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Platelet SR-PSOX/CXCL16 and CXCR6 axis influences thrombotic propensity and prognosis in coronary artery disease

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List of abbreviations

ACD	All-cause death
ABCG4	ATP-binding cassette sub-family G member 4
ACS	Acute coronary syndrome
ADAM	Disintegrin and metalloproteinase domain-containing protein
ADP	Adenosine 5'-diphosphate
AHF	Acute heart failure
AMI	Acute myocardial infarction
ApoE	Apolipoprotein E
AS	Atherosclerosis
ATP	Adenosine triphosphate
AUC	The area under the curve
CAD	Coronary artery disease
CCS	Chronic coronary syndrome
CCL	CC chemokine ligand
CD36	Platelet glycoprotein 4
CD40	Cluster of differentiation 40
CD62P	P-Selectin
CHF	Chronic heart failure
CK	Creatine kinase
CPDA	Citrate-phosphate-dextrose solution with adenine
CRP	C-reactive protein
CVD	Cardiovascular disease
CXCL	CXC chemokine ligand
EGF	Epidermal growth factor
EGFR	Estimated glomerular filtrate rate
ELISA	Enzyme linked immunosorbent assay

ESC	European Society of Cardiology
G	Gravity-force
GBD	Global burden disease
GP	Glycoprotein
HDL	High-density lipoprotein
HF	Heart failure
Hs-CRP	High-sensitivity C-reactive protein
IFN-γ	Interferon-γ
IL	Interleukin
ISH	Ischemic heart disease
LDL	Low-density lipoprotein
LOX	Lectin-type oxidized LDL receptor
LV	Left ventricular
LVEF	Left ventricular ejection fraction
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
MFI	Median fluorescence intensity
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
MPV	Mean platelet volume
NADPH	Nicotinamide-adenine dinucleotide phosphate
NETosis	Neutrophil extracellular trap formation
NK cells	Natural killer cells
NSTEMI	Myocardial infarction without ST elevation
NT-proBNP	N-terminal pro-brain natriuretic peptide
Ox-LDL	Oxidized low-density lipoprotein
PAC-1	Procaspase Activating Compound-1

PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PDGF	Platelet-derived growth factor
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PF	Platelet factor
PI3K	Phosphatidylinositol 3 kinase
PMA	Platelet-monocyte-aggregate
PSGL	P-selectin glycoprotein ligand
ROS	Reactive oxygen species
SAP	Stable angina pectoris
SCD	Sudden cardiac death
SD	Standard deviation
SDF	Stromal cell derived factor
SMC	Smooth muscle cell
SR	Scavenger receptor
STEMI	Myocardial infarction with ST-elevation
TC	Total cholesterol
Tc cell	Cytotoxic T cell
TG	Triglyceride
Th cell	T helper cell
TNF-α	Tumor necrosis factor-α
TnI	Troponin I
TRAP	Thrombin receptor activating peptide
UAP	Unstable angina pectoris
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
VWF	Von Willebrand factor

1. Introduction

1.1. Coronary artery disease

Coronary artery disease (CAD), also known as ischemic heart disease (ISH), refers to the chronic and acute heart disease of myocardial ischemia and hypoxia caused by coronary artery stenosis or occlusion. This disease occurs most often in the population over 40 years of age, 49% in males and 32% in females [1]. Global Burden Disease (GBD) Survey in 2019 showed that CAD has affected 200 million people worldwide [2], causing more than 9 million deaths and being the leading cause of human death [3].

CAD was formerly classified as "stable coronary disease" and acute coronary syndrome (ACS), according to whether discomforts may be relieved by rest until 2013 [4]. Angina is one of the most typical symptoms of CAD, which is characterized by discomfort in the jaw, shoulder, chest, back or arm, resulting from inadequate myocardial supply and oxygen demand [4]. Likewise, patients with CAD can also feel short of breath or even have no symptoms. In 2019, ESC has expanded the categorization of "stable coronary artery disease" to "chronic coronary syndrome (CCS)" instead. CCS refers not only to a stable angina symptom but also to other clinical situations (shown in Table 1) that may lead to an acute atherothrombotic event and ultimately cause ACS [5]. Acute coronary syndrome (ACS) consists of three clinical situations that patients can encounter: unstable angina pectoris (UAP), myocardial infarction without ST-elevation (NSTEMI), and myocardial infarction with ST-elevation (STEMI). Their manifestations are shown in the table below (Table 2) [6]. Compared to the CCS, ACS may be more fatal and even cause sudden cardiac death (SCD).

Table 1: 6 most frequent clinical manifestations of CCS.

(Based on 2019 ESC Guidelines for the diagnosis and management of CCS)

Patients with suspected CAD and 'stable' anginal symptoms, and/or dyspnea

Patients with new-onset of HF or LV dysfunction and suspected CAD

Asymptomatic and symptomatic patients with stabilized symptoms <1 year after an ACS or patients with recent revascularization

Asymptomatic and symptomatic patients >1 year after initial diagnosis or revascularization

Patients with angina and suspected vasospastic or microvascular disease

Asymptomatic subjects in whom CAD is detected at screening

Table 2: Clinical manifestations of ACS.

(Based on 2020 ESC Guidelines for the management of ACS in patients presenting without persistent ST-segment elevation)

ACS	ECG	Troponin
UAP	Normal, ST depression, transient ST elevation, T-inversion, flat/pseudo-normalization of T waves	Negative
NSTEMI	Normal, ST depression, transient ST elevation, T-inversion, flat/pseudo-normalization of T waves	Positive
STEMI	Persistent (>20min) ST elevation or new LBBB	Positive

1.2. Atherosclerosis

Atherosclerosis (AS) plays a vital role in the progression of CAD and human studies on AS have been carried out for over a hundred years. Nevertheless, the mechanism of AS is not yet entirely clear. Studies in 2000 have found that 70% of ACS are caused by AS plaque rupture (only 20% are stenosis plaques and 50% are non-stenosis plaques) and subsequent thrombosis [7], and the remaining 30% might be caused by other unknown factors. With significant advancement in

research related to AS, it is now well acknowledged that AS is not simply caused by the accumulation of lipids, but is also a systemic disease induced by the accumulation of inflammatory cells in the intima layers of arteries [8]. These suggest that the risk of AS plaque to the development of ACS is primarily dependent on the intrinsic composition of the plaque rather than the severity of lumen stenosis. These plaques, which predispose to subsequent acute cardiovascular events and are named "vulnerable plaques" [9], are characterized by large central lipid cores, abundant inflammatory cells, thin fibrous caps and few smooth muscle cells (SMCs) [10].

The main steps in developing AS are as follows. First, diverse factors such as hypertension and hyperlipidemia, lead to vascular endothelial injury, dysfunction, which is accompanied by increased endothelial permeability and adhesiveness for immune cells and platelets, representing the starting point for subendothelial deposition and modification of low-density lipoprotein (LDL) particles and chemokine-driven cell recruitment [11]. Lipids (phospholipids, lipoprotein, cholesterol) then deposit continuously in the subendothelial matrix and trigger endothelial activation and the release of chemokines [12, 13]. Meanwhile, monocytes interact with the activated endothelium (e.g. initial tethering, leukocyte rolling) and adhesion to endothelium firmly via monocyte integrins and endothelial ligands [14]. These processes lead to the accumulation of immune cells (e.g. macrophages, dendritic cells, lymphocytes, NK-cells, mast cells and neutrophils) in the subendothelial space, which are called fatty streaks [15]. Meanwhile, platelets also gradually accumulate and adhere to the injured endothelium. Growth factors and cytokines (PDGF, fibroblast growth factor-β, TNF-α, and EGF) released by these phagocytes, endothelial cells and platelets, stimulate vascular smooth muscle cells (VSMC) to proliferate and synthesize collagen fibers [16]. Then foam cells and extracellular lipid droplets form a core region surrounded by a cap of VSMC and collagen-rich matrix. As the lipids keep

depositing and inflammatory cells (T cells, macrophages, and mast cells) continue infiltrating, the fibrous cap becomes thinner, and the atherosclerosis plaque slowly evolves into an unstable one. Finally, thrombosis is triggered as the plaque rupture or erosion happens [17].

It is widely accepted that the interaction of endothelium and circulatory lipids initiate the development of AS. The endothelium is a selectively permeable barrier with tight intercellular junctions. Apolipoprotein-B-containing-lipoprotein up to 70 nm in diameter, particularly low-density lipoprotein (LDL), the main vehicle of blood cholesterol transport, may cross the endothelium and accumulate in the subendothelial bed as the permeability of the endothelium increases. The mechanism of this process is quite complex and three main compartments have been discovered to be involved: (i) glycocalyx, which creates a scaffold on the endothelial surface [18]; (ii) transcellular pathway, which is an energy-dependent vesicular trafficking [19]; and (iii) the paracellular pathway, also known as the opening of the cell to cell junctions [20]. Increased endothelial permeation may be induced by hyperlipidemia, hypertension, diabetes, or other metabolic diseases, and then lead to further modification of LDL particles like glycation, oxidation, or nitrosylation.

Oxidized LDL (ox-LDL) then initiates a sterile inflammatory response that triggers monocyte recruitment into the arterial wall, which then differentiates into macrophages [21]. Exposed to a high concentration of oxidized cholesterol, these macrophages lose their ability to move into circulation where they may have transferred cholesterol to high-density lipoproteins (HDL). Instead, they phagocytose ox-LDL and remain in situ as foam cells. Other modifications of LDL (i.e., micropinocytosis, enzymatic degradation, and aggregation) also facilitate its uptake by macrophages, leading to cholesterol-laden foam cells [22, 23]. In addition, platelets may also enter AS lesions through neovessels and internalize lipids via their scavenger receptors [24, 25], and modulate AS plaque progression

by regulating differentiation of progenitor cells into macrophages or endothelial cells, transmigration and conversion of monocytes into foam cells via chemokines (e.g. CCL5, CXCL4, CXCL12). Platelets can also modulate essential functions of dendritic cells, including their activation, differentiation and apoptosis [26].

The endothelial intima is gradually replaced by a compact layer of SMCs and collagen-rich matrix as the lipid core grows into the depth of the intima, forming the fibrous cap [27]. Plaque rupture and plaque erosion are considered the major causes of atherothrombosis. Studies suggest that plaque rupture which is found in nearly 70% of STEMI survivors [28], is more likely to occur in the population predisposed to hypertriglyceridemia, diabetes mellitus, among women and in the elderly [29]. And these AS plaques tend to be constituted by fibrous caps up to 65μm, which are defined as thin-cap fibroatheroma [30]. Then due to LDL-induced plaque inflammation, matrix decomposition caused by the production of cathepsins and metalloproteases by macrophages (i.e. elastases, collagenases) [31], together with local SMCs death [32] and cholesterol crystals penetrating the cap tissue [33], contribute to AS plaque rupture. Conversely, lesions with thick fibrous caps or without lipid cores are more likely to produce thrombus due to plaque erosion. They exhibit large quantities of plate necrosis and are characterized by the accumulation of hyaluronans and proteoglycans under the endothelium [34]. Both plaque rupture and erosion may induce neutrophil recruitment and NETosis (neutrophil extracellular trap formation) and finally contribute to thrombosis [35].

AS is known to be an inflammatory disease, and the inflammatory response is a chronic one that expands throughout the entire atherosclerotic process. There are a large number of inflammatory cells that infiltrate into atherosclerotic plaques, such as macrophages, activated T lymphocytes and mast cells [36]. These cells can not only form foam cells, but also secrete cytokines, growth factors, matrix-degrading enzymes, and chemokines, which play a significant role

in the occurrence and development of AS. Various types of immune cells migrate into the plaque and inflammatory cell generated cytokines such as interferon-γ (IFN-γ) and TNF-α contribute to plaque progression. TNF-α and IFN-γ also contribute to macrophage and VSMC apoptosis, promoting the thinning of the fibrous cap and the growth of the necrotic core, which activate an intracellular complex, the NLRP3 inflammasome, leading to the local production of IL-1β and IL-18. Additionally, macrophages may increase oxidative stress through the production of superoxide, neopterin, and hydrogen peroxide, which initiate cellular apoptosis by increasing ox-LDL and endoplasmic reticulum stress. These radicals also weaken the fibrous cap by activating matrix metalloproteinases (MMP-2, MMP-9) [37]. The inflammatory processes mentioned above are associated with a high risk of plaque rupture.
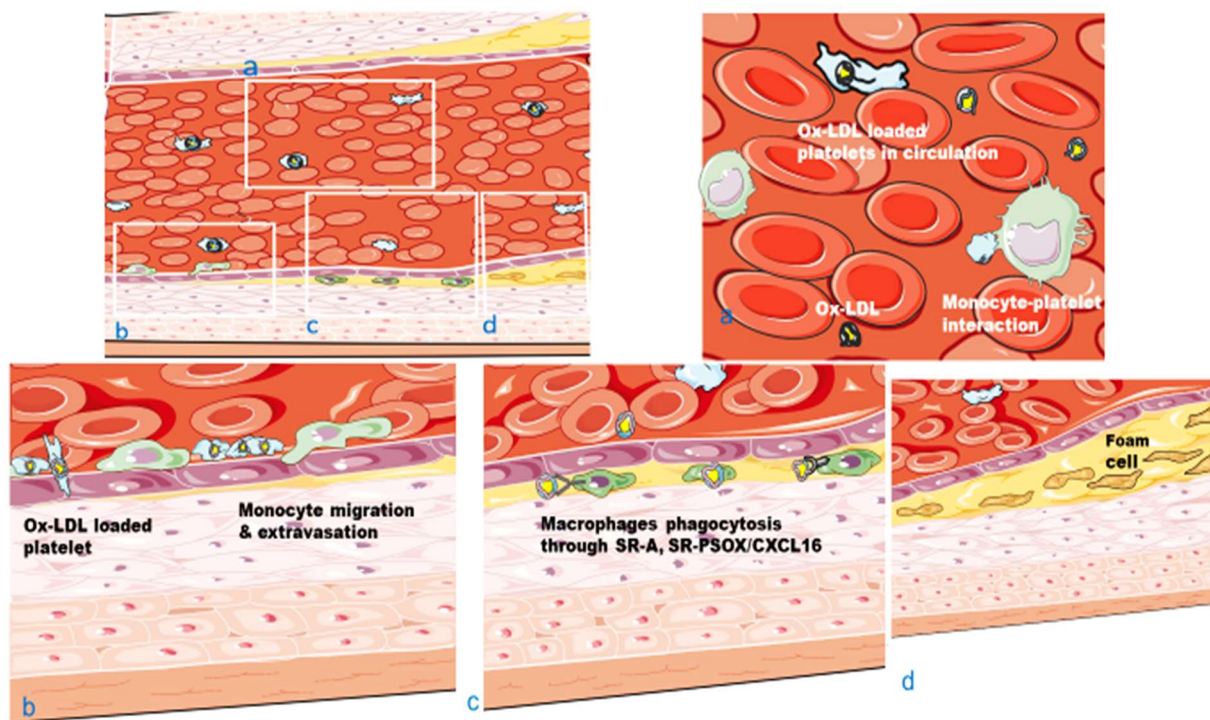
Figure 1. Progression of atherosclerosis. a. High concentrations of ox-LDL may be loaded into platelets and induce monocyte-platelet interaction. b. ox-LDL loaded platelets and monocytes may be recruited to the injured endothelium and infiltrate into the subendothelial space. c. Macrophages are differentiated from monocytes and phagocyte extracellularly stored cholesterol (ox-LDL) through scavenger receptor A (SR-A) and membrane-bound CXCL16 (SR-PSOX /CXCL16). d. Macrophages filled with lipids are transformed into foam cells, which develop fatty streaks and finally form the core of the AS plaque [38].

1.3. Platelets

Platelets are disk-shaped cells that stem from megakaryocytes of mammalian bone marrow, only 2-5μm in diameter and 0.5μm in thickness [39, 40]. Platelets are anucleated cytoplasmic fragments that have no genomic DNA, but have a complete transcriptome and can translate the mRNA into proteins [41]. Platelets also contain three main secretory granules: α-granules, dense granules, and lysosomal granules. These granule components are released during platelet activation, which are crucial for the initiation of coagulation and recruiting cells during the process of inflammation [42].

Although platelets play a primary role at the site of vascular lesion to assist in the formation of platelet plug and hemostasis, they are also responsible for the acute formation of thrombi that lead to thrombo-ischemic events as seen in ACS patients [43]. The healthy and intact endothelium serves as a barrier that separates the circulating platelets from the collagen located in the subendothelial matrix. Endothelial lesions result in the exposure of collagen [44] that promotes platelet adherence to the injured endothelium, subendothelial matrix and promotes recruitment and interaction with leukocytes. A variety of glycoproteins on the platelet surface facilitate platelet binding to collagen (e.g. GPIa/IIA, GP VI) [45], fibronectin (e.g. GP IIb/IIIa) [46] and von Willebrand factor (e.g. GP IIb/IIIa, GP Ib-IX-V complex) [47]. The binding of collagen to GPVI causes platelet activation and the release of platelet granule components. Components of α-granules are factors responsible for platelet adhesion, which include integrins (e.g., αIIb, α6, β3), cellular adhesion molecules (e.g., CD62P, CD63), hemostatic factors and cofactors (e.g., fibrinogen, vWF, fibronectin), and chemokines (e.g., CXCL4, CCL5, CXCL12, CXCL16). Dense granules, however, contain high concentrations of platelet activation factors such as ADP, ATP, Ca^{2+}, Mg^{2+}, K^+ and polyphosphate [48].

Platelet activation also leads to conformational changes of platelets from

smooth discs to irregular spheroids with extrusion of filopodia, degranulation, rapid calcium influx and thromboxane production. Next, this process leads to the positive feedback of platelet activation and aggregation, which may amplify the initial response and recruit more platelets to form a growing hemostatic plug, eventually generating a stable thrombus as the plasma coagulation cascade is triggered by thrombin, all of which may consequently influence the onset of ACS [49, 50].

Platelets may be activated not just at the site of a vascular lesion, but also in many pro-inflammatory conditions such as AS, diabetes, cancer and the central nervous system diseases (Alzheimer's disease, multiple sclerosis) [24, 51-53]. Inflammation acts as a potent thrombotic stimulus that can upregulate procoagulant factors (IL-6, which increases both platelet count and their responsiveness to agonists like thrombin), downregulate natural anticoagulants (e.g. thrombomodulin, free protein S and the endothelial cell protein C receptor, which are involved in the protein C pathway), and inhibit fibrinolytic activity by increasing plasminogen activator inhibitor levels [54].

1.3.1. Platelet lipid interplay in atheroprogression

In the blood, circulating platelets are continuously exposed to lipids. Besides their classical role in thrombosis, platelets are shown as a major player in the initiation of the atherosclerotic process [55, 56] and are influenced by circulating lipoproteins in the blood [57, 58]. Platelet count [59], mean platelet volume (MPV) [60] and soluble mediators released by activated platelets are associated with atherosclerosis. MPV has been reported to be increased in hypertension, dyslipidemia, and inflammation and may be reduced by statins [61]. Platelets express several receptors for lipoproteins: SR-BI, SR-BII, SR-BIII(CD36), LOX-1, apoE-receptor-2 and SR-PSOX /CXCL16 that contribute to these processes.

In the development of AS, platelets can initialize modified LDL which is then

stored in dense granules via specific scavenger receptors such as CD36 [62] or LOX-1 [63], contribute to the further oxidation of lipoproteins by a substantial release of ROS, and modulate plaque progression by regulating differentiation of progenitor cells to macrophages or endothelial cells and foam cells and modulate essential functions of dendritic cells, including their activation, differentiation and apoptosis [26].

Hypercholesterolemia increases the number of circulating neutrophils and monocytes but also platelet count by enhanced production via the cholesterol-efflux transporter ABCG4 on megakaryocyte progenitors [64]. Curtiss et al. [65] showed that platelets mediate the conversion of monocytes into foam cells by enhancing both the rate of cholesteryl esters formation and the total cholesteryl esters accumulation in cultured peripheral blood mononuclear cell-derived macrophages. They also demonstrated that this process can be induced by products released from activated platelets.

Elevated circulating LDL exerts 'pro-atherogenic' activating effects on platelet function resulting in hyperaggregability while HDL desensitizes platelets and has 'anti-atherogenic' effects. HDL influences platelet reactivity through binding to SR-BI indirectly, which is via maintaining normal plasma cholesterol homeostasis and generating an inhibitory signal for platelet activation. In contrast, ox-LDL activates platelets followed by quick changes in shape and aggregation contributing to thrombus formation after plaque rupture [66, 67].

