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Detection of oxLDL levels in platelets and its relation to CXCR4 and CXCR7 receptors surface expression in patients with coronary artery disease

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1. Abbreviations

ACE inhibitors	Angiotensin-converting enzyme inhibitors
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ASA	Acetylsalicylic acid
AT II	Angiotensin II receptor antagonists
BMI	Body mass index
CAD	Coronary artery disease
CD36	Cluster of differentiation 36
CHD	Coronary heart disease
CK-MB	Creatine Kinase muscle/brain
CMP	Common megakaryocytes -erythroid progenitor
CPDA	Citrate-phosphate-dextrose-adenine
CRP	Collagen-related peptide
CV	Cardiovascular
CyPA-PPIase	Cyclophilin A peptidylproline isomerase
DNA	Deoxyribonucleic acid
ECG	Electrocardiography
EPCs	Endothelial progenitor cells
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FSC	Forward scatter small angel
HDL	High-density lipoproteins
HIF-1 α	Hypoxia-inducible factor 1-alpha
i-MK	Immature megakaryocytes
IDL	Intermediated density lipoproteins
IHD	Ischemic heart diseases
LAD	Left anterior descending artery
LCX	Left circumflex

LDL	Low-density lipoproteins
LM	Left main coronary artery
MFI	Mean fluorescence intensity
MK-p	Megakaryocytes progenitor
MK	Megakaryocytes
NHLBI	National Heart, Lung, and Blood Institute
NO	Nitric oxide
NSTEMI	Non-ST-segment elevation myocardial infarction
OxLDL	Oxidized low-density lipoprotein
PAR-1	Protease-activated receptors-1
PBS	Phosphate buffered saline
PBSF	Pre-B-cell growth-stimulating factor
PCI	Percutaneous coronary intervention
PE	Phycoerythrin
PMA	Platelet-monocyte aggregates
PRP	Platelet-rich plasma
RMP	Renal multipotent progenitor cells
SAP	Stable angina pectoris
SCC	Side scatter
SDF-1	Stromal cell-derived factor 1
SR	Scavenger receptors
STEMI	ST-segment elevation myocardial infarction
TPO	Thrombopoietin
TRAP	Thrombin receptor agonist peptide
VLDL	Very low-density lipoproteins
vWf	Von Willebrand factor

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4. Introduction

4.1. Coronary artery disease

4.1.1. Definition of coronary artery disease

Coronary artery disease (CAD), also known as coronary heart diseases (CHD), is caused mainly by the pathophysiological development of atherosclerosis in the coronary arteries, leading to narrowing of coronary arteries, which supply the heart with oxygen. (1) The atherosclerotic plaques accumulate within the coronary artery lumen resulting in myocardial blood flow impairment and artery lumen dynamic narrowing. (2) In most cases, the coronary blood flow cutback results in symptomatic CAD, during physical exertion or rest condition might trigger unexpected cardiac death or myocardial infarction. This is dependent on the arterial blockage's progression and severity. (3) Turning of smooth and elastic lumen into narrow and rigid one causes lumen obstruction and reduces the flow of blood through the coronary artery. This in turn results in deficiency in nutrient and oxygen supply that play a critical role in heart muscle. (4) A group of diseases like ST-elevation myocardial infarction, sudden coronary death, stable angina, non-ST-elevation myocardial infarction and unstable angina is referred to as CAD and is the world's main cause of death. (4, 5, 6)

4.1.2. Pathophysiology of CAD

The recent evidence strongly proposes that coronary artery disease is a result of vessel occlusion through lipid deposition; it is also a presentation of a chronic inflammatory response to infection or injury. (3) Moreover, it is considered as a cholesterol storage disease, since a strong relation was seen between elevated plasma cholesterol level and coronary heart disease as an important risk factor for CHD. (7) Atherogenesis is currently understood as a complex interplay of risk factors inclusive of artery wall cells and the blood and its molecular messages which they interchange. (7, 8) Thus, the process of atherosclerosis considers the fundamental pathophysiologic mechanism that begins CHD, which advances for decades, preceding the acute events such as acute coronary syndrome. (8) This process is accelerated by well-known cardiovascular risk factors like arterial hypertension, smoking, diabetes, high cholesterol, and genetics. (9) Moreover, it was shown that inflammation plays a significant role in atherogenesis and it also takes

part in local, systemic and myocardial complications of atherosclerosis. (8) (Figure 1) When the arterial endothelium comes in close contact with bacterial products or various components of cardiovascular risk factors, this leads to increase expression of adhesion molecules by these components, which enhance the adherence of blood leukocytes to the internal surface of the arterial wall. The components of cardiovascular risk factors include vasoconstrictor hormones involved in hypertension, dyslipidemia, the outcome of glycoxidation affiliated to hyperglycemia or proinflammatory cytokines acquired from excess adipose tissue. (9) The expression of chemoattractant cytokines significantly controls the transmigration of the adherent leukocytes, which is managed by signals related to atherosclerosis risk factors. (10)

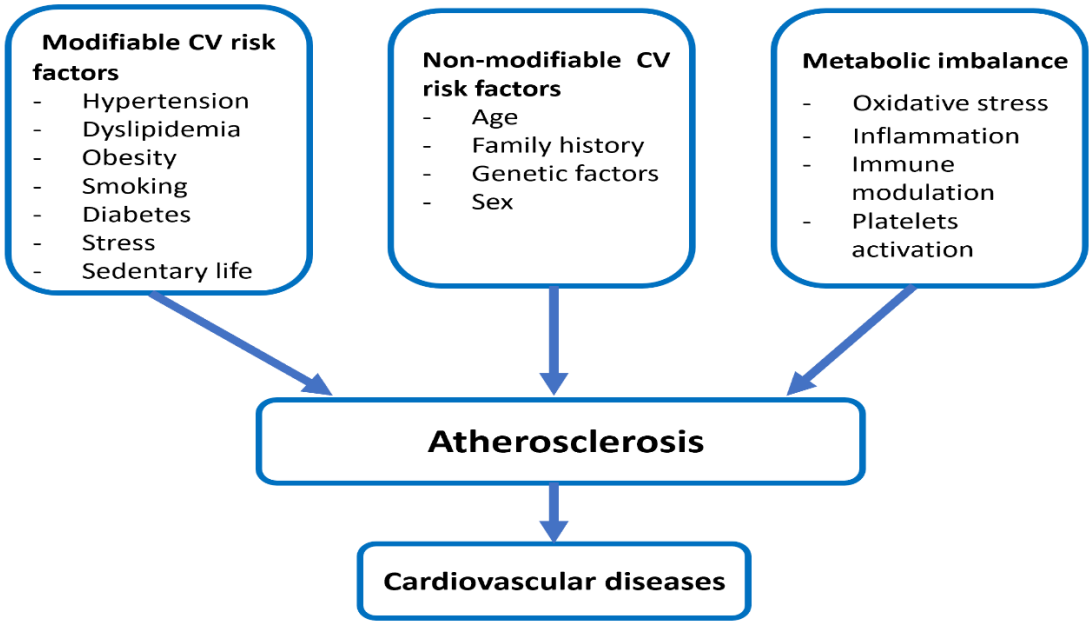

Figure 1: Schematic diagram showing different atherosclerosis risk factors. The diagram shows different modifiable and non-modifiable risk factors and metabolic imbalance. All these factors accelerate atherosclerosis progression. Modified after Hunziker et al., (11) and Park et al. (12)

