. The importance of such receptors in

cholesterol homeostasis still needs further investigations; however, they showed a significant role in the metabolism of triglycerides-rich lipoproteins (e.g. chylomicrons). In PCSK9-knockout and PCSK9-transgenic mice, PCSK9 downregulates the triglycerides and free-fatty acids entrances in visceral adipocytes via degradation of adipose tissue VLDLR and CD36, leading to an increased chylomicron clearance and a reduced post-prandial hypertriglyceridemia.[168] Plasma PCSK9 has been positively associated with triglycerides in the general population; moreover, the pharmacological inhibition of PCSK9 has been related to a reduction in triglycerides levels.[169]

Furthermore, recombinant PCSK9 increases cholesterol uptake by human intestinal epithelial cells, suggesting that PCSK9 is also involved in lipid absorption.[167]

All these effects of circulating PCSK9 on lipids clearly suggest that PCSK9 plays a central role in lipid metabolism beyond the regulation of LDL-cholesterol levels. The mechanisms of action still need to be clarified; however, they seem regulated by intracellular trafficking.

PCSK9 and inflammation

Strong evidence suggested that PCSK9 is involved in non-lipid pathways, such as inflammation.[12, 170, 171] Animal models showed that using inflammatory stimuli, such as lipopolysaccharides (LPS), zymosan or turpentine, PCSK9 mRNA expression markedly increases in mice liver and kidney.[172] Several *in vitro* studies also showed that pro-inflammatory factors modulate the expression of PCSK9. LPS increases PCSK9 expression in human endothelial cells and VSMCs in a dose-dependent manner.[173-176] Tumor necrosis factor alpha (TNF-α), one of the major pro-inflammatory cytokines implicated in systemic inflammation, is associated with increased PCSK9 mRNA and protein expression in hepatic HepG2 cells line.[177] Oxidized-LDL also enhances PCSK9 expression by affecting the secretion of inflammatory chemokines, such as TNF-α, interleukin-6, and interleukin-1α, in a particular line of derived macrophages in a dose-dependent way.[178] All these findings have suggested that PCSK9 may contribute to inflammatory processes underlying the pathogenesis of atherosclerosis.

PCSK9 expression has also been identified in cells within the arterial wall directly implicated in the formation of atherosclerotic lesions.[174, 179-181] This local PCSK9 plays a role in:

1) circulating monocytes inflammatory activity enhancement by increasing the expression of TNF-α and C–C chemokine receptor type 2 (involved in monocytes recruitment to the arterial wall), and by decreasing anti-inflammatory interleukin-10 secretion;[176, 182]
2) endothelium inflammation and damage by activating Bax/Bcl-2 axis (pro-apoptotic and anti-apoptotic proteins, respectively) and by increasing reactive oxygen species and LOX-1 (a protein that binds, internalizes and degrades oxidized-LDL) levels;[183]
3) circulating inflammatory monocytes adhesion to the endothelium by enhancing the expression of VCAM-1 and ICAM-1 (adhesion molecules on cell surface) in endothelial cells;[173, 184, 185]
4) inflammation initiated by monocytes-derived macrophages within atherosclerotic lesion by enhancing pro-inflammatory cytokines secretion;[180, 186]
5) inflammatory activation of VSMCs.[175]

Clinical evidence suggests that circulating PCSK9 is also associated with soluble inflammatory markers of atherosclerosis. For example, a positive relationship has been found between PCSK9 and hs-CRP level,[187-189] and between PCSK9 and WBC count and its subset of lymphocytes.[188, 190] More recently, it has been shown that PCSK9 is linearly associated with index of continuous inflammation, i.e. the amount and fraction of necrotic core tissue within coronary plaques.[19] This finding suggests that this secreted proprotein also contributes to inflammation/composition within atherosclerotic plaques.

According to all these studies, PCSK9 may be considered as an inflammatory mediator in atherosclerosis and, therefore, its pharmacological inhibition is important for both lipid-lowering and anti-inflammatory activities.

MATERIALS AND METHODS

THE IMPROVE STUDY

All these issues have been analyzed taking advantage of databases, biobanks and imaging-bank of “IMPROVE”, a European, multicenter, longitudinal, and observational study designed to identify novel biomarkers of subclinical and clinical atherosclerosis.[191]

Design of the IMPROVE study

The IMPROVE study, funded by the Vth European Union framework program, was carried out in seven centres of five European countries [Italy (Milan and Perugia), France (Paris), the Netherlands (Groningen), Sweden (Stockholm) and Finland (two centres in Kuopio)]. The IMPROVE complies with the rules of Good Clinical Practice and with the ethical principles established in the Helsinki Declaration, and was approved by local Ethics Committees in each study centre. Each participant provided two written informed consents: one for the general participation in the study and one for genotyping.

Participants recruitment

Almost 21,000 subjects were screened: 3,400 in Milan, 3,804 in Perugia, 1,800 in Paris, 4,050 in Groningen, 4,239 in Stockholm, 1,450 in the first Kuopio center and 2,354 in the second one. Among these, a total of 3,703 subjects (1,774 men and 1,929 women; aged from 55 to 79 years) at high cardiovascular risk for the presence of ≥ three VRFs were considered as eligible for the study. Subjects were considered to be exposed to a VRF when one of the following criteria was satisfied:

- male sex or, at least, 5 years after menopause for women;
- hypercholesterolemia (mean calculated LDL-cholesterol levels > 160 mg/dL or treatment with lipid lowering drugs);
- hypertriglyceridemia (triglycerides levels > 200 mg/dL after diet or treatment with triglycerides lowering drugs);
- hypoalphalipoproteinemia (HDL-cholesterol levels < 40 mg/dL);

- hypertension (diastolic blood pressure, or DBP, > 90 mmHg and/or systolic blood pressure, or SBP, > 140 mmHg or treatment with anti-hypertensive drugs);
- diabetes or impaired fasting glucose (blood glucose levels > 110 mg/dL or treatment with insulin or oral hypoglycemic drugs);[192]
- smoking habits (at least 10 cigarettes/day for, at least, thirty months);
- family history of CVDs.

Due to its observational nature, participation did not require any change in medication.

Subjects excluded from the study presented, at least, one of the following characteristics:

- age under 55 or over 79 years;
- any condition that might limit cIMT visualization (i.e. abnormal anatomical configuration of neck and muscles; marked tortuosity and/or depth of the carotid vessels, and/or uncommon location of arterial branches);
- CVDs outcomes (i.e. personal history of myocardial infarction, angina pectoris, stroke, TIAs, aortic aneurysm or claudicatio; re-vascularization in carotid, coronary or peripheral arteries);
- personal history of congestive heart failure (III-IV NYHA Class);
- personal history of serious medical conditions that might limit longevity.

Participants were followed-up for 36 months. Clinical, biochemical, genetic, socio-economic, psychological, nutritional and educational data, personal and family history of CVDs, chronic drugs intake, and physical activity were recorded at baseline visit. Each subject underwent ultrasonographic scans of extracranial carotid arteries at baseline, at 15 and at 30 months. Clinical chemistry was centralized in Stockholm, where plasma, serum, and DNA samples are stored. Carotid scans were recorded on sVHS videotapes and stored in the IMPROVE imaging bank in Milan. To prevent loss of information due to unexpected accidents (thawing of the biologic samples

or deterioration of videotapes), biologic samples were fractioned and separately stored in two different biobanks, and ultrasonographic scans were digitalized.

Laboratory analyses

Venous blood samples were obtained after an overnight fast. The Stockholm biobank contains 14 aliquots of 0.5 mL EDTA plasma and 8 aliquots of 0.5 mL serum for each participant. Moreover, 2 X 5 mL whole blood for each subject was stored for DNA extraction.

Plasma glucose concentration, blood cells count, hematocrit and differential count had already been measured locally. To assess all the other biochemical variables, blood was kept at room temperature for a minimum of 30 min in order to allow clotting to occur. Serum, prepared by centrifugation at 2000g for 20 min, was dispensed in polypropylene tube and frozen at –80°C before shipment to Stockholm.

Lipids, uric acid, creatinine, and soluble inflammatory markers

Serum concentrations of total, HDL- and LDL-cholesterol (by Friedewald's formula), triglycerides, uric acid, creatinine and hs-CRP were analyzed (in the Department of Clinical Chemistry, Karolinska University Hospital Solna, Stockholm, Sweden) with the use of LX Beckman instruments. Cholesterol, triglycerides and uric acid were measured with enzymatic methods, creatinine with colorimetry-alkaline picrate analysis, and hs-CRP with turbidimetry (NIPIA methods).

Intra-assay and inter-assay variability tests were performed to test precision and validity of the humoral/biochemical biomarkers measurements.[191]

PCSK9 ELISA

Aliquots of blood samples were sent from Stockholm to the Italian center of Milan for PCSK9 measurements. Plasma PCSK9 levels were measured in 2 Italian laboratories: 1) Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, and 2) Dipartimento di Scienze del Farmaco, Università degli Studi di Padova, Padova.

Plasma PCSK9 concentrations were measured by a commercial ELISA kit (R&D Systems, MN) able to recognize free and LDLR-bound PCSK9. Plasma samples were diluted 1:20, according to the manufacturer's instructions, and incubated onto a microplate pre-coated with a monoclonal antibody specific for human PCSK9. Sample concentrations were obtained by generating a four-parameter logistic curve-fit. The minimum detectable concentration was 0.219 ng/mL. Intra- and inter-assay coefficients of variability (CVs) were 5.4±1.2% and 4.8±1.0%, respectively.

Measurement of ultrasonographic variables

Most of the ultrasonographic variables considered in the present study have been measured on the ultrasonographic scans stored in the IMPROVE imaging-bank in the contest of other studies.[86, 92, 191] All measurements were made in the ultrasonographic lab of the Milan center. By contrast, carotid echolucency and the Length of the Acoustic Shadow (LAS) were measured in the contest of the present project.

Carotid IMT, carotid plaques and their changes over time

Carotid ultrasound was performed using seven ultrasonographic machines calibrated with an appropriate phantom before the start of the IMPROVE study and checked after 1 year. Sonographers and readers were trained and certified by the coordinating center in Milan. Most participants (62.2%) were followed throughout the study by the same sonographer, and all scans of each patient were assigned to a single reader after coding and were read blindly. All measurements were made using a dedicated software (M'Ath, Metris SRL France),[193] which allows a semi-automatic edge detection of the distance between the blood-intima and media-adventitia interfaces, thus identifying the intima-media complex (Figure 10).

Figure 10. Semi-automatic edge detection of the distance between the blood-intima and media-adventitia interfaces (i.e. carotid intima-media thickness, cIMT) by using M'Ath software.

Sonographer sat behind the patient's head. The far walls of left and right common carotids (CCs), bifurcations (Bifs), and internal carotid arteries (ICAs) were visualized at 3 scan angles (lateral, anterior and posterior). Images were recorded with the subject's head rotated approximately 45° opposite to the side being imaged, and the three angles were acquired with the transducer held, approximately, at 45°, 20° and 60° from the vertical angle. Each segment of CCs (in their entire length), Bifs, and the first proximal centimeter of the ICAs were measured in, at least, 3 different frames. The cIMT was measured by incorporating plaques in the measurement; in particular, a plaque was defined as a maximum cIMT > 1.5 mm. This ultrasonographic protocol allowed to visualize about 30 carotid segments (3 images on CC + one image on Bif + one image on ICA = 5 images for a single projection; 5 images for a single projection x 3 projections x 2 carotids = 30 images).

To evaluate the fastest changes of cIMT and plaque-size over time, ultrasonographic exams and measurements were repeated after 15 months using the same ultrasonographic protocol (positions and angles of ultrasound transducer with respect to the neck) used at baseline. Carotid IMT and plaque-size changes, expressed in mm/year, were calculated as:

$$\frac{\text{(the 15}^{\text{th}}\text{ month measurement) – (the corresponding baseline value)}}{\text{the length of the intervening time period}}$$

The variables used for the statistical analyses were: IMT_{mean} (i.e. average of 1st CC-IMT_{mean}, CC-IMT_{mean}, Bif-IMT_{mean}, and ICA-IMT_{mean} of left and right carotid arteries), IMT_{max} (i.e. the highest value out of 1st CC-IMT_{max}, CC-IMT_{max}, Bif-IMT_{max}, and ICA-IMT_{max} of left and right carotid arteries), $IMT_{mean\text{-}max}$ [i.e. the average of maximal IMT measured in 8 segments (1st CC, CC, Bif, and ICA in left and right carotid arteries)], plaque-free (PF) CC-IMT_{mean} (i.e. IMT_{mean} measured in the 2nd cm of CCs in plaque-free areas), and Fastest-$IMT_{max\text{-}progr}$ (the 15th month progression of IMT_{max} detected in the whole carotid tree regardless of location).

Results of intra- and inter-assay variability tests have been previously reported.[86, 92, 191]

Echolucency of cIMT and carotid plaques

Starting from ultrasonographic scans, the best frames among the free projections were selected by using a specific program (Avidemux version 2.6) and echolucency measurements were performed by using a semi-automatic dedicated software (MIA Carotid Analyzer version 6.7.5) that provides a densitometric analysis of images.

The quantitative evaluation of echolucency is based on the analysis of the grey scale median (GSM)[100, 105, 194-196] of pixels distribution of a manually placed ROI (i.e. carotid IMT or plaque), normalized to the GSM value of blood (e.g. zero for maximum black) and to the GSM value of vessel adventitia (e.g. 255 for maximum white) (Figure 11). Once defined, such parameters are applied to the entire sequence of frames.

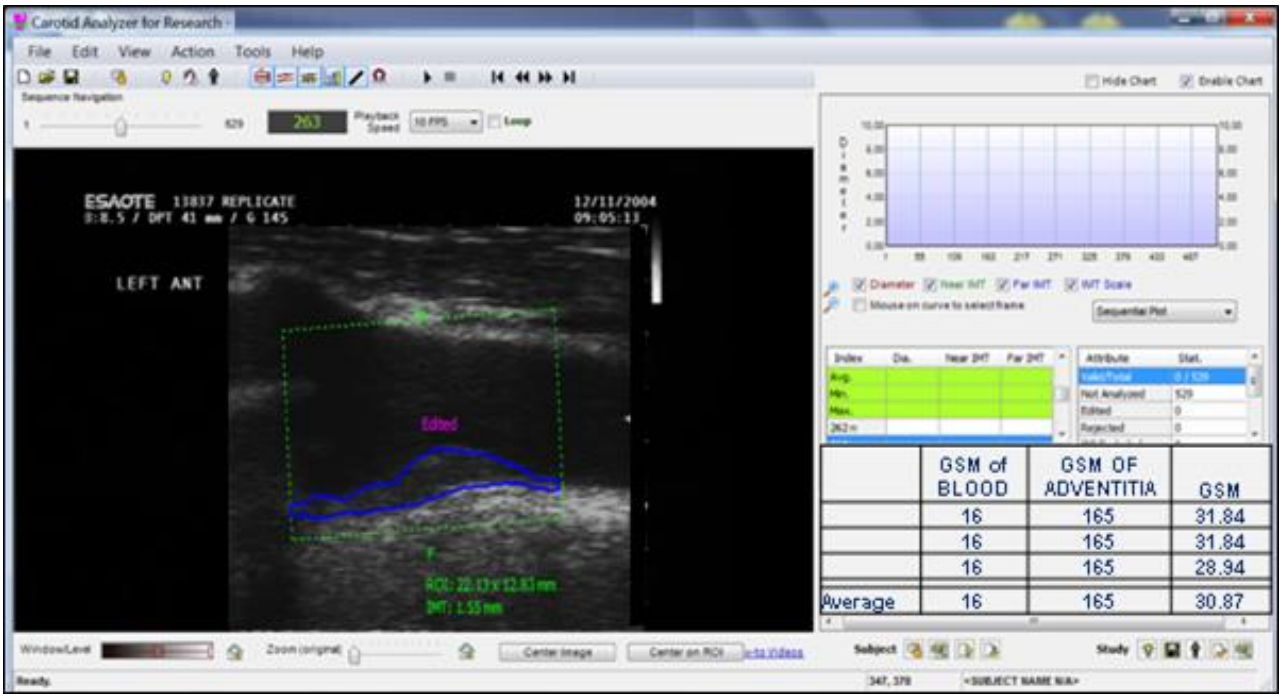

Figure 11. Semi-automatic evaluation of echolucency of a carotid plaque by using MIA Carotid Analyzer software. The analysis is based on the Grey Scale Median (GSM) of pixels distribution of a specific region of interest (e.g. in this case, the plaque in the carotid bifurcation).