It is well known that vascular cells, such as endothelial cells and smooth muscle cells, can convert native LDL to oxidized LDL resulting in scavenger receptor-mediated uptake of ox-LDL by macrophages and its intracellular degradation. However, ox-LDL can also be generated by platelets by lipid peroxidation [68]. Several studies showed that platelets are activated by ox-LDL mainly via scavenger receptors SR-A and CD36, accompanied by enhanced adhesiveness and thrombus formation [69, 70]. Thus, CD36 may link

hyperlipidemia with a prothrombotic state and increased platelet reactivity [71, 72]. Activated platelets may also promote the oxidation of LDL particles following the production of reactive oxygen species (ROS) by NADPH oxidase [68]. In summary, platelets interact with LDL particles leading to a positive feedback mechanism with enhanced platelet activation and cumulative LDL oxidation. Stimulation of platelets with ox-LDL results in the formation of platelet-monocyte-aggregates (PMA) and promotes ox-LDL uptake, foam cell formation and transmigration across endothelial monolayers, which is dependent on platelet CD36 and the release of CXCL4, a cationic chemokine within α-granules [73]. Activation of platelets leads to intraplatelet conversion of LDL to ox-LDL.

1.3.2. Platelet-derived chemokines in atheroprogression

In the latest research related to atherosclerosis, chemokines and their receptors are unanimously recognized to play important regulatory roles in the development of AS plaque formation and have been used as markers of disease trigger, progression and targets for anti-atherosclerotic therapy [74]. Chemokines are small secretory proteins produced by virtually all nucleated tissue cells. All known chemokines contain four conserved cysteine (C) molecules and can be divided into four families according to the structure of their amino terminus (N-terminus): CC (no amino acid inserted between the first two cysteine amino acids), CXC (1 amino acid inserted between the first two cysteine acids), C (only 1 cysteine acid in N-terminus) and CX3C (3 amino acids inserted between the first two cysteine acids). They can effectively mediate inflammatory responses by aggregating and activating leukocyte subsets such as T and B lymphocytes, macrophages, eosinophils, basophils and neutrophils, and participate in immune regulation and immunopathology [75].

Activated platelets secrete a plethora of chemokines including CXCL4 or platelet factor 4 (PF4), CCL5, CXCL12 or stromal cell derived factor-1α (SDF-1α),

CXCL16 and others at sites of vascular injury. These processes are mainly mediated by the recruitment of circulating hematopoietic stem cells, neutrophils, monocytes or lymphocytes on the vascular walls.

CXCL4

CXCL4 (also called platelet factor 4, PF4), the most abundant platelet chemokine, is secreted by α-granules as a product of activated platelets and various immune cells [76]. Platelet CXCL4 is present in advanced carotid plaque and its levels correlate with plaque severity [77].

CXCL4 has multiple proatherogenic functions, e.g., increasing the uptake of ox-LDL, inducing endothelial cell apoptosis and altering the differentiation of T-cells and macrophages by inhibiting neutrophil and monocyte apoptosis [78].

CXCL4 is involved in the retention of LDL on cell surfaces by inhibiting the binding and degradation of LDL by the formation of multimolecular complexes and thereby predisposing LDL to proatherogenic modifications [79]. Simultaneously, CXCL4 mediates ox-LDL binding to vascular cells and monocytes via interactions with surface glycosaminoglycans and promotes ox-LDL uptake by macrophages, thus promoting foam cell development [80]. CXCL4 is also responsible for the differentiation of monocytes to macrophages [81] and induces macrophage polarization towards a non-characteristically macrophage phenotype, which is called M4 [82]. In the M4 phenotype, the expressions of scavenger receptors are reduced whereas expressions of cholesterol efflux transporters are higher, resulting in lower LDL content.

CXCL4 has demonstrated to stimulate monocytes selectively to induce apoptosis in endothelial cells via the release of ROS [83]. Besides, CXCL4 forms heterodimers with CCL5 and enhances CCL5-mediated monocyte arrest on endothelial cells [84].

CCL5

The chemokine CCL5 is also referred to as RANTES and it is known to bind

multiple receptors: CCR1, CCR3 as well as CCR5 [85]. Platelet-derived CCL5 on activated endothelium was shown to elicit firm adhesion and transmigration of monocytes through inflamed endothelium by interacting with platelet P-selectin (CD62P) [86, 87] and trigger atherogenic monocyte recruitment.

CXCL12

Human platelets release and present CXCL12 on their surface after activation by collagen or adhesion to endothelial cells. CXCL12 exhibits proatherogenic properties by promoting dyslipidemia, insulin resistance, vascular inflammation, neointimal hyperplasia and angiogenesis [88, 89]. Platelet-derived CXCL12 interacts with monocytes via CXCR4/7 and regulates several monocytic functions including migration, survival, adhesion to activated platelets and monocyte polarization in vitro, thereby contributing to monocyte recruitment [90]. Additionally, CXCL12 induces monocyte polarization into predominantly CD163+ M2-like macrophages and promotes foam cell formation [91].
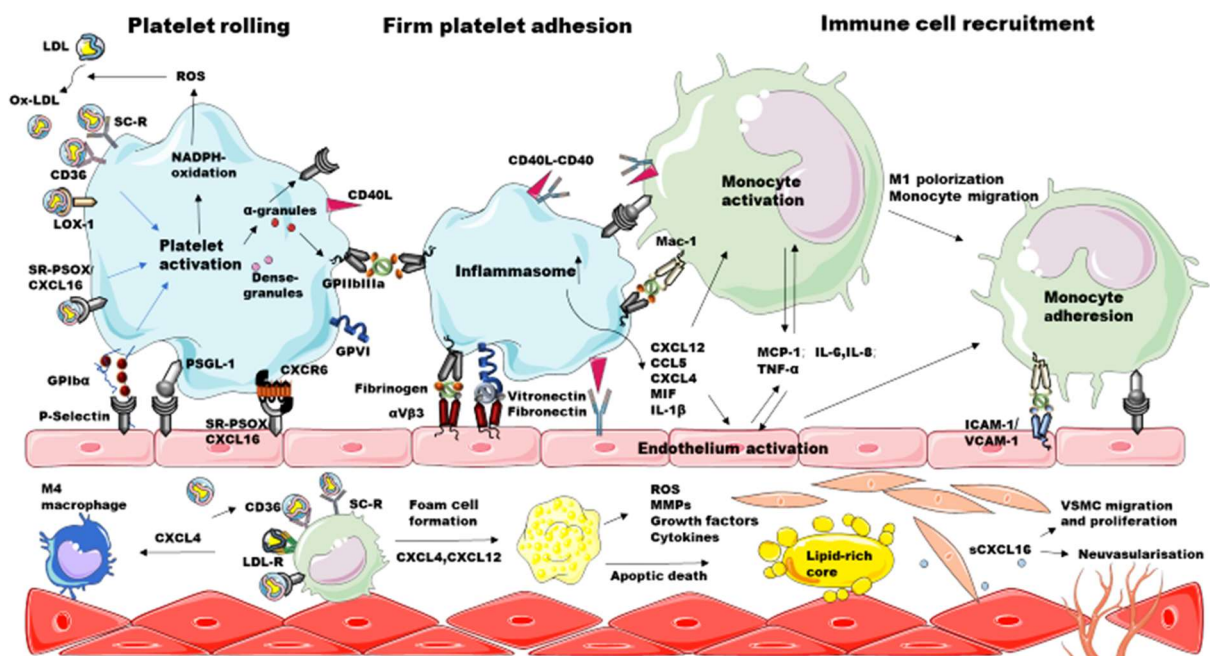

Figure 2. Role of platelets and chemokines in AS [38].

In the platelet rolling phase, ox-LDL loaded by various receptors (SC-R, CD36, LOX-1, SP-PSOX/CXCL16) on platelets, and the initial reversible contact of platelets and endothelium mediated by GP I bα, PSGL-1& P-selectin, CXCR6 & SP-PSOX/CXCL16, enhance platelet activation. Activated platelets express P-selectin, CD40L, GPVI，

GPⅡbⅢa upon α-granules release, increase platelet-platelet aggregation and lead to the increased adhesion of platelets to the endothelium.

In the firm platelet adhesion phase, platelet-endothelial interactions via receptors (GPⅥ& GPⅡbⅢa) and platelet-leukocyte interactions via receptors (GPⅡbⅢa, P-selectin, CD40-CD40L) promote endothelial activation and immune cell recruitment to the subendothelial space via direct cell-cell interactions and soluble mediators (CXCL12, CCL5, CXCL4, MIF, IL-1β). Platelets adhere to the endothelium in a sequential process leading to endothelial activation accompanied by increased adhesiveness and cytokine secretion

In the immune cell recruitment phase, stimulated platelets also activate monocytes and induce a proinflammatory phenotype in monocyte-derived macrophages, which is associated with enhanced adhesiveness to endothelial cells via receptors MAC-1 (complement receptor type 3, CD11b/CD18) and PSGL-1, and the secretion of proinflammatory cytokines (IL-1, TNF-α, MCP-1, IL-8).

Monocytes infiltrate into the subendothelial space, uptake ox-LDL via receptors (CD36, SP-PSOX/CXCL16, SC-R), transform into foam cells induced by CXCL4&CXCL12, and finally become the lipid core of AS. Besides, soluble CXCL16 induces the neovascularization, migration and proliferation of VSMC.

1.4. CXCL16

In 2000, a new chemokine called CXCL16 was identified by Mathoubian, which is capable of activating its sole receptor, CXCR6 (also known as BONZO, STRL33 or TYMSTR), thereby regulating the transfer of T cells in the body [92, 93]. Around the same time, a new scavenger receptor that may bind ox-LDL was termed SR-PSOX (scavenger receptor binds phosphatidylserine and oxidized lipids), which turned out to have sequence homology with CXCL16. CXCL16 was later characterized to exist in two forms: the transmembrane and soluble form. The transmembrane receptor, mainly expressed in macrophages, SMCs and vascular endothelial cells in the atherosclerotic lesions [94], is responsible for the uptake of ox-LDL [53, 95] and promoting adhesion of cells expressing CXCR6 [52, 96, 97]. The transmembrane form acts as a scavenger receptor while the soluble secreted type is mainly distributed in the bloodstream and acts as a chemokine. Studies in 2005 showed that a polymorphism within exon 4 of CXCL16, which is located within the spacer region between the chemokine and transmembrane region, is associated with increased severity of coronary stenosis and with reduced minimum luminal diameter [98]. This suggests CXCL16 may be involved in the progression of CAD.

1.4.1. Transmembrane CXCL16 (SR-PSOX/CXCL16)

Human transmembrane CXCL16 is mainly expressed in immune system cells, macrophages, platelets, keratinocytes, SMCs and vascular endothelial cells in atherosclerotic lesions [94, 99, 100], and is composed of a 30KDa type I glycoprotein containing 254 amino acids. It consists of four structural domains: the chemotactic domain structure, glycosylated mucins area, single-pass transmembrane nut-bolts domain and cytoplasmic domain. SR-PSOX/CXCL16 is the only member of the type G scavenger receptor, which has no homologous parallel with other scavenger receptors. Scavenger receptors are a group of molecules that were originally identified as regulatory molecules in macrophages that mediate uptake of modified lipoproteins [101]. Indeed, these receptors participate in chemotaxis, atherogenesis, regulating the clearance of apoptotic cells and triggering intracellular signal transduction. Consequently, they affect both chemotactic infiltration of cells promoting atherosclerosis and participate in anti-atherosclerotic actions [102, 103].

In addition to its function as a scavenger receptor mentioned above, membrane-type CXCL16 may also act as an adhesion molecule, increasing cellular adhesion of CXCR6+ cells, which can play the role of mitogen, that is, CXCL16/CXCR6 can stimulate and regulate immune cells in paracrine or autocrine ways. SR-PSOX/CXCL16 also mediates the adhesion of phagocytosis of gram-positive and negative bacteria [104]. Moreover, we have previously shown that platelets express SR-PSOX/CXCL16 as transcript and protein, which is increased upon activation with agonists like ADP, TRAP, ox-LDL and assists in ox-LDL binding to TRAP-activated platelets. And we also observed significantly enhanced platelet SR-PSOX/CXCL16 surface expression among ACS patients as compared to CCS, which correlated with pro-inflammatory CRP, and creatine kinase (CK) at baseline in a small cohort of 61 patients undergoing coronary angiography [105].

Transmembrane-type CXCL16 can be subjected to proteolytic enzyme cleavage and converted into soluble secreted type, which has both different and inter-related biological effects [53]. Disintegrin and metalloproteinase domain-containing protein (ADAM)-10 and ADAM-17 (enzymes formed by TNF-transformation) are known to be involved in this process. ADAM-10 regulates the production and decomposition of SR-PSOX/CXCL16 [106-108], while ADAM-17 only guides its decomposition [109].

1.4.2. Soluble CXCL16

Soluble CXCL16 (sCXCL16), as a member of the CXC chemokine family, has the characteristics of both CC- and CX3C chemokine families. CXCR6 is the only receptor of sCXCL16, belonging to the G protein coupled receptor superfamily member [110, 111]. CXCR6 is mainly expressed on fibroblasts, SMCs, CD4+T lymphocytes, CD8+lymphocytes, B cells, macrophages, dendritic and NK cells [105, 112, 113].

Soluble CXCL16 operates primarily as a chemotactic factor and interacts with cytokines such as the proinflammatory factors TNF-α and IFN-γ [114]. Soluble CXCL16 recruits activated T cells and NKT lymphocytes expressing CXCR6, resulting in the accumulation of immune cells at the inflammatory site [104]. Then these lymphocytes secrete cytokines such as TNF-α, IFN-γ, IL-2 and IL-6, which affect the functional activities of surrounding cells [115], ultimately favoring the development of inflammatory diseases (chronic inflammatory liver diseases inflammatory bowel diseases, diabetes, autoimmune arthritis, and sepsis). Moreover, most CXCR6-expressing cells in circulation are Th or Tc cells capable of producing IFN-γ [116], which can regulate the expression of CXCL10, CCL2, and CX3CL1 [117], affecting the recruitment of macrophages and T-cells into the atherosclerotic aortic wall.

In addition to chemokine functions, sCXCL16 can stimulate activation of the

phosphatidylinositol-3 kinase/Akt (PI3K/Akt) pathway [118], which plays an important role in the progression of AS [119]. PI3K/Akt signaling promotes monocyte-endothelial cell adhesion monocyte chemotaxis, reduces macrophage apoptosis, mediates macrophage polarization and phenotypic transition [120], induces intracellular lipid accumulation, promotes VSMC-derived foam cell formation by inhibiting autophagy [121], and platelet activation [122]. Soluble CXCL16 also participates in promoting angiogenesis. The study found that sCXCL16 may not only induce proliferation and differentiation of vein endothelial cells to form a new blood vessel cavity but may also induce expression of VEGF, promoting the formation of blood vessels [123]. Soluble CXCL16 also promotes angiogenesis in AS plaques [94], accelerating the progression of plaques, and even causing plaque hemorrhaging, rupture and serious thrombotic complications.

1.4.3. CXCL16 and platelets

Both transmembrane and soluble CXCL16 have effects on platelet aggregation and activation. Previously we have reported the expression of CXCR6, the receptor for soluble CXCL16, in platelets as transcript and protein. Moreover, we demonstrated that soluble CXCL16 triggered platelet CXCR6-dependent PI3K/Akt signaling, leading to platelet degranulation and αIIbβIII-integrin activation, synergistically stimulating aggregatory response to ADP, which substantiated platelet adhesion to the endothelial monolayer *in vitro*, and injured carotid arteries *in vivo*. These effects were significantly ablated in mice deficient in CXCR6, Akt1, and Akt2 [124]. Another study in 2015 [125] shows SR-PSOX/CXCL16 are richly expressed at atherosclerotic lesions of carotid endarterectomy specimens or on TNF-α, IFN-γ-inflamed human radial artery segment. And SR-PSOX/CXCL16 acts as an endothelial adhesion ligand and efficiently captures CXCR6 expressing platelets, inducing PBMC-adhesion to the

AS-prone vessel wall and thus promoting the progression of AS [126]. Unlike soluble CXCL16, SR-PSOX/CXCL16 triggers intraplatelet calcium mobilization and induces irreversible platelet aggregation, even in absence of an additional stimulus, and thus may contribute to both platelet deposition and thrombus formation.

Hence, CXCL16 as a molecule with multiple functions of chemokines, scavenger receptors, and adhesion molecules, is implicated in multiple steps of the immune response, from antigen recognition to transmission of immune cells to inflammatory mediator aggregation. Consequently, CXCL16 has become a hot topic in the field of atherosclerosis research soon after its discovery.

In 2007 [127], Lehrke M et al. studied the association of sCXCL16 with atherosclerosis in macrophages, mice, and ACS in humans, which suggests that CXCL16 may play a pro-inflammatory role in human atherosclerosis, particularly in ACS. Sun Y et al. [128] found that sCXCL16 increased significantly in ACS patients as compared to control and SAP patients. Soluble CXCL16 levels in patients with SAP were higher than in control subjects, although no statistical significance was found. Studies thereafter showed that the increase in sCXCL16 levels correlates with long-term mortality in ACS patients [129], patients with acute ischemic stroke [130], and adverse events (all-cause death, non-fatal myocardial infarction, revascularization, and angina pectoris requiring re-hospitalization) among high-risk patients with intermediate coronary artery lesions [131]. Additionally, sCXCL16 has been suggested to provide valuable information on the risk of eventually succumbing to MI, tracked over 11.3 years among 58,761 healthy men and women free of cardiovascular disease included in the large population-based HUNT cohort [132].

Moreover, it has been shown that serum CXCL16 levels negatively correlated with eGFR, creatinine clearance rate, and blood albumin, and positively with 24 h proteinuria, blood urea nitrogen (BUN), creatinine, and uric

acid levels in patients with diabetic nephropathy [133] and hs-CRP in patients with diabetic coronary artery disease [134]. Recently a subgroup of 5142 patients randomized in the PLATO trial [135] (Platelet Inhibition and Patient Outcome) showed statistically positive associations of the admission level of serum sCXCL16 with leukocytes, cystatin C, NT-proBNP (N-terminal pro-brain natriuretic peptide), and troponin T (TnT). Serum sCXCL16 was also shown in this study to be independently associated with cardiovascular death and morbidity in ACS patients.

As mentioned above, various studies have explored the diagnostic and prognostic significance of sCXCL16, which suggests its potential to aid risk stratification in cardiovascular disease patients. However, the roles of platelet expression of transmembrane-SR-PSOX/CXCL16 and CXCR6, which interact with sCXCL16, are yet to be revealed. Since platelets express transmembrane-SR-PSOX/CXCL16 and may contribute to serum sCXCL16, we have extended these clinical findings by exploring the relevance of platelet SR-PSOX/CXCL16 and CXCR6 in influencing thrombotic potential and prognosis among CAD patients over 3 years.
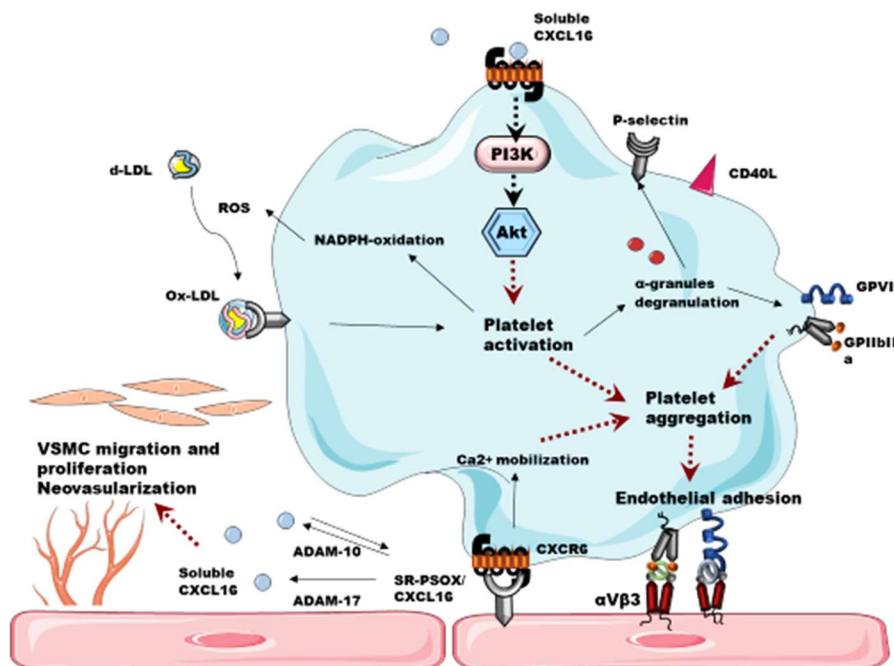
Figure 3. Platelet CXCL16-CXCR6 axis. Soluble CXCL16 decomposed from SR-PSOX/CXCL16 contributes to platelet activation by binding to CXCR6 on platelet and activating PI3K/Akt pathway. Soluble CXCL16 also leads to neovascularization, VSMC migration, and proliferation. SR-PSOX/CXCL16 mediates the adhesion of platelet to endothelium leading to platelet aggregation via Ca^{2+} mobilization [38].