When blood leukocytes especially T-lymphocytes and mononuclear phagocytes adhere to the arterial intima, they come in contact with the arterial wall endogenous cells, endothelial and smooth muscle cells (SMCs). (3, 13) This results in an exchange of vital messages between the types of cells, which is associated with atherogenesis based on immunity and inflammation mediators, incorporated small molecules that include

mediators of lipids such as arachnoid acid derivatives, such as leukotrienes and prostanoids. (14) The recent studies focus on inflammation and immunity protein mediators, such as cytokines and complement components. The migration of SMCs from tunica media into intima is an important result of the inflammatory ferment carried out in early atheroma. (14) These cells enhance the development of complex and rich extracellular matrix through its proliferation. (15) In cooperation with monocytes and endothelial cells, they discharge matrix metalloproteinases (MMPs) as a result of different hemodynamic, oxidative, autoimmune and inflammatory signals, in relation to their endogenous tissue inhibitors, adjusting multiple vascular cells functions, containing proliferation, activation, migration and cell death besides formation of new vessels and remodeling, healing of extracellular matrix of arteries and myocardium destruction. (15) Binding of specific components of the extracellular matrix (particularly proteoglycans) to lipoproteins leads to prolongation of their adherence to the intima and makes them more prone to oxidative modification and glycation (nonenzymatic conjugation with sugars). (16) Lipoprotein modification products, such as oxidized phospholipids, and end products of glycation promote the inflammatory response. (17, 18) When the lesion progresses, it may lead to calcification by a mechanism similar to that of bone formation. (19) Programmed cell death (apoptosis) frequently occurs, along with proliferation, in the established atherosclerotic lesion. (20) Extracellular deposition of tissue factor, in a particular form, can occur as a result of the death of lipid-laden macrophages. (21) The accumulation of extracellular lipid in the intima coalesces and shape a classic, lipid rich necrotic core of the atherosclerotic plaque. It was recently shown that intact non-activated endothelium prevents the adhesion of platelets to the extracellular matrix. (22) The adhesion of platelets through its receptor to the subendothelial matrix can occur, for example, as a result of atherosclerotic plaque rupture; the exact molecular mechanism that enhances the interaction between platelets and endothelium isn’t completely understood. (23) However, major progress is made in understanding the molecular mechanism of platelets and its interplay with the endothelial cells of the arterial wall. The understanding of the interaction between platelets and atherosclerosis may help to develop novel therapeutic strategies. (24) Moreover, genomics and proteomics are recently introduced in platelets research; these will contribute significantly to

understanding the pathology of platelets and its role in the progression of atherosclerosis. (24)

4.1.3. Prevalence

CVD is the number one cause of death globally. (25) Most deaths from coronary heart diseases are caused by myocardial infarction. In the United States, CAD is the primary cause of death for both men and women. (26, 27) Also in Germany, cardiovascular diseases are the most common cause of death with a prevalence of 48.2%, according to statistical analysis in 2013. (28) The number of people died from chronic heart diseases alone is 73176, which is 20.6% of total deaths. For the myocardial infarction, the number of deaths was 52044, which is 14.7% of total deaths, including 11.5% women and 18.9% men. (28) There is speculation that CAD is more dangerous and causes more deaths every year, than the four leading causes of death taken together: cancer, chronic lower respiratory diseases, accidents, and diabetes mellitus. (29)

4.1.4. Manifestations of CAD

The primary symptoms of the CAD are chest pain known as angina, which by itself is not a disease but a symptom, which is caused as a result of ischemia when the heart muscle does not get enough oxygen-rich blood, which is caused by arterial occlusion by atherosclerosis or thrombosis. (4) The patient complains of pain located in the chest (retrosternal pain) that may radiate to the whole chest, neck, lower jaw, and the left arm, or feeling of discomfort described as pressure or heaviness in the chest. Physical activity, emotional stress, following a heavy meal and cold weather are trigger factors for angina; it fades and subsides with the disappearance of the provoking factors. (30)

4.1.5. Types of CAD

Stable Angina pectoris: SAP refers to a clinical syndrome whose characteristics include chest pain and discomfort that is triggered by emotional stress, exertion and exercise and relieves after a period of less than ten minutes, when the trigger disappears or immediately after nitro-glycerine or at rest condition. (4) It is a chronic disease as it progresses gradually as a result of atherosclerosis. (4) When atherosclerosis affects coronary arteries, the artery lumen narrows progressively. This results in an imbalanced myocardial oxygen consumption and supply thus causing myocardial ischemia. (31) In SAP, this imbalance arises as the demand for oxygen escalates, for instance, because

of increased heart rate and exercise. (32, 33) As mentioned before, the patient complains of anginal chest pain and discomfort that is described as a sensation of heaviness or tightness, shortness of breath and other less common symptoms, such as faintness, nausea, and restlessness. (34) The severity of angina is classified using multiple classification systems, such as the Canadian cardiovascular society classification, the Duke specific activity index or the Seattle angina questionnaire. The grading system of angina pectoris according to Canadian cardiovascular society is as follows: (32)

I. Ordinary activities, such as walking and climbing stairs, do not cause angina
II. Slight limitation of ordinary activity
III. Marked limitation of ordinary physical activity
IV. Inability to carry out any physical activity without discomfort or angina at rest.

The gold standard for treatment and diagnosis of SAP is coronary angiography, but it is considered as an invasive technique. Other non-invasive methods of SAP diagnosis include rest ECG or stress ECG or stress echocardiography. (35) Life changes and treating such modifiable cardiovascular risk factors as diet control, weight loss, smoking cessation, exercise, as well as hypertension and diabetes control are very critical in SAP prevention and treatment. These also apply to all CAD. (34)

Acute coronary syndrome: ACS is an absolute medical emergency as it results in a sudden decrease in blood flow to the heart and may cause sudden death (cardiac arrest) if not handled and diagnosed quickly. (36) ACS caused by arterial thrombosis that may rupture and lead to partial or complete occlusion of the artery lumen. (37) This sudden reduction in myocardial blood flow can lead to myocardial necrosis. The most common symptoms are anginal chest pain, sweating, shortness of breath and nausea; it is related to coronary thrombosis. (38) ACS is divided into three types: unstable angina pectoris, ST-segment elevation myocardial infarction, and non-ST elevation myocardial infarction (they are named according to their appearance in the electrocardiogram). (38) (Figure 2)

Unstable angina pectoris: it is also known as rest angina, new onset angina or rapidly progressing angina**.** It differs from stable angina pectoris in many aspects; unstable angina pectoris appears at rest; it also lasts for more than 20 minutes with increasing chest pain, which doesn't respond quickly to nitroglycerine. (39) Different diagnostic tools can be used to differentiate between unstable angina pectoris and myocardial infarction,

including ECG and measuring blood levels of cardiac enzymes such as creatine kinase MB (CK-MB) and cardiac troponin I, T, which are not elevated in unstable angina pectoris as opposed to myocardial infarction. (40)