Low GSM values represent an echolucent (dark) cIMT or plaque (Figure 12, top panels), whereas high GSM values represent an echorich (bright) cIMT or plaque (Figure 12, bottom panels).

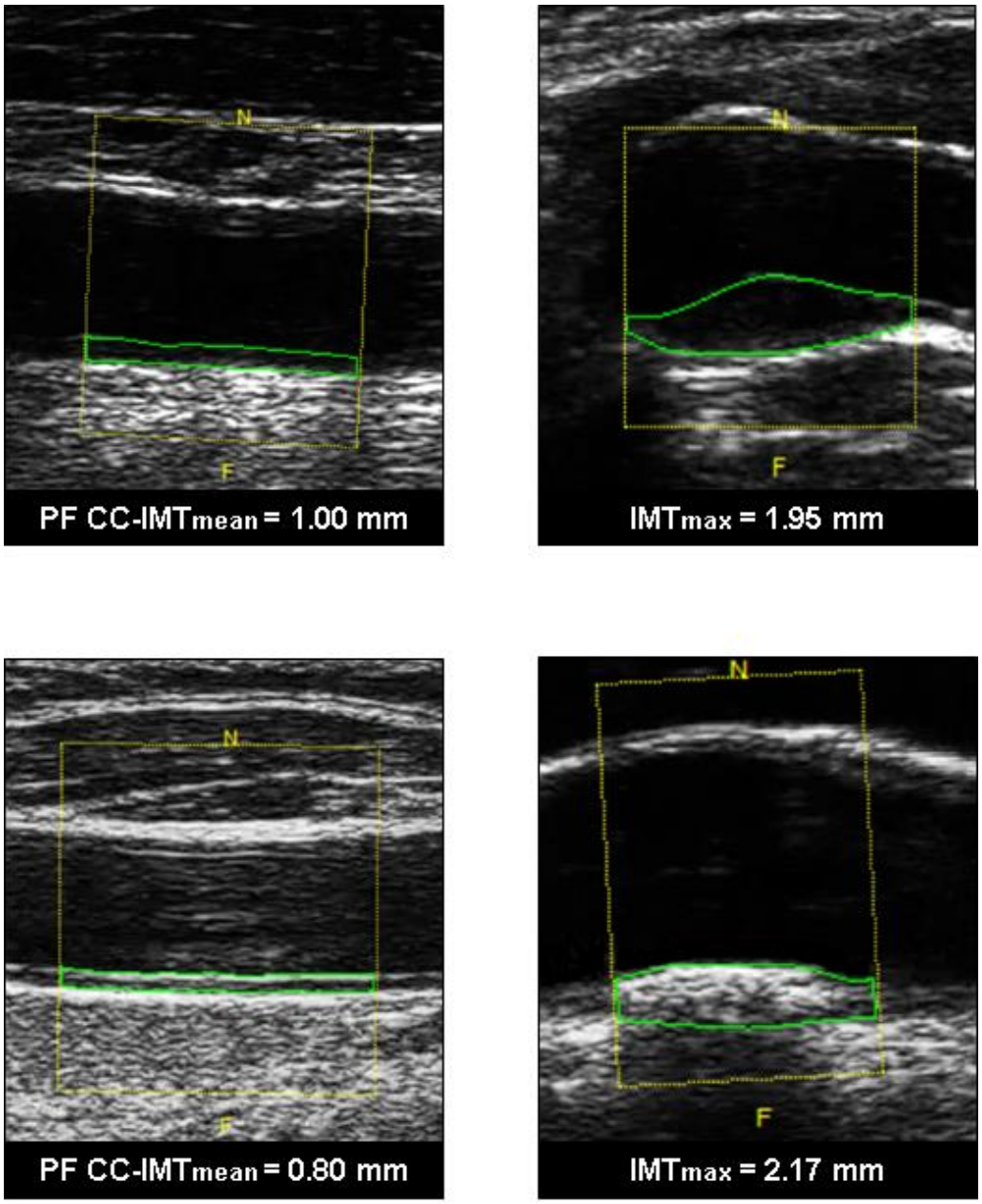

Figure 12. Echolucent (dark) PF CC-IMT$_{mean}$ and plaque (i.e. IMT$_{max}$) (top panels; left and right, respectively), and echorich (bright) PF CC-IMT$_{mean}$ and plaque (bottom panels; left and right, respectively). Echolucent PF CC-IMT$_{mean}$ and plaque are characterized by low GSM values (e.g. in this case, 22.50 and 16.15, respectively); whereas echorich PF CC-IMT$_{mean}$ and plaque are characterized by high GSM values (e.g. in this case, 82.78 and 89.96, respectively). PF CC-IMT$_{mean}$ has been detected in the 2nd cm of common carotid arteries in plaque-free areas; the biggest carotid plaque has been detected in the whole carotid tree (e.g. in this case, in carotid bifurcations).

For each ultrasonographic scan, 1) GSM of PF CC-IMT$_{mean}$ detected in lateral projection, and 2) GSM of the biggest plaque detected in the whole carotid tree among the 3 projections were measured on the far walls of both left and right carotid arteries.

The variables used for the statistical analyses were: mean GSM of left and right PF CC-IMT$_{mean}$, and GSM of the blackest one between left and right biggest carotid plaques.

Reproducibility results are reported in this manuscript.

Carotid calcium score (cCS)

To calculate the cCS, the length of acoustic shadow (LAS) generated by plaque calcium was measured on selected frames (by Avidemux version 2.6) by using the same software used for the evaluation of GSM (Figure 13).

Figure 13. Semi-automatic evaluation of the length of the acoustic shadow (LAS) generated by plaque calcium by using MIA Carotid Analyzer software.

The LAS of each calcified plaque, when present, was measured in 2 different frames and averaged. Plaques were looked for in the far and the near walls in each carotid segment (CC, Bif and ICA) and in each insonation angle (anterior, lateral, and posterior) on both left and right

carotids. The sum of the LAS mean of all plaques considered (i.e. cCS) was used for the statistical analysis. Results of reproducibility tests are reported in this manuscript.

Genetic data

Selection of *PCSK9* single nucleotide polymorphisms (SNPs)

The selection of four SNPs in the *PCSK9* locus (i.e. rs11583680, rs11591147, rs2479409, and rs11206510) was based on three criteria,[22] namely:

1) a robust association with LDL-cholesterol (as observed by the Global Lipids Genetics Consortium);[197]
2) a low pairwise linkage disequilibrium (LD) ($r^2 \leq 0.30$) with other variants in that region (using 1000 Genomes CEU data);
3) a Combined Annotation Dependent Depletion score[198] (ranging from 1 to 99) that evaluates potential functionality.

Genotyping

DNA was purified from all subjects who signed written informed consent for genetic studies. Subjects were genotyped using both Illumina Cardio-Metabo 200k[199] and Immunochip 200k[200] array platforms. Standard quality control was conducted on the individual chips as well as the combined dataset, including: exclusion of samples with cryptic relatedness, low call rate (<95%) or ambiguous sex and exclusion of variants for low call rate (<95%), failing Hardy-Weinberg Equilibrium ($p<1x10^{-5}$) or low call rate [minor allele frequency (MAF) >1%]. Multi-dimensional scaling (MDS) components 1–3 were calculated in PLINK[201] to enable adjustment for population structure. The four *PCSK9* variants included in the present study have a MAF ≥ 0.017 and satisfy the Hardy-Weinberg equilibrium (p>0.16).

DATA FROM THE UK BIOBANK

The UK Biobank cohort

The UK Biobank recruited over 500,000 volunteer participants (age range 40–69 at baseline) from 22 centers across the UK between 2006 and 2010.[202] All participants completed a wide variety of baseline assessments and questionnaires, and provided blood samples for standard biochemical assays and genotyping. All subjects provided informed consent, and the study was conducted according to the Helsinki Declaration.

To compare the IMPROVE study cohort with data from the UK Biobank, a subset of 22,179 white British ancestry individuals with cIMT measures were included in the present project. The use of UK Biobank data for the present study was permitted under the generic approval granted by the National Health Service National Research Ethics Service (approval letter dated 13 May 2016, Ref 16/NW/0274) and under UK Biobank applications #6553 (PI Daniel Smith) and #17689 (PI Donald Lyall).

Carotid IMT and carotid plaques in the UK Biobank cohort

Carotid IMT measures were recorded during a follow-up visit (4 to 8 years after the recruitment), during which also anthropometric measurements and questionnaires were repeated from the baseline visit in order to be completed.

In a pilot phase, subjects underwent carotid ultrasound in 18 centers (with 8 centers representing for 98% of the sample) with an extensive manual quality control being conducted. Subsequently, the manual quality control was considered unnecessary, and all centers started to recruit and record automated measures (with 10 centers representing for 93% of the sample).

The far walls of left and right distal CCs were visualized at 2 scan angles (120° and 150° for left CCs; 210° and 240° for right CCs) with sonographer sat in front of the patient.

An automated software was used to record images and to measure mean and maximum cIMT (UK Biobank data fields 22670-22681).

The variables used for the statistical analyses were: IMT_{mean} [i.e. the average of 4 mean measures (2 for each of left and right carotid arteries)]; IMT_{max} (i.e. the average of maximum cIMT values, where the highest of the 4 maximum cIMT measures was used); $IMT_{mean-max}$ [i.e. the mean of 4 maximum measures (2 for each of left and right carotid arteries)]. If >1 value was missing because of the poor quality of the image, the subject was excluded from analyses.

Genotyping

UK Biobank participants were genotyped using either the Affymetrix BiLEVE Axion or the Affymetrix UK Biobank Axion arrays and imputed to 1000 Genomes, UK10K and Haplotype Reference Consortium (March 2018 release) reference panels.[203] Standard genetic quality control (consistent with that described above), pre- and post-imputation, was conducted centrally by UK Biobank. The four variants included in this study have a MAF ≥ 0.017 and satisfy the Hardy-Weinberg equilibrium ($p>0.12$).

STATISTICAL ANALYSIS

All quantitative variables were reported as mean ± SD. Variables with approximately log-normal distributions were reported as median and inter-quartile range, and log-transformed before all parametric analyses. Although a moderately skewed distribution, PCSK9 levels were also reported as mean ± SD, thus allowing comparisons with the existing literature. Categorical variables were reported as count and percentage. Trends across PCSK9 quintiles for continuous and categorical variables were assessed by ANCOVA and Mantel-Haenszel $\chi 2$-test, respectively.

Spearman correlation coefficient was calculated to assess the correlations between PCSK9 levels and both lipids (total, LDL- and HDL-cholesterol, and triglycerides) and inflammatory markers (hs-CRP, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and WBC count). For the relationships between PCSK9 and both LDL-cholesterol and hs-CRP, statins users were excluded. Multivariable analysis with stepwise selection was performed to investigate the independent determinants of PCSK9 levels. Circulating PCSK9 was entered as dependent variable; latitude,

gender, age, height, weight, waist, hip, SBP, DBP, educational level, personal history of hypercholesterolemia, of hypertriglyceridemia, of hypoalphalipoproteinemia, of hypertension, and of diabetes, current smoker, pack-years, family history of coronary, cerebral, and/or peripheral vascular diseases, of hyperlipidemia, of hypertension, and of diabetes, physical activity, total cholesterol, HDL-cholesterol, triglycerides, uric acid, blood glucose, creatinine, hs-CRP, WBC count, red blood cells (RBC) count, consumption of wine, beer, spirits, fruit, milk, coffee, tea, meat, fish, and eggs, and therapies with statins, and/or fibrates, fish oil, other lipid-lowering drugs, beta-blockers, calcium antagonists, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, diuretics, anti-platelet agents, insulin, and/or estrogen supplement were entered as independent variables. A sensitivity analysis, aimed at evaluating whether pharmacological treatments may modify the effects of predictors, was also performed by excluding subjects treated with statins, or fibrates or with a combination of both these drugs from the original multivariable analysis. The multivariable relationship between PCSK9 levels and ultrasonographic variables (i.e. carotid IMT_{mean}, IMT_{max}, $IMT_{mean-max}$, PF CC-IMT_{mean}, Fastest-$IMT_{max-progr}$, GSM of PF CC-IMT_{mean}, GSM of carotid plaque, and cCS) was analyzed by running several models: adjusted for age, sex, and latitude (Model 1); adjusted for the common independent determinants between PCSK9 and each ultrasonographic variable (Model 2); and adjusted for other possible confounders (Model 3 and/or Model 4). Bland-Altman was performed to evaluate the repeatability of GSM and cCS measurements. Cox regression analyses were performed to estimate crude and adjusted hazard ratios (HRs) of the associations between plasma PCSK9 levels and different types of VEs [combined VEs (i.e. coronary- plus cerebral- plus peripheral-VEs); single district events (i.e. or coronary VEs or cerebral VEs); hard events (i.e. myocardial infarction, sudden death and stroke)]. Also in this case, the analysis was carried out by running several models: unadjusted (Model 1); adjusted for age, sex, and stratified by latitude (Model 2); and adjusted as Model 2 + PCSK9 independent determinants (i.e. statins or fibrates treatment, hip circumference, personal history of hypercholesterolemia and of hypertriglyceridemia, pack-years, family history of diabetes, total and HDL-cholesterol, uric acid, fish and wine consumption) (Model 3). All analyses were carried out

with SAS statistical package v. 9.4 (SAS Institute Inc., Cary, NC, USA). P_{values} <0.05 were considered statistically significant.

Linear regression for continuous variables was used in PLINK 1.07 to investigate the effect of each *PCSK9* variant (additive genetic model) on cIMT variables in common between IMPROVE and UK Biobank (i.e. IMT_{mean}, IMT_{max}, and $IMT_{mean-max}$). In the IMPROVE study, *PCSK9* variants were tested for associations with LDL-cholesterol and cIMT variables. All the analyses were adjusted for sex, age, lipid-lowering drugs (yes vs not), and Multi-Dimensional Scaling (MDS) components 1-3 (MDS1-3). In the UK Biobank cohort, *PCSK9* SNPs were tested for associations with cIMT variables only. Analyses were adjusted for sex, age, lipid-lowering drugs (yes vs not), population structure (PGC1-8), and genotyping chip. All P_{values} corrected by Bonferroni ≤0.01 were considered statistically significant. A SNPs score was constructed by coding genotypes as 0, 1 and 2 relating to the number of LDL-cholesterol lowering alleles and considering an additive allelic effect. Prior to analysis, the SNP score was standardized. Multiple linear regression analysis was used in IBM SPSS 26.0 to investigate whether the standardized SNP score was independently associated with cIMT measures in the IMPROVE and UK Biobank cohorts. In the IMPROVE and UK Biobank, analyses were adjusted for the same covariates used in PLINK, respectively. P_{values} corrected by Bonferroni ≤0.025 were considered statistically significant.