1.5. Aims and objectives

Recent studies on sCXCL16 have linked sCXCL16 to ACS, however, the role of SR-PSOX/CXCL16 in CAD is yet to discovered. As platelets play an essential role in CAD, we investigated platelet expression of SR-PSOX/CXCL16, CXCR6, and sCXCL16 in patients with CAD.

The aims of the current analysis were the following:

1. To analyze the surface expression of SR-PSOX/CXCL16 and CXCR6 on circulating platelets in CCS and ACS patients. To assess the levels of soluble CXCL16 in serum samples of patients with either CCS or ACS.

2. To address the potential significance of platelet SR-PSOX/CXCL16-CXCR6 axis and serum levels of sCXCL16 in influencing disease severity among CAD patients.

3. To validate the prognostic significance of platelet SR-PSOX/CXCL16, CXCR6 and serum levels of sCXCL16 in CAD patients.

2. Materials and methods

2.1. Study subjects:

Platelet surface expression of the transmembrane chemokine/scavenger receptor SR-PSOX/CXCL16 and the receptor CXCR6 for the soluble chemokine CXCL16 was evaluated in a cohort of n=240 consecutive patients with symptomatic CAD. Blood samples were collected during PCI and immediately analyzed for surface expression of CXCR6, SR-PSOX/CXCL16, PAC-1, CD62P, and GPIb (CD42b) by flow cytometry. All subjects gave written informed consent. CCS and ACS were defined as elaborated previously (Table 1 and Table 2) [125, 136]. All patients admitted to the Department of Cardiology and Angiology, University Hospital Tübingen, gave written informed consent. The study was approved by the institutional ethics committee (270/2011BO1 and 237/2018BO2), which complies with the declaration of Helsinki and good clinical practice guidelines. Patients' characteristics (age, gender, cardiovascular risk factors, co-medication) of the subgroups of CCS and ACS are provided in Table 3.

Table 3: Baseline characteristics of the overall cohort of all patients (n=240)

	CCS (n=62)	ACS (n=178)	p value
Age, years (mean ± SD)	70 (±10.0)	70 (±11.1)	0.942
Male, n(%)	50 (80.6%)	141 (79.2%)	0.810
Cardiovascular risk factors, n(%)			
Arterial hypertension	50 (80.6%)	145 (81.5%)	0.893
Dyslipidemia	44 (71.0%)	95 (53.4%)	**0.015**
Diabetes mellitus	19 (30.6%)	47 (26.4%)	0.520
Current smokers	22 (35.5%)	47(26.4%)	0.173
Obesity	11 (17.7%)	23 (12.9%)	0.349

Family history	15 (24.2%)	37 (20.8%)	0.576
Echocardiography			
Left ventricular function, start % (mean ± SD)	53(±9.7)	52(±10.7)	0.443
Left ventricular function, Follow-up % (mean ± SD)	53(±9.8)	51(±10.5)	0.445
Admission laboratory, median (25th percentile - 75th percentile)			
Platelets, 1000/µl	211.0(182.0-258.8)	210.0(173.5-255.0)	0.559
eGFR, ml/m²	81.8(67.9-94.0)	74.4(61.0-92.5)	0.284
hs-CRP, mg/dl	0.13(0.04-0.48)	0.4(0.09-1.19)	**0.002**
Troponin I, ng/dl	0.03(0.03-0.03)	0.03(0.03-0.41)	**<0.001**
NT pro-BNP, ng/l	99 (21-387)	393(162-2696)	0.088
CK, U/l	107 (62-143)	101 (71-184)	0.150
HDL, U/l	49 (39-56)	45 (36-59)	0.345
LDL, U/l	95.5(65.5-127.5)	106.0(83.0-133.0)	0.057
TC, U/l	166.5(127.5-192.5)	167.0(146.0-201.0)	0.337
Trigylcerides, U/l	124.0(93.8-161.3)	128.0(87.0-181.0)	0.776
Medication at admission, n(%)			
ASA	31 (50.0%)	75 (42.1%)	0.515
Clopidigrel	15 (23.1%)	19 (10.7%)	**0.016**
Prasugrel	2 (3.2%)	3 (1.7%)	0.521
Ticagrelor	7 (11.3%)	8 (4.5%)	0.081
DAPT	19 (30.6%)	25 (14.0%)	**0.007**
ACEi	23 (37.1%)	51 (28.7%)	0.359
ARB	19 (30.6%)	44 (24.7%)	0.543

Aldosterone inhibitors	5 (8.1%)	9 (5.1%)	0.467
Thiazides Diuretics	10 (16.1%)	24 (13.5%)	0.784
Loop Diuretics	8 (12.9%)	21 (11.8%)	0.992
Calcium channel blockers	13 (21.0%)	22 (12.4%)	0.086
Beta-blockers	35 (56.5%)	76 (42.7%)	0.127
Statins	37 (59.7%)	70 (39.3%)	**0.011**
Oral anticoagulation	12 (19.4%)	23 (12.9%)	0.281

2.2. Materials

Platelet surface expression-Flowcytometry	
Mouse anti-human CXCL16-PE Mouse anti-human CXCR6-PE	R&D systems
Anti-human CD62P-FITC Anti-human CD42b-FITC	Beckman Coulter,
Anti-human CD42b-PE Anti-human PAC-1-FITC	BD Biosciences
Enzyme-linked immunosorbent assay (ELISA)	
Human CXCL16 Quantikine ELISA kit	R&D systems

2.3. Methods

2.3.1. Study design

20ml of blood was drawn through the catheter sheet into a common, sterile 20ml syringe from all participants. Immediately, blood was put into tubes containing the following anticoagulants: citrate-phosphate-dextrose solution with adenine (CPDA) (for flow cytometric analysis), Hirudin (for impedance aggregometry). Furthermore, blood was filled into specific tubes without anticogulants to collect serum. Experiments were conducted in the laboratories

of the University Hospital Tübingen, Department of Cardiology and Angiology (Internal Medicine III). All experiments were conducted at room temperature.

2.3.2. Platelet surface expression measured by FACS

2.3.2.1. Surface expression of SR-PSOX/CXCL16 and CXCR6 on platelets

Platelets in whole blood were analyzed for platelet surface-associated SR-PSOX/CXCL16 and CXCR6, gating for the platelet specific marker GPIb (CD42b). Blood collected in CPDA from CAD patients (n=240) was diluted 1:50 with PBS (Gibco), 20µl of the whole blood was put into a Cellstar tube and diluted with 980 µl of PBS. Then, 40µl of this dilution was transferred into flow cytometry tubes and incubated with 5 µl the respective fluorochrome-conjugated antibodies-mouse monoclonal anti-human SR-PSOX/CXCL16-PE, 5µl mouse monoclonal anti-human CXCR6-PE, and 5 µl mouse anti-human GPIb (CD42b)-FITC for 30 minutes in dark at room temperature, respectively. After staining, the samples were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (FACS-Calibur flow cytometer BD Biosciences) [105, 124].

2.3.2.2. Platelet activation markers CD62P and PAC-1

Platelet activation markers CD62P surface expression (for degranulation from α-granules) and PAC-1 binding (αIIbβIII-integrin activation) at baseline were also checked by whole blood flow cytometry gating for platelet specific marker GPIbα (CD42b). CPDA anticoagulated blood from CAD patients (n=240) was diluted 1:50 with PBS (Gibco) as described above and incubated with 5µl anti-human CD62P-FITC, 5µl PAC-1-FITC, and 5µl mouse anti-human GPIbα (CD42b)-PE for 30 minutes at room temperature. Samples were fixed with 0.5% paraformaldehyde and analyzed by flow cytometry (FACS-Calibur flow cytometer BD Biosciences) [105, 124].

2.3.3. Enzyme-linked immunosorbent assay (ELISA)

Levels of soluble CXCL16 were estimated in serum samples which were collected from 228 CAD patients and centrifuged at 2500 Xg for 10 minutes at room temperature and stored at -80°C until analysis, using the Quantikine-ELISA kit (R&D systems) as instructions [135].

2.3.4. Follow up of left ventricular function

A parameter that can be measured quite easily and gives good information about myocardial function is the left ventricular ejection fraction (LVEF%). However, in an emergency like ACS, detailed echocardiography is sometimes not possible. Therefore, the intra-hospital course of LVEF% was evaluated in CAD patients using transthoracic echocardiography. After a median of 3 years, we re-evaluated LVEF% in ACS patients. Two-dimensional echo LVEF was assessed using Simpson's biplane method of discs by manual planimetry of the endocardial border in end-diastolic and end-systolic frames [137].

2.3.5. Follow-up for clinical endpoints

All patients enrolled in the study were followed up for all-cause death (ACD) and LVEF for 1080 days after study inclusion. Follow-up was performed by telephone interview and/or review of patients' charts on readmission by investigators blinded to laboratory results [138]. 23 out of 240 patients (9.6%) were lost to follow-up.

2.3.6. Statistical analysis

All data were analyzed using SPSS version 26.0 (SPSS Inc., ChicagoIL). Normally distributed data are presented as mean values ± standard deviation (SD) and compared using independent Student's t-test. Non-parametric data, including

MFIs, are presented as median values (25th; 75th percentile) in the form of box plots and compared using the Mann–Whitney U test. Correlations were assessed using Spearman's rank correlation coefficient (ρ) and are presented as scatter plots for optical evaluation. The level of significance was determined as $p < 0.05$.

Cox proportional hazard (PH) regression was applied to investigate associations between survival endpoints and CXCL16 as well as CXCR6. The time-dependent covariate method was used to check the proportional hazard assumption of the Cox model. Survival functions were estimated by Kaplan-Meier curves. The log-rank test was applied to compare survival functions between high vs low CXCL16 as well as CXCR6 levels. All statistical tests were two-tailed and statistical significance level was defined as $p < 0.05$.

3. Results

3.1. Expression of platelet SR-PSOX/CXCL16, CXCR6 and serum levels of sCXCL16 in CAD

In the current clinical cohort of CAD patients, both SR-PSOX/CXCL16 and CXCR6 are expressed on the platelet surface, and platelet SR-PSOX/CXCL16 expression showed a strong and significant positive correlation with platelet surface expression of CXCR6 ($\rho=0.800$, $p<0.001$). However, neither platelet SR-PSOX/CXCL16 nor platelet CXCR6 showed an evident correlation with serum sCXCL16 ($\rho=0.028$, $p>0.05$ & $\rho=-0.028$, $p>0.05$) (**Figure 4**).
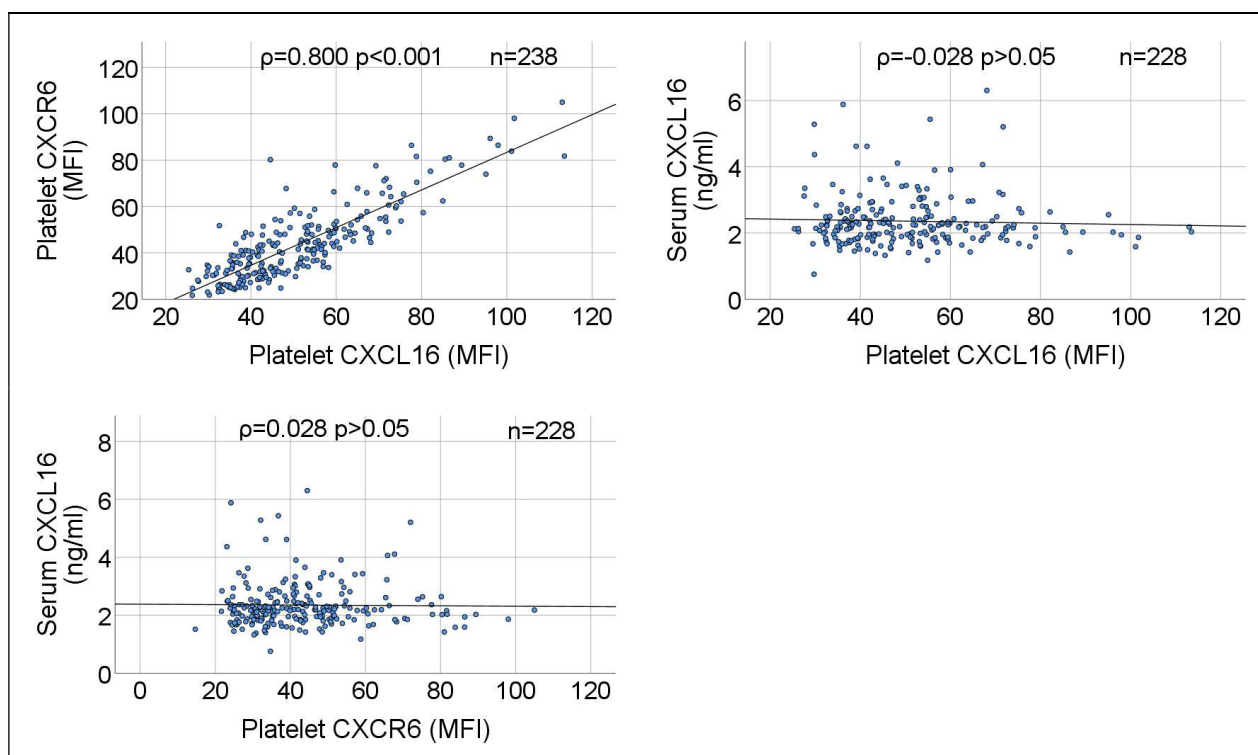

Figure 4. Correlation between platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, and serum levels of soluble CXCL16 in CAD patients. Correlations were assessed by Spearman's rank correlation coefficient (ρ); p: level of significance; MFI: mean fluorescence intensity.

Platelet SR-PSOX/CXCL16 and CXCR6 correlate with MPV ($\rho=0.357$, $p<0.001$ and $\rho=0.373$, $p<0.001$), and platelet count significantly ($\rho=-0.216$, $p=0.001$ and $\rho=-0.263$, $p<0.001$) (**Figure 5A & 5B**). Moreover, we observed a significant correlation between MPV and sCXCL16 ($\rho=0.134$, $p=0.043$). We also observed a significant inverse correlation ($\rho=-0.149$; $p=0.025$) between platelet
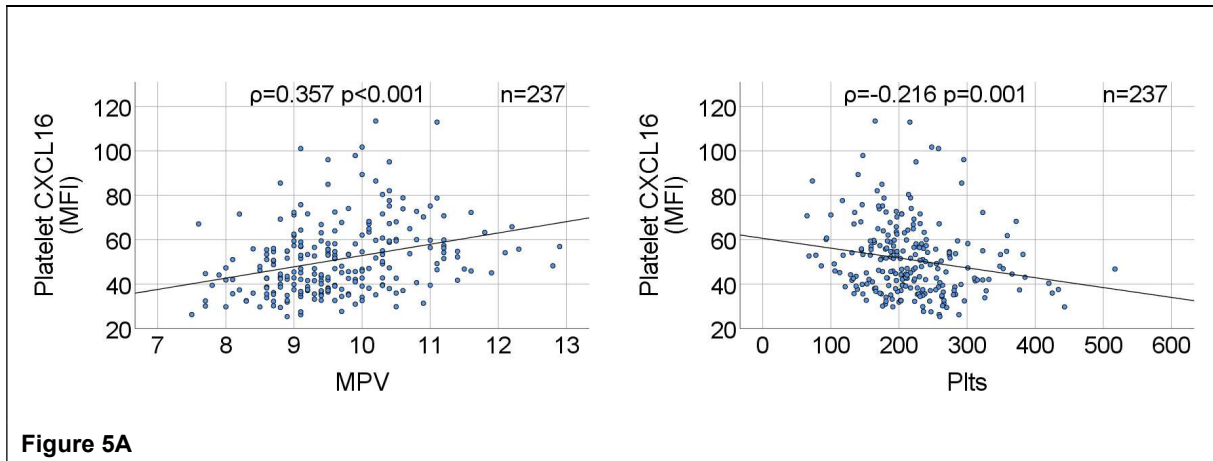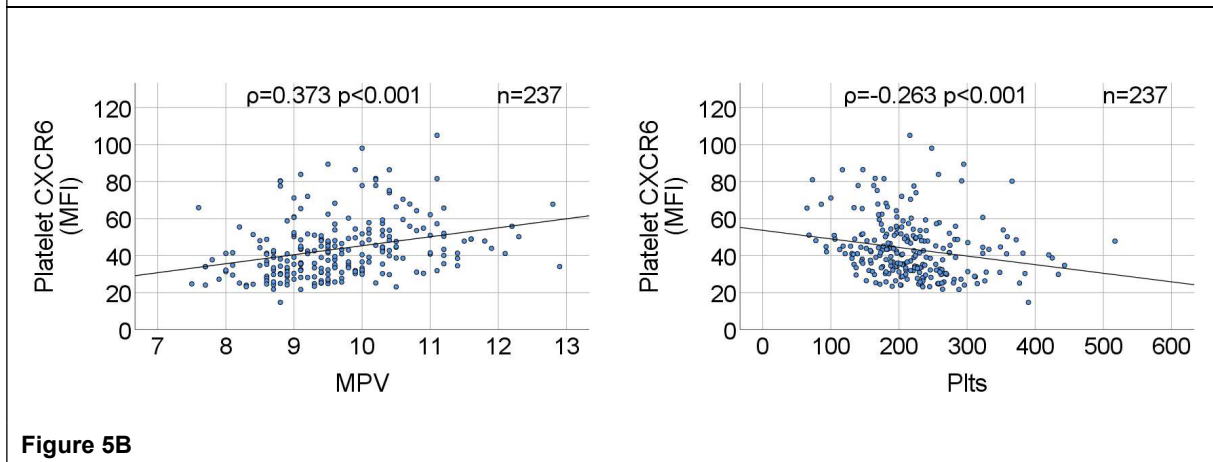
count and sCXCL16 (**Figure 5C**).
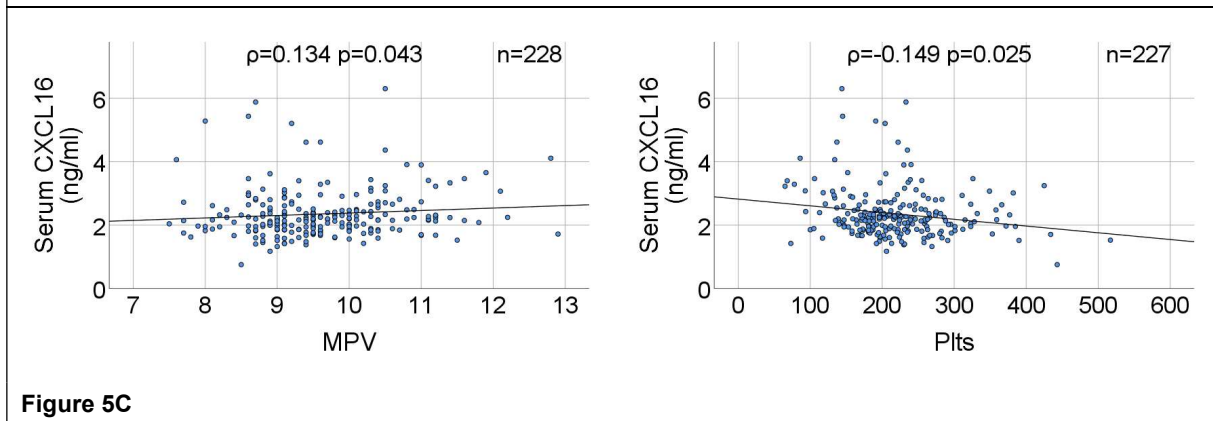

Figure 5A

Figure 5B

Figure 5C

Figure 5A-5C. Correlation between platelet surface-associated SR-PSOX/CXCL16 (5A), platelet-CXCR6 (5B), and serum levels of sCXCL16 (5C) with MPV and platelet count in CAD patients. Correlations were assessed by Spearman's rank correlation coefficient (ρ); p: level of significance; MFI: mean fluorescence intensity.