STEMI: it results from complete occlusion of coronary arteries, accompanied by persistent ST elevation in ECG in more than one lead, depending on the site of infarcted area with an elevation of cardiac enzymes. (41) These cardiac enzymes consider biomarkers to myocardial necrosis as creatine kinase MB (CK-MB) and cardiac troponin I, T. (42) STEMI patients need rapid intervention in the catheter lab by coronary angiography in two hours or it may result in sudden death or permanent damage of cardiac muscles, if there is going to be a long delay, fibrinolytic therapy can be used. (39)

Non-STEMI: NSTEMI results from partial occlusion of coronary arteries. It is diagnosed by elevation of cardiac enzymes and transient ST-segment depression. (43) It requires intervention in the first 24 hours or immediately depending on patient clinical status. (39)

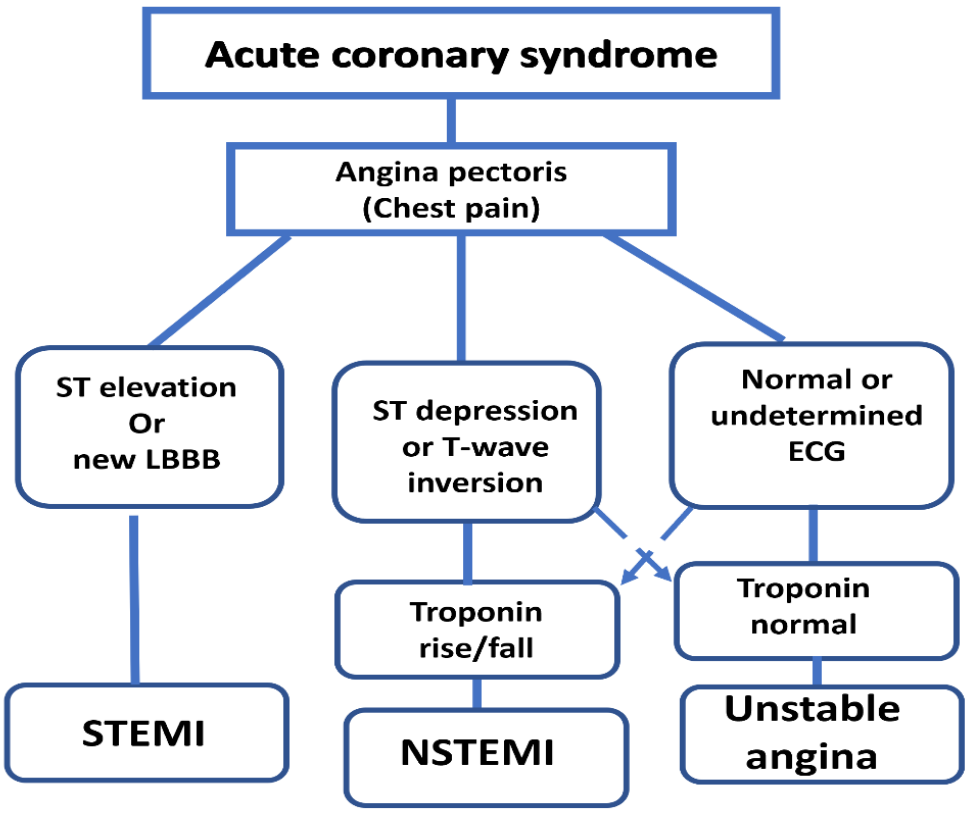

Figure 2: Schematic diagram demonstrating the classification of ACS: We can make a differentiation between ACS types using ECG and laboratory markers especially cardiac markers

(Troponin and CK). The management of ACS can be decided using ECG. If STEMI was detected, the patients should receive PCI immediately. If ST depression or T-inversion were identified, the decision of treatment is dependent on laboratory results and clinical status. Modified after Eisen et al. (44) and Crea et al. (45)

4.2. Assessment of angiographic severity of CAD

CAD's angiographic severity can be measured in a diverse scoring system. A risk score system referred to as TIMI or Thrombolysis in Myocardial Infarction is commonly used in evaluating CAD's angiographic severity. (46, 47) Gensini score is another scoring system that was used in the study for evaluating CAD's angiographic severity. The Gensini score could be utilized for measuring the atherosclerotic burden among CAD patients. Within the score, the stenosis severity is denoted by a decrease in lumen diameter alongside the cumulative impact of several obstructions; additionally, it relies on the stenosis' geographical location. (48) Different multipliers are applied depending on the area of the lesion and its functional importance. The Gensini score considers a sum of the lesions score. The severity of CAD was measured using the angiographic assessment system of the Gensini score. The Gensini score was defined according to the severity of stenosis as 1 point for < 25% stenosis, 2 points for 26% – 50% stenosis, 4 points for 51% – 75% stenosis, 8 points for 75% – 90% stenosis and 32 points for total occlusion. (49, 50) Depending on the importance of the lesion's position in the coronary artery system, the score was multiplied by a factor. (49) For example, 1 for the distal LAD, 1.5 for the mid-region and 2.5 for the proximal LAD or proximal LCX, 5 for the LM. (49) The severity was calculated by the sum of all segments score. (50) (Figure 3)

Degree of stenosis (DS)	Score
25	1
50	2
75	4
90	8
99	16
100	32

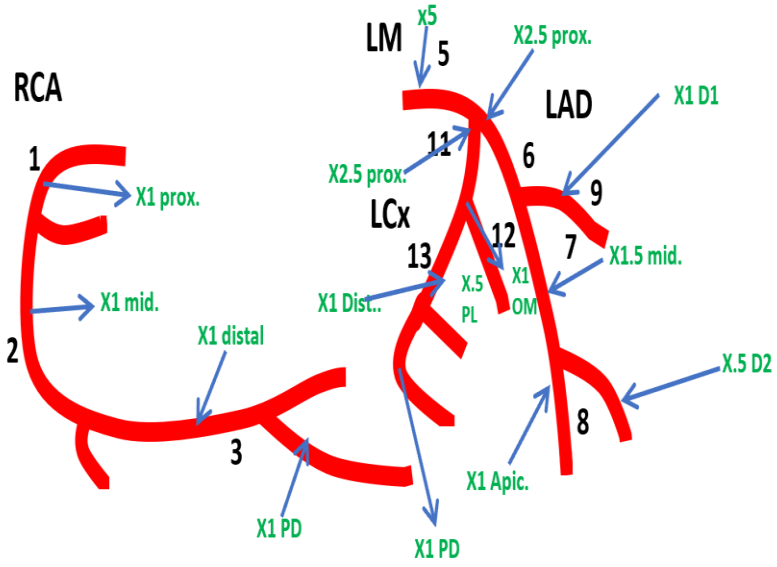

Figure 3: Schematic diagram showing the Gensini scoring system to measure the angiographic severity of CAD. LM =Left main, LAD= left anterior descending, LCX=left circumflex, RCA=right coronary artery, D= Diagonal branch, OM= Obtuse marginal, PD= Posterior descending, PL= Posterior lateral). Modified after Parsa et al. (51)