RESULTS

Plasma PCSK9 levels were successfully measured in 3,673 (1,915 women and 1,758 men) out of 3,703 participants included in the IMPROVE study cohort.[204] PCSK9 measurements showed a moderately right-skewed distribution with a mean concentration of 310.8 ± 108.6 ng/mL and a broad range, from 20.1 ng/mL to 1,133.9 ng/mL.

The baseline characteristics of the IMPROVE study participants, stratified by quintiles of PCSK9, are shown in Table 1. For some continuous variables [i.e. age, height, weight, BMI, waist and hip circumferences, waist/hip ratio, DBP, SBP, educational level, uric acid, blood glucose, creatinine, RBC count, haemoglobin, haematocrit, mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and estimated glomerular filtration rate (GFR)] and some categorical variables (i.e. latitude, sex, personal history of low HDL-cholesterol, personal history of hypertension and personal history of diabetes, family history of coronary and peripheral vascular diseases, family history of diabetes, physical activity, consumption of beer, spirits, tea, and/or eggs, and therapies with antiplatelet and/or insulin) a negative trend across PCSK9 quintiles was observed.

Conversely, total, HDL- and LDL-cholesterol, triglycerides, basophils and platelets (among the continuous variables), and personal history of hypercholesterolemia and of hypertriglyceridemia, family history of hyperlipidaemia and of hypertension, consumption of wine and therapies with statins, fibrates, and/or fish oil (among the categorical variables) were positively associated with PCSK9 quintiles.

Table 1. Baseline characteristics of the IMPROVE study cohort stratified by quintiles of plasma PCSK9 levels (ng/mL).

	1st quintile (n=734)	2nd quintile (n=735)	3rd quintile (n=735)	4th quintile (n=735)	5th quintile (n=734)	Pvalue
PCSK9 (ng/mL)	183 (155; 203)	251 (238; 264)	300 (287; 312)	356 (340; 373)	449 (416; 503)	
Recruitment center						
Perugia, n (%)	25 (3.4)	70 (9.5)	114 (15.5)	159 (21.6)	173 (23.6)	
Milan, n (%)	82 (11.2)	85 (11.6)	91 (12.4)	89 (12.1)	205 (27.9)	
Paris, n (%)	35 (4.8)	91 (12.4)	109 (14.8)	136 (18.5)	125 (17.0)	**<0.0001**
Groningen, n (%)	51 (6.9)	133 (18.1)	147 (20.0)	109 (14.8)	75 (10.2)	
Stockholm, n (%)	107 (14.6)	134 (18.2)	119 (16.2)	111 (15.1)	60 (8.2)	
Kuopio, n (%)	434 (59.1)	222 (30.2)	155 (21.1)	131 (17.8)	96 (13.1)	
Anthropometric variables						
Male, n (%)	482 (65.7)	381 (51.8)	347 (47.2)	277 (37.7)	271 (36.9)	**<0.0001**
Age (years)	64.7 ± 5.3	64.4 ± 5.1	64.4 ± 5.5	63.7 ± 5.4	63.7 ± 5.8	**<0.0001**
Height (m)	1.70 ± 0.10	1.69 ± 0.10	1.67 ± 0.10	1.66 ± 0.10	1.65 ± 0.10	**<0.0001**
Weight (Kg)	80.2 ± 14.6	78.5 ± 15.6	76.5 ± 15.0	74.9 ± 15.4	73.3 ± 14.4	**<0.0001**
Body mass index (Kg/m^2)	27.6 ± 4.2	27.4 ± 4.4	27.3 ± 4.3	27.2 ± 4.4	26.9 ± 4.0	**0.0004**
Waist (mm)	96.1 ± 12.5	94.7 ± 12.7	94.4 ± 12.5	92.9 ± 12.7	92.1 ± 12.0	**<0.0001**

Hip (mm)	102.4 ± 8.4	103.0 ± 9.7	102.7 ± 10.2	102.4 ± 10.0	101.2 ± 9.4	**0.01**
Waist/hip ratio	0.94 ± 0.10	0.92 ± 0.10	0.92 ± 0.10	0.91 ± 0.10	0.91 ± 0.10	**<0.0001**
DBP (mmHg)	84.1 ± 10.0	82.6 ± 9.9	81.5 ± 9.6	81.1 ± 9.8	80.7 ± 9.2	**<0.0001**
SBP (mmHg)	146.2 ± 19.1	142.6 ± 18.9	141.7 ± 17.8	140.8 ± 18.2	138.4 ± 17.7	**<0.0001**
Educational level (study yrs)	10.8 ± 3.8	10.5 ± 3.9	10.3 ± 3.8	10.4 ± 3.9	10.3 ± 4.1	**0.03**
Personal history of						
Hypercholesterolemia, n (%)	398 (54.2)	491 (67.0)	513 (69.9)	577 (78.6)	656 (89.4)	**<0.0001**
Hypertriglyceridemia, n (%)	125 (17.0)	149 (20.3)	200 (27.2)	196 (26.7)	279 (38.0)	**<0.0001**
Low HDL-cholesterol, n (%)	144 (19.6)	102 (13.9)	93 (12.7)	62 (8.4)	84 (11.4)	**<0.0001**
Hypertension, n (%)	577 (78.6)	497 (67.8)	514 (69.9)	484 (65.9)	464 (63.2)	**<0.0001**
Diabetes, n (%)	227 (30.9)	174 (23.7)	200 (27.2)	156 (21.2)	146 (19.9)	**<0.0001**
Smoking habits						
Current smokers, n (%)	117 (15.9)	110 (15.0)	95 (12.9)	108 (14.7)	114 (15.5)	
Former smokers, n (%)	272 (37.1)	290 (39.5)	274 (37.3)	261 (35.5)	263 (35.8)	0.57
Never smokers, n (%)	345 (47.0)	335 (45.6)	366 (49.8)	366 (49.8)	357 (48.6)	
Pack-years	0.7 (0.0; 18.0)	1.5 (0.0; 18.5)	0.0 (0.0; 19.0)	0.0 (0.0; 18.0)	0.6 (0.0; 17.3)	0.57
Family history of						

CHD, n (%)	501 (71.1)	469 (67.5)	456 (64.0)	448 (62.4)	418 (58.6)	**<0.0001**
CVD, n (%)	277 (37.7)	263 (35.8)	263 (35.8)	250 (34.0)	262 (35.7)	0.69
PVD, n (%)	99 (13.5)	91 (12.4)	64 (8.7)	91 (12.4)	94 (12.8)	**0.045**
Hyperlipidemia, n (%)	237 (32.3)	279 (38.0)	311 (42.3)	335 (45.6)	401 (54.6)	**<0.0001**
Hypertension, n (%)	459 (62.5)	405 (55.1)	420 (57.1)	426 (58.0)	449 (61.2)	**0.03**
Diabetes, n (%)	301 (41.0)	260 (35.4)	253 (34.4)	270 (36.7)	221 (30.1)	**0.001**
Physical activity						
Low, n (%)	91 (12.4)	119 (16.2)	154 (21.1)	159 (21.6)	205 (28.0)	
Medium, n (%)	276 (37.6)	352 (48.0)	345 (47.3)	335 (45.6)	320 (43.7)	**<0.0001**
High, n (%)	367 (50.0)	262 (35.7)	231 (31.6)	241 (32.8)	207 (28.3)	
Biochemical variables						
Total cholesterol (mg/dL)	203.9 ± 40.8	211.9 ± 43.5	213.7 ± 44.0	214.7 ± 44.8	215.5 ± 44.1	**<0.0001**
HDL-cholesterol (mg/dL)	47.0 ± 14.1	48.5 ± 14.2	48.6 ± 13.5	49.9 ± 13.5	50.1 ± 14.4	**<0.0001**
Triglycerides (mg/dL)	104 (77; 156)	110 (78; 159)	120 (84; 168)	117 (84; 168)	122 (86; 177)	**0.0003**
LDL-cholesterol (mg/dL)	132.4 ± 36.0	137.6 ± 38.2	138.0 ± 39.2	137.3 ± 41.0	137.3 ± 39.6	**0.04**
Uric acid (mg/dL)	5.5 (4.7; 6.3)	5.3 (4.5; 6.1)	5.2 (4.4; 6.0)	5.1 (4.3; 5.9)	5.0 (4.2; 5.8)	**<0.0001**
Blood glucose (mmol/L)	6.23 ± 1.6	5.97 ± 1.6	5.93 ± 1.8	5.69 ± 1.4	5.76 ± 1.7	**<0.0001**
Creatinine (µmol/L)	84.1 (73.8; 96.4)	79.4 (68.8; 90.4)	78.0 (68.4; 89.4)	76.5 (65.6; 87.2)	76.8 (66.9; 88.9)	**<0.0001**

hs-CRP (mg/L)	1.7 (0.7; 3.4)	2.1 (0.8; 3.7)	1.9 (0.7; 3.8)	2.0 (0.8; 3.8)	1.8 (0.8; 3.3)	0.60
Leucocytes (WBC) (x 10^9/L)	6.02 ± 1.6	6.16 ± 1.7	6.12 ± 1.6	6.19 ± 1.6	6.21 ± 2.3	0.053
Neutrophils (%)	56.85 ± 9.7	58.02 ± 9.1	57.21 ± 8.9	57.75 ± 9.0	55.98 ± 8.8	0.058
Lymphocytes (%)	33.57 ± 8.6	32.28 ± 8.1	33.00 ± 8.2	32.67 ± 8.2	33.95 ± 8.4	0.24
Monocytes (%)	6.82 ± 2.2	6.74 ± 2.2	6.67 ± 2.1	6.49 ± 2.1	7.00 ± 2.2	0.50
Eosinophils (%)	3.16 ± 2.2	3.03 ± 2.0	3.06 ± 2.1	2.94 ± 1.9	2.96 ± 1.9	0.13
Basophils (%)	0.4 (0.0; 0.7)	0.4 (0.0; 0.7)	0.5 (0.2; 0.8)	0.5 (0.2; 0.7)	0.5 (0.3; 0.8)	**0.02**
Erythrocytes (RBC) (x 10^{12}/L)	4.69 ± 0.4	4.66 ± 0.4	4.65 ± 0.4	4.65 ± 0.4	4.63 ± 0.4	**0.01**
Haemoglobin (g/dL)	14.45 ± 1.2	14.26 ± 1.2	14.14 ± 1.1	14.11 ± 1.1	13.99 ± 1.1	**<0.0001**
Haematocrit (%)	42.58 ± 3.4	42.19 ± 3.7	42.18 ± 4.4	42.12 ± 3.6	41.95 ± 3.2	**0.002**
MCV (fl)	90.98 ± 4.5	90.44 ± 4.5	90.51 ± 5.2	90.75 ± 5.1	90.73 ± 4.4	0.74
MCH (pg)	30.92 ± 1.8	30.64 ± 1.7	30.54 ± 1.7	30.48 ± 1.7	30.28 ± 1.7	**<0.0001**
MCHC (g/dL)	33.98 ± 0.9	33.88 ± 1.1	33.69 ± 1.1	33.54 ± 1.1	33.36 ± 1.0	**<0.0001**
Platelets (x 10^9/L)	225.5 ± 56.0	236.4 ± 56.9	237.6 ± 57.2	241.1 ± 58.1	246.5 ± 55.0	**<0.0001**
Estimated GFR (mL/min)	84.3 ± 22.8	86.0 ± 23.3	83.9 ± 22.4	83.9 ± 22.5	80.2 ± 21.7	**<0.0001**
Food items						
Wine, n (%)	278 (7.59)	273 (7.45)	295 (8.05)	287 (7.83)	363 (9.91)	**<0.0001**
Beer, n (%)	177 (4.83)	140 (3.82)	124 (3.38)	110 (3)	93 (2.54)	**<0.0001**

Spirits, n (%)	158 (4.31)	124 (3.38)	96 (2.62)	90 (2.46)	72 (1.96)	**<0.0001**
Fruit, n (%)	679 (18.51)	679 (18.51)	699 (19.06)	695 (18.95)	698 (19.03)	0.06
Milk, n (%)	590 (16.12)	566 (15.46)	580 (15.84)	553 (15.11)	550 (15.02)	0.06
Coffee, n (%)	663 (18.1)	664 (18.13)	658 (17.96)	661 (18.05)	656 (17.91)	0.98
Tea, n (%)	326 (8.9)	345 (9.42)	271 (7.4)	267 (7.29)	236 (6.44)	**<0.0001**
Meat, n (%)	719 (19.65)	716 (19.57)	722 (19.73)	721 (19.7)	720 (19.68)	0.50
Fish, n (%)	669 (18.28)	655 (17.9)	658 (17.98)	674 (18.42)	674 (18.42)	0.19
Eggs, n (%)	619 (16.92)	577 (15.77)	553 (15.11)	571 (15.61)	515 (14.07)	**<0.0001**
Pharmacological therapies						
Statins, n (%)	169 (23)	234 (32)	279 (38)	369 (50)	423 (58)	**<0.0001**
Fibrates, n (%)	19 (2.6)	25 (3.4)	50 (6.8)	53 (7.2)	136 (19)	**<0.0001**
Fish oil, n (%)	24 (3.3)	20 (2.7)	20 (2.7)	21 (2.9)	40 (5.4)	**0.02**
Other lipid-lowering drug (%)	3 (0.4)	9 (1.2)	2 (0.3)	4 (0.5)	5 (0.7)	0.23
Beta-blockers, n (%)	194 (26)	165 (22)	183 (25)	168 (23)	162 (22)	0.23
Calcium antagonists, n (%)	122 (17)	111 (15)	136 (19)	109 (15)	119 (16)	0.33
ACE inhibitors, n (%)	160 (22)	128 (17)	131 (18)	154 (21)	145 (20)	0.15
ARB, n (%)	134 (18)	98 (13)	110 (15)	110 (15)	108 (15)	0.11
Diuretics, n (%)	160 (22)	159 (22)	168 (23)	181 (25)	184 (25)	0.39

Anti-platelet agents, n (%)	150 (20)	106 (14)	114 (16)	110 (15)	134 (18)	**0.01**
Insulin, n (%)	40 (5.4)	33 (4.5)	26 (3.5)	17 (2.3)	24 (3.3)	**0.02**
Estrogen supplement, n (%)	52 (7.1)	47 (6.4)	46 (6.3)	52 (7.1)	29 (4.0)	0.08

PCSK9, proprotein convertase subtilisin/kexin type 9; DBP, diastolic blood pressure; SBP, systolic blood pressure; HDL, high-density lipoproteins; CHD, coronary heart disease; CVD, cerebrovascular disease; PVD, peripheral vascular disease; LDL, low-density lipoproteins; hs-CRP, high-sensitivity C-reactive protein; WBC, white blood cells; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; GFR, glomerular filtration rate; ACE, angiotensin-converting enzyme; ARB, angiotensin-2 receptor blockers. Data are n (percentage) or mean ± SD, except for PCSK9, pack-years, triglycerides, uric acid, creatinine, hs-CRP, and basophiles which are summarized as median (1^{st} and 3^{rd} quartiles). Group differences were assessed by Student's t-test for the numerical variables, by $\chi 2$-test or Fisher for the categorical ones, and by Kruskal-Wallis for pack-years, triglycerides, uric acid, creatinine, hs-CRP and basophiles. Estrogen supplementation was calculated only in women. The P_{values} refer to the trends across PCSK9 quintiles.