3.2. Influence of SR-PSOX/CXCL16, CXCR6 and serum sCXCL16 on the activation status of circulating platelets

In the current clinical cohort of CAD patients, we observed a significant correlation between platelet surface-associated SR-PSOX/CXCL16, and platelet

CXCR6 surface expression with the indicators of circulatory platelet activation i.e., CD62P surface exposure denoting degranulation (ρ=0.284, p<0.001 and ρ=0.321, p<0.001), and PAC-1 binding suggesting αIIbβIII-integrin activation (ρ=0.276, p<0.001 and ρ=0.279, p<0.001), (**Figure 6A & 6B**). However, the ligand for platelet CXCR6, i.e., serum sCXCL16 did not show any correlation with basal platelet activation in the absence of additional *ex vivo* stimulation (**Figure 6C**).
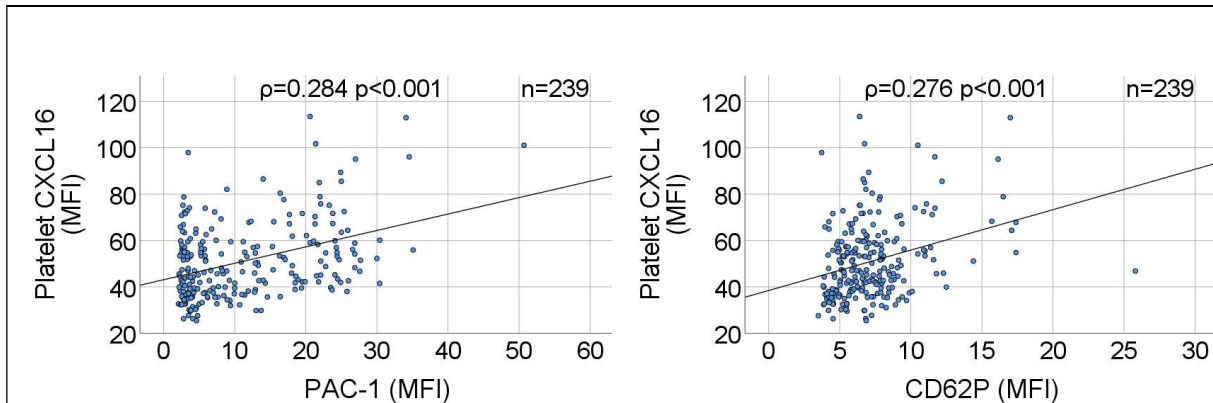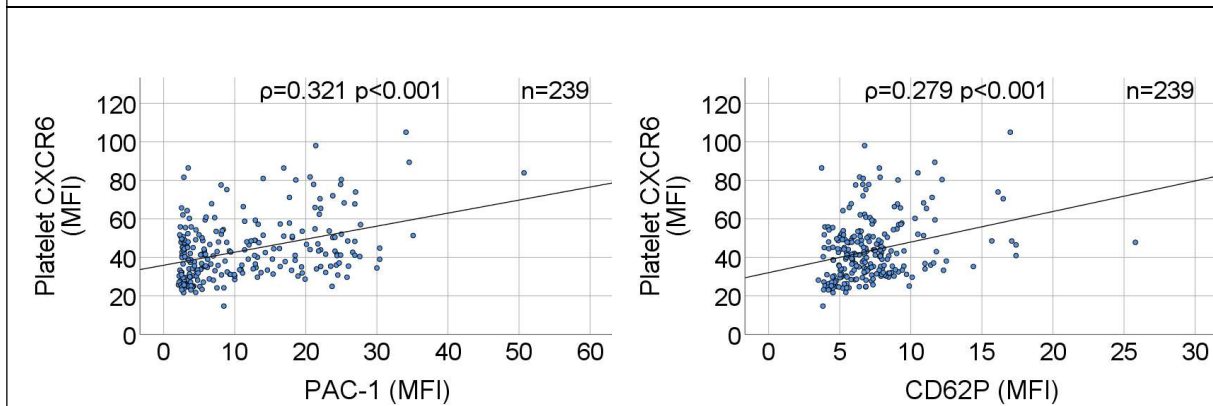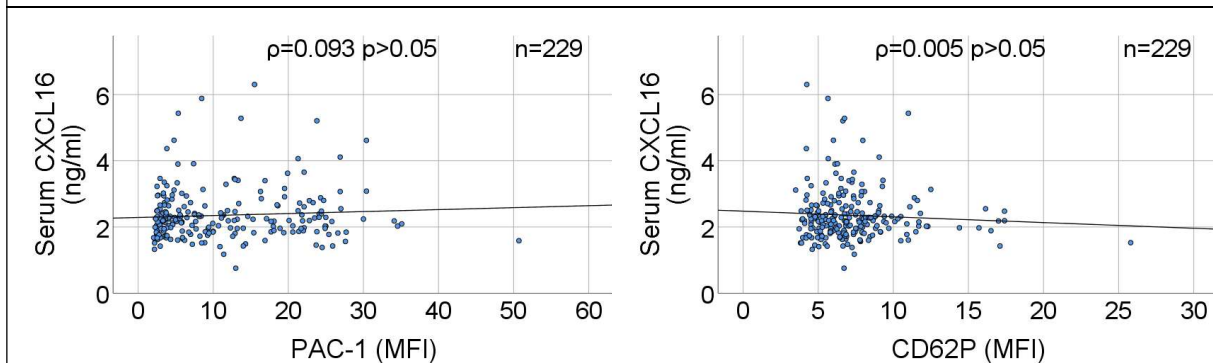

Figure 6A

Figure 6B

Figure 6C

Figure 6A-6C. Correlation between platelet surface-associated CXCL16 (6A), platelet-CXCR6 (6B), and serum

levels of sCXCL16 (6C) with platelet activation markers PAC-1 and CD62P binding in CAD patients. Correlations were assessed by Spearman's rank correlation coefficient (ρ); p: level of significance; MFI: mean fluorescence intensity.
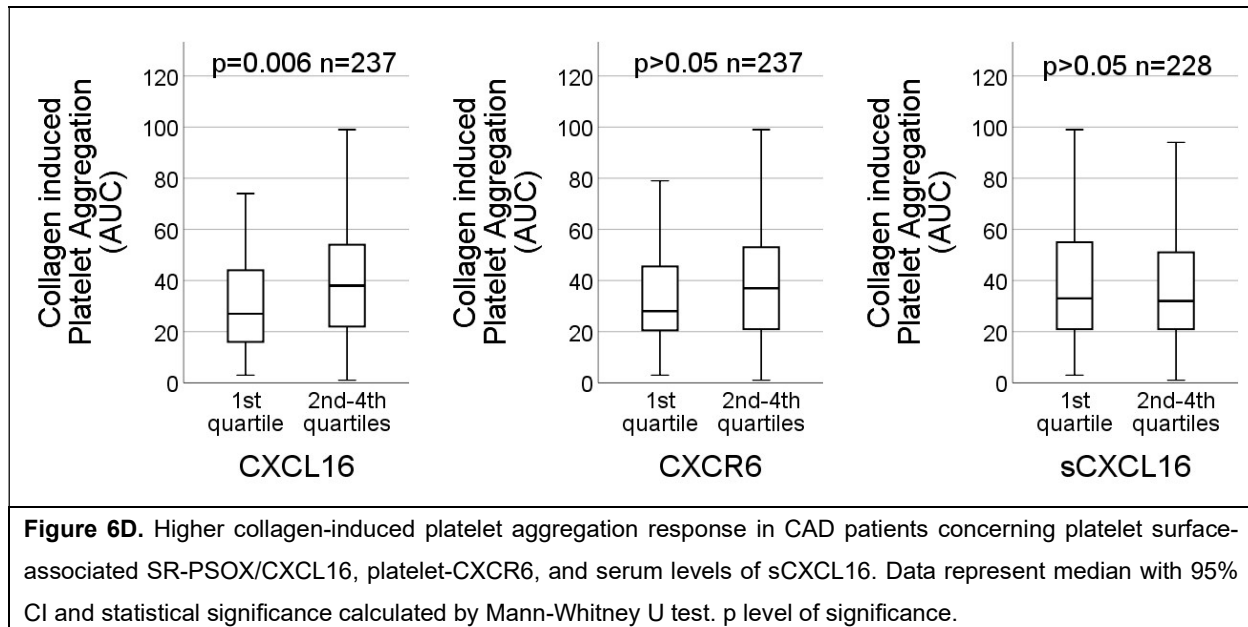

Figure 6D. Higher collagen-induced platelet aggregation response in CAD patients concerning platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, and serum levels of sCXCL16. Data represent median with 95% CI and statistical significance calculated by Mann-Whitney U test. p level of significance.

We also explored collagen-induced platelet aggregation *ex vivo*. Patients with relatively enhanced (1st quartile vs 2-4th quartiles) platelet surface-associated SR-PSOX/CXCL16 showed significantly increased aggregation response to collagen [median CXCL16 MFI (25th; 75th percentile) 27.00 (16.00; 44.50) in the 1st quartile vs. 38.00 (22.00; 54.00) in the 2-4th quartiles, p=0.006] , which were not observed with elevated CXCR6 or serum sCXCL16 levels [median CXCR6 MFI (25th; 75th percentile) 28.00 (20.25; 45.75) in the 1st quartile vs. 37.00 (21.00; 53.50) in the 2-4th quartiles, p>0.05] & [median (25th; 75th percentile) 33.00 (21.00; 55.00) in the 1st quartile of serum sCXCL16 vs. 32.00 (21.00; 51.00) in the 2-4th quartiles of serum sCXCL16, p>0.05] **(Figure 6D)**.

3.3. Expression of platelet SR-PSOX/CXCL16, CXCR6, and serum levels of sCXCL16 in dyslipidemia

Although sCXCL16 is a chemokine, transmembrane SR-PSOX/CXCL16 is a scavenger receptor for ox-LDL [107], the expression of which in cellular sources like endothelium [94], smooth muscle cells [94], macrophages [107], and platelets

[124] might be influenced by circulating lipids. In the current CAD-cohort, dyslipidemia, TG, or TC levels did not have any impact on platelet SR-PSOX/CXCL16, CXCR6, or serum sCXCL16 levels (**Figure 7A**).
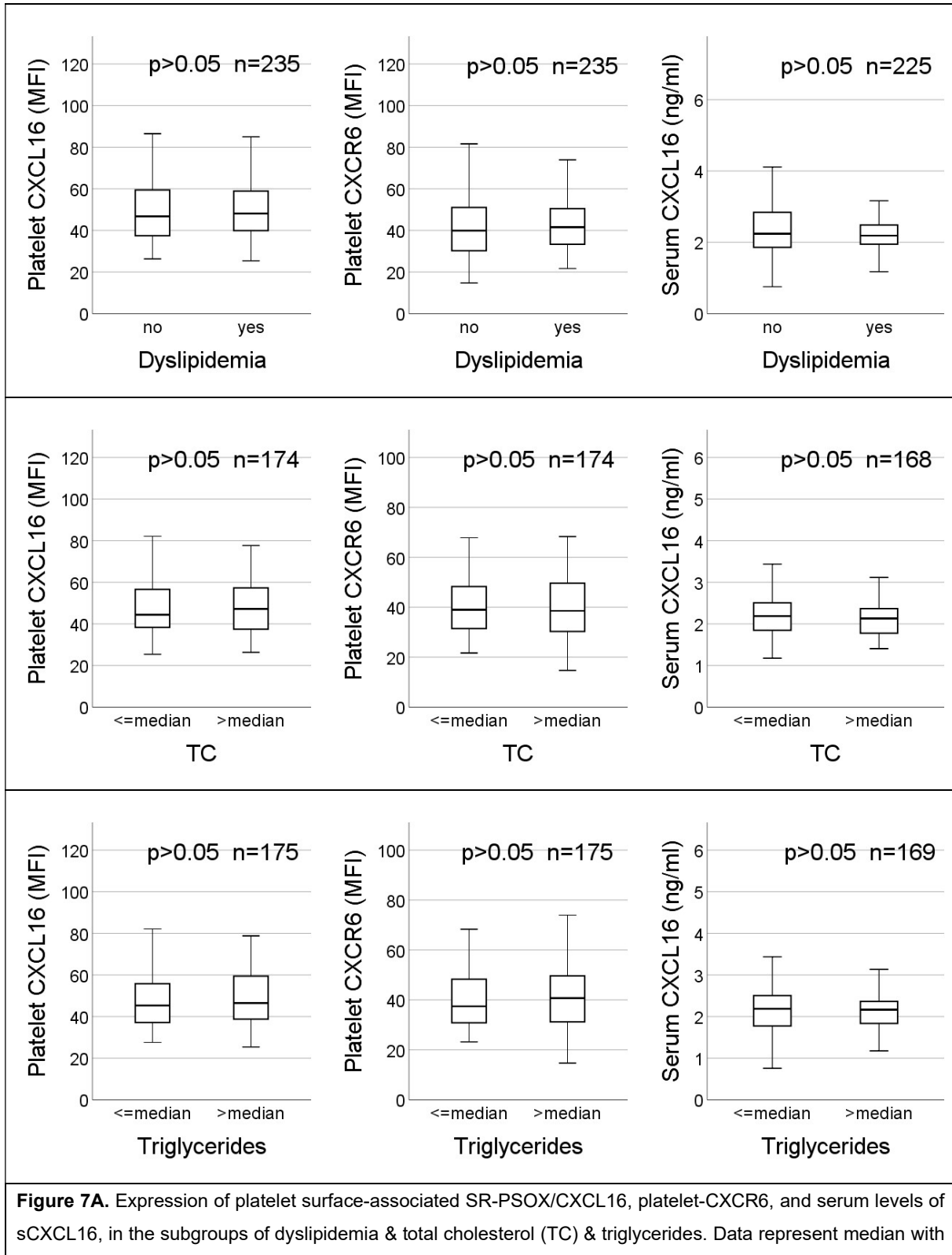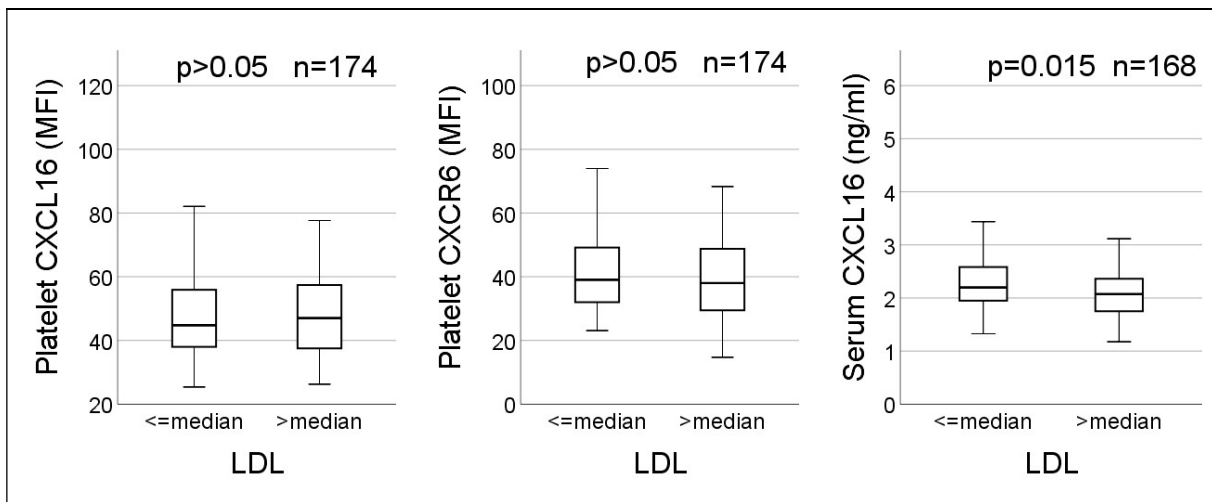
Figure 7A. Expression of platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, and serum levels of sCXCL16, in the subgroups of dyslipidemia & total cholesterol (TC) & triglycerides. Data represent median with

However, serum sCXCL16 levels significantly decreased (p=0.015) in the LDL>median subgroup [median (25th; 75th percentile) 2.07(1.74;2 .36) ng/ml] as compared to patients with the LDL≤median [median (25th; 75th percentile) 2.20(1.94; 2.62) ng/ml]. Platelet SR-PSOX/CXCL16 and CXCR6 MFI showed no difference (p>0.05) when compared within different LDL subgroups (**Figure 7B**). HDL being associated with reduced platelet hyper-reactivity, patients with higher (>median) plasma HDL levels showed significantly decreased (p=0.004) platelet SR-PSOX/CXCL16 [median MFI (25th; 75th percentile) 50.20 (39.80; 61.98) for HDL≤median and 43.15 (36.08; 53.40) for HDL>median], and CXCR6 [median MFI (25th; 75th percentile) 41.10 (32.05; 50.90) for HDL≤median and 35.25 (29.85; 47.75) for HDL>median, p=0.011], which is consistent with platelet PAC-1 & CD62P, but HDL did not influence circulatory sCXCL16 levels [median (25th; 75th percentile) 2.17 (1.84; 2.39) ng/ml for HDL≤median and 2.19 (1.76; 2.49) ng/ml for HDL>median, p>0.05] (**Figure 7C**).

Figure 7B

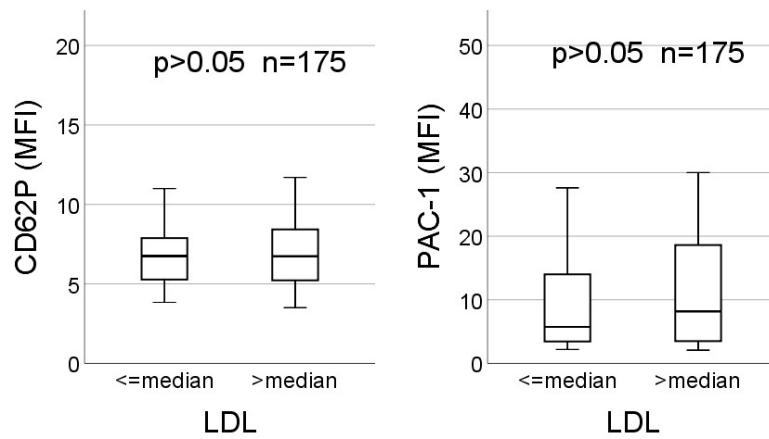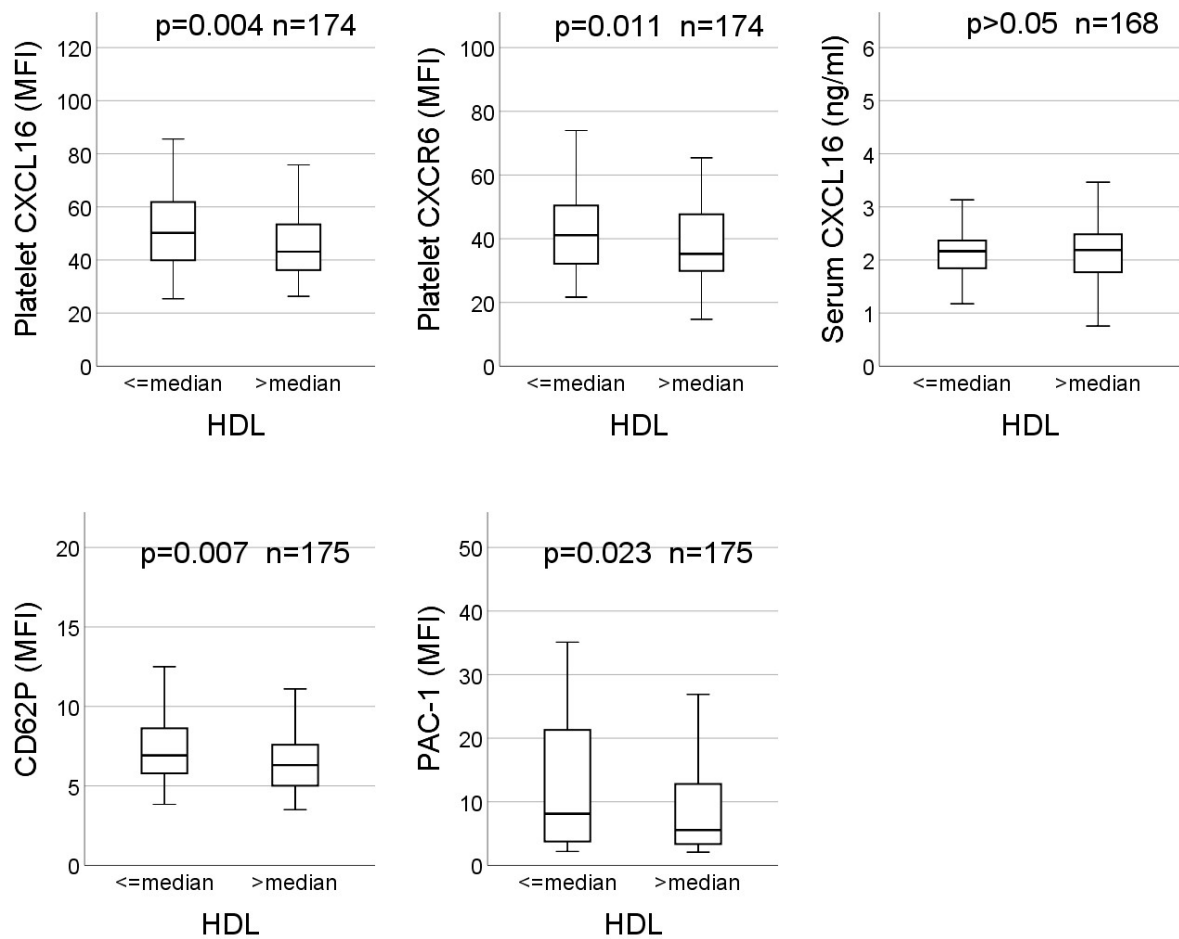**Figure 7C**

Figure 7B & 7C. Expression of platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, and serum levels of sCXCL16, platelet activation markers PAC-1 MFI and CD62P MFI in the subgroups of LDL (7B) & HDL (7C). Data represent median with 95% CI and statistical significance calculated by Mann-Whitney U test. p: level of significance; MFI: mean fluorescence intensity.