4.3. The contribution of platelets to the pathophysiology of coronary artery disease

4.3.1. Platelets morphology and origin

Platelets are small anuclear cells. The approximate diameter of a platelet is 2.0 to 5.0 μm, its thickness is 0.5 μm, and the mean cell volume is 6 – 10 femtoliters. (52) The lifespan of platelets ranges from 7 to 10 days after leaving the bone marrow and moving into the circulation; in the peripheral blood. If platelets were not a part of hemostasis or thrombosis processes, it would be eliminated by the reticuloendothelial system in liver and spleen. (53, 54) A normal platelet count ranges from 150,000 to 450,000 platelets per microliter of blood. (55) Platelets originate from their precursor cells, megakaryocytes, in the bone marrow. The platelets are the smallest cell in the blood, while the megakaryocytes are the largest and one of the rarest cells in the bone marrow. Megakaryocytes are containing a nucleus and DNA. (56)

4.3.2. Platelets function, activation, and aggregation

Platelets not only play a significant role to keep the vascular integrity through the balance between bleeding and coagulation (hemostasis process), but it is also proven that they play a role in inflammation, cancer biology and innate immunity. (57) Under normal physiological conditions, platelets play an important role in the process of homeostasis, following tissue injury by stopping the resulting bleeding. (58) This exact process can also harm the body through thrombosis and vessel occlusion, resulting in severe, deadly ischemic thrombotic diseases, such as myocardial infarct and stroke. (59) In the event of an injury within the endothelium, the platelets migrate to the injured site, attach to the endothelium, become active, aggregated, and commence the process of halting bleeding via a cohesive and adhesive function, which culminates in hemostatic plug formation that is regarded as the initial defense line against loss of blood, and that is called the primary hemostasis. (60, 61) Many cellular receptors on the platelet surface, such as selectins and integrins, alongside different adhesive proteins, such as, fibrinogen and vWF, play a fundamental role in the process. (62) After platelets activation, they secrete a number of factors that are contained within the dense and alpha granules that help in aggregating platelets which in turn results in the formation of thrombi. (63) Moreover, activated platelets play an important role in secondary homeostasis process that refer to localized coagulation aggravated by surface phospholipids expression on the activated platelets thus leading to the generation of thrombin and formation of fibrin. (64) Besides the role of activated platelets in hemostasis, they also take part in innate immunity and inflammation by recruiting leucocytes through lysosomes. Lysosomes are secreted by activated platelets and contain enzymes, such as acid proteases, glycosidases, and cationic proteins, that may participate in inflammation and extravasation of leukocytes through their cytotoxic and proteolytic activity on the sites of platelet accumulation at the inflamed tissue. (65)

4.3.3. The contribution of platelets to the pathophysiology of coronary artery disease

Platelets play a significant role in hemostasis to prevent blood loss as a response to injury under physiological conditions. (60) They also contribute to pathogenic thrombi formation. Improperly activated platelets may cause thrombosis and vessel occlusion, which can

lead to acute life-threatening conditions, such as acute coronary syndrome, ischemic stroke, and symptomatic peripheral artery disease. (66) Platelets play a pivotal role of a mediator in inflammation and immunity, contributing to atherogenesis, causing chronic diseases, such as stable coronary artery disease. (67) Arterial thrombosis is formed from chronic atherosclerotic lesions. (68) Fibrin and platelets are considered the main elements of thrombi (clots) formation that leads to arterial occlusion and also contributes to the progression of atherosclerotic plaques. (69) Consequently, the platelets are considered as the main component in atherothrombosis process, which represent a mixture of acute and chronic artery diseases. (69) The mechanisms underlying the role of platelets in the initiation of plaque rupture are still to be investigated. (69) Nevertheless, the subendothelial layer after the disruption of plaque is exposed to the circulation (70, 71), leading to direct contact of the subendothelial layer with circulating platelets with its contents. (68) The formation of stable platelet plug necessitates the arrest of more platelets on the site of subendothelium and amplification of their response to cross-link and stabilize the new plug. (68, 72) In fact, these described steps happen simultaneously. VWF interaction with GPIb/IX/V and the interaction of collagen with GPVI are needed to initiate platelet adhesion. (73) Subsequently, the activated platelet secretes soluble agonists (ADP, thrombin, thromboxane A2), which lead to more enhancement of platelets activation. (74) The binding of thromboxane to its receptors and the binding of APD to P2Y12 and P2Y1 elicit alternation in the platelets shape their degranulation. Moreover, the cleavage of thrombin leads to activation of PAR-1 and PAR-4 in high concentrations, which in turn results in the oligomerization and a conformational change of the integrin αIIβ3. (75, 76) These changes with integrin lead to increase receptor's affinity to bind to its ligands. (77) A significant amount of integrin αIIβ3 in platelets shows the biological significance of this process because it leads to the gathering of large platelet aggregates. (78) The aggregation of platelets on the site of ruptured plaque leads to secretion of a significant number of cytokines and other mediators that enhance inflammatory reaction and activation of leukocytes. (79) Furthermore, it releases the growth factor from platelets granules, which initiate angiogenesis, the formation of matrix and tissue remodeling. Understanding of platelets interaction with its receptor helps in developing the therapeutic and diagnostic strategies in CAD patients. (70) This mechanism also shows the benefit

of using antiplatelet therapy and their effect on improving the clinical prognosis of patients with CAD. (80) The recent studies target the inflammatory biomarkers of platelets and mediators to understand and diagnose the predisposition of atherosclerosis and eventually help in the prevention of thromboembolic complications. (80)

4.4. The interplay of platelets and lipids in contributing to atherosclerosis

4.4.1. Atherosclerosis

Atherosclerosis is composed of two Greek words: “athero,” which means gruel (lipid accumulation) and “sclerosis,” which means hardening. (81) It is a multifactorial immune-inflammatory disease of medium-sized and large arteries in the body; it is the leading cause of cerebral and coronary infarction. The pathogenesis related to maladaptive immune response appears as inflammation of the intima (arterial inner lining) of the arteries; besides that, there is an imbalance in lipid metabolism. (82) In addition, certain cells play a significant role in atherosclerosis, such as leukocytes, endothelial cells, and intimal smooth muscle cells. (83) All these factors lead to plaque formation, which consists of different cell types and is filled with lipid. There are two types of plaques: lipid-rich plaque and collagen-rich plaque. The first one is susceptible to rupture and causes acute coronary syndrome or conversion of stable atherosclerotic plaque to an unstable one; this is known as fissuring, rupture, and disruption. The second type of plaque tends to cause stable artery diseases. (84)