CORRELATIONS BETWEEN PLASMA PCSK9 LEVELS AND LIPIDS OR INFLAMMATORY BIOMARKERS

Univariate correlation between PCSK9 as continuous variable and lipids or inflammatory markers are shown in Figure 14 and 15, respectively. PCSK9 was positively correlated with total, LDL-, and HDL-cholesterol, as well as with triglycerides (Figure 14), and basophils (Figure 15). PCSK9 was also negatively correlated with neutrophils and eosinophils (Figure 15). The correlations with hs-CRP (log) and WBC count were just close to the statistical significance (Figure 15).

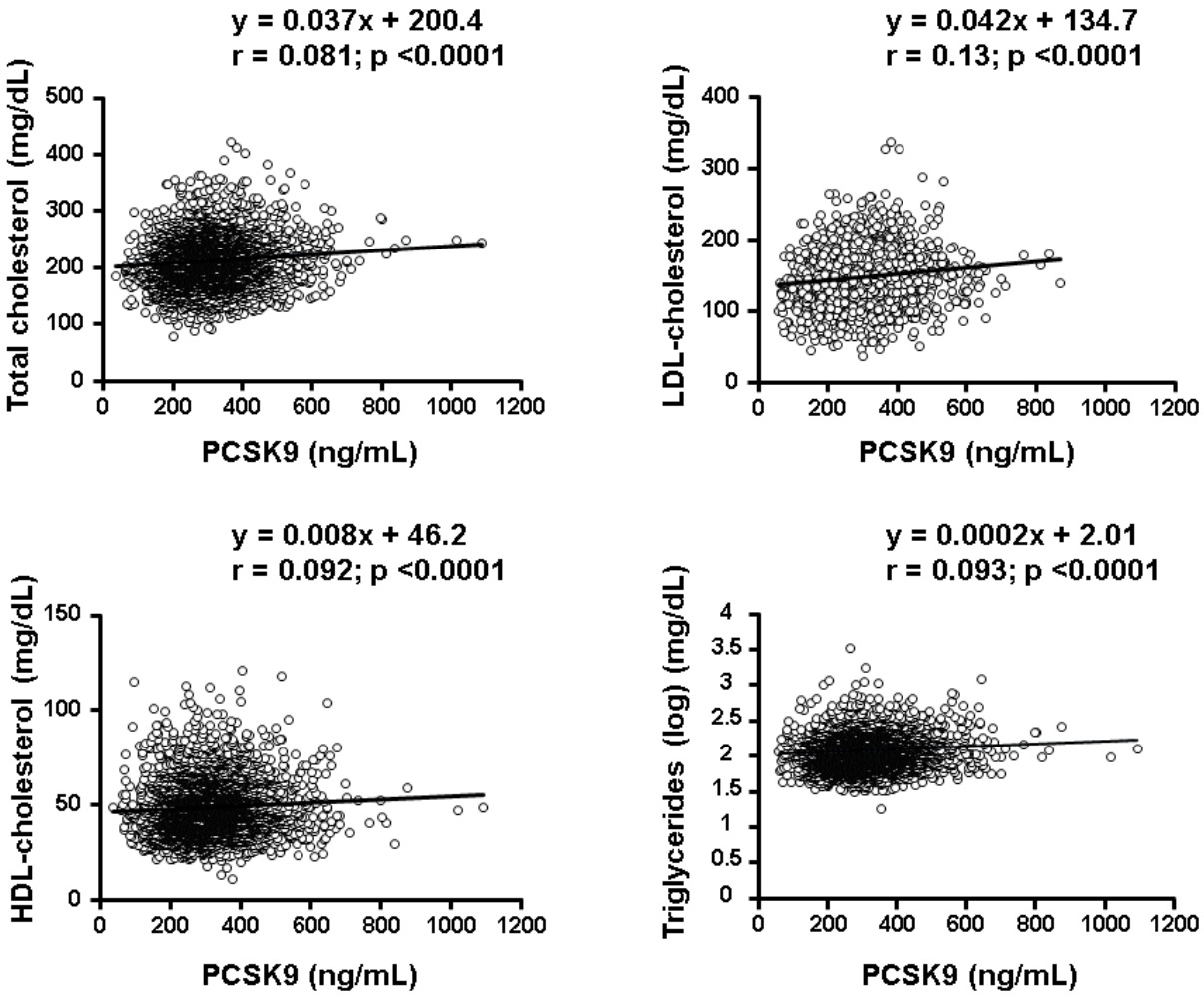

Figure 14. Correlations between plasma PCSK9 levels and lipids in the whole group. A) Total cholesterol; B) LDL-cholesterol; C) HDL-cholesterol; D) Triglycerides (log). For the analysis of LDL-cholesterol, statins users were excluded. PCSK9, proprotein convertase subtilisin/kexin type 9; LDL, low-density lipoproteins; HDL, high-density lipoproteins.

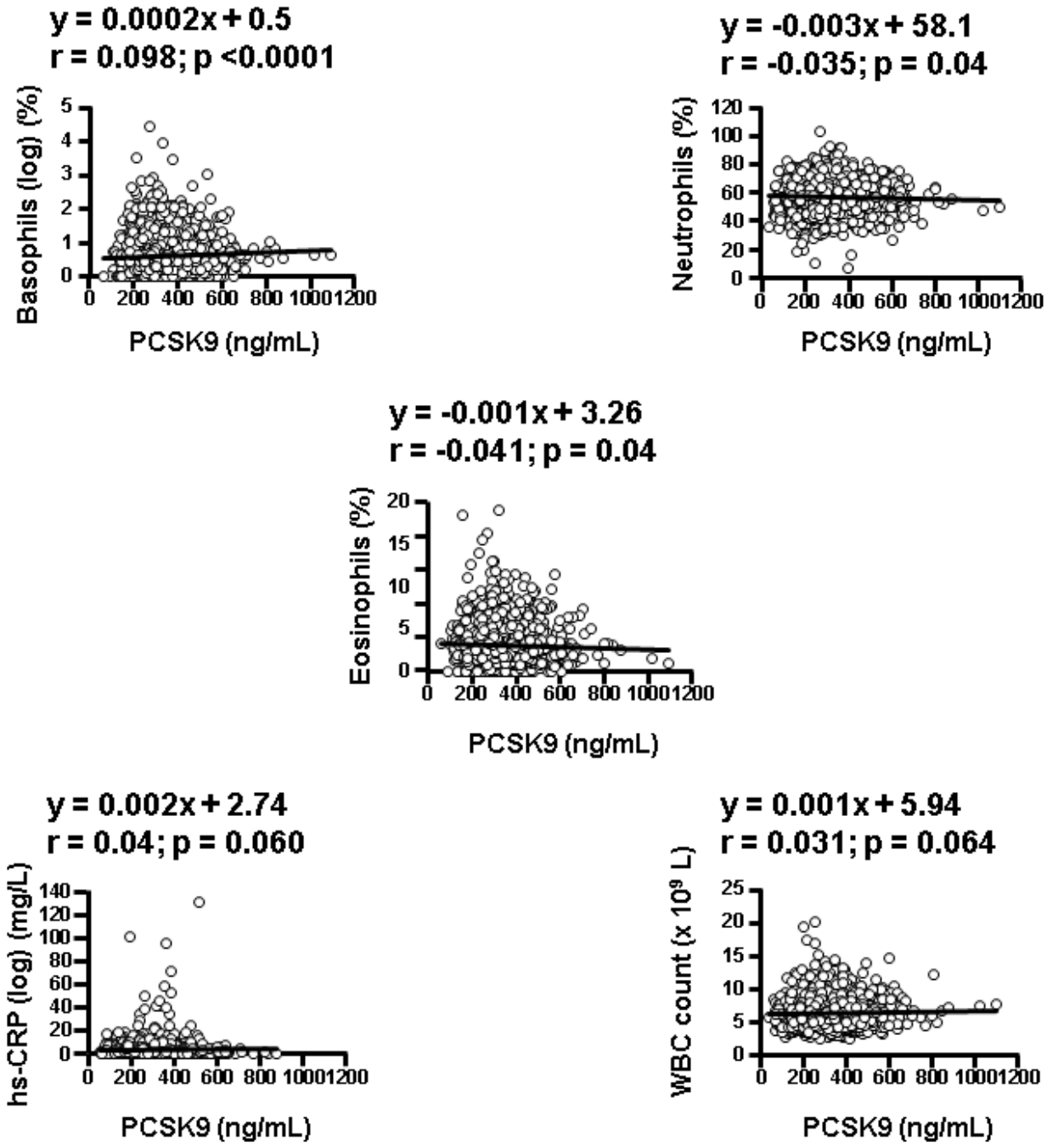

Figure 15. Correlations between plasma PCSK9 levels and markers of inflammation in the whole group. A) Basophils (log); B) Neutrophils; C) Eosinophils; D) hs-CRP (log); E) WBC count. For the analysis of hs-CRP, statins users were excluded. PCSK9, proprotein convertase subtilisin/kexin type 9; hs-CRP, high-sensitivity C-reactive protein; WBC, white blood cells.

DETERMINANTS OF PLASMA PCSK9 LEVELS

The results of a multivariable analysis investigating the independent predictors of PCSK9, among all the variables listed in Table 1 (see Table 1, pages 50-55), are shown in Table 2. Treatments (fibrates or statins), personal history of hypercholesterolemia and hypertriglyceridemia, wine consumption, hip circumference, pack-years, total and HDL-cholesterol were independent positive predictors of PCSK9 (Beta values ranging from 0.10 to 78.6; all $p < 0.05$); whereas latitude, fish consumption, uric acid, family history of diabetes, and male sex were negative predictors (Beta values ranging from -3.4 to -26.4; all $p < 0.05$). The two first strongest positive predictors of PCSK9, among the variables considered, were therapies (Beta: 78.6, and 53.4 for fibrates and statins, respectively; both $p < 0.0001$), whereas the two first strongest negative predictors were male sex (Beta: -26.4; $p < 0.0001$) and family history of diabetes (Beta: -7.3; $p = 0.03$).

Table 2. Variables independently associated with plasma PCSK9 levels by multivariable linear regression with stepwise selection in the total IMPROVE cohort.

Variables	Beta (SE); P_{value}
Fibrates treatment	78.6 (6.9); <0.0001
Statins treatment	53.4 (4.3); <0.0001
Personal history of hypercholesterolemia	14.8 (4.6); 0.001
Personal history of hypertriglyceridemia	10.7 (4.3); 0.01
Wine consumption	3.0 (0.96); 0.002
Hip circumference	0.84 (0.19); <0.0001
Pack-years (log)	0.31 (0.11); 0.003
HDL-cholesterol	0.30 (0.14); 0.03
Total cholesterol	0.10 (0.05); 0.03
Latitude	-3.4 (0.3); <0.0001
Fish consumption	-3.5 (1.4); 0.01
Uric acid (log)	-4.1 (1.5); 0.01
Family history of diabetes	-7.3 (3.4); 0.03
Gender (male sex)	-26.4 (4.0); <0.0001

PCSK9, proprotein convertase subtilisin/kexin type 9; HDL, high-density lipoproteins; SE, standard error. Beta values indicate the percent change in PCSK9 levels associated with a unit increment of the predictor. Variables not significantly associated were: age, height, weight, waist, SBP, DBP, educational level, personal history of hypoalphalipoproteinemia, of hypertension, and of diabetes, current smoker, family history of CHD, of CVD, of PVD, of hyperlipidemia, and of hypertension, physical activity, triglycerides, blood glucose, creatinine, hs-CRP, WBC count, RBC count, consumption of beer, spirits, fruit, milk, coffee, tea, meat, and eggs, and therapies with fish oil, or other lipid-lowering drugs, beta-blockers, calcium antagonists, ACE-inhibitors, ARB, diuretics, anti-platelet agents, insulin, or estrogen supplement.

Due to the strong associations of fibrates and statins with PCSK9, we ran a sensitivity analysis by excluding subjects treated with fibrates, or statins (40.1% of the total cohort), or both (Table 3). The results showed that wine consumption, pack-years, total cholesterol, latitude, and male sex remained significantly associated with PCSK9 in all three groups (all $p \leq 0.01$); fibrates or statins

treatment, personal history of hypercholesterolemia, hip circumference, HDL-cholesterol, fish consumption, uric acid, and family history of diabetes remained significantly associated with PCSK9 at least in one group (all $p < 0.05$); whereas personal history of hypertriglyceridemia was no longer associated with PCSK9. In this sensitivity analysis, triglycerides, creatinine, weight, and family history of hypertension were independent predictors of PCSK9 levels in at least one of these three groups (all $p < 0.05$).

Table 3. Variables independently associated with plasma PCSK9 levels by multivariable linear regression with stepwise selection after exclusion of subjects in therapy with fibrates or statins, or with both fibrates and statins.

	No subjects in therapy with fibrates	No subjects in therapy with statins	No subjects in therapy with fibrates + statins
Variables	**Beta (SE); P_{value}**	**Beta (SE); P_{value}**	**Beta (SE); P_{value}**
Fibrates treatment	Not applicable	97.3 (7.0); <0.0001	Not applicable
Statins treatment	59.7 (4.4); <0.0001	Not applicable	Not applicable
P.H. of hypercholesterolemia	10.9 (4.7); 0.02	ns	ns
P.H. of hypertriglyceridemia	ns	ns	ns
Wine consumption	3.5 (1.0); 0.001	3.3 (1.2); 0.005	3.2 (1.2); 0.01
Hip circumference	1.3 (0.34); 0.0002	1.4 (0.40); 0.001	ns
Pack-years (log)	0.41 (0.11); 0.0002	0.50 (0.12); <0.0001	0.62 (0.12); <0.0001
HDL-cholesterol	0.29 (0.14); 0.04	ns	ns
Total cholesterol	0.14 (0.05); 0.01	0.25 (0.05); <0.0001	0.28 (0.05); <0.0001
Triglycerides (log)	0.05 (0.02); 0.003	ns	ns
Creatinine (log)	ns	ns	-0.27 (0.14); 0.045
Weight	-0.58 (0.24); 0.02	-0.61 (0.29); 0.04	ns
Latitude	-3.0 (0.31); <0.0001	-3.4 (0.36); <0.0001	-2.7 (0.34); <0.0001
Fish consumption	-3.2 (1.4); 0.02	ns	ns
Uric acid (log)	ns	-5.9 (1.9); 0.002	ns
Family history of hypertension	ns	ns	-9.2 (4.0); 0.02
Family history of diabetes	-7.0 (3.4); 0.04	ns	ns
Gender (male sex)	-21.6 (5.0); <0.0001	-15.4 (6.0); 0.01	-24.5 (5.0); <0.0001

PCSK9, proprotein convertase subtilisin/kexin type 9; P.H., personal history; HDL, high-density lipoproteins; SE, standard error; ns, not significant. Beta values indicate the percent change in PCSK9 levels associated with a unit increment of the predictor. Variables not significantly associated after exclusion of subjects in therapy with **fibrates, or statins, or with fibrates and**

statins were: age, height, waist, SBP, DBP, educational level, personal history of hypertriglyceridemia, of hypoalphalipoproteinemia, of hypertension, and of diabetes, current smoker, family history of CHD, of CVD, of PVD, of hyperlipidemia, physical activity, blood glucose, hs-CRP, WBC count, RBC count, consumption of beer, spirits, fruit, milk, coffee, tea, meat, and eggs, and therapies with fish oil, or other lipid-lowering drugs, beta-blockers, calcium antagonists, ACE-inhibitors, ARB, diuretics, anti-platelet agents, insulin, or estrogen supplement. Variables not significantly associated after exclusion of subjects in therapy with **fibrates or statins** were: family history of hypertension and creatinine. Variable not significantly associated after exclusion of subjects in therapy with **fibrates or with fibrates and statins** was uric acid. Variables not significantly associated after exclusion of subjects in therapy with **statins or with both fibrates and statins** were: personal history of hypercholesterolemia, family history of diabetes, HDL-cholesterol, triglycerides, consumption of fish. Variables not significantly associated after exclusion of subjects in therapy with both **fibrates and statins** were: weight, and hip.