3.4. Association between platelet SR-PSOX/CXCL16, CXCR6, and serum levels of sCXCL16 with disease severity in CAD patients

As are shown in the CCS and ACS groups (**Figure 4A**), platelet expression of SR-PSOX/CXCL16 [median MFI (25th; 75th percentile) 45.65 (39.10; 58.58) in CCS vs. 50.33 (38.30; 59.56) in ACS], and CXCR6 [median MFI (25th; 75th percentile) 41.80 (30.89; 48.51) in CCS vs. 41.00 (32.00; 51.00) in ACS] showed no significant differences (p>0.05) with respect to disease severity. In contrast, serum CXCL16 was significantly (p=0.002) elevated in ACS patients as compared to CCS [median (25th; 75th percentile) 2.02 (1.77; 2.35) ng/ml in ACS vs. 2.25 (1.94; 2.73) ng/ml in CCS], confirming previous reports [127, 129, 132, 135].

Since ACS consists of UAP, NSTEMI and STEMI, we performed a more specific subgroup analysis. Platelet SR-PSOX/CXCL16 MFI [median MFI (25th; 75th percentile) 49.73 (39.10; 59.46) in UAP vs. 48.70 (36.94; 57.89) in NSTEMI vs. 54.18 (39.60; 63.83) in STEMI], platelet CXCR6 MFI [median MFI (25th; 75th percentile) 39.50 (32.09; 51.05) in UAP vs. 41.25 (29.76; 53.14) in NSTEMI vs. 41.65 (35.05; 50.20) in STEMI] and soluble CXCL16 [median (25th; 75th percentile) 2.19 (1.95; 2.39) ng/ml in UAP vs 2.34 (1.95; 3.03) ng/ml in NSTEMI vs. 2.29(1.86; 2.93) ng/ml in STEMI] showed no statistically significant differences (p>0.05) between these subgroups (**Figure 8B**).
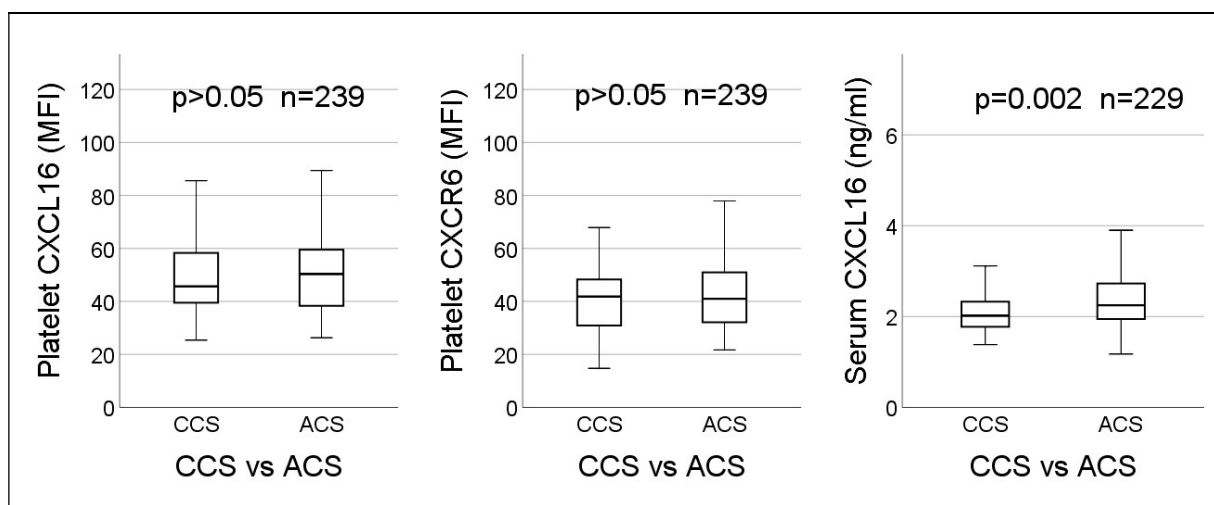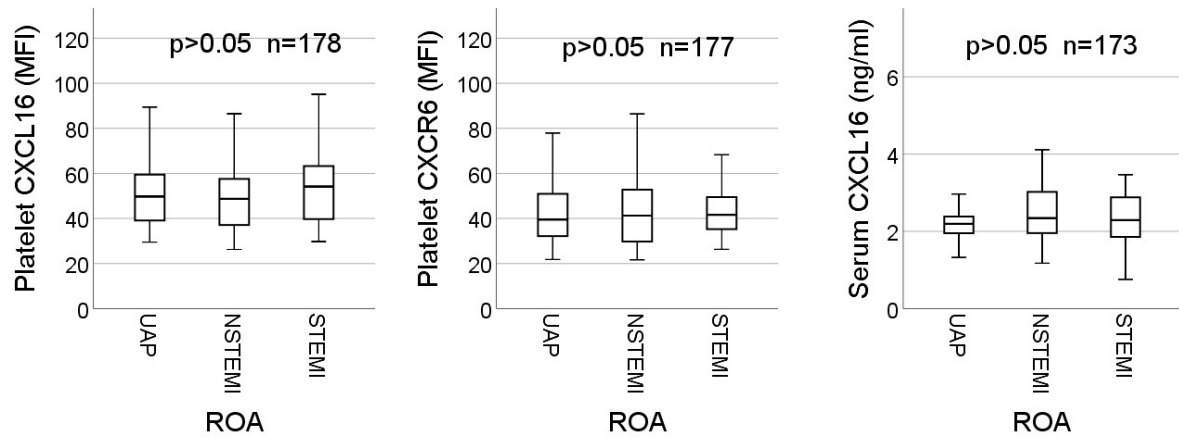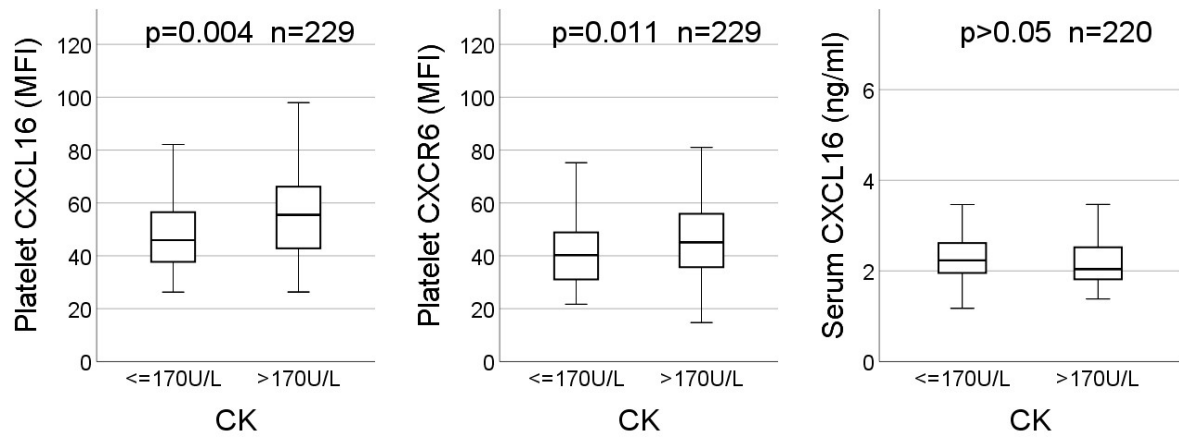
Figure 8A

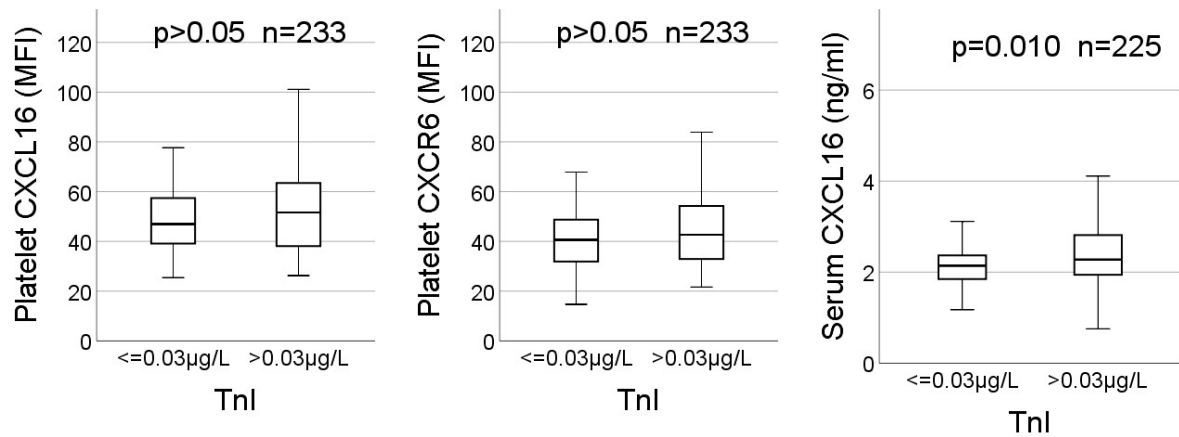**Figure 8B**

Figure 8C

Figure 8D

Figure 8. Association of platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, and serum levels of

sCXCL16 with disease severity (8A), reason of recruitment (8B), creatine kinase (8C), Troponin I (TnI) (8D) in CAD patients. Data represent median with 95% CI and statistical significance calculated by Mann-Whitney U test. p: level of significance; MFI: mean fluorescence intensity.

Troponin I (TnI) and creatine kinase (CK), as sensitive markers of ACS, are commonly used in the clinical diagnosis of ACS. sCXCL16 levels were marginally but significantly (p=0.010) elevated in patients with increased troponin I [median (25th; 75th percentile) 2.14 (1.85; 2.37) ng/ml for TnI<=0.03µg/L vs. 2.28 (1.93; 2.82) ng/ml for TnI>0.03µg/L]. However, patients with enhanced (>0.03µg/L) troponin levels did not show any significant alternations (p>0.05) in platelet SR-PSOX/CXCL16 [median MFI (25th; 75th percentile) 46.95 (39.10; 57.78) for TnI<=0.03µg/L vs. 51.63 (38.04; 63.94) for TnI>0.03µg/L] and CXCR6 [median MFI (25th; 75th percentile) 40.60 (31.89; 48.80) for TnI<=0.03µg/L vs. 42.65 (32.10; 54.80) for TnI>0.03µg/L] (**Figure 8D**).

However, it was not the case for the CK groups. We found that platelet SR-PSOX/CXCL16 MFI and platelet CXCR6 MFI were significantly increased in the CK elevated subgroup as compared to patients in the CK normal subgroup [median SR-PSOX/CXCL16 MFI (25th; 75th percentile) 45.93 (37.68; 56.53) for CK<=170U/L vs. 55.50 (42.60; 67.30) for CK>170U/L, p=0.004] and CXCR6 [median CXCR6 MFI (25th; 75th percentile) 40.23 (31.01; 48.88) for CK<=170U/L vs. 45.10 (35.45; 57.00) for CK>170U/L, p=0.011]. In contrast, sCXCL16 showed a slightly but no significant decrease (p>0.05) in CK elevated subgroup as compared to CK normal subgroup [median (25th; 75th percentile) 2.23 (1.95; 2.63) ng/ml for CK<=170U/L vs. 2.03 (1.81; 2.55) ng/ml for CK>170U/L] (**Figure 8C**).

3.5. Differential expression of platelet SR-PSOX/CXCL16, CXCR6, and serum levels of sCXCL16 under the influence of P2Y12 antagonists

As shown in **Figure 9,** none of the P2Y12 antagonists (ticagrelor, clopidogrel) showed any significant impact (p>0.05) on platelet SR-PSOX/CXCL16 [median MFI (25th; 75th percentile) 45.15 (38.00; 54.30) with ticagrelor vs. 47.65 (38.60; 58.89) w/o ticagrelor], CXCR6 [median MFI (25th; 75th percentile) 35.60 (30.35;

42.65) with ticagrelor vs. 41.10 (32.20; 50.95) w/o ticagrelor].

However, patients administered with ticagrelor showed significantly reduced levels of sCXCL16 [median (25th; 75th percentile) 1.92 (1.66; 2.22) ng/ml with ticagrelor vs 2.19 (1.90; 2.58) ng/ml w/o ticagrelor, p=0.039].
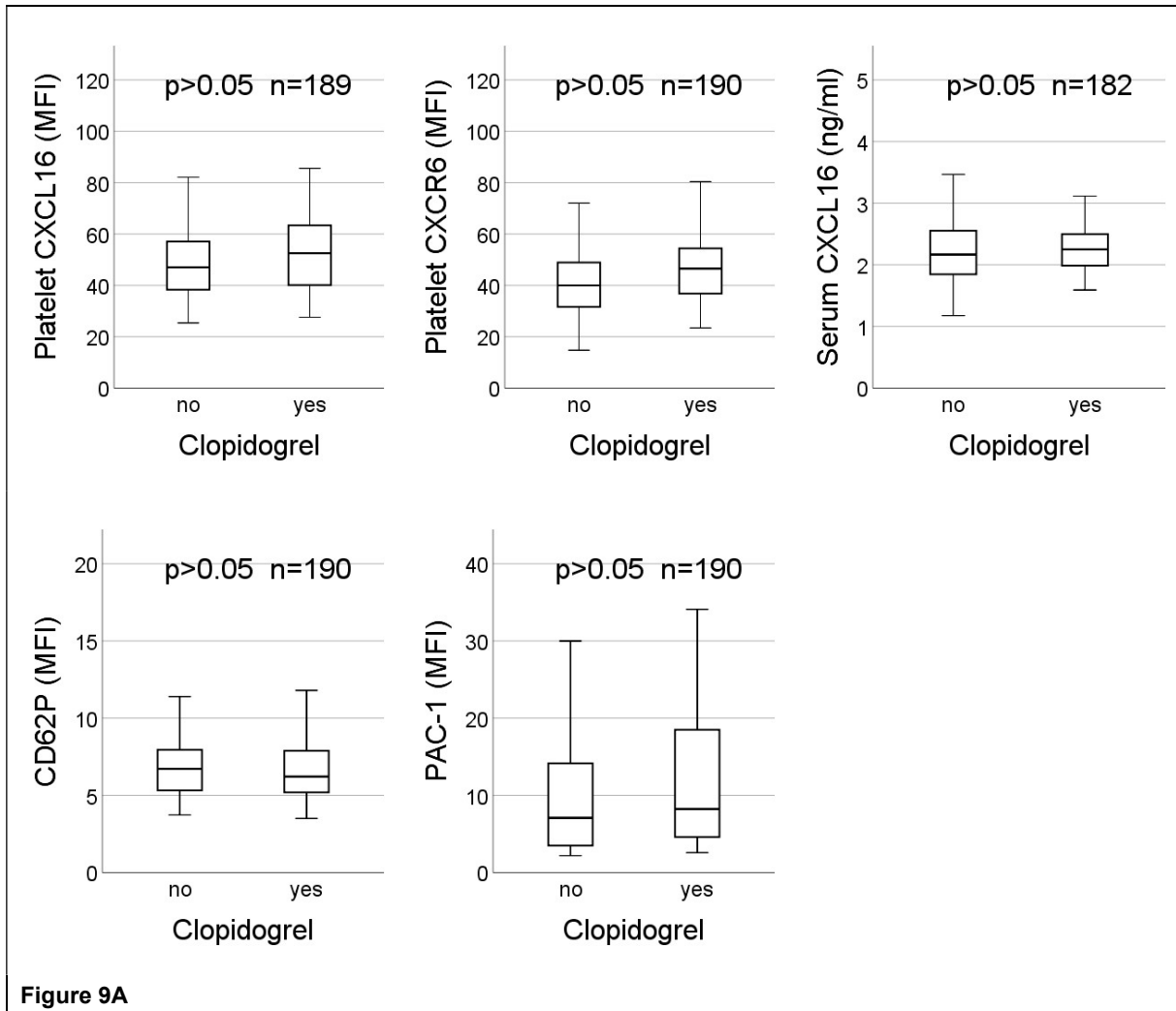
Figure 9A
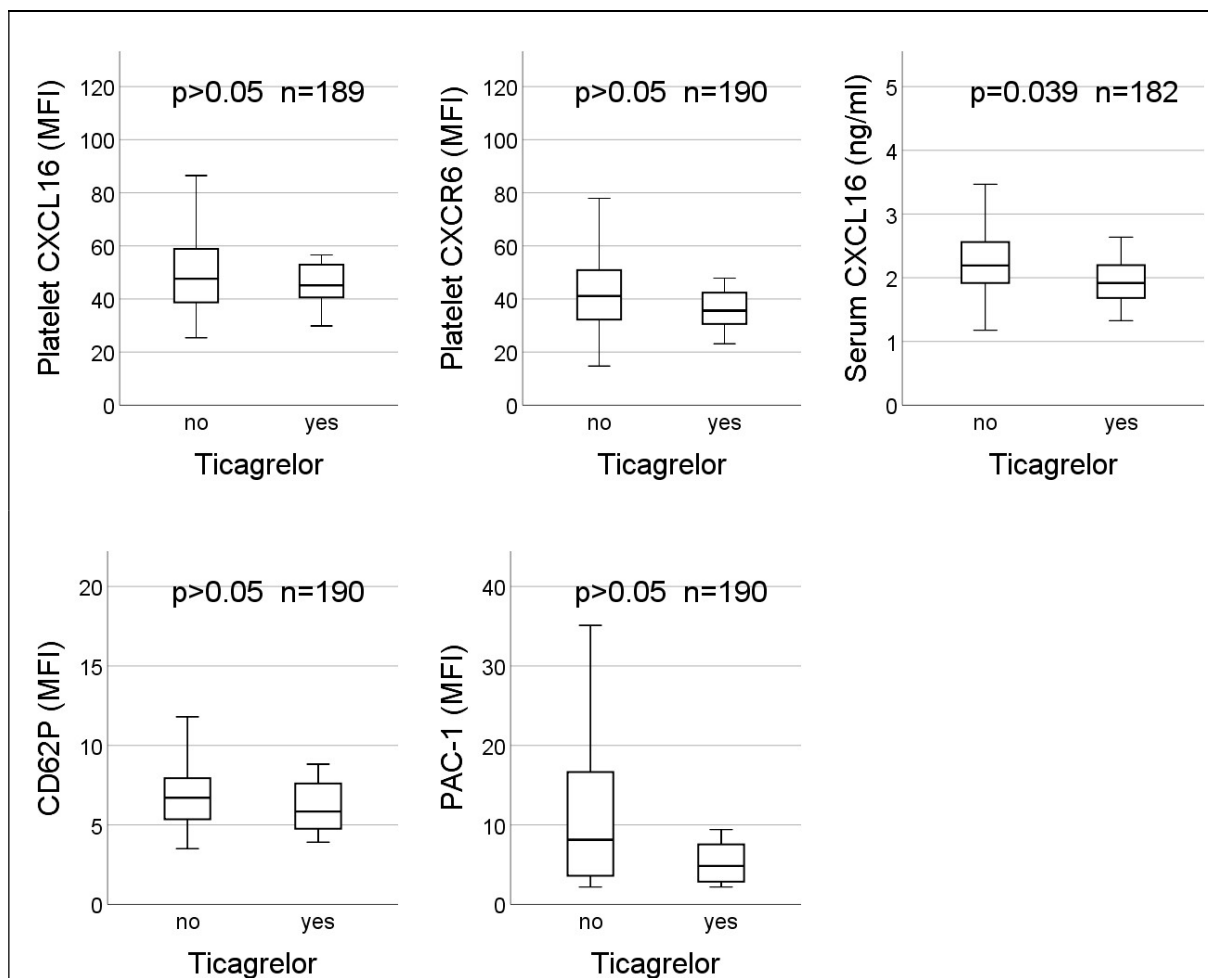
Figure 9B

Figure 9. Expression of platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, serum levels of sCXCL16, platelet activation markers PAC-1 MFI and CD62P MFI in clopidogrel (9A) and ticagrelor (9B) subgroups in CAD patients. Data represent median with 95% CI and statistical significance calculated by Mann-Whitney U test. p: level of significance; MFI: mean fluorescence intensity.