4.4.2. Platelet lipid interaction in contributing to cardiovascular disease

Recent studies showed that platelets contributed towards atherosclerotic plaque formation via atherogenesis activation, arterial wall remodeling and raising vascular inflammation. (85, 86) Platelets that have been activated secrete varying inflammatory mediators after sticking on endothelial monolayer. (87) Platelet-derived inflammatory mediators recruit blood cells in circulation such as monocytes and the endothelial progenitor cells (EPCs). In addition, these mediators activate the endothelial monolayer. (88) Furthermore, platelets help in foam cell and macrophage formation through stimulation of EPC and monocyte differentiation. (88) Consequently, an inflammatory “hot spot” is formed by accumulating platelets on the walls of the arteries, causing atherosclerotic lesion formation. (88) It has been proven that platelets are very useful in the vascular injury healing process. They also enhance the repair of endothelial

monolayer through differentiation and recruitment of EPCs that circulate. (88) Platelets and such atherosclerotic cofactors as oxLDL (oxidized LDL) enhance the repair of vascular lesions (healing) and lesion progression and formation. (89) These mechanisms including inflammatory mediators and receptors contribute to the balancing of opposing factors involved in either vascular injury or repair through platelets. Several studies have demonstrated a beneficial effect of prolonged use of antiplatelet therapy on the prognosis in atherosclerotic disease patients. (90) This has opened the door to elucidate the mechanisms of platelet-mediated impacts on atherosclerotic diseases to identify novel biomarkers and therapeutic targets in order to develop new diagnostic tools and therapeutic strategies for thromboischaemic diseases. (85)

4.5. Oxidized LDL

Lipoproteins play an essential role in atherogenesis as they alter the properties of various cells that take part in thrombosis and atherosclerosis, including low-density lipoproteins (LDL) and its oxidized form oxLDL, which bind to the platelet surface via scavenger receptors SR-B, CD36, LOX-1 or CXCL16. (91) Both oxLDL and LDL are taken up and stored by platelets in significant amounts. They also lead to activation of platelets and formation of thrombus. Macrophages phagocytose lipid-laden platelets, which induces differentiation of foam cell from monocytes and CD34+ progenitor cells. (92) The platelet-dependent generation of foam cells *in vitro* and the atheroprogression in Apoe $^{-/-}$ mice were shown to be inhibited by the soluble scavenger receptor CD68. (93) Moreover, the oxLDL-laden platelets activate the endothelium and reduce the number of CD 34+ cells (colony forming cells, which transform into endothelial cells). (92) Patients with stable coronary artery disease in comparison to ACS patients show significantly oxLDL binding on stimulated platelets. The degree of platelet activation and plasma oxLDL levels show a positive correlation with platelet-bound oxLDL. (94) OxLDL also enhances adhesion of platelet to collagen and activated endothelial cells under shear stress *in vitro*. (95) Recently, it was demonstrated that platelet apoptosis is induced by oxLDL uptake by the platelets. (93) CXCL12 acting through CXCR4 and CXCR7 on monocytes and M1-M2 macrophages promote phagocytosis of lipid-laden platelets and enhance their differentiation into foam cells. (96) It was shown that, if platelets were activated with

oxLDL, this leads to the formation of platelet-monocyte aggregates (PMA), as well as phagocytosis of platelets and also enhances oxLDL uptake by monocytes, depending on the release of platelet CXCL4 and CD36. (84) This means that platelets can store and transfer a significant amount of oxLDL to atherosclerotic lesions, which shows the significant role of platelets in atherogenesis. (97)

4.6. Plasma lipids and platelets

Cholesterol and triglycerides are hydrophobic, and to be transported in water, they must be transported in association with proteins. Lipoproteins are composed of a core containing cholesterol esters and triglycerides surrounded by a coat of free cholesterol, phospholipids, and apolipoproteins. (98) There are different types of lipoproteins, which are differentiated from each other depending on size, density, and electrophoretic characteristics and divided into five types. (99) These are chylomicrons (density <0.95 gm/ml), very low-density lipoproteins (VLDL, density 0.95 to 1.006 gm/ml), intermediated density lipoproteins (IDL, density 1.006 to 1.019 gm/ml), low density lipoproteins (LDL, density 1.019 to 1.063 gm/ml) and high density lipoproteins (HDL, density 1.063 to 1.210 gm/ml). (99) Specific apolipoprotein (apo-) constituents of the different lipoprotein classes control and regulate the metabolism of plasma. These apolipoproteins contain apoE, apoB, apoA-I, apoA-II, apo IV, apoC-I, apoC-II, and apoC-III. Lipoprotein metabolism is regulated by specific apolipoprotein function. (100) Apolipoproteins are involved in the transport and redistribution of lipids among various cells and tissues, and they contribute as cofactors for enzymes of lipid metabolism and maintain the structure of lipoprotein particles. (101)

Lipoproteins and platelets contribute intimately to a wide range of diseases, such as atherosclerosis, thrombosis and coronary heart disease. Many studies in the past years showed the direct relation between the hemostatic function of platelets and plasma lipoproteins. Especially relevant in this regard is the fact that a lot of patients with familial type IIa hyperlipoproteinemia showed abnormal platelet function. (102) This disease is characterized by predisposition and early onset of atherosclerosis. In these patients, platelets show enhanced aggregation and secretory response to a different physiological stimulus. Plasma lipoprotein levels are reduced by nicotinic acid and clofibrate. (103)

Moreover, nicotinic acid and clofibrate decrease in these patients the platelets hypersensitivity as well as reduce the responsiveness of platelets from the control group. (104) Moreover, the platelets in patients with type II hyperlipoproteinemia have elevated cholesterol and phospholipid content. The elevated cholesterol level is associated with platelets hypersensitivity and stimulation. (105) These changes may contribute to the basis for the abnormalities in platelets' function in this disease. Recent studies show that lipoprotein can alter the aggregation response of platelets. It was also demonstrated that HDL reduced thrombin-induced platelet aggregation, while LDL enhanced thrombin-induced platelet aggregation. (100) Moreover, plasma LDL levels correlated with platelet aggregation and enhanced platelet sensitivity by epinephrine. The observation from different studies suggests that the interaction of lipoprotein with platelet surface initiates the mechanism for altering platelet function. (106) Hyperlipidemia stimulates platelets and enhances platelet activation in response to different agonist. Hypercholesterolemia contributes to platelet activation more potently than hypertriglyceridemia; thus, cholesterol plasma level seems to play an important role in modulating platelets activity. (106) OxLDL and oxidized phospholipids increase hyperlipidemia, thus play a role in platelet activation. (107) Moreover, a recent study showed the important role of targeting cholesterol plasma levels in cardiovascular patients as a form of secondary prevention of atherosclerosis. (85) Up to now, statins and PCSK9 inhibitors are considered as the main therapies for atherosclerosis and its clinical progression. In addition to lipid-lowering therapies, antiplatelet therapy plays a major role in treating patients with advanced atherosclerosis. (85) P2Y12 inhibitors (clopidogrel, prasugrel, ticagrelor) or PAR-1 antagonists (vorapaxar) are well-known antiplatelets therapies, which are used either alone or in combination with therapies such as secondary prevention for CAD patients. (87) (Figure 4) Antiplatelet therapy does not just decrease thromboischemic risk but also reduces the platelet driven systemic inflammation and possibly affects atheroprogression. (24)