The ultrasonographic variables measurements, stratified by quintiles of PCSK9, are shown in Table 4. All these variables were related with PCSK9 quintiles. In particular, all cIMT variables and cCS showed a negative trend across PCSK9 quintiles, whereas GSM of both PF CC-IMT$_{mean}$ and carotid plaque was positively associated.

Table 4. Ultrasonographic variables of the IMPROVE study cohort stratified by quintiles of plasma PCSK9 levels (ng/mL).

	1st quintile (n=734)	2nd quintile (n=735)	3rd quintile (n=735)	4th quintile (n=735)	5th quintile (n=734)	P_{value}
PCSK9 (ng/mL)	183 (155; 203)	251 (238; 264)	300 (287; 312)	356 (340; 373)	449 (416; 503)	
IMT_{mean} (mm)	0.89 (0.78; 1.02)	0.85 (0.75; 1.01)	0.86 (0.74; 0.99)	0.82 (0.73; 0.97)	0.81 (0.72; 0.96)	**<0.0001**
IMT_{max} (mm)	2.03 (1.55; 2.68)	1.85 (1.45; 2.5)	1.85 (1.45; 2.5)	1.76 (1.35; 2.48)	1.76 (1.3; 2.41)	**<0.0001**
$IMT_{mean-max}$ (mm)	1.43 (1.19; 1.69)	1.35 (1.14; 1.67)	1.36 (1.13; 1.63)	1.3 (1.09; 1.6)	1.27 (1.07; 1.6)	**<0.0001**
PF CC-IMT_{mean} (mm)	0.72 (0.66; 0.78)	0.71 (0.65; 0.77)	0.7 (0.65; 0.76)	0.69 (0.64; 0.75)	0.69 (0.63; 0.74)	**<0.0001**
Fastest-$IMT_{max-progr}$ (mm/y)	0.22 (0.11; 0.37)	0.22 (0.11; 0.34)	0.18 (0.11; 0.32)	0.17 (0.1; 0.31)	0.18 (0.09; 0.36)	**0.01**
cCS (mm)	1.74 (0; 8.01)	0.47 (0; 6.48)	0.93 (0; 7.65)	0.3 (0; 6.67)	0 (0; 5.5)	**0.0003**
GSM of PF CC-IMT_{mean}	40.9 (33.7; 52.3)	42.7 (34.1; 52.8)	44.4 (35.6; 54.6)	43.7 (35; 53.6)	44.7 (37; 54.1)	**<0.0001**
GSM of carotid plaque	29.9 (22.9; 38.1)	30.4 (23.6; 37.4)	31.6 (24.8; 40.1)	31.1 (24.1; 39.8)	32.3 (25.6; 39.8)	**0.0001**

PCSK9, proprotein convertase subtilisin/kexin type 9; IMT_{mean}, average of mean of intima-media thickness in left and right carotid arteries; IMT_{max}, highest value of maximum of intima-media thickness in left and right carotid arteries; $IMT_{mean-max}$, mean of maximum intima-media thickness in left and right carotid arteries; PF CC-IMT_{mean}, IMT_{mean} measured in the 2nd cm of common carotids in plaque-free areas; Fastest-$IMT_{max-progr}$, the 15th month progression of IMT_{max} detected in the whole carotid tree regardless of location; cCS, carotid calcium score, i.e. the sum of the mean of length of acoustic shadow generated by plaque calcium in left and right carotids; GSM, grey scale median of pixels distribution of a region of interest (IMT or plaque). Data are expressed as median (1st and 3rd quartiles). Group differences were assessed by Kruskal-Wallis. The P_{values} refer to the trend across PCSK9 quintiles.

CORRELATIONS BETWEEN PLASMA PCSK9 LEVELS AND ULTRASONOGRAPHIC VARIABLES

Univariate correlations between continuous PCSK9 levels and ultrasonographic variables are shown in Figure 16. A negative trend was observed between PCSK9 levels and both cIMT variables and cCS, whereas a positive trend was observed between PCSK9 and GSM of both PF CC-IMT$_{mean}$ and carotid plaque.

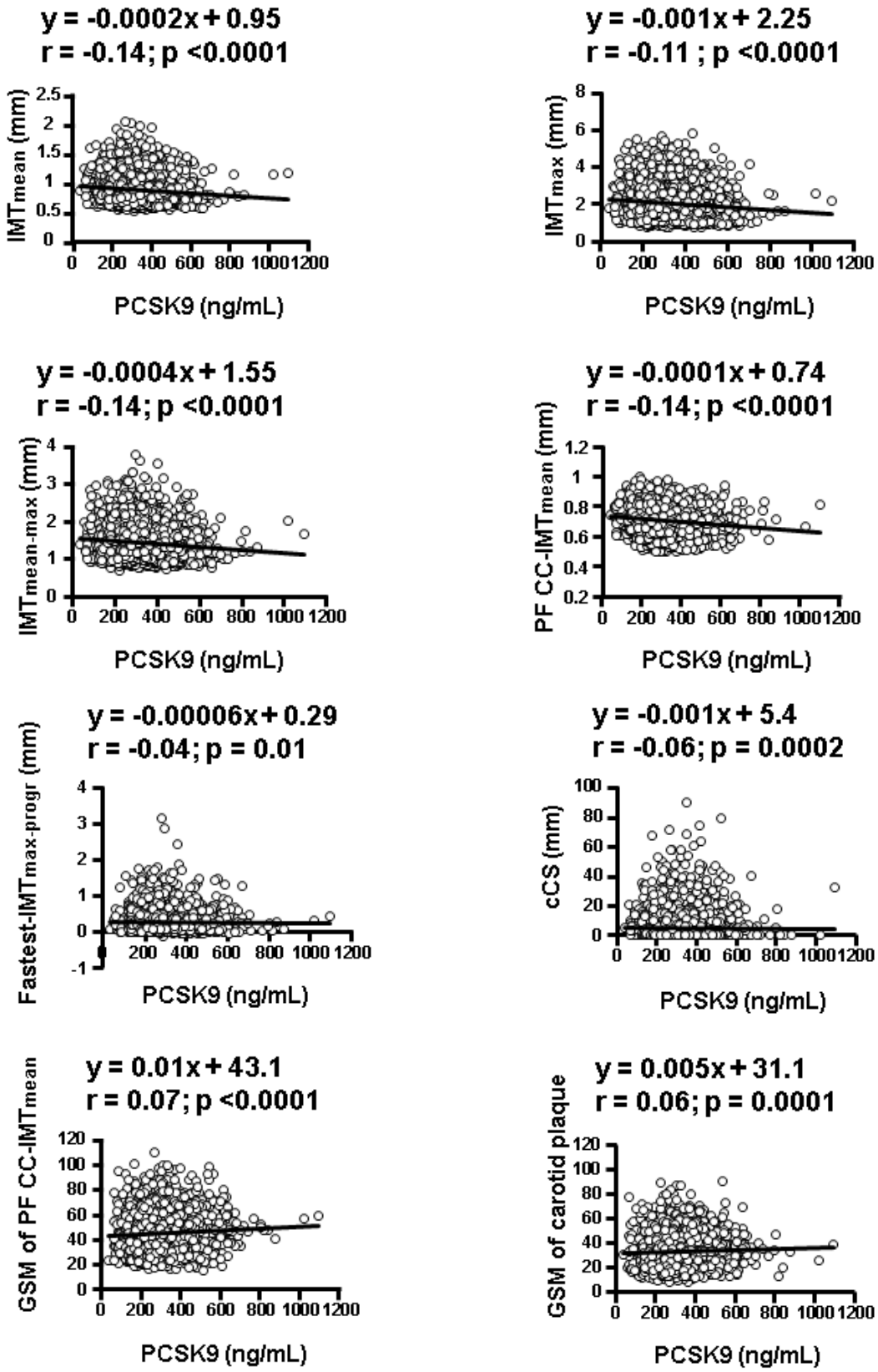

Figure 16. Correlations between plasma PCSK9 levels and carotid ultrasonographic variables. A) IMT_{mean}; B) IMT_{max}; C) $IMT_{mean\text{-}max}$; D) PF CC-IMT_{mean}; E) Fastest-$IMT_{max\text{-}progr}$; F) cCS; G) GSM of PF CC-IMT_{mean}; H) GSM of carotid plaque. PCSK9, proprotein convertase subtilisin/kexin type 9; IMT_{mean}, average of mean of intima-media thickness in left and right carotid arteries; IMT_{max}, highest value of maximum of intima-media thickness in left and right carotid arteries; $IMT_{mean\text{-}max}$, mean of maximum intima-media thickness in left and right carotid arteries; PF CC-IMT_{mean}, IMT_{mean} measured in the 2nd cm of common carotids in plaque-free areas; Fastest-$IMT_{max\text{-}progr}$, the

15th month progression of IMT_{max} detected in the whole carotid tree regardless of location; cCS, carotid calcium score, measured as the sum of the mean of length of acoustic shadow generated by plaque calcium in left and right carotids; GSM, grey scale median of pixels distribution of a region of interest (IMT or plaque).

MULTIVARIABLE RELATIONSHIPS BETWEEN PLASMA PCSK9 LEVELS AND cIMT VARIABLES

Table 5 shows the multivariable relationships between PCSK9 and cIMT variables.

In Model 1 (adjusted for age, sex and latitude), none correlation was found with carotid IMT_{mean}, IMT_{max}, $IMT_{mean-max}$, and PF CC-IMT_{mean} Even the adjustment for other potential confounders (Model 2; list of variables in the table legend) did not change the results, and the relationships with PCSK9 remained statistically not significant.

Table 5. Multivariable relationships between plasma PCSK9 levels and cIMT variables.

	Model 1	**Model 2**
	Beta (SE); P_{value}	**Beta (SE); P_{value}**
IMT_{mean} (log)	0.00005 (0.00003); 0.08	0.00003 (0.00003); 0.28
IMT_{max} (log)	0.0002 (0.0001); 0.12	0.00003 (0.0001); 0.84
$IMT_{mean-max}$ (log)	0.0001 (0.00006); 0.11	0.00004 (0.00006); 0.56
PF CC-IMT_{mean} (log)	-0.00001 (0.00001); 0.4	-0.000003 (0.00001); 0.81

PCSK9, proprotein convertase subtilisin/kexin type 9; IMT_{mean}, average of mean of intima-media thickness in left and right carotid arteries; IMT_{max}, highest value of maximum of intima-media thickness in left and right carotid arteries; $IMT_{mean-max}$, mean of maximum intima-media thickness in left and right carotid arteries; PF CC-IMT_{mean}, IMT_{mean} measured in the 2nd cm of common carotids in plaque-free areas; SE, standard error. Beta values indicate the percent change in carotid IMT variable associated with a unit increment of plasma PCSK9 levels (ng/mL). **Model 1**: adjusted for age, sex, and latitude. **Model 2**: as model 1 + pack-years, HDL-cholesterol and statins for IMT_{mean} and $IMT_{mean-max}$; as model 1 + pack-years, statins and fibrates for IMT_{max}; as model 1 + pack-years, HDL-cholesterol, uric acid, blood glucose and statins for PF CC-IMT_{mean}.

The index of carotid IMT progression (Fastest-$IMT_{max\text{-}progr}$) was not associated with PCSK9 levels neither in Model 1 nor in Model 2 of the multivariable analysis (Table 6).

Table 6. Multivariable relationship between plasma PCSK9 levels and carotid Fastest-$IMT_{max\text{-}progr}$.

	Model 1	**Model 2**
	Beta (SE); P_{value}	**Beta (SE); P_{value}**
Fastest-$IMT_{max\text{-}progr}$ (log)	0.00007 (0.00004); 0.09	0.00008 (0.00004); 0.08

PCSK9, proprotein convertase subtilisin/kexin type 9; Fastest-$IMT_{max\text{-}progr}$, the 15th month progression of IMT_{max} detected in the whole carotid tree regardless of location; SE, standard error. Beta values indicate the percent change in carotid Fastest-$IMT_{max\text{-}progr}$ associated with a unit increment of plasma PCSK9 levels (ng/mL). **Model 1**: adjusted for age, sex, and latitude. **Model 2**: as model 1 + HDL-cholesterol.

RELATIONSHIP BETWEEN PLASMA PCSK9 LEVELS AND GSM OF BOTH cIMT AND PLAQUES

The repeatability of GSM measurements of PF CC-IMT$_{mean}$ (Figure 17) and of the biggest plaque detected in the whole carotid tree (Figure 18) were assessed in 158 subjects who underwent two scans two weeks apart. The analysis was performed considering left and right carotid arteries separately. For both PF CC-IMT$_{mean}$ and carotid plaque, a significant correlation was observed between GSM measurements at basal and replicate scans (Figure 17 and Figure 18; top panels). Bland-Altman plots (Figure 17 and Figure 18; bottom panels) also revealed that the bias between the two measurements was not significant.