3.6. Influence of cardiovascular risk factors on the expression of platelet SR-PSOX/CXCL16, CXCR6, and serum levels of sCXCL16 in CAD patients

As hs-CRP is an important marker used to evaluate inflammation in patients, we also compared the expression of platelet SR-PSOX/CXCL16, CXCR6, and serum levels of sCXCL16 with hs-CRP. SR-PSOX/CXCL16 and CXCR6 MFIs showed no significant difference (p>0.05) between the hs-CRP>median and the hs-CRP<=median subgroups [median SR-PSOX/CXCL16 MFI (25th; 75th percentile) 46.63 (37.85; 59.34) for hs-CRP<=median vs. 47.23 (38.19; 56.95) for hs-CRP>median] and [median CXCR6 MFI (25th; 75th percentile) 42.30 (32.00;

50.90) for hs-CRP<=median vs. 39.03 (30.94; 49.41) for hs-CRP>median]. In contrast, serum sCXCL16 was significantly (p=0.001) elevated in the hs-CRP>median subgroup [median (25th; 75th percentile) 2.31 (2.02; 2.94) ng/ml] as compared to patients with hs-CRP<median subgroup [median (25th; 75th percentile) 2.13 (1.85; 2.37) ng/ml] (**Figure10A**).

Serum sCXCL16 increased significantly as the eGFR(ml/min/1.73m^2) decreased [median (25th; 75th percentile) 3.15 (2.61; 4.25) ng/ml in eGFR<=30ml/min/1.73m^2 vs. 2.49 (2.27; 3.05) ng/ml in 30<eGFR<=60 (ml/min/1.73m^2) vs. 2.15 (1.82; 2.43) ng/ml in 60<eGFR<90 (ml/min/1.73m^2) vs. 2.07 (1.84; 2.37) ng/ml in eGFR>=90ml/min/1.73m^2, p<0.001]. Platelet expression of CXCR6 also showed slight changes among the compared eGFR subgroups [median MFI (25th; 75th percentile) 41.10 (31.58; 51.25) in eGFR<=30ml/min/1.73m^2 vs. 45.05 (36.00; 54.80) in 30<eGFR<=60 (ml/min/1.73m^2) vs. 41.43 (33.44; 49.35) in 60<eGFR<90 (ml/min/1.73m^2) vs. 34.70 (28.75; 48.71) in eGFR>=90ml/min/1.73m^2, p=0.031], but the impact was not as evident as that observed for sCXCL16. In contrast, platelet SR-PSOX/CXCL16 showed no significant differences (p>0.05) among the compared eGFR subgroups [median SR-PSOX/CXCL16 MFI (25th; 75th percentile) 54.15 (33.43; 67.40) in eGFR<=30ml/min/1.73m^2 vs. 49.15 (42.70; 63.35) in 30<eGFR<=60 (ml/min/1.73m^2) vs. 50.33 (39.33; 59.11) in 60<eGFR<90 (ml/min/1.73m^2) vs. 42.78 (34.94; 55.80) in eGFR>=90ml/min/1.73m^2] (**Figure 10B**).

Diabetic patients in the current cohort (30.6% of CCS, and 26.4% of ACS patients) showed marginally but significantly (p=0.023) elevated levels of sCXCL16 [median (25th; 75th percentile) 2.18 (1.86; 2.43) ng/ml for w/o diabetes vs. 2.31 (1.96; 2.88) ng/ml for w/diabetes], confirming previous reports [133, 134], but exhibited no influence (p>0.05) on platelet SR-PSOX/CXCL16 [median MFI (25th; 75th percentile) 47.95 (38.50; 57.38) for w/o diabetes vs. 46.95 (38.60;

63.24) for w/diabetes] or CXCR6 [median MFI (25th; 75th percentile) 40.10 (34.28; 49.45) for w/o diabetes vs. 42.78 (34.94; 53.53) for w/diabetes] (**Figure 10C**).
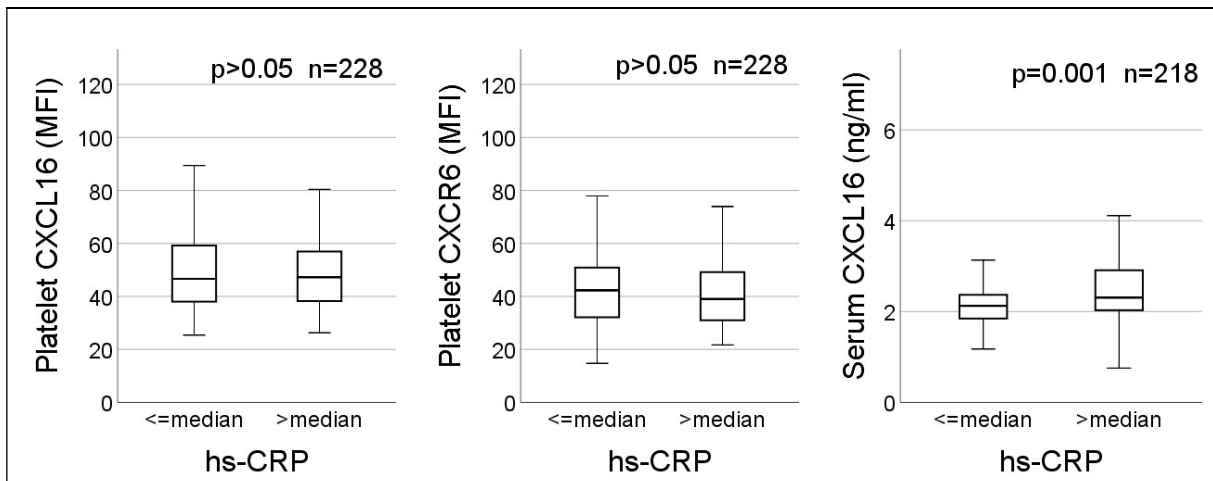
Figure 10A
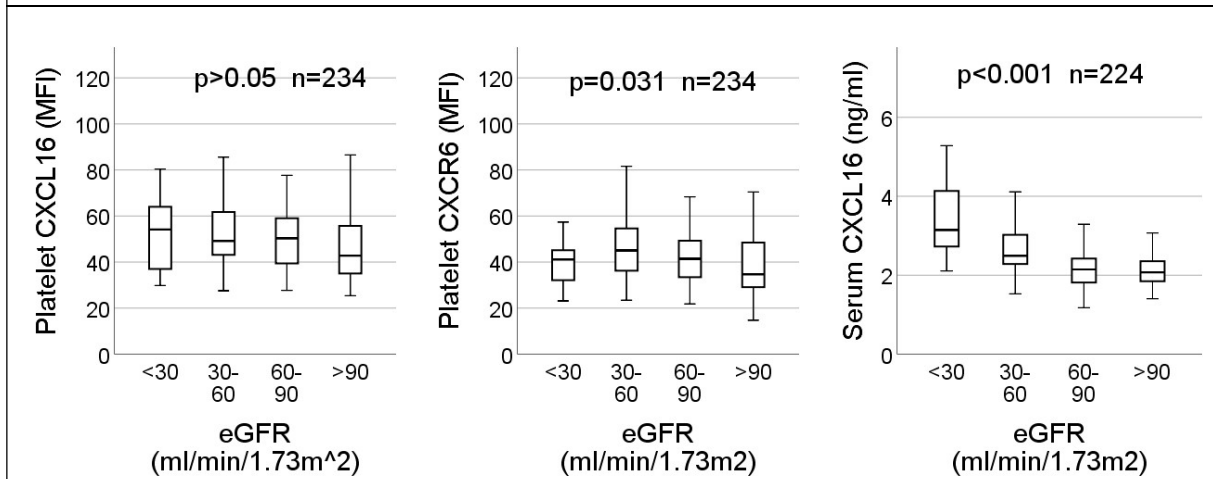
Figure 10B

Figure 10C

Figure 10A-10C. Expression of platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, serum levels of sCXCL16 in the different subgroups of CRP (10A), eGFR (10B), diabetes (10C). Data represent median with 95% CI and statistical significance calculated by Mann-Whitney U test. p: level of significance; MFI: mean fluorescence intensity.

We also verified the influence of age and NT-proBNP on platelet SR-PSOX/CXCL16, CXCR6, and serum sCXCL16. Both platelet CXCR6 MFI and sCXCL16 showed a significant positive correlation with advanced age (CXCR6 ρ=0.150, p=0.020 & sCXCL16 ρ=0.251, p<0.001). However, platelet SR-PSOX/CXCL16 MFI showed no evident correlation with age (ρ=0.018, p>0.05) (**Figure 10D**).

Platelet SR-PSOX/CXCL16 MFI, platelet CXCR6 MFI and sCXCL16 showed a moderate and significant positive correlation with NT-proBNP (ρ=0.495, p=0.027 & ρ=0.466, p=0.038 & ρ=0.667, p=0.002) (**Figure10E**).

Figure 10D

Figure 10E

Figure 10D & 10E. Correlation of age (10D), NT-proBNP (10E) with platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, serum levels of sCXCL16. Correlations were assessed by Spearman's rank correlation coefficient (ρ); p: level of significance; MFI: mean fluorescence intensity.

3.7. Differential influence of platelet SR-PSOX/CXCL16, CXCR6, and serum levels of sCXCL16 on LVEF

Both platelet SR-PSOX/CXCL16 MFI and CXCR6 MFI showed a negative and significant correlation with LVEF in CAD patients [median SR-PSOX/CXCL16 MFI (25th; 75th percentile) 56.95 (41.65; 70.75) in LVEF<40% vs. 44.83 (38.23; 66.21) in 40%<=LVEF<=49%   vs. 48.10 (39.70; 63.50) in 49%<LVEF<=59% vs. 46.70 (37.08; 55.55) in LVEF>59%, p=0.027] & [median CXCR6 MFI (25th; 75th percentile) 44.80 (37.05; 62.55) in LVEF<40% vs. 45.30 (31.94; 60.50) in 40%<=LVEF<=49% vs. 41.98 (33.46; 52.46) in 49%<LVEF<=59% vs. 38.85 (31.19; 47.70) in LVEF>59%, p=0.010] (**Figure 11A**) and the deterioration course of LVEF in ACS patients after 3 years [median SR-PSOX/CXCL16 MFI (25th; 75th percentile) 56.50 (40.00; 71.95) in LVEF decreased group vs. 47.00 (38.50; 57.28) in LVEF non-decreased group, p=0.011] & [median CXCR6 MFI (25th; 75th percentile) 48.00 (32.40; 58.78) in LVEF decreased group vs. 40.63 (32.23; 49.45) in LVEF non-decreased group, p=0.042] (**Figure 11B**).

Serum sCXCL16 showed a significant but modest correlation with LVEF [median (25th; 75th percentile) 2.43 (1.88; 3.10) ng/ml in LVEF<40% vs. 2.30 (2.03; 3.01) ng/ml in 40%<=LVEF<=49% vs. 2.25 (2.01; 2.74) ng/ml in 49%<LVEF<=59% vs. 2.14 (1.81; 2.41) ng/ml in LVEF>59%, p=0.021], but no

significant differences are found between LVEF-follow-up groups [median (25th; 75th percentile) 2.15 (1.95; 2.49) in LVEF decreased group vs. 2.24 (1.91; 2.64) in LVEF non-decreased group, p>0.05] (**Figure 11**).

Figure 11A

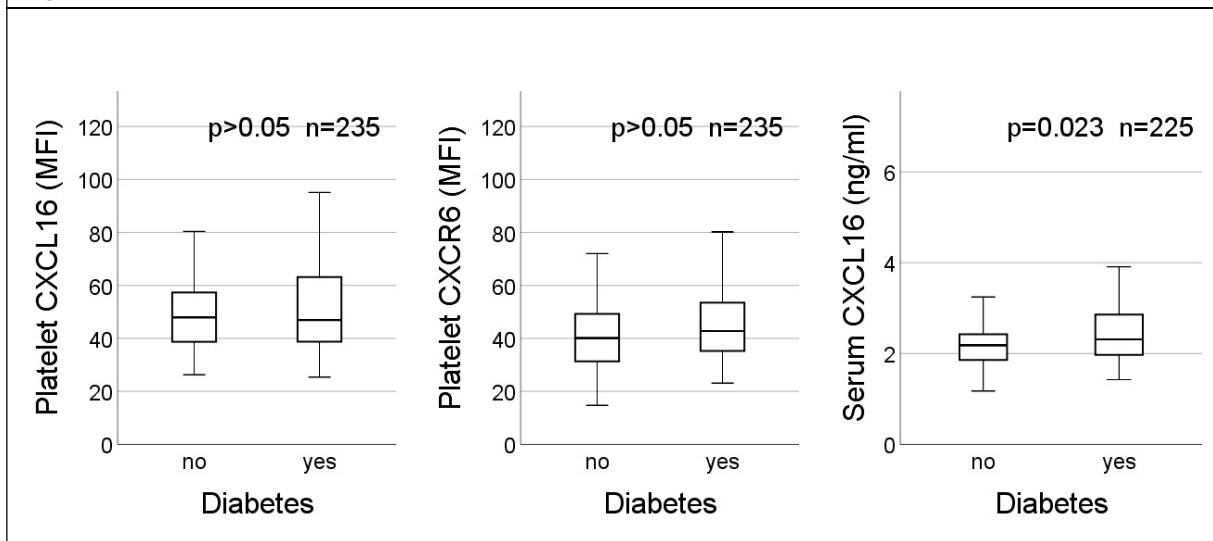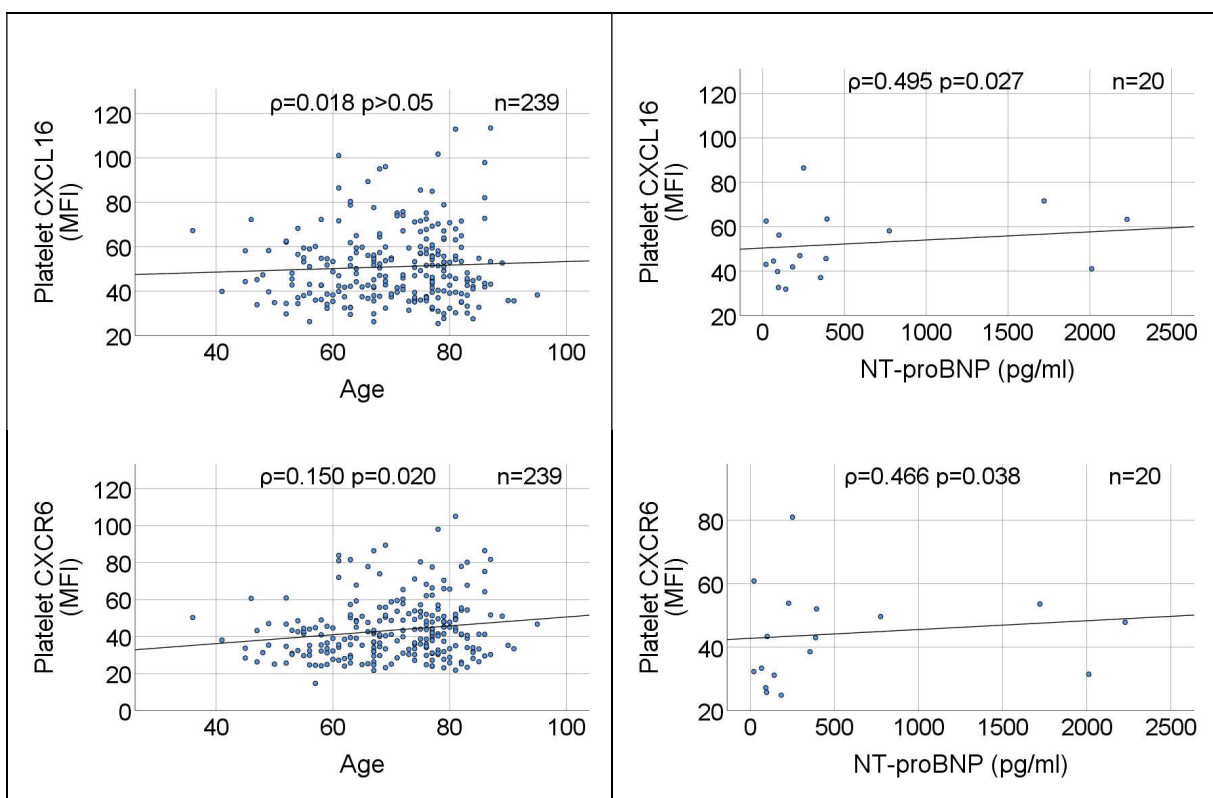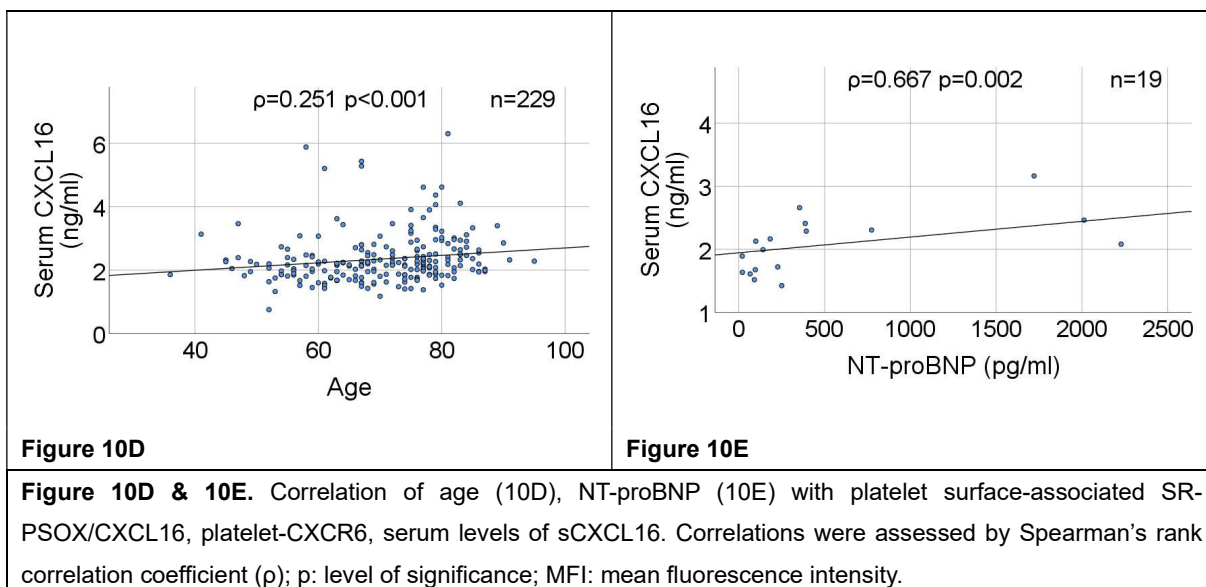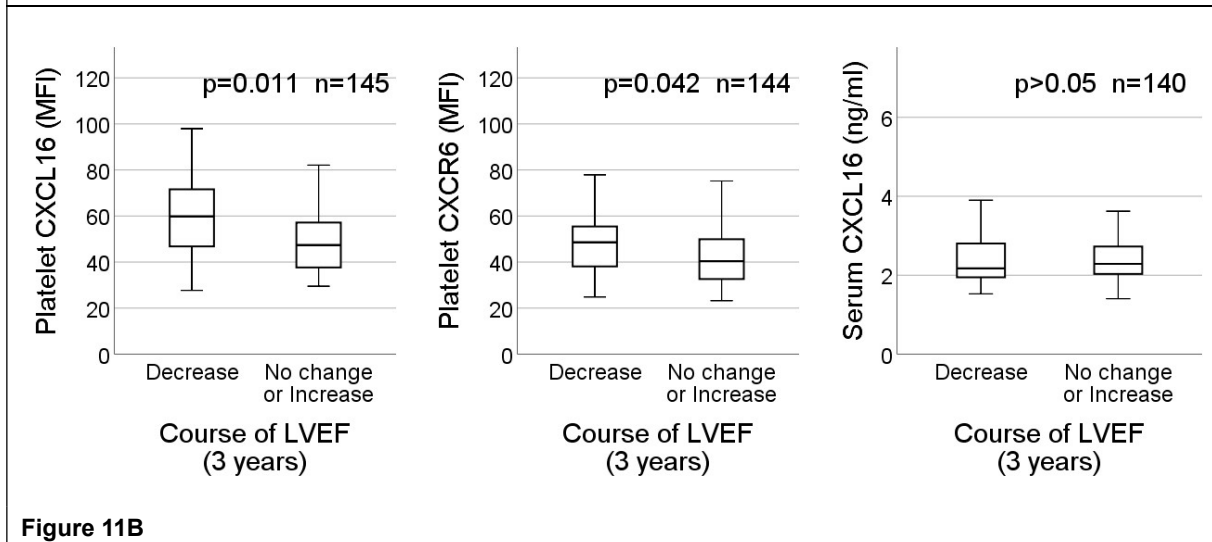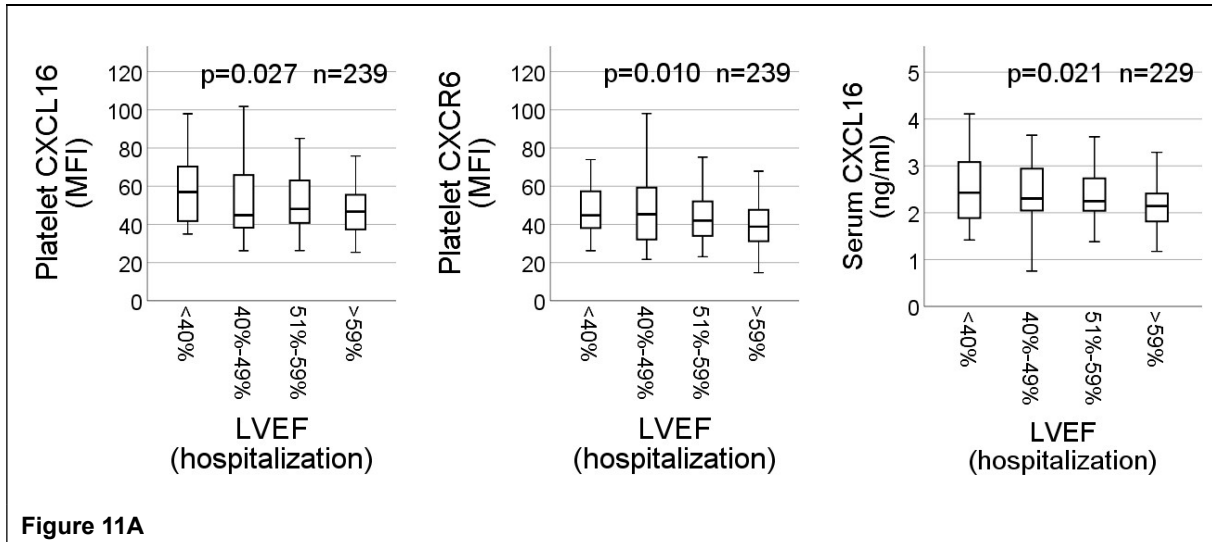**Figure 11B**