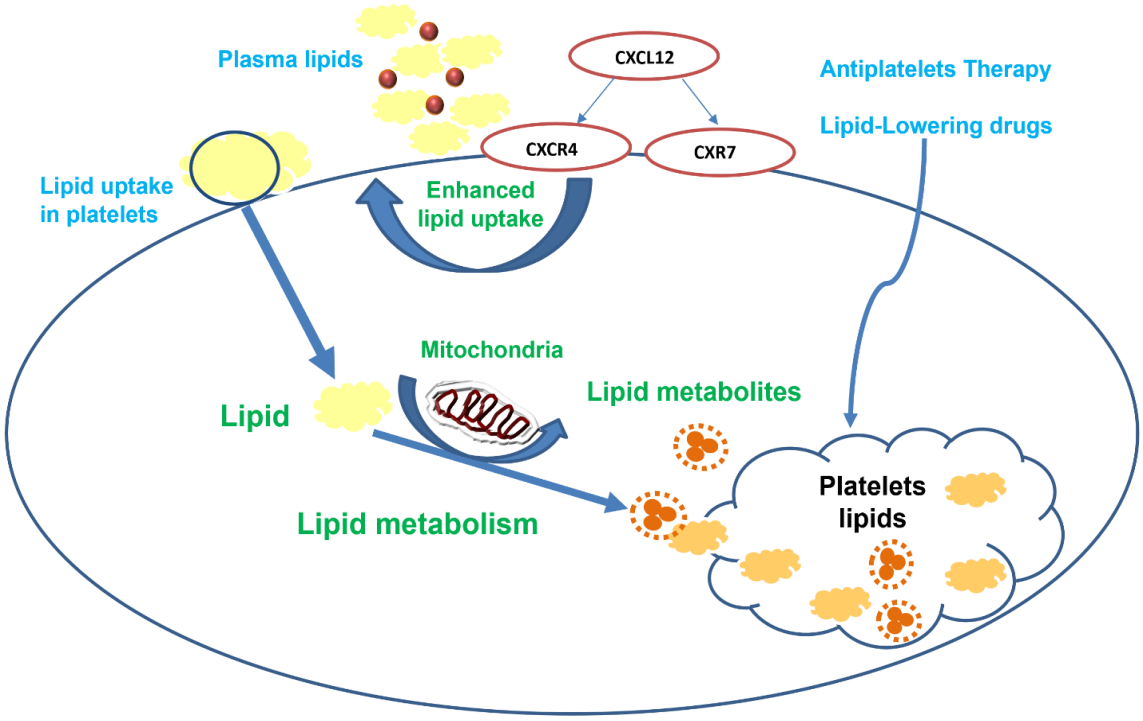

Figure 4: Schematic diagram showing platelets-lipid interactions through the CXCL12/CXCR4-CXCR7 axis and the role of lipid-lowering therapies and antiplatelet in treating patients with advanced atherosclerosis. Modified after Chatterjee et al. (108)

4.7. CXCL12-CXCR4-CXCR7 axis in the context of cardiovascular pathophysiology

4.7.1. Chemokines and chemokine receptors

Chemokines refer to a group of small cytokines that have chemotactic characteristics, which attach to and activate chemokine receptor families. (109) They have been found to play a major role in CADs, malignant tumors, infection, tissue injury, inflammation, and all atherosclerosis development phases. (110, 111) Chemokines mediate immune cell recruitment and stimulation. They also control cellular homeostasis. (109) Additionally, they have been found to play different roles in inflammation, infection, allergy, tumors, and tissue injury. (110, 112) Until now, more Over fifty chemokines have so far been identified with 4 subgroup classifications i.e., C, CC, CXC, and CX3C depending on the N-terminal cysteine residue. (110, 113) They attach onto many different G-protein-coupled transmembrane receptors. (114) When a ligand binds to the extracellular binding site of the transmembrane receptor, it activates a cascade of signal transduction events. (115) The number of chemokine receptors that have been identified has reached 20. The

specificity profile of the binding potential of the chemokines to their receptors varies between different chemokines. On the one hand, some chemokines can bind only to one receptor, while others can bind to several receptors. On the other hand, some receptors can bind only to one chemokine, while other receptors can bind to several chemokines. (116)

4.7.2. SDF-1 (CXCL12)

CXCL12 is one of the CXC chemokines that was shown to be significantly expressed on activated platelets and released from platelets adhering to the site of vascular damage leading to the recruitment of $CD34^+$ cells and the modification of their proliferation and differentiation to different cell types including endothelial cells, foam cells, and macrophages. (117, 118) CXCL12 was first identified in bone-marrow-derived stromal stem cells. It was shown to act as a pre-B-cell growth-stimulating factor (PBSF). (112) Its expression profile includes a wide range of tissues. It acts as a chemoattractant for hematopoietic stem cells as well as stimulates their translocation in the bone marrow and controls the proliferation and survival of stem cells in humans and rodents. (119, 120) The production of CXCL12 in the bone marrow is mainly from osteoblasts. (121) It controls the CD34+ cells' migration. (122) CXCR4 is one of the CXCL12 receptors, and its blocking was shown to impair the chemoattractant activity of CXCL12. (123) Following tissue injury or hypoxia, the hypoxia-inducible factor 1-alpha (HIF-1α) expression is increased. The expression of HIF-1α on endothelial cells have been shown to be associated with enhancing the expression of CXCL12. (124) The increase of CXCL12 leads to the attraction of CXCR4+ stem cells to damaged tissues, which enhances the regenerative potential. (122) Platelets considered as a major source of CXCL12; it is stored as part of their α granules secretome. The release and surface expression of CXCL12 is enhanced through activation of platelets. (125, 126) Studies have shown the roles of CXCL12 in mediating $CD34^+$ progenitor cells recruitment *in vivo* to promote vascular remodeling and repair. (127) Moreover, the pivotal role of CXCL12 from activated platelets was recently demonstrated in the differentiation of circulating monocytes into multipotential cells. (125) These multipotentials cells have the capacity to be transformed into the endothelial and mesenchymal lineage. (128) Additionally, platelets derived CXCL12 regulates paracrine

mechanisms like adhesion, proliferation, chemotaxis, and differentiation of nucleated cells inclusive of progenitor cells. (126) Regardless of these insights, the potential effect of CXCL12 on exerting autocrine/paracrine signaling regulating activation of platelets relevant to thrombosis or hemostasis has not been completely studied. (129) CXCL12 was previously known to signal exclusively through the Gαi-coupled G-protein-coupled receptor (GPCR), CXC chemokine receptor type 4 (CXCR4). (126, 130) The recent studies have recognized CXCR7 as a higher affinity receptor for CXCL12. (131) It is believed that CXCR7 receptor works as a decoy receptor. (132) CXCR7 can internalize bound CXCL12 to modulate CXCL12 gradients, which is important for optimal signals through CXCR4. (133) Moreover, the regulation CXCL12 mediated functions need the heterodimerization of both receptors. (130) CXC4 and CXCR7 are both expressed on the platelets surface, while CXCR4 seems functional, the CXCR7 importance in the physiology of platelets is only starting to be understood. (126, 134, 135) Platelet-derived CXCL12 might act as a potent autocrine agonist besides its paracrine inflammatory roles. (136) Platelet activation through ADP-receptor (P_2Y_{12}), glycoprotein VI (GPVI) and protease-activated receptors (PAR)1 ligation drives the preferential release of CXCL12 which mediates their regenerative functions. (136) Experimental evidence accumulated over the years established the potential of platelets for protein synthesis, and several transcripts have been identified in platelet polysomes. (136) Interestingly resting platelets do not harbor mature mRNAs for CXCL12; whereas activation with agonists like thrombin triggers the maturation process of CXCL12 pre-mRNA, subsequently leading to *de novo* protein synthesis. (129) Secreted from activated or adherent platelets, it triggers migration and differentiation of $CD34^+$ progenitor cells into endothelial progenitor cell contributing to vascular regeneration but also macrophage foam cell phenotype causing vascular inflammation. (129) Platelet surface expression of CXCL12 is enhanced in patients with CAD. (96, 131) Moreover, surface expression of platelet CXCL12 significantly associates with ACS and is particularly elevated in patients with ST-elevation myocardial infarction. (96, 131) Additionally, It was shown that the overexpression of CXCL12 has a cardioprotective role of enhancing the myocardial function following infarction. (137, 138) CXCL12 expression was recently demonstrated in activated platelets and was shown to be decreased in STEMI as compared to CAD patients. (139, 140) Furthermore, It was