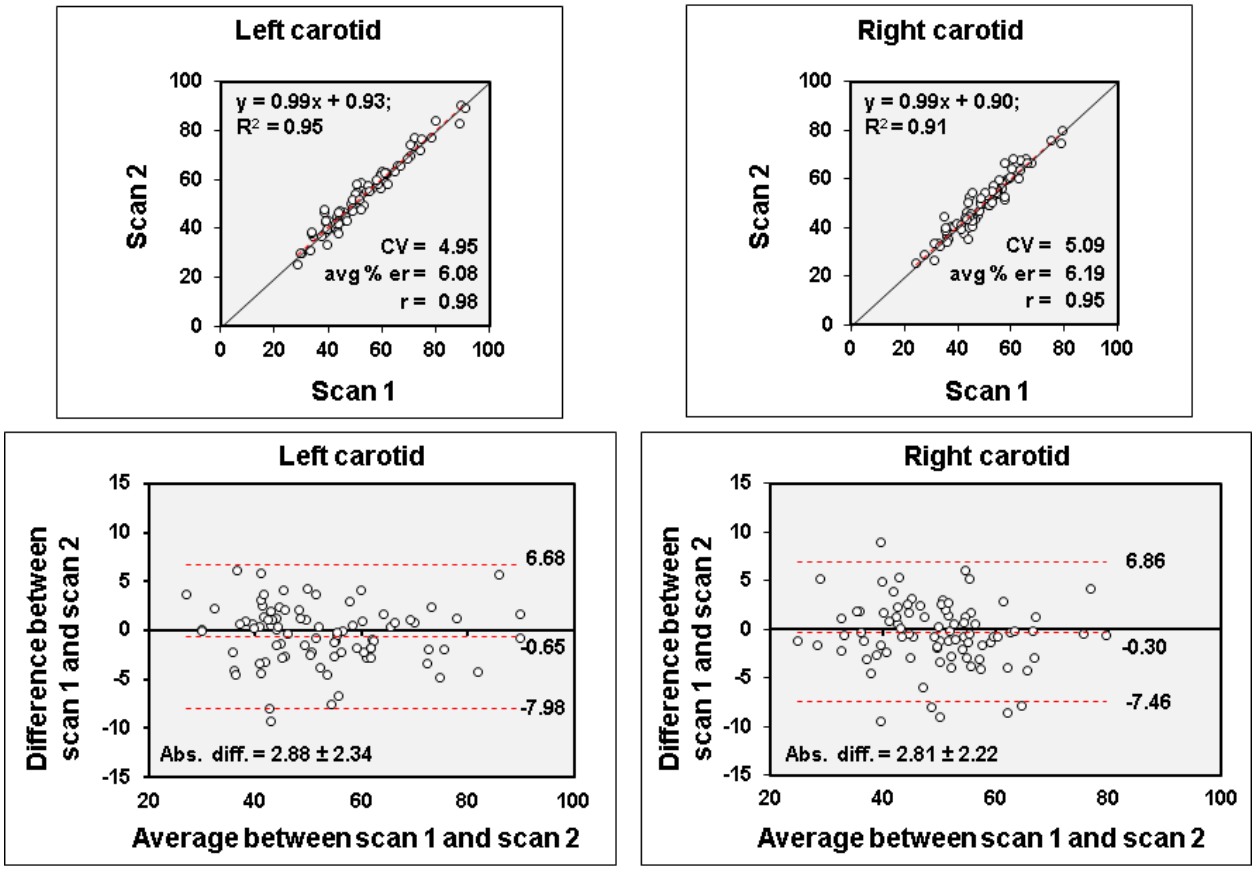

Figure 17. Linear regression analyses (top panels) and Bland-Altman plots (bottom panels) assessing the agreement between PF CC-IMT$_{mean}$ GSM measurements made on basal (visit 1) and replicate (visit 2) scans in left carotid (left panels) and right carotid (right panels).

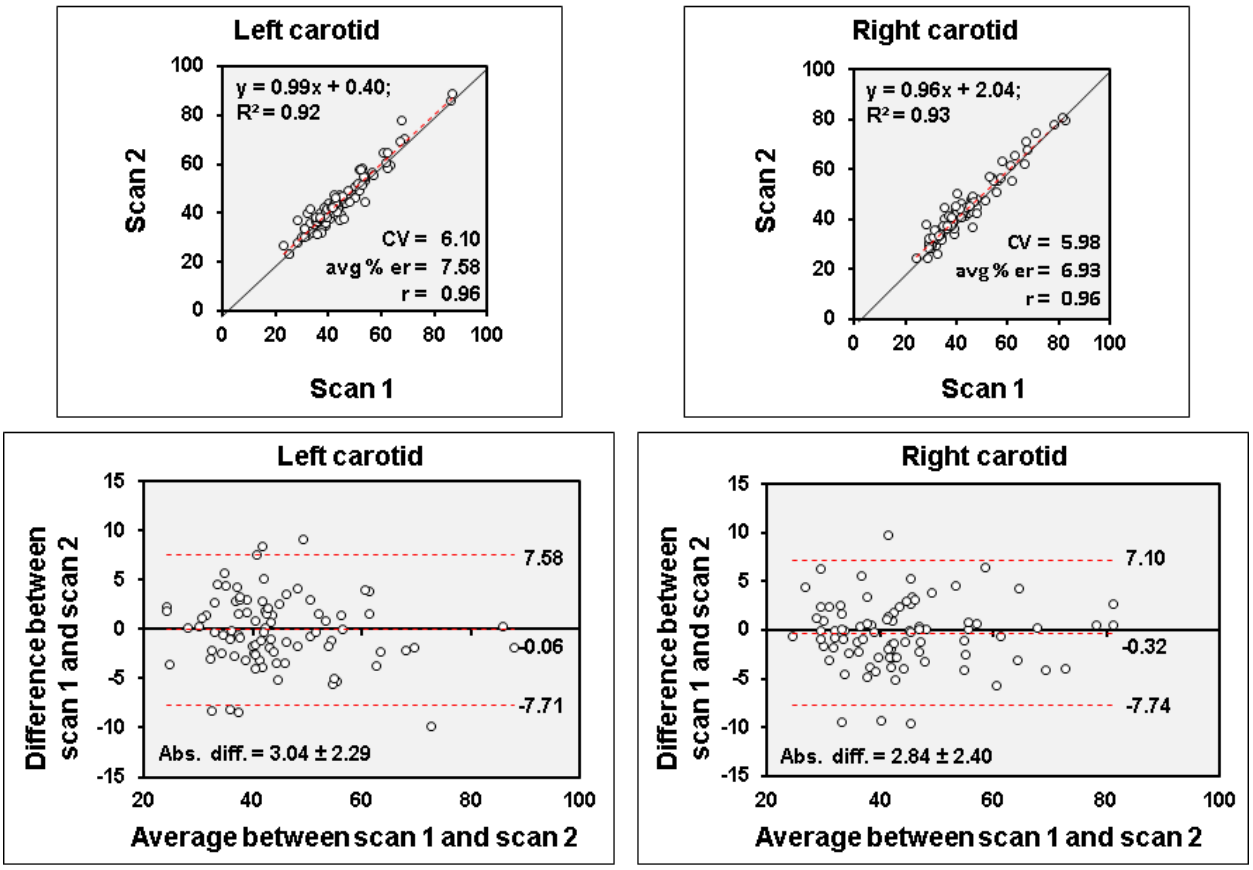

Figure 18. Linear regression analyses (top panels) and Bland-Altman plots (bottom panels) assessing the agreement between carotid plaque GSM measurements made on basal (visit 1) and replicate (visit 2) scans in left carotid (left panels) and right carotid (right panels).

The multivariable relationships between PCSK9 and GSM of PF CC-IMT$_{mean}$ and of the biggest plaque detected in the whole carotid tree are reported in Table 7. PCSK9 was positively correlated with GSM of PF CC-IMT$_{mean}$ in Model 2 and Model 3 (covariates are listed in the table legend), whereas was not correlated with that of carotid plaque.

Table 7. Multivariable relationships between plasma PCSK9 levels and GSM of both PF CC-IMT$_{mean}$ and carotid plaque.

	Model 1	**Model 2**	**Model 3**
	Beta (SE); P$_{value}$	**Beta (SE); P$_{value}$**	**Beta (SE); P$_{value}$**
GSM of PF CC-IMT$_{mean}$	0.0016 (0.0019); 0.39	0.005 (0.002); **0.01**	0.005 (0.002); **0.01**
GSM of carotid plaque	0.0002 (0.002); 0.92	0.003 (0.002); 0.08	0.003 (0.002); 0.064

PCSK9, proprotein convertase subtilisin/kexin type 9; GSM, grey scale median of pixel distribution of a region of interest (IMT or plaque); PF CC-IMT$_{mean}$, IMT$_{mean}$ measured in the 2nd cm of common carotids in plaque-free areas; SE, standard error. Beta values indicate change in the grey scale median (GSM), an index of echolucency, associated with a unit increment of plasma PCSK9 levels (ng/mL). **Model 1**: adjusted for age, sex, latitude, GSM of blood and GSM of adventitia. **Model 2**: as model 1 + blood glucose, personal history of hypercholesterolemia, fibrates treatment, and fish consumption. **Model 3**: as model 2 + PF CC-IMT$_{mean}$ for GSM of PF CC-IMT$_{mean}$; as model 2 + IMT$_{max}$ for GSM of carotid plaque.

RELATIONSHIP BETWEEN PLASMA PCSK9 LEVELS AND CAROTID CALCIUM SCORE (cCS)

The repeatability of cCS measurements (Figure 19), generated by the sum of LAS_{mean} due to carotid plaque calcium detected in the whole left and right carotid trees, was assessed in the same 158 subjects used to evaluate GSM repeatability. 67 subjects did not have any calcified plaque, so have been excluded from the analyses. In the remaining 91 subjects, calcified plaques were present in 42 left and 49 right carotids. The two top panels of Figure 19 showed a significant correlation between the first and the second measurements for left and right carotids. Bland-Altman plots (Figure 19; bottom panels) also revealed that the bias between the two measurements was negligible.

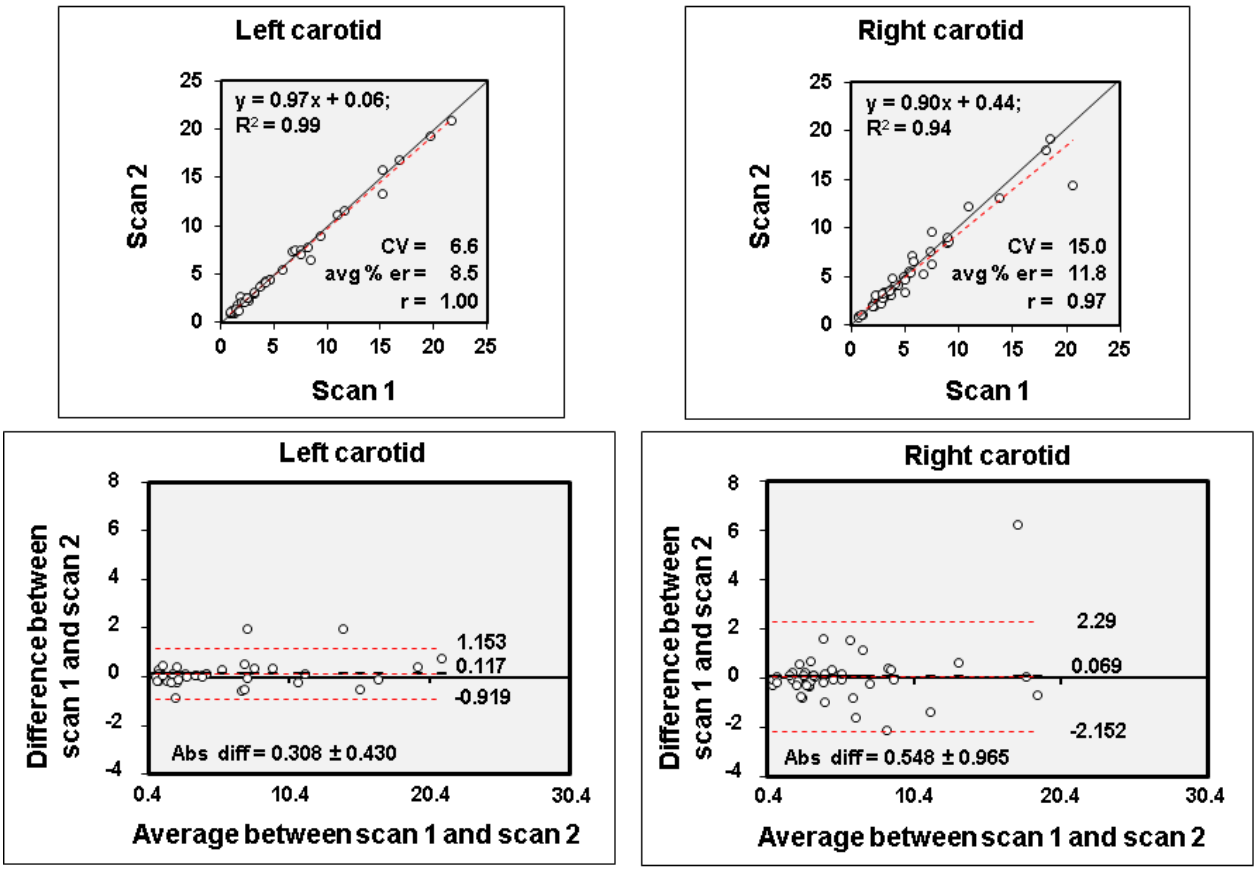

Figure 19. Linear regression analyses (top panels) and Bland-Altman plots (bottom panels) assessing the agreement between carotid calcium score (i.e. cCS) measurements made on basal (visit 1) and replicate (visit 2) scans in left carotid (left panels) and right carotid (right panels).

The multivariable relationship between PCSK9 levels and cCS is shown in Table 8. The cCS was associated with PCSK9 in the model adjusted for age, sex and latitude (p <0.0001; Model 1). Such association become weaker, but still remained close to the statistical significance (p=0.064) when adjusted for other confounders (Model 2; list of variables in the table legend). The addition among covariates of an index of atherosclerotic burden (e.g. IMT_{max}; Model 3) did not change the results (p=0.054).

Table 8. Multivariable relationship between plasma PCSK9 levels and cCS.

	Model 1	**Model 2**	**Model 3**
	Beta (SE); P_{value}	**Beta (SE); P_{value}**	**Beta (SE); P_{value}**
cCS (mm)	0.005 (0.001); **<0.0001**	0.003 (0.001); 0.064	0.003 (0.001); 0.054

PCSK9, proprotein convertase subtilisin/kexin type 9; cCS, carotid calcium score, i.e. the sum of the mean of length of acoustic shadow generated by plaque calcium in left and right carotids; SE, standard error. Beta values indicate change in the carotid calcium score (cCS in mm) associated with a unit increment of plasma PCSK9 levels (ng/mL). **Model 1**: adjusted for age, sex and latitude. **Model 2**: as model 1 + pack-years, uric acid, statins and fibrates treatment. **Model 3**: as model 2 + IMT_{max}.

PLASMA PCSK9 LEVELS AND INCIDENCE OF VEs

Participants were followed-up for a median of 3.01 years (inter-quartile range: 2.98 to 3.12 years). During such follow-up, 215 VEs were recorded: 125 were coronary, 73 cerebral and 17 were peripheral VEs. Among all these, 37 were hard events.

Table 9 shows the HRs (95% CI) of the associations between PCSK9 and VEs. In the unadjusted analysis (Model 1), PCSK9 was significantly associated with combined and coronary events (both p <0.01), but not with cerebrovascular events (p=0.58). The relationships with both combined and coronary events completely lose their statistical significance when the analyses were adjusted for age, sex, and stratified by latitude (Model 2). The addition to the model of independent determinants of PCSK9 (listed in the table legend; Model 3) did not change the results.

Table 9. HR (95% CI) of the associations between plasma PCSK9 levels and incidence of combined (coronary + cerebrovascular + peripheral events), coronary, and cerebrovascular events by Cox regression analyses.