Figure 11. Expression of platelet surface-associated SR-PSOX/CXCL16, platelet-CXCR6, serum levels of sCXCL16 in different LVEF subgroups of CAD patients (11A) and LVEF follow-up groups of ACS patients (11B). Data represent median with 95% CI and statistical significance calculated by Mann-Whitney U test. p: level of significance; MFI: mean fluorescence intensity.

3.8. Prognostic implications of platelet SR-PSOX/CXCL16, CXCR6, and serum levels of sCXCL16 in CAD patients

As indicated in the LVEF-follow up section, we evaluated the prognostic role of platelet SR-PSOX/CXCL16, CXCR6, and serum sCXCL16 on all-cause-death in CAD patients (n=217). Higher baseline expression of platelet SR-

PSOX/CXCL16, CXCR6, and serum sCXCL16 was related to a poor prognosis of CAD patients (**Figure 12**).
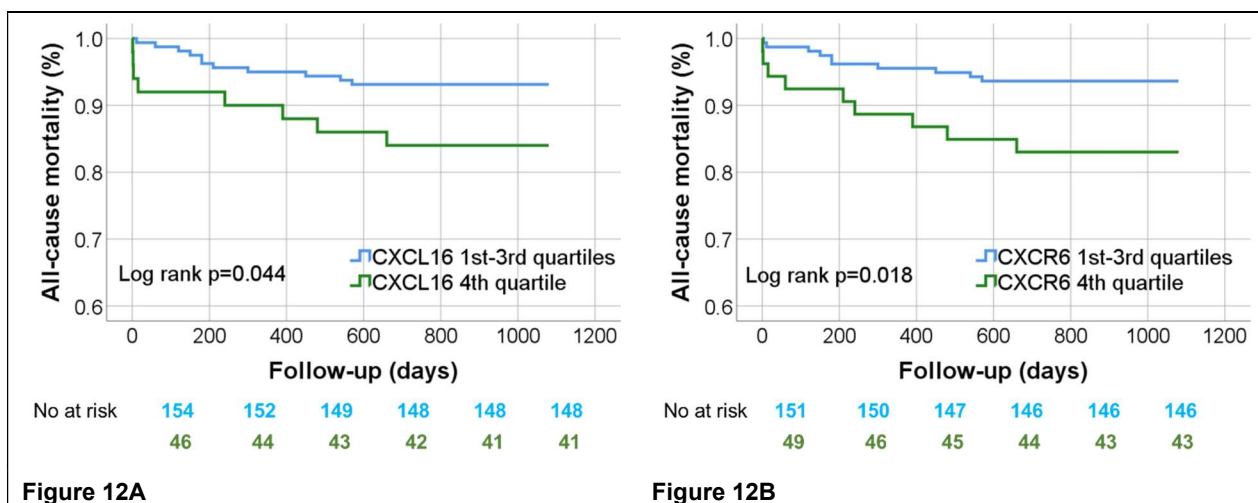
Figure 12A

Figure 12B
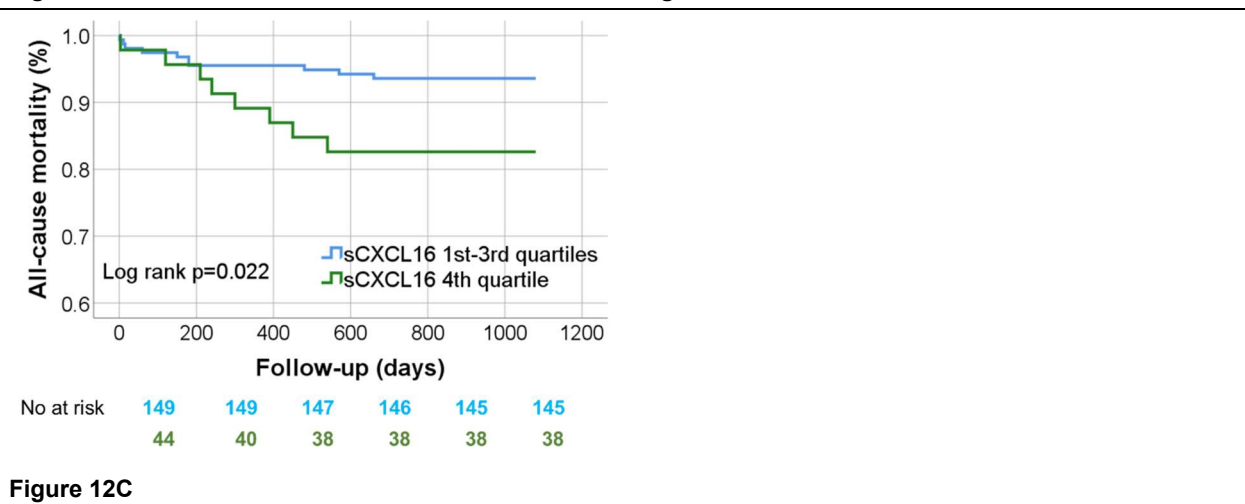
Figure 12C

Figure 12. Kaplan–Meier curves illustrating the likelihood of ACD stratified according to platelet surface-associated SR-PSOX/CXCL16 MFI quartile subgroups (12A), platelet-CXCR6 MFI quartile subgroups (12B), serum levels of sCXCL16 quartile subgroups (12C).   p: level of significance.

3.9. Regression analysis of various factors related to all cause of death (ACD)

The expression of platelet SR-PSOX/CXCL16, platelet CXCR6, and serum levels of sCXCL16 seemed to influence prognosis in CAD patients for all-cause-death. Therefore, regression analysis was employed to seek out the independent factors. We included several cardiovascular risk factors (e.g., age, hypertension, dyslipidemia, diabetes mellitus type II, ROA) in our model. As shown in **Table 4**, besides ages, platelet SR-PSOX/CXCL16 but not CXCR6 or sCXCL16 is an

independent factor influencing prognosis in CAD (p=0.039).

Table 4. Multivariable Cox PH regression analyses with time to all-cause mortality as the independent variable and clinical factors as covariates

Variable	HR (95% CI)	p
Age	1.09 (1.03-1.16)	**0.004**
Arterial hypertension	0.77 (0.21-2.82)	0.693
Dyslipidemia	0.43 (0.17-1.11)	0.082
Diabetes mellitus type II	1.99 (0.79-5.03)	0.144
CCS vs ACS	1.83 (0.52-6.41)	0.345
Platelet CXCR16 (4th quartile vs rest)	2.72 (1.05-7.03)	**0.039**
Variable	**HR (95% CI)**	**p**
Age	1.08 (1.02-1.15)	**0.007**
Arterial hypertension	0.81 (0.22-2.97)	0.810
Dyslipidemia	0.47 (0.18-1.21)	0.117
Diabetes mellitus type II	1.99 (0.78-5.04)	0.148
CCS vs ACS	1.73 (0.49-6.10)	0.488
Platelet CXCR6 (4th quartile vs rest)	2.23 (0.92-5.74)	0.075
Variable	**HR (95% CI)**	**p**
Age	1.09 (1.03-1.16)	**0.005**
Arterial hypertension	0.80 (0.22-2.98)	0.804

Dyslipidemia	0.53 (0.20-1.41)	0.206
Diabetes mellitus type II	1.99 (0.73-5.44)	0.179
CCS vs ACS	1.50 (0.42-5.39)	0.537
sCXCL16 (4th quartile vs rest)	1.45 (0.50-4.17)	0.491

4. Discussion

4.1. Implications for platelet SR-PSOX/CXCL16, CXCR6 and serum levels of sCXCL16 in CAD

Previously we have documented a synergistic impact of recombinant-sCXCL16 on platelet-driven thrombotic response acting through CXCR6 in experimental studies with human (*in vitro*) and murine systems (arterial thrombosis model *in vivo*) [124]. In the current clinical cohort of CAD patients (n=240; CCS n=62; ACS n=178), both SR-PSOX/CXCL16 and CXCR6 are expressed on the platelet surface, and the strong and significant positive correlation of platelet surface expression of SR-PSOX/CXCL16 and platelet CXCR6 suggests that their expression on platelet surface may be driven by the same pathway signals or factors.

The poor correlation between serum sCXCL16 and platelet SR-PSOX/CXCL16 suggested that sCXCL16 in circulation maybe also shed from other circulatory (e.g. T cells, B cells) and vascular cells (e.g. endothelium, smooth muscle cells) apart from activated platelets [108]; moreover, circulatory sCXCL16 levels may not influence the expression of its cognate receptor CXCR6 on platelets.

The significant correlation between mean platelet volume (MPV) and sCXCL16 and the inverse correlation between platelet count and sCXCL16 (Figure 5C) suggest that platelet may be contributing to plasma levels of sCXCL16 and then a fraction of sCXCL16 in the soluble plasma phase (detected with ELISA) may bind back to platelet CXCR6. MPV, a routine measure of platelet size, has been shown to reflect increased platelet activation [139] and inflammation [140], and to be an independent risk factor associated with adverse cardiovascular events following MI [141, 142] and mortality [143]. In our study, both platelet SR-PSOX/CXCL16, CXCR6, and serum sCXCL16 showed significant positive correlations with MPV, which indicate their potential roles in

platelet activation then we explored their relations with the markers of activated platelets: PAC-1 and P-selectin.

4.2. Platelet SR-PSOX/CXCL16, CXCR6, serum levels of sCXCL16 and activation status of circulating platelets

CD62P (P-selectin), which belongs to granule membrane glycoproteins (GP), is primarily stored in α-granules of resting platelets and in the vascular endothelium of tissues such as the heart, liver, lung, colon, and kidney. CD62P is expressed only on the surface of activated platelets or exists as a soluble (sP-selectin) form in plasma.

PAC-1 is a monoclonal antibody that recognizes the activated GPIIbIIIa complex (αIIbβIII-integrin), which is the most abundant platelet membrane glycoprotein, recognizing the fibrinogen (Fg) binding site [144]. PAC-1 is at its peak within 10 seconds after platelets are activated [145] and reflects the early activation of platelets [146, 147], whereas CD62P does not change with time and is thus considered the "golden standard" of platelet activation markers [148].

In the current clinical cohort of CAD patients (n=240; CCS n=62; ACS n=178), we observed a strong correlation of platelet surface-associated SR-PSOX/CXCL16 and platelet CXCR6 surface expression with indicators of circulatory platelet activation, i.e., CD62P surface exposure denoting degranulation, and platelet expression of PAC-1 suggesting αIIbβIII-integrin activation. This validated an association between platelet SR-PSOX/CXCL16-CXCR6 axis and platelet activation in CAD, as platelet expression of SR-PSOX/CXCL16 and CXCR6 were found to correlate with markers of platelet activation. And to our expectation, serum sCXCL16 may also increase as platelet SR-PSOX/CXCL16 is shed upon platelet activation. However, serum sCXCL16, the ligand for platelet CXCR6, did not show any correlation with basal platelet activation in absence of additional *ex vivo* stimulation. This set of results further

exemplifies the hypothesis that only platelet surface-associated CXCL16 may contribute to platelet activation. Studies have suggested SR-PSOX/CXCL16 to act as a receptor of sCXCL16 "inversely" in malignant glioma cells [149] and in cultured human meningioma cells [150]. In this way, a part of sCXCL16 may bind to the up-regulated SP-PSOX/CXCL16 in turn and may not be detected in circulation. This may explain the lack of correlation between sCXCL16 with platelet activation markers.

We further explored collagen induced platelet aggregation *ex vivo*. Patients with relatively enhanced (1st quartile vs 2-4th quartiles) platelet surface-associated SR-PSOX/CXCL16 showed significantly increased aggregation response to collagen (**Figure 6D**), which were not observed with elevated platelet CXCR6 or platelet-free serum sCXCL16 levels. This observation signifies that it is platelet surface-associated SR-PSOX/CXCL16 interacting with CXCR6 that may mediate the pro-thrombotic response [124]. Predictably, the platelet surface association of sCXCL16 is relevant for showing a significant effect on platelet responsiveness i.e. aggregatory response. Nevertheless, elevated levels of circulatory sCXCL16 or SR-PSOX/CXCL16 deposited at atherosclerotic lesions and plaques [95, 129, 130, 151] may exaggerate platelet aggregation by engaging the cognate receptor CXCR6 [129] on the platelet surface, which may not be observed in circulation.

Taken together, this indicates a possibility that the expression of platelet SR-PSOX/CXCL16, CXCR6, and sCXCL16 is related to the platelet activation and may also be a predictor of prognosis in CAD.

4.3. Platelet SR-PSOX/CXCL16, CXCR6, serum levels of sCXCL16 in dyslipidemia

Dyslipidemia which refers to an increase in total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and a decrease in

high-density lipoprotein cholesterol (HDL-C), is one of the major risk factors for ACS [152].

Since the membrane-bound SR-PSOX/CXCL16 is an ox-LDL scavenger receptor, we explored the relationship of platelet SR-PSOX/CXCL16, CXCR6, and sCXCL16 with lipids (TC, TG, LDL, HDL). Contrary to our expectations, neither platelet SR-PSOX/CXCL16 nor platelet CXCR6 correlated with plasma LDL levels. As ox-LDL decreased the expression of SR-PSOX/CXCL16 in human visceral adipocytes [153], the decline of serum sCXCL16 in the LDL>median group suggests that sCXCL16 in circulation is mainly converted from SR-PSOX/CXCL16 in other cells (except for platelets), and that there is an interaction of LDL with sCXCL16 and membrane-bound SR-PSOX/CXCL16.

Moreover, we observed decreased expression of platelet SR-PSOX/CXCL16 and CXCR6 in the higher HDL group, along with the decrease of platelet activation markers (PAC-1, CD62P). HDL has been suggested to reduce the aggregation and activation of platelets [154, 155], which also indicates platelet expression of SR-PSOX/CXCL16 and CXCR6 have interaction with platelet activation.

Combined with what we have discussed above, platelet expression of SR-PSOX/CXCL16 and CXCR6 seem to be related to the activation status of platelets instead of dyslipidemia, TC, TG or LDL.

4.4. Association between platelet SR-PSOX/CXCL16, CXCR6, serum levels of sCXCL16 with disease severity in CAD patients

Both TnI and CK are cardiac injury markers, and CK can be detected in someone with a heart attack within 3–6 hours after the onset of acute chest pain, peaks by 24 hours and normalizes within 48 to 72 hours. However, TnI is elevated in 2-4 hours after myocardial injury, peaks in 24 to 48 hours, and stays elevated for 1-2 weeks. As the timing of blood sample collection from patients after ACS

affects the value of CK and TnI, in this study, we analyzed peak CK and TnI while hospitalization to evaluate the severity of ACS.

Platelet expression of SR-PSOX/CXCL16 and CXCR6 increased significantly in the higher CK group compared with the normal CK group, but showed no differences between the higher TnI group and normal TnI group. Interestingly, only serum levels of sCXCL16 showed a significant positive correlation with ACS compared to CCS which is consistent with previous studies, and elevated significantly in the higher TnI group as compared to the normal TnI group. CK is stored in the brain, myocardium, and skeletal muscle [156] [157]. Elevation of CK is associated with inflammation/damage of these tissues and is more sensitive to acute hypoxia [158]. Hypoxia may induce platelet activation [159], which may explain the significant association of SR-PSOX/CXCL16, CXCR6, and CK. TnI only elevated in serum after the damage of myocardial cells, is a more sensitive and specific ACS marker compared to CK [160]. Cardiac troponin is the recommended cardiac biomarker to diagnose myocardial infarction (Table 2) and has been proven to have higher sensitivity and specificity than CK in stratifying the risk of ACS [161]. However, elevated cardiac troponin concentrations in patients with chronic renal failure without any signs of cardiovascular disease have often been observed in clinical practice [162], and the mechanism is still unclear.