shown in mice that the administration of recombinant CXCL12 leads to a decrease in infarct size following transient ischemia. (126) There was a correlation between enhanced platelet CXCL12 surface expression and circulating CD34+ progenitor cell quantity, infarct size amelioration alongside enhanced LVEF% following acute myocardial infarction (AMI) among patients with ACS. (96, 131) Moreover, circulating CXCL12 levels from platelets alongside other sources of cells could affect CXCR4-CXCR7 progressive surface availability on platelets. (126) CXCL12/CXCR4 axis regulates megakaryopoiesis, platelet functions, and survival. (129) Platelets transmigrate through endothelial layer towards CXCL12, a response inhibited by the CXCR4 antagonist, inhibitors of the PI3K pathway and by disruption of actin polymerization. (129) Notably, platelets selectively assemble at high CXCL12 sites under conditions of flow and show flow-directed migration. (141) Although considered a weak platelet agonist, CXCL12 enhances platelet activation through Gαi coupled CXCR4 but not CXCR7. (128, 129) CXCL12 strongly antagonizes adenylate cyclase activity in platelets and counteracts prostaglandin (PG)I_2 analog induced cyclic adenosine monophosphate (cAMP) levels. (129) CXCL12-induced platelet aggregation is further supported by granular release, phospholipase C (PLC) activation leading to full aggregation. (126) On the one hand, at lower concentrations initiates CXCL12 the primary phase of aggregation response, on the other hand at increased concentrations induces both primary and secondary response through the PI3K pathway in the initial primary phase of aggregation and other downstream kinases and prostanoids to achieve maximal irreversible aggregation and granule secretion. (108, 141) CXCL12 also potentiate aggregation induced by the sub-threshold concentration of ADP and thrombin under arterial and lower shear conditions. (126) CXCL12 fails to activate washed platelets, presumably requiring the presence of plasma components like epinephrine serotonin or a synergistic effect from thromboxane (Tx)A2, or ADP released in platelet-rich-plasma (PRP) preparations to instigate a biphasic aggregation response. (129, 141) Similarly, CXCL12 does not mobilize intracellular calcium in washed platelet preparations but triggers TxB2 and CXCL4 release in PRP preparations under stirring conditions. (126, 129) In spite of, CXCL12 does not alter the exposure of P-selectin, αIIbβ3 activation under non-stirring conditions, exposure of platelets to CXCL12 in the presence of sub-threshold ADP concentrations and under low shear drives P-selectin

exposure. (141) CXCL12 does not induce serotonin secretion from dense granules either alone or in the presence of low ADP concentrations but instigates adenosine triphosphate (ATP) release from dense granules. (126, 129) CXCL12 also induces morphological changes leading to the formation of blebs, which could potentially enhance the likelihood of blood coagulation. (129) CXCL12 enhances adhesion of platelets to collagen type IV and fibrinogen under arterial flow conditions. (129, 141) The autocrine effect of collagen-activated platelet-derived-CXCL12 was evaluated on platelet aggregation and thrombus formation, which shows CXCR4 as the mediating receptor. (129) Significant CXCL12 released following collagen-GPVI stimulation leads to ATP release from dense granules, TxA2 production, which promotes aggregation and thrombus formation under dynamic flow conditions. (129) Recently we have also shown that essentially acting as a pro-thrombotic mediator CXCR12 also enhances lipid-induced free radical generation in platelets, synergistically increases platelet activation and thrombus formation in the presence of LDL/oxLDL which makes it a prime target to be considered to regulate platelet hyper-responsiveness to hyperlipidemia. (108)

4.7.3. CXCR4

CXCR4 is a chemokine conserved transmembrane receptor that is activated by CXCL12 binding and, consequently, mediates the migration of leukocytes and hematopoietic progenitor cells. (140, 142-144). The activation of CXCR4 through CXCL12 was shown to play a role in the translocation of the early hematopoietic cells in the bone marrow in humans. CXCR4 knock-out mice do not survive and die *in utero* due to extreme hypocellularity in bone marrow. (139, 145) Mature human platelets were shown to have lesser responsiveness to CXCL12 as compared to progenitor cells. (146) Megakaryocytes and platelets express CXCR4, which is the major receptor for CXCL12. The interaction between CXCL12 and CXCR4 regulates circulating platelets functions and megakaryopoiesis. (147, 148) Binding of CXCL12 to CXCR4 in platelets enhances intracellular calcium mobilization and augments aggregation promoted by ADP or thrombin. (129, 149) In previous studies, it was shown the possible autocrine/paracrine signaling role of CXCL12 acting mainly through CXCR4. (129) Furthermore, CXCL12/CXCR4 axis culminates in induced TxA_2 production and secretion of dense granules to promote thrombus formation. (129) The enhancing effects of CXCL12 on

platelets responsiveness to collagen are mainly driven through TxA_2 dependent signaling. On the other hand, ADP mediated signaling contribute minimally to the enhancing effects of platelets of CXCL12. (129) This evidence suggests the potential of platelet-released CXCL12 in a wide array of other platelets derived inflammatory agents in mediating autocrine activation events which could significantly contribute to thrombosis and hemostasis. (129) Both CXCR4 and CXCR7 are expressed constitutively in platelets. However, CXCR4 surface expression at basal level is higher as compared to CXCR7. (96) The relative surface availability of CXCR4/CXCR7 might influence their comparative participation in driving pathophysiological processes. (96, 126) Presence of ligands like CXCL12, CXCL11, and MIF influences a dynamic alteration in CXCR4/CXCR7 surface expression as CXCR4 is internalized and CXCR7 is preferentially externalized in response to CXCL12 but not by CXCL11 and MIF. (141) CXCL12-induced CXCR4 internalization which precedes and is coupled to CXCR7 translocation to the platelet surface and is counteracted by CXCR4-blocking antibody or antagonist-AMD3100. (96, 131, 141) CXCL12/CXCR4-triggered CXCR7 externalization is executed through Erk1/2 and the intracellular molecular chaperone CyPA. (131) Thus the initial ligation of CXCR4 by CXCL12 or SDF1 contributes to the relative surface availability of both the receptors and drive subsequent CXCL12 driven response from platelets. (131, 141)