	Model 1	**Model 2**	**Model 3**
	HR (95% CI) P_{value}	**HR (95% CI) P_{value}**	**HR (95% CI) P_{value}**
Combined events (n = 215)	0.31 (0.14; 0.72) **0.006**	0.81 (0.32; 2.09) 0.67	0.83 (0.29; 2.39) 0.74
Coronary events (n = 125)	0.19 (0.06; 0.54) **0.002**	0.61 (0.19; 2.01) 0.42	0.69 (0.18; 2.59) 0.58
Cerebrovascular events (n = 73)	1.53 (0.34; 7.00) 0.58	3.38 (0.57; 19.95) 0.18	5.48 (0.75; 39.74) 0.09

PCSK9, proprotein convertase subtilisin/kexin type 9; HR, hazard ratio; CI, confidential interval. HR (95% CI) of combined, coronary and cerebrovascular events associated with a unit increment of plasma PCSK9 levels (ng/mL). **Model 1**: unadjusted. **Model 2**: adjusted for age, sex, and stratified by latitude. **Model 3**: as model 2 + PCSK9 independent determinants (i.e. statins or fibrates treatment, hip circumference, personal history of hypercholesterolemia and of hypertriglyceridemia, pack-years, family history of diabetes, total cholesterol, HDL-cholesterol, uric acid, fish and wine consumption).

The lack of a significant association between plasma PCSK9 levels and VEs was confirmed also when the analyses were limited to hard VEs (Table 10).

Table 10. HR (95% CI) of the associations between plasma PCSK9 levels and incidence of hard vascular events (myocardial infarction, stroke, and sudden death) by Cox regression analyses.

	Model 1	**Model 2**	**Model 3**
	HR (95% CI); P_{value}	**HR (95% CI); P_{value}**	**HR (95% CI); P_{value}**
Hard vascular events (n=37)	0.56 (0.07; 4.83); 0.60	1.10 (0.11; 11.11); 0.93	0.89 (0.07; 11.55); 0.93

PCSK9, proprotein convertase subtilisin/kexin type 9; HR, hazard ratio; CI, confidential interval. HR (95% CI) of hard vascular events associated with a unit increment of plasma PCSK9 levels (ng/mL). **Model 1**: unadjusted. **Model 2**: adjusted for age, sex, and stratified by latitude. **Model 3**: as model 2 + PCSK9 independent determinants (i.e. statins or fibrates treatment, hip circumference, personal history of hypercholesterolemia and of hypertriglyceridemia, pack-years, family history of diabetes, total cholesterol, HDL-cholesterol, uric acid, fish and wine consumption).

IMPROVE STUDY COHORT vs UK BIOBANK COHORT

Table 11 shows the demographic features of the IMPROVE and UK Biobank participants used as covariates in genetics analyses.

Table 11. Baseline demographic features of the IMPROVE and UK Biobank participants used as covariates in genetics analyses.

	IMPROVE (n=3,468)	**UK Biobank (n=22,179)**
Age (years)	64.2 ± 5.41	55.23 ± 7.46
P.H. of stroke, n (%)	Not applicable	185 (0.8)
P.H. of ischemic heart disease, n (%)	Not applicable	278 (1.3)
St. SNP score	1.56 ± 1	1.6 ± 1.07
LDL-cholesterol (mg/dL)	136.8 ± 38.7	Not applicable
Lipid lowering drugs, n (%)	1676 (49.5)	2718 (12.3)
IMT_{mean} (mm)	0.85 (0.74; 1.00)	0.66 (0.59; 0.75)
IMT_{max} (mm)	1.85 (1.40; 2.50)	0.89 (0.77; 1.01)
$IMT_{mean-max}$ (mm)	1.20 (1.04; 1.41)	0.77 (0.68; 0.88)

P.H., personal history; St. SNP score, standardized single nucleotide polymorphism score; LDL, low-density lipoproteins. For the IMPROVE, IMT_{mean} was defined as the average of 1st CC-IMT_{mean}, CC-IMT_{mean}, Bif-IMT_{mean}, and ICA-IMT_{mean} of left and right carotid arteries; IMT_{max} as the highest value out of 1st CC-IMT_{max}, CC-IMT_{max}, Bif-IMT_{max}, and ICA-IMT_{max} of left and right carotid arteries; $IMT_{mean-max}$ as the average of maximal IMT measured in 8 segments (1st CC, CC, Bif, and ICA in left and right carotid arteries). For the UK Biobank, IMT_{mean} was defined as the average of 4 mean measures (2 for each of left and right carotid arteries); IMT_{max} as the average of maximum carotid IMT values, where the highest of the 4 maximum carotid IMT measures was used; $IMT_{mean-max}$ as the mean of 4 maximum measures (2 for each of left and right carotid arteries). Data are mean ± SD or n (%), except for carotid IMT variables that are expressed as median (1st and 3rd

quartiles) because of the skewed distribution. Standardized SNP score was calculated as SNP score value/1.243.

PCSK9 SNPS AND LDL-CHOLESTEROL LEVELS IN THE IMPROVE STUDY COHORT

To further investigate the role of PCSK9 on subclinical atherosclerosis, we also assessed the association between some *PCSK9* genetic variants, known to be associated with low LDL-cholesterol levels, and the cIMT. The genetic variants identified for the analysis are shown in Table 12.

Table 12. Four *PCSK9* SNPs associated with the presence of low LDL-cholesterol levels.

SNPs	MAF	Alleles	Minor alleles associated with low LDL-cholesterol levels
rs11591147	0.01	T/G	T
rs11583680	0.14	T/C	T
rs11206510	0.17	C/T	C
rs2479409	0.36	G/A	A

PCSK9, proprotein convertase subtilisin/kexin type 9; LDL, low-density lipoproteins; SNPs, single nucleotide polymorphisms; MAF, minor allele frequency.

As a first analysis we have assessed whether such genetic variants were associated with low LDL-cholesterol levels also in the IMPROVE study cohort. To this aim, a multivariable analysis adjusted for sex, age, lipid-lowering drugs (yes vs not) and Multi-Dimensional Scaling (MDS) components 1-3 (MDS1-3) was performed. A significant inverse association was found only for the rs11591147 SNP ($p < 0.000001$; Table 13).

Table 13. Multivariable relationships between four *PCSK9* SNPs and LDL-cholesterol levels in the IMPROVE cohort (n=3,377).

	CHR	SNPs	BP	Allele	Beta (SE); P_{value}
LDL-cholesterol (mg/dL)	1	rs11206510	55496039	C	-1.75 (1.11); 0.12
	1	rs2479409	55504650	A	-1.56 (0.88); 0.08
	1	rs11591147	55505647	T	-16.7 (3.31); **<0.000001**
	1	rs11583680	55505668	T	-1.07 (1.32); 0.42

PCSK9, proprotein convertase subtilisin/kexin type 9; LDL, low-density lipoproteins; CHR, chromosome; SNPs, single nucleotide polymorphisms; BP, base pair; SE, standard error. Beta values indicate change in LDL-cholesterol (mg/dL) associated with the presence of *PCSK9* genetic variants. The analysis was adjusted for sex, age, lipid lowering drugs (yes vs not), and Multi-Dimensional Scaling (MDS) components 1-3 (MDS1-3). The P_{values} corrected by Bonferroni ≤0.01 were considered statistically significant.

MULTIVARIABLE RELATIONSHIP BETWEEN THE SELECTED *PCSK9* VARIANTS AND cIMT VARIABLES IN IMPROVE AND UK BIOBANK COHORTS

In the IMPROVE study cohort, no significant correlation was identified when the relationships among cIMT variables and the four selected *PCSK9* variants were investigated in multivariable models adjusted for sex, age, lipid-lowering drugs (yes vs not) and MDS1-3 (Table 14).

Table 14. Multivariable relationship between each cIMT variable and the four selected *PCSK9* variants in the IMPROVE study cohort (n=3,383).

	CHR	SNPs	BP	Allele	Beta (SE); P_{value}
IMT_{mean} (log)	1	rs11206510	55496039	C	-0.0032 (0.003); 0.22
	1	rs2479409	55504650	A	-0.0017 (0.002); 0.42
	1	rs11591147	55505647	T	-0.0083 (0.008); 0.29
	1	rs11583680	55505668	T	-0.002 (0.003); 0.63
IMT_{max} (log)	1	rs11206510	55496039	C	-0.008 (0.005); 0.12
	1	rs2479409	55504650	A	-0.0001 (0.004); 0.98
	1	rs11591147	55505647	T	-0.014 (0.015); 0.34
	1	rs11583680	55505668	T	-0.0062 (0.006); 0.29
$IMT_{mean-max}$ (log)	1	rs11206510	55496039	C	-0.003 (0.0028); 0.28
	1	rs2479409	55504650	A	-0.001 (0.002); 0.75
	1	rs11591147	55505647	T	-0.011 (0.008); 0.20
	1	rs11583680	55505668	T	-0.002 (0.003); 0.53

PCSK9, proprotein convertase subtilisin/kexin type 9; CHR, chromosome; SNPs, single nucleotide polymorphisms; BP, base pair; SE, standard error; IMT_{mean}, average of mean of intima-media thickness in left and right carotid arteries; IMT_{max}, highest value of maximum of intima-media thickness in left and right carotid arteries; $IMT_{mean-max}$, mean of maximum intima-media thickness in left and right carotid arteries. Beta values indicate the percent change in carotid IMT variable associated with the presence of *PCSK9* genetic variants. Carotid IMT variables were log transformed prior to analyses. The analysis was adjusted for sex, age, lipid lowering drugs (yes vs not), and Multi-Dimensional Scaling (MDS) components 1-3 (MDS1-3). The P_{values} corrected by Bonferroni ≤0.01 were considered statistically significant.

When an analogous analysis was performed with UK Biobank data (Table 15) with a model adjusted for sex, age, lipid-lowering drugs (yes vs not), population structure (PGC1-8), and type of chip used for genotyping, the rs11591147 variant was significantly associated with IMT_{max}, but not with IMT_{mean} or with $IMT_{mean-max}$.

Table 15. Multivariable relationship between each cIMT variable and the four selected *PCSK9* variants in the UK Biobank cohort (n=19,135).

	CHR	SNPs	BP	Allele	Beta (SE); P_{value}
IMT_{mean} (ln)	1	rs11206510	55496039	C	-0.004 (0.002); 0.059
	1	rs2479409	55504650	A	-0.004 (0.002); 0.02
	1	rs11591147	55505647	T	-0.013 (0.006); 0.04
	1	rs11583680	55505668	T	-0.003 (0.002); 0.28
IMT_{max} (ln)	1	rs11206510	55496039	C	-0.005 (0.003); 0.050
	1	rs2479409	55504650	A	-0.004 (0.002); 0.04
	1	rs11591147	55505647	T	-0.024 (0.008); **0.002**
	1	rs11583680	55505668	T	-0.004 (0.003); 0.18
$IMT_{mean-max}$ (ln)	1	rs11206510	55496039	C	-0.004 (0.002); 0.046
	1	rs2479409	55504650	A	-0.004 (0.002); 0.04
	1	rs11591147	55505647	T	-0.015 (0.006); 0.02
	1	rs11583680	55505668	T	-0.003 (0.002); 0.25

PCSK9, proprotein convertase subtilisin/kexin type 9; CHR, chromosome; SNPs, single nucleotide polymorphisms; BP, base pair; SE, standard error; IMT_{mean}, average of 4 mean measures of carotid intima-media thickness (2 for each of left and right carotid arteries); IMT_{max}, average of maximum carotid intima-media thickness values, where the highest of the 4 maximum carotid intima-media thickness measures was used; $IMT_{mean-max}$, mean of 4 maximum measures of carotid intima-media thickness (2 for each of left and right carotid arteries). Beta values indicate the percent change in carotid IMT variable associated with the presence of *PCSK9* genetic variants. Carotid IMT variables were ln transformed prior to analyses. The analysis was adjusted for sex, age, lipid lowering drugs (yes vs not), population structure (PGC1-8), and genotyping chip. The P_{values} corrected by Bonferroni ≤0.01 were considered statistically significant.

The results did not change even when the four variants were joined in a standardized score (Table 16 and Table 17). Indeed, in the IMPROVE cohort the results remained substantially not significant, whereas in the UK Biobank cohort the standardized SNP score was independently and negatively associated with carotid IMT_{mean}, IMT_{max}, and $IMT_{mean-max}$.

Table 16. Multivariable relationship between each cIMT variable and standardized SNP score in the IMPROVE cohort (n=3,383).

	Beta (SE); P_{value}
IMT_{mean} (log)	-0.002 (0.001); 0.19
IMT_{max} (log)	-0.0032 (0.003); 0.22
$IMT_{mean-max}$ (log)	-0.0016 (0.0015); 0.29

PCSK9, proprotein convertase subtilisin/kexin type 9; SNP, single nucleotide polymorphisms; SE, standard error; IMT_{mean}, average of mean of intima-media thickness in left and right carotid arteries; IMT_{max}, highest value of maximum of intima-media thickness in left and right carotid arteries; $IMT_{mean-max}$, mean of maximum intima-media thickness in left and right carotid arteries. Beta values indicate the percent change in carotid IMT variable associated with a standard deviation increment of SNP score. Carotid IMT variables were log transformed prior to analyses. The analysis was adjusted for sex, age, lipid lowering drugs (yes vs not), and Multi-Dimensional Scaling (MDS) components 1-3 (MDS1-3). The P_{values} corrected by Bonferroni ≤0.025 were considered statistically significant.

Table 17. Multivariable relationship between each cIMT variable and standardized SNP score in the UK Biobank cohort (n=19,135).

	Beta (SE); P_{value}
IMT_{mean} (ln)	-0.003 (0.001); **0.008**
IMT_{max} (ln)	-0.004 (0.001); **0.006**
$IMT_{mean-max}$ (ln)	-0.003 (0.001); **0.009**

PCSK9, proprotein convertase subtilisin/kexin type 9; SNP, single nucleotide polymorphisms; SE, standard error; IMT_{mean}, average of 4 mean measures of carotid intima-media thickness (2 for each of left and right carotid arteries); IMT_{max}, average of maximum carotid intima-media thickness values, where the highest of the 4 maximum carotid intima-media thickness measures was used; $IMT_{mean-max}$, mean of 4 maximum measures of carotid intima-media thickness (2 for each of left and right carotid arteries). Beta values indicate the percent change in carotid IMT variable associated with a standard deviation increment of SNP score. Carotid IMT variables were ln transformed prior to analyses. The analysis was adjusted for sex, age, lipid lowering drugs (yes vs not), population structure (PGC1-8), and genotyping chip. The P_{values} corrected by Bonferroni ≤0.025 were considered statistically significant.

DISCUSSION

Our results, obtained in European subjects with at least 3 VRFs but asymptomatic for CVDs, show that fibrates or statins therapies (positively), and male sex and family history of diabetes (negatively) are the strongest independent predictors of PCSK9 plasma levels. Moreover, PCSK9 correlates with lipids and some inflammatory markers, and plays a role in determining carotid wall composition, and carotid calcium score. By contrast, PCSK9 levels are not associated with VEs in multivariable analyses. We also observed that the lack of association between cIMT and the four *PCSK9* variants considered in IMPROVE cohort is probably due to the IMPROVE sample size (n ~3,400), too small to appreciate such weak associations. Indeed, when the same analyses were repeated in the much larger UK cohort (n ~20,000), the associations investigated appeared highly significant.