Therefore, sCXCL16 is considered as a potential marker of disease severity for ACS because of its significant association with TnI. However, neither platelet SR-PSOX/CXCL16 nor CXCR6 fulfills this criterion. Because of their significant positive correlation with CK, platelet SR-PSOX/CXCL16 and CXCR6 seem to be associated with the severity of systemic hypoxia instead.

4.5. Platelet SR-PSOX/CXCL16, CXCR6, serum levels of sCXCL16 are affected by P2Y12 antagonists

We also compared the expressions of platelet SR-PSOX/CXCL16, CXCR6, serum levels of sCXCL16, platelet PAC-1, and platelet CD62P in different groups of patients receiving different P2Y12 antagonists. The P2Y12 receptor belongs to the G protein-coupled purinergic receptor family and is distributed on the surface of platelets. As ADP binds to the P2Y12 receptor, PI3K is activated, thereby resulting in platelet aggregation and thrombus formation. Both ticagrelor and clopidogrel are P2Y12 receptor antagonists and are applied as anti-platelet medications in clinics. Ticagrelor binds to the P2Y12 receptor without metabolic activation and takes effect immediately after oral administration [163]. However, clopidogrel is a prodrug that needs to undergo a metabolic two-step bioactivation for its pharmacological efficacy, which takes 2-8 hours to take effect.

We assumed CD62P and PAC1 would decrease in P2Y12 antagonists (ticagrelor, clopidogrel) administered groups because of their antiplatelet effect. However, in our study, none of the P2Y12 antagonists showed any significant impact on platelet SR-PSOX/CXCL16 or CXCR6 surface association/expression. Similarly, the markers of platelet activation, CD62P, and PAC1, showed no differences in P2Y12 antagonist administered groups. This is not surprising as we had no chance to measure the changes of the parameters before and after the administration of P2Y12 antagonists in the same patients.

However, we found that patients administered with ticagrelor showed significantly reduced levels of serum sCXCL16. Ticagrelor has demonstrated its superiority in reducing vascular inflammatory reactions and stabilizing endothelial function due to its direct effect on P2Y12 receptors [164, 165], thereby further suppressing the release of inflammatory factors. Li et al. found that ticagrelor may better decrease inflammatory factors such as IL-6, CRP, and endothelial cell-specific molecule 1 [166], and it may also exert anti-inflammatory, anti-

arteriosclerotic, anti-fibrotic, and cardiac protective effects by increasing the concentration of ADP in the blood [167].

This might be indicative of the possibility that antiplatelet medications may reduce platelet-derived sCXCL16 contributing to circulatory levels via reducing inflammation. Thus, the correlation between sCXCL16 and ticagrelor may further suggest that sCXCL16 is an inflammatory factor.

4.6. Platelet SR-PSOX/CXCL16, CXCR6, serum levels of sCXCL16 and risk factors in CAD patients

Then we explored the relationship of platelet SR-PSOX/CXCL16, CXCR6 sCXCL16, and risk factors (diabetes, age, CRP, eGFR, and NT-proBNP) which have a strong correlation with inflammation and poor prognosis in CAD patients.

It is widely recognized that advancing age and diabetes are both major risk factors for the development and poor prognosis of CVD. The pathological mechanisms of these factors are extremely complex. However, two main mechanisms are included: oxidative stress and inflammatory processes [168, 169]. Only serum levels of sCXCL16 showed a significant correlation with both of these factors, which suggested sCXCL16 to be involved in the complicated processes of oxidative stress and inflammation. Studies confirmed that sCXCL16 guides the migration of activated T cells into inflamed tissue, promote matrix degradation [151] and ROS production [170]. Meanwhile, sCXCL16 also interacts with other inflammatory cells to promote myocardial fibrosis through enhanced MMPs degradation, which is closely related to the development of inflammatory cardiomyopathy and heart failure [171, 172].

As everything mentioned above indicates an inflammatory role of sCXCL16, we further explored the relationship between sCXCL16 and hs-CRP. CRP, which is a commonly recognized indicator of inflammation, serves as a predictive factor for ACS and is of great importance in the prediction of the stability of AS plaques,

the severity of vascular diseases, and the occurrence of cardiovascular events in CAD [173, 174]. Levels of circulating CRP levels in serum ≥3 µg/mL are unspecific markers for inflammation, infection, and tissue injury with acute-phase response in the clinic [175]. Circulating CRP, which is released to the circulation after inflammatory stimuli, dissociates irreversibly into non-soluble monomers CRP (mCRP) involved in activating the complement cascade [176], angiogenesis [176], and thrombosis [177]. Circulating CRP has no involvement in thrombogenesis, whereas mCRP, only detectable in the vessel wall [178], can promote thrombosis by inducing platelet activation [179], platelet adhesion by upregulating P-selectin [157], and thrombus growth [180]. As our results show, sCXCL16 but not platelet SR-PSOX/CXCL16 or CXCR6, highly correlated with hs-CRP, confirming previous studies that the level of sCXCL16 in patients with rheumatoid arthritis is proportional to the degree of joint injury [181], and may be downregulated by anti-inflammatory therapies (e.g. aspirin, NF-κB blocker, PPAR-γ agonist) [127]. This is in accordance with the fact that sCXCL16 is an inflammatory chemokine and therefore may be influenced by the inflammatory status of patients. In contrast, neither platelet SR-PSOX/CXCL16 nor CXCR6 showed a significant correlation with circulating hs-CRP suggesting a poor relationship of platelet SR-PSOX/CXCL16 and CXCR6 with inflammation.

Glomerular Filtration Rate (eGFR) is widely used to evaluate renal function. It has been shown that cardiovascular mortality was twice as high in patients with eGFR (30–59mL/min/1.73m^2) and three times higher with lower eGFR(15–29mL/min/1.73m^2) when compared to those with normal renal function. eGFR<60mL/min/1.73m^2 was also associated with an increased risk of CHF, stroke, peripheral artery disease, coronary heart disease, and atrial fibrillation [182]. Patients with lower eGFR present with a varying degree of inflammation [183], which leads to the elevation of sCXCL16 in the circulation. Moreover, as sCXCL16 showed a significant negative correlation with eGFR which leads to an

elevation of TnI, the role of sCXCL16 in evaluating the severity of ACS is restricted.

NT-proBNP in circulation is a sensitive and specific marker for the identification of congestive heart failure patients presenting with acute dyspnea to the emergency department [184]. Heart failure (HF) happens when the heart fails to pump sufficiently oxygenated blood to meet the metabolic needs of body tissues [184]. NT-proBNP is also well known as a marker for systematic inflammation [185], and and correlates significantly with CRP concentration in patients with heart failure [186]. It is not surprising that serum sCXCL16 showed a strong correlation with NT-proBNP in our study, which further indicated a proinflammatory role of serum sCXCL16.

Patients with chronic heart failure (CHF) and AHF present with hypoxia, enhanced platelet activation and aggregation, along with increasing MPV and the expression of platelet-derived adhesion molecules (platelet/endothelial cell adhesion molecule-1, PECAM-1) [187-191]. Elevation of P-selectin is correlated with the severity of AHF[192]. Platelet SR-PSOX/CXCL16, CXCR6 which had no correlation with hs-CRP, however, showed significant relationships of with NT-proBNP. This further suggests the platelet expression of SR-PSOX/CXCL16 and CXCR6 is involved in the pathway of platelet activation and also indicate their potential roles in evaluating HF.

4.7. Platelet SR-PSOX/CXCL16, CXCR6, serum levels of sCXCL16 and LVEF

Platelets remain activated long after clinical stabilization post ACS [193], and abnormal platelet activation has been proven to correlate with associated comorbidities (re-thrombosis) in CHF and poor prognosis in acute congestive heart failure [191,192]. Although we had no chance to reevaluate the parameters after a 3-year follow-up period, platelet expression of SR-PSOX/CXCL16, CXCR6 at baseline were decreased significantly in CAD patients

with better LVEF during the hospital stay and better 3-year LVEF recovery after ACS in the present study. Combined with the significant positive relationship of SR-PSOX/CXCL16, CXCR6 with CK and NT-proBNP in our study, these indicate that expression of platelet SR-PSOX/CXCL16, CXCR6 may be potential clinical markers of AHF, and may predict LVEF recovery after ACS.

In contrast, serum CXCL16 only showed slight differences between different LVEF subgroups during the hospital stay in CAD patients, but failed to show any differences in the 3-year-course of LVEF after ACS. This may suggest serum sCXCL16 to be a sensitive inflammatory marker in CAD at baseline, but not a specific marker of progressively deteriorated LVEF after ACS, although its prognostic role in predicting all-cause death and cardiovascular death after ACS has been shown in several studies [129, 135] and our study **(Figure 12C)**.

4.8. Platelet SR-PSOX/CXCL16, CXCR6, serum levels of sCXCL16 and prognosis

Kaplan-Meier analysis showed a significant relationship between the expression of platelet SR-PSOX/CXCL16, platelet CXCR6, soluble CXCL16, and ACD following three years of the follow-up period. Based on multivariable analyses, after adjusting for hypertension, diabetes, and dyslipidemia, platelet SR-PSOX/CXCL16 remained an independent prognostic factor for ACD besides age, which may also suggest that platelet SR-PSOX/CXCL16 is a specific long-term prognosis marker in CAD.

Taken together, platelet expression of SR-PSOX/CXCL16, CXCR6 may represent independent markers for platelet activation, and platelet SR-PSOX/CXCL16-CXCR6 axis may influence thrombotic propensity and thereby prognosis specifically for long-term survival in CAD patients.

5. Summary

Background: CAD has affected 200 million people worldwide, causing more than 9 million deaths and being the leading cause of human death. CAD is classified as CCS and ACS according to different clinical situations. Platelets and chemokines play a vital role in AS which is responsible for the progression of CAD. Platelets express the transmembrane chemokine SR-PSOX/CXCL16, which is a scavenger for the atherogenic mediator ox-LDL, and phosphatidylserine exposed on the surface of apoptotic cells and procoagulant platelets. Proteolytic cleavage of its extracellular domain by metalloprotease ADAM10 generates the soluble-(s) chemokine sCXCL16, which engages CXCR6 on platelets to synergistically propagate degranulation, aggregation, and thrombotic response.

Objectives: We assessed the pathophysiological and prognostic association of platelet SR-PSOX/CXCL16-CXCR6 axis in CAD patients.

Methods: Platelet surface-associated SR-PSOX/CXCL16 and CXCR6, platelet activation markers CD62P (degranulation from α-granules), and PAC-1 binding (αIIbβ3-integrin activation) were evaluated by flow cytometry in n=240 consecutive patients with symptomatic CAD (CCS n=62; ACS n=178) at baseline upon admission. Blood samples were collected during PCI. sCXCL16 was evaluated by ELISA in serum samples. The course of left ventricular ejection fraction (LVEF) was followed up during intrahospital stay in CAD patients and at 3 years in ACS patients. All patients enrolled in the study were followed up for all-cause death (ACD).

Results: Currently, we observed a strong correlation of platelet surface-associated SR-PSOX/CXCL16, and surface-expressed CXCR6 with markers of circulatory (basal) platelet activation i.e. CD62P surface exposure, and PAC-1 binding, which validated an association between platelet SR-PSOX/CXCL16-CXCR6 axis and thrombotic response in CAD patients. Patients with relatively

enhanced (1st quartile vs 2nd-4th quartiles) platelet surface-associated SR-PSOX/CXCL16 showed significantly increased aggregation response to collagen assessed by whole blood impedance aggregometry. Platelet SR-PSOX/CXCL16 and CXCR6 expression did not alter with dyslipidemia, triglyceride, total cholesterol, or LDL levels. Patients with higher (>median) plasma HDL levels showed significantly decreased platelet SR-PSOX/CXCL16 and CXCR6 expression. HDL is associated with reduced platelet hyper-reactivity. Serum sCXCL16 showed a stronger positive correlation with deteriorated eGFR, age, and NT-proBNP when compared with platelet SR-PSOX/CXCL16 and CXCR6. Platelet surface-associated SR-PSOX/CXCL16 and CXCR6 were not significantly altered with disease severity in ACS vs CCS patients, in contrast to serum sCXCL16 levels which were significantly elevated in ACS patients confirming previous reports. Although platelet SR-PSOX/CXCL16 and CXCR6 expression did not change significantly with troponin I levels, with hs-CRP, or with diabetes, they corresponded with higher Creatine Kinase-(CK) activity and deteriorated LVEF upon admission and over a 3-year follow-up of LVEF. P2Y12-antagonists did not influence platelet SR-PSOX/CXCL16 or CXCR6 surface expression, but ticagrelor was associated with significantly reduced serum sCXCL16, suggesting an impediment on activated platelet-derived circulatory levels of sCXCL16. Finally, elevated (4th quartile) platelet SR-PSOX/CXCL16 and CXCR6 measured upon admission were significantly associated with ACD.

Conclusion: Platelet expression of SR-PSOX/CXCL16 and CXCR6 may be markers of platelet activation and platelet SR-PSOX/CXCL16-CXCR6 axis may influence thrombotic propensity and thereby prognosis specifically for long-term survival in CAD patients.

Zusammenfassung

Hintergrund: Weltweit leiden 200 Millionen Menschen an einer KHK. Diese verursacht mehr als 9 Millionen Todesfälle jährlich und stellt damit die häufigste Todesursache beim Menschen dar. Die KHK kann je nach klinischer Situation in CCS und ACS unterteilt werden. Thrombozyten und Chemokine spielen eine wichtige Rolle bei der Entstehung der Atherosklerose, die für das Fortschreiten der KHK verantwortlich ist. Thrombozyten exprimieren das Transmembran-Chemokin SR-PSOX/CXCL16, das ein „Scavenger" für den atherogenen Mediator oxLDL ist, und Phosphatidylserin, das auf der Oberfläche apoptotischer Zellen und prokoagulierender Thrombozyten exponiert ist. Durch proteolytische Spaltung seiner extrazellulären Domäne durch die Metalloprotease ADAM10 entsteht das lösliche Chemokin sCXCL16, das an CXCR6 auf Thrombozyten bindet, um Degranulation, Aggregation und thrombotische Reaktion synergistisch zu fördern.

Zielsetzungen: Wir untersuchten pathophysiologische und prognostische Implikationen der SR-PSOX/CXCL16-CXCR6-Achse bei KHK-Patienten.

Methoden: Die auf der Thrombozytenoberfläche exprimierten Marker SR-PSOX/CXCL16 und CXCR6, die CD62P (Degranulation aus α-Granula) und die PAC-1 (αIIbβ3-Integrin-Aktivierung) wurden bei n=240 konsekutiven Patienten mit symptomatischer KHK (CCS n=62; ACS n=178) zu Studieneinschluss mittels Durchflusszytometrie untersucht. Blutproben wurden während der PTCA entnommen. sCXCL16 wurde mittels ELISA in Serumproben untersucht. Die linksventrikuläre Auswurffraktion (LVEF) wurde bei KHK-Patienten während des Krankenhausaufenthalts und bei ACS-Patienten nach 3 Jahren bestimmt. Alle Patienten wurden für 3-Jahre auf den Endpunkt Gesamtmortalität nachverfolgt.

Ergebnisse: Wir beobachteten eine starke Korrelation von auf der Thrombozytenoberfläche exprimierten SR-PSOX/CXCL16 und CXCR6 mit Markern der zirkulierenden (basalen) Thrombozytenaktivierung, d. h. CD62P-

Oberflächenexposition und PAC-1-Bindung, was einen Zusammenhang zwischen der thrombozytären SR-PSOX/CXCL16-CXCR6-Achse und der thrombotischen Reaktion bei KHK-Patienten nahelegt. Patienten miterhöhter Thrombozytenoberflächen-assoziierter SR-PSOX/CXCL16 Expression zeigten eine signifikant erhöhte Aggregationsreaktion auf Kollagen, die mittels Vollblut-Impedanzaggregometrie gemessen wurde. Die Expression von SR-PSOX/CXCL16 und CXCR6 in den Blutplättchen änderte sich nicht in Abhängigkeit von Dyslipidämie, Triglycerid-, Gesamtcholesterin- oder LDL-Spiegel. Bei Patienten mit höherenHDL-Plasmaspiegeln war die Expression von SR-PSOX/CXCL16 und CXCR6 in den Blutplättchen deutlich geringer. HDL wird mit einer geringeren Hyperreaktivität der Blutplättchen in Verbindung gebracht. CXCL16 im Serum wies im Vergleich zu Thrombozyten- SR-PSOX/CXCL16 und CXCR6 eine stärkere positive Korrelation mit verschlechterter eGFR, Alter   und NT-proBNP auf. Thrombozytenoberflächen-assoziiertes- SR-PSOX/CXCL16 und CXCR6 waren bei ACS-Patienten im Vergleich zu Patienten mit CCS nicht signifikant verändert, im Gegensatz zu den sCXCL16-Spiegeln im Serum, die bei ACS-Patienten signifikant erhöht waren, was frühere Berichte bestätigt. Obwohl sich die Expression von Thrombozyten- SR-PSOX/CXCL16 und CXCR6 nicht signifikant mit den Troponin-I-Spiegeln, mit hsCRP oder mit Diabetes änderte, korrespondierten sie mit einer höheren Kreatinkinase-(CK)-Aktivität und einer schlechteren LVEF bei Aufnahme und zum Zeitpunkt der 3-Jahres-Nachbeobachtung der LVEF. P2Y12-Antagonisten hatten keinen Einfluss auf die Oberflächenexpression von Thrombozyten- SR-PSOX/CXCL16 oder CXCR6, aber Ticagrelor war mit einer signifikanten Verringerung von sCXCL16 im Serum assoziiert, was auf eine Beeinträchtigung der von aktivierten Thrombozyten stammenden zirkulierenden sCXCL16-Spiegel schließen lässt. Schließlich waren erhöhte Thrombozyten- SR-PSOX/CXCL16-und CXCR6-Spiegel, die bei der Aufnahme gemessen wurden, signifikant mit der Gesamtmortalität assoziiert.

Schlussfolgerung: Die Expression von SR-PSOX/CXCL16 und CXCR6 auf Thrombozyten kann ein Marker für die Thrombozytenaktivierung sein, und die thrombozytäre SR-PSOX/CXCL16-CXCR6-Achse kann möglicherweise das thromboembolische Risiko und damit die Prognose speziell für das Langzeitüberleben von KHK-Patienten beeinflussen.

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7. Declaration of contributions

The study was conducted in the Department of Internal Medicine III - Cardiology and Angiology - German Heart Competence Centre Tübingen under the supervision of PD Dr. med. Dominik Rath and Dr. Madhumita Chatterjee.

- The study design, identification and recruitment of patients were carried out in collaboration with Dr. Madhumita Chatterjee and co-ordinated by PD Dr. med. D. Rath.

- Sample acquisition for the study cohort of healthy volunteers, CCS and STEMI patients was performed as part of the TuePIC cohort DFG-KFO-274 during the period 2015-2017.

- Isolation of sample material (serum isolation) and flow cytometry measurements were performed by Ms Lydia Laptev (medical-technical assistant) using standardized laboratory protocol set by Dr. Chatterjee.

-ELISA for serum sCXCL16 levels was performed by Frederic Emschermann.

-Analysis of flow cytometry data for the clinical cohort, assessment of clinical criteria, statistical evaluation of all data sets was done by me under the supervision of Dr. Chatterjee (flow cytometry, ELISA) and Dr. Rath (clinical characteristics).

-Collection of patients data for prognostic analysis was conducted by telephonic interviews and from clinical records by by Dr. Patrick GrogaBada. Statistical evaluation of prognostic association was done by me under the supervision of PD Dr. med. D. Rath.

-Preparation of all data sets, graphical presentation for the thesis and the manuscript (submitted) was done by me under the supervision of Dr. Chatterjee. Manuscript writing, presentation of data (poster titled `Platelet SR-PSOX/CXCL16 and CXCR6 axis influences thrombotic propensity and prognosis

in Coronary Artery Disease`) at the Atherothrombosis Winterschool 2022 (Organized by AG19 of Deutsche Gesellschaft für Kardiologie-DGK) was done by me under the supervision of Dr. Chatterjee.

I certify that I have written the dissertation independently according to the instructions of PD Dr. med. Dominik Rath and Dr. Madhumita Chatterjee and that I have not used any sources other than those indicated.

Tuebingen, den 18.05.2022

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