4.7.4. CXCR7

CXCR7 is a relatively newly identified transmembrane chemokine receptor of CXCL12. (145, 150) It was shown in previous studies that CXCR7 is expressed on activated endothelial cells, vascular endothelium, and fetal liver cells. (145, 151-153) Its expression was demonstrated to be increased in the endothelial cells of certain tumors. In acute renal failure, CXCR7 neutralization led to the reduction of the number of the renal multipotent progenitor cells (RMP). CXCR7 is seen to be responsible for the survival of RMPs and their migration and adhesion to endothelial cells. (154) Following ischemic brain lesion in rodents, it was observed that CXCR7 expression increased in the tissues surrounding the lesion, postulating a neuronal protective role of CXCL12 via CXCR7. (155) A role in mediating the survival of the endothelial progenitor cells was also observed. (156) Other functions of the CXCR7's mediated effects of CXCL12 still need to be investigated. Previous studies demonstrated that elevated platelet surface expression of CXCR7 is

closely related to ACS patients. (157) Moreover, it was also seen that CXCR4 and CXCR7 correlate with CXCL12 surface expression. (157) ACS patients have an insignificant increase of CXCR4 in comparison to patients with stable CAD. (158) Hence, the hypothesis that this increase in CXCR7 expression is induced by hypoxia in ACS patients. (159) Presence of influences a dynamic alteration in the CXCR4/CXCR7 surface expression on platelets as CXCR4 is internalized and CXCR7 is preferentially externalized in response to CXCL12. (96) In platelets, ubiquitination of CXCR7 is dynamically up-regulated upon CXCL12 exposure, involving Erk1/2, cyclophilin A peptidyl proline isomerase (CyPA-PPIase) and E1-ligase activity, culminating in CXCR7 externalization. (160) Enhanced availability of CXCR7 upon CXCL12 exposure can further perpetuate these anti-apoptotic effects. (131) CXCL12-CXCR7 exerted prosurvival effect is counteracted by pharmacological inhibition of Erk1/2 pathway also CyPA-PPIase activity thus coupling the dynamic trafficking of CXCR4-CXCR7 to the resultant anti-apoptotic effect mediated through CXCR7. (131, 160) Macrophage migration inhibitory factor (MIF) is another chemokine-like cytokine which also binds to CXCR7 on platelets and exerts a prosurvival effect through the PI3K-Akt kinase pathway. (96, 161) Although both MIF and CXCL12 bind to CXCR4, ligand-specific CXCR4-mediated signaling in platelets are executed. (96) CXCL11 does not affect the CXCL12-induced CXCR4 internalization but externalizes CXCR7 and therefore counteracts CXCL12-induced CXCR7 externalization. (160) Under inflammatory circumstances or at the site of CXCL12/MIF enriched atherosclerotic plaques this dynamic receptor trafficking could result as a paracrine effect or mediate by activated platelet-derived-CXCL12/MIF in an autocrine fashion. (131) Therefore, the relative availability of CXCR4-CXCR7 with or without the ligands can have serious psychological impacts at the vascular injury sites or inflammation where chemokines are secreted, and their levels are enriched by platelets which also guide regeneration and vascular inflammation course. (96) Researchers have recently established that oxLDL increased CXCL12 surface expression that reduced the surface exposure of CXCR4. (108) The interaction between CXCR7 surface expression and elevated platelet-oxLDL was observed. It is known that CXCR7 receptor induces the uptake of cholesterol in adipose tissue to evaluate the levels of plasma cholesterol. (162) The correlation between the plasma levels of CXCL12 and cholesterol hyperlipidemia

was previously described. This shows the possible effect of the CXCL12- CXCR4 axis on circulatory lipid turnover in platelets. (108, 163, 164) These novel findings speculate a prothrombotic and prooxidative role of the platelet-derived chemokines in hyperlipidaemic conditions, proposing the CXCL12/CXCR4/CXCR7 as a possible future therapeutic target in the modulation of the platelet-lipoprotein mediated progression of the CAD. (108)

4.7.5. The significance of platelet CXCR4/CXCR7 expression in clinical prognosis

CXCL12 mediates the regenerative influence of platelets. (126) The balance between initial inflammatory and successive regenerative mechanisms and reperfusion injury level determine prognosis and functional recovery after ACS. (129) Surface expression of CXCL12 is enhanced in CAD patients but its relationship with levels of plasma is poor. Nevertheless, this suggests that activated circulating platelets can act as a principal source of plasma CXCL12. (141) The surface expression of Platelet CXCL12 increases among patients with ACS, especially with ST elevated myocardial infarction than stable CAD patients. (163) Peripheral mobilization of bone marrow-derived regenerative CD34+ cells associated with enhanced platelet CXCL12 surface expression and correlated with LVEF recovery in ACS patients. (165) Accordingly, patients with enhanced platelet CXCL12 expression showed a significant amelioration of infarct size and improved LVEF% after AMI in contrast to patients with lower CXCL12 expression levels. (166) We have previously demonstrated that platelet CXCR7 surface expression is upregulated in ACS patients as compared to stable CAD; further, it significantly correlates with CXCL12 surface expression. Elevated platelet-CXCR7 levels are related to functional recovery (improved LVEF%). (96, 167) On contrary, CXCR4 surface expression is comparable between ACS and CAD patients. (96, 163) However, the prognostic significance of platelet CXCR4 surface expression is revealed in a 12 month follow up among patients with symptomatic CAD. (157) CXCR4 baseline levels are considerably lower among patients facing MI and all-cause mortality. Additionally, CXCR7 and CXCR4 baseline surface expressions have a significant correlation with all cause –death among symptomatic CAD patients. (167) There was an independent and significant correlation between lower progressive CXCR4 levels and all-cause death, all-cause mortality's integrated primary endpoint, as well as MI. (157) In view of this, it could be inferred that

the surface expression of CXCR7 shares in short-term myocardial repair among patients with ACS. (141, 167) On the other hand, the surface expression of CXCR4 could affect long-term outcomes within the chronic stage of CAD by controlling recruitment of inflammatory cells. (96) Such clinical observations confirm the notion that more comprehensive, mechanistic comprehension of CXCR7/ CXCR4/ CXCL12 axis might offer valuable insights of designing targeted therapies to improve recovery and survival among patients with CAD. (96, 141, 157)

5. Objectives of the study

Coronary artery disease is the is a major cause of mortality globally. (6) Atherosclerosis constitutes one major risk factor underlying CAD, whereas hyperlipidemia has a correlation with high susceptibility to thrombosis among ACS and CAD patients. (84) In contrast, platelets are found to play an important role within atherosclerosis pathogenesis and thrombosis. (168) Additionally, many studies have confirmed that oxLDL influences CAD severity, particularly among patients with ACS than stable CAD. (169) The aim of this study is to detect platelets levels of oxLDL in ACS and CAD patients and to examine the correlation between the oxLDL status and CXCL12 acting through its receptors CXCR4 and CXCR 7, as well as the correlation between markers of plasma lipids and platelet oxLDL. (108)

Aims

1. To analyze the levels of oxLDL in platelets of patients with symptomatic coronary artery disease.