PLASMA PCSK9 LEVELS

The present project is one of the largest analyses on circulating PCSK9 conducted in European subjects (n=3,673). Indeed, only two observational studies of analogous size were carried out: the Dallas Heart Study (DHS) (n=3,138),[205] and the study of Chernogubova *et al.*,[206] carried out in 4 cohorts of healthy, middle-aged, mainly Swedish subjects (n=5,722). Comparing our results with those of the DHS, a clear difference in the mean levels of circulating PCSK9 emerges (i.e., 310.8 ng/mL vs 517 ng/mL in the IMPROVE and in the DHS, respectively). A similar wide range variation in PCSK9 levels (~50-fold range) was also found in the 4 cohorts included in the Chernogubova *et al.* study.[206] The maximum plasma level observed in the IMPROVE was 1,134 ng/mL and the maximum plasma level observed in the DHS (2,988 ng/mL) was about three times higher, thus suggesting that the above mentioned differences might be due to the use of different ELISA assays. A confirmation that the between-study differences may be due to methodological reasons (e.g. assays used or other confounding factors) is corroborated by Leander *et al.*,[20] and Chernogubova *et al.*,[206] who reported mean PCSK9 levels (~100 ng/mL) much lower than those documented in the present study. Consistently, Gencer *et al.*, in 2,030 subjects with acute coronary syndrome (ACS),[187] and Ridker *et al.*, in the Women's Health Study,[21] obtained comparable mean

PCSK9 values by using the same commercially available kit as in our project (323 ng/mL and 302.1 ng/mL, respectively).

PCSK9 INDEPENDENT DETERMINANTS

In this study, among all the variables considered, fibrates and statins treatments were the two first strongest positive predictors of PCSK9, whereas male sex and family history of diabetes were the two first strongest negative predictors. Our results are in line with those of several previous studies demonstrating that statins treatment increases PCSK9 levels by a negative feedback mechanism in response to lower cholesterol concentrations.[207-210] Regarding fibrates treatment, previous reports yielded mixed results, although most of the published data agree with our results suggesting that also fibrates may raise the circulating levels of PCSK9.[211-213] Since PCSK9 is also positively associated with triglycerides levels, we may hypothesize that fibrates treatment acts in the same manner as statins therapy, i.e. fibrates increase PCSK9 levels by a negative feedback mechanism in response to lower triglycerides concentrations.

Our finding that PCSK9 concentration is higher in women than in men, in both the univariate and the multivariable analyses, is in agreement with finding of other studies performed in a large, multiethnic general population,[205] in children and adolescents,[214] and in several cohorts of patients with ACS or stable coronary artery disease (CAD).[215, 216]

By contrast, only two studies did not report any gender difference in small cohorts.[217, 218]

As far as we know, the independent association between family history of diabetes and PCSK9 levels is a completely novel observation. Until now, it has been demonstrated that PCSK9 synthesis and insulin concentrations are positively correlated: the higher the PCSK9 levels, the higher the fasting plasma glucose and the insulin resistance. However, this association was not consistent across several studies.[190, 214, 219-221]

PCSK9 AND LIPIDS

Studies performed in healthy, middle-aged subjects of European descent,[211, 222-224] in children as well as in adolescents,[214] in Chinese[221] and ethnically different[205] cohorts confirmed the association between circulating PCSK9 and plasma LDL-cholesterol level among seemingly healthy individuals. In some, but not all,[211, 216, 225-228] studies, PCSK9 has also been positively associated with triglycerides,[15, 221, 226, 229, 230] total cholesterol,[221, 230] and HDL-cholesterol.[214, 231-233] Our results are in line with those of all these studies demonstrating that circulating PCSK9 is positively correlated with not only LDL-cholesterol, but also with all the other lipids considered.

PCSK9 AND INFLAMMATORY BIOMARKERS

Clinical evidence suggests that circulating PCSK9 is associated with inflammation in atherosclerosis. In a Swiss prospective cohort (n=2,030) with ACS, higher plasma PCSK9 concentrations were independently and positively associated with hs-CRP levels.[187] Two other smaller studies are in line with the just mentioned paper, reporting a positive association between serum PCSK9 and hs-CRP levels in stable CAD patients without a history of lipid-lowering pharmacological treatment for ≥ three months.[188, 189] Plasma PCSK9 also showed a positive and independent correlation with WBC count and its subsets (neutrophils and lymphocytes) in stable CAD patients not treated with lipid-lowering drugs.[188, 190] In our study, we found the same association between PCSK9 and hs-CRP or WBC count, even if the relationships were not fully significant (both p=0.06).

By contrast, the negative correlation we observed with neutrophils is in the opposite direction to that observed in the aforementioned studies.[188, 190]

In addition, our study adds another piece of information by reporting a positive correlation with basophils and a negative correlation with eosinophils, never evaluated before.

PCSK9 AND cIMT

The discovery of the association between plasma PCSK9 and both lipid and non-lipid risk factors for CVDs has promoted studies on the relationship between this proprotein and markers of clinical and subclinical atherosclerosis. Among the latter markers, the relationship with cIMT has been one of the most investigated. PSCK9 has been positively associated with cIMT independently from traditional VRFs in asymptomatic individuals (n=295),[13] in hypertensive subjects (n=126),[14] and in patients with familial hypercholesterolemia (n=267).[16] However, in larger studies (e.g. the FATE study, performed in 1,527 middle-aged men in primary prevention), PCSK9 was correlated with traditional VRFs, but not with cIMT.[15] Our results are in line with those of this latter study.

It should be stressed that the ultrasonographic variables used in all the aforementioned studies were the mean IMT value of common carotid artery (CC-IMT_{mean}), and/or the maximal IMT detected in the whole carotid tree. In our study, we measured the IMT in all the segments of the whole left and right carotid trees, so we have been able to produce results not only considering the IMT of common carotid arteries, but also of bifurcation and internal carotid arteries. Moreover, several authors doubt that CC-IMT is an index of atherosclerosis. Our measurements, instead, since have been carried out considering also the segments mostly affected by atherosclerotic plaques and incorporating the plaque into the measurements, are without doubt surrogate markers of subclinical and even clinical atherosclerosis. All these variables, even if significantly correlated to PCSK9 in univariate analyses, lost their association after adjustment for main possible confounders, thus suggesting that the same correlations observed in the aforementioned studies are likely spurious correlations. Our results, therefore, do not allow to confirm that circulating PCSK9 plays a role in subclinical atherosclerosis development, at least when such disease is indexed by variables referring to the size of arterial wall.

PCSK9 AND ECHOLUCENCY

B-mode ultrasound detected GSM of PF CC-IMT_{mean} is an index of the presence of fibrous tissue in arterial wall. GSM of atherosclerotic plaque, instead, in an index of plaque vulnerability to rupture.

To the best of our knowledge, our study is the first providing findings about the relationship between these ultrasonographic variables and circulating levels of PCSK9. Most of the studies published so far showed that GSM is negatively associated with most of VRFs,[107, 108, 111] and that plaques characterized by a low GSM (rich in lipids and macrophages and appearing darker on ultrasonographic images) are those considered more vulnerable.[103, 104, 234-236] Therefore, we expected to find a negative relationship between plasma levels of PCSK9 and GSM of plaque and/or IMT because this proprotein is a widely recognized atherosclerosis risk factor.[12, 171, 237] Actually, we did not observe any correlation between PCSK9 and GSM of carotid plaques and, surprisingly, we found a positive correlation with the GSM of PF CC-IMT_{mean}. Such unexpected positive association, however, is in line with data observed in other studies showing a similar positive association between GSM of plaques and another VRF, i.e. smoking.[107, 238] So, according to what speculated in these studies, there is the chance that, also in our study, high levels of PCSK9 will associate to a higher content of fibrotic tissue in the PF CC-IMT_{mean} that, consequently, will appear ultrasonographically whiter. This hypothesis, however, requires deep investigations in ad hoc designed studies.

PCSK9 AND cCS

Besides echolucency, another index of carotid plaque composition is the quantification of calcium within plaques. Previous studies demonstrated that a low carotid calcium score (cCS) detected by CT is related to biological factors involved in plaque stability and inflammation,[239] and may improve CVDs risk prediction.[240] However, CT is a rather expensive technique, and requires X-ray exposure, that makes the procedure at least in part invasive. In search of new biomarkers of atherosclerosis, we tried to calculate carotid calcification by measuring the length of acoustic shadow (LAS; see Figure 5, page 21), generated by plaque calcium on B-mode ultrasonographic scans, in an easy, cheap and completely non-invasive manner. Consequently, LAS measurement allowed us to investigate, for the first time, the relationship between PCSK9 levels and cCS. The relationship between circulating PCSK9 and the amount of calcium within atherosclerotic plaques had already been investigated by considering the coronary artery calcium score (CAC score).[18] In

a cohort of 161 FH subjects who underwent CAC score assessment by CT, PCSK9 was an independent determinant of CAC score; the higher the PCSK9 level, the higher the amount of calcium in coronary arteries. On this basis, we hypothesized that PCSK9 levels may predict also the B-mode ultrasound detected calcification of carotid plaque. Our findings, even not reaching the fully significance, are in line with that observed at coronary level. Indeed, we found a highly significant ($p < 0.0001$) direct association in the analysis adjusted for age, sex and latitude, and a not fully significant direct association in both Model 2 (adjusted for some risk factors and pharmacological treatments known to affect PCSK9 levels; $p=0.064$) and Model 3 [where the analysis was also adjusted for the atherosclerotic burden as indexed by the size of the biggest plaque detected in the whole carotid tree (i.e. IMT_{max}); $p=0.054$].

PCSK9 AND VEs

Another finding of the present study is that circulating PCSK9 is not a predictor of future VEs (both hard and non-hard VEs) in high-risk subjects in primary prevention.

Our results are in line with some previous reports. In the FATE study, the association between plasma PCSK9 and clinical outcomes was investigated in middle-aged men (n=1,527) in primary prevention at baseline, and followed-up for a period of 7.2 ± 1.7 years.[15] Although PCSK9 was associated with some VRFs (i.e. LDL-cholesterol, triglycerides, and insulin), no significant association was found with cardiovascular events (i.e. composite of cardiovascular death; resuscitated cardiac arrest; nonfatal myocardial infarction; stroke or transient ischemic attack; symptomatic vascular disease with a greater than 50% stenosis; and revascularization in coronary, carotid, or peripheral arteries). Similarly, Ridker *et al.* showed that PCSK9 levels did not predict first VEs (myocardial infarction, cardiovascular death, and ischemic stroke) in a sub-cohort of initially healthy women (n=716, ≥45 years old) enrolled in CVDs prevention programs and followed-up for 17 years.[21] This lack of association between PCSK9 levels and first clinical outcomes of atherosclerosis is in contrast with the study of Leander *et al*.[20] They found that, in a cohort of general sixty-years old men and women (n=4,232) followed-up for 15 years, the higher the PCSK9 levels, the higher the risk of incident VEs (composite outcome of fatal or non-fatal myocardial

infarction, angina, chronic ischemic heart disease, sudden cardiac death and fatal or non-fatal ischemic stroke), even after adjustment for established VRFs.

To note, all these studies were performed in very different populations, and mean PCSK9 levels detected were different (e.g. ~300 ng/mL in the IMPROVE and Ridker *et al.* study, ~286 ng/mL in the FATE study, and ~94 ng/mL in Leander *et al.* study). Whatever the reason of the conflicting results reported in the aforementioned studies, at the moment neither our data nor data present in the literature allow to drawn a reliable conclusion on the existence of a significant relationship between PCSK9 levels and clinical atherosclerosis.

PCSK9 AND GENETICS

Studies of genetic variants that specifically decrease plasma LDL-cholesterol would provide an ideal system to assess the consequences of low LDL-cholesterol levels independently from other factors modifying atherosclerosis development.

Some *PCSK9* genetic variants have been associated with a phenotype characterized by a reduction in LDL-cholesterol levels in both black[241] and white[242] subjects. Consequently, some studies have investigated the effect of such *PCSK9* variants on carotid atherosclerosis, showing that carotid IMT_{mean} was significantly lower in carriers than in non-carriers.[5, 243]

We also investigated the relationship between 4 similar *PCSK9* variants and carotid atherosclerosis in the IMPROVE-study participants. To validate the results observed, the same analysis was also repeated by using data of the UK Biobank. We observed that at least one of these 4 selected *PCSK9* genetic variants and their standardized SNPs score are negatively associated with at least one cIMT variables in the UK Biobank, but no one of the variants considered nor their SNPs score were associated with ultrasonographic variables in the IMPROVE study. The discrepancy between the results observed in the IMPROVE study and in the UK Biobank study is quite difficult to be explained. Indeed, the differences observed between the two studies can be due either to simple methodological aspects, such as the sample size (too small in the IMPROVE study to detect very weak correlations), but also to the fact that the UK cohort has characteristics slightly healthier than those of the IMPROVE cohort (selected on the basis of the

presence of at least three VRFs). However, being the mean IMT values rather similar in the two cohorts, the second hypothesis seems to be rather unlikely.

STRENGTHS AND LIMITS OF THE STUDY

The present study has several strengths: firstly, it analyzes one of the largest samples so far available; second, it is carried out in 5 European countries, thus increasing the results generalizability. Furthermore, the acquisition method of carotid images, standardized across the 7 IMPROVE centers (operators were all trained and certified, and all scans were blindly analyzed in a single reading center), examined a huge number of cIMT variables, thus allowing to identify, if any, the most informative cIMT segment and/or the best cIMT summary variable for the predictive analyses. Another strength is that follow-up data were obtained on 93.7% of subjects and, therefore, the results were minimally affected by “loss to follow-up” bias. The present study also includes some limitations. First, the use of a simple and cheap commercial ELISA method, instead of the mass spectrometry, that is much more precise but also much more expensive and complex. Second, the fact that the commercial ELISA assay used does not allow to discriminate between the two plasma forms of PCSK9, the first one, circulating free in the plasma and less biologically active, and the second one, bound to LDL or to Lp(a) and much more biologically active. Third, the fact that the acoustic shadow generated by calcified plaque present on the carotid near wall masks and makes it difficult to measure the cCS in the carotid artery far wall. Finally, the carotid ultrasonographic protocol used for UK Biobank cohort, focused on just one segment and just two angles, is much more rough from the one used for IMPROVE participants, where the arterial walls were investigated in the whole length, in both carotid arteries, and in three different angles of insonation.

CONCLUSION

In conclusion, in European subjects defined at high cardiovascular risk for the presence of ≥ 3 VRFs, plasma PCSK9 levels are not associated with clinical atherosclerosis (i.e. vascular events).

Regarding markers of subclinical atherosclerosis, plasma PCSK9 levels are associated neither with lesion size nor with carotid plaque echolucency, but are associated with echolucency of carotid wall thickness and with carotid calcium score. Therefore, further studies are needed to better understand the role of such circulating proprotein in carotid wall thickness echolucency and in carotid calcium score.

Fibrates or statins therapies (positively), and male sex and family history of diabetes (negatively) are the strongest independent predictors of PCSK9 levels. Moreover, the association between circulating PCSK9 and both lipids and some inflammatory markers (i.e. hs-CRP and WBC count), already observed in previous studies, has been confirmed. However, the relationship between plasma PCSK9 levels and other inflammatory markers (i.e. neutrophils, basophils and eosinophils) deserves further investigations.

Finally, the role of the four selected *PCSK9* variants on cIMT variables in the UK Biobank cohort warrants further studies, aimed at investigating the role of this circulating proprotein in atherosclerosis preventive strategies.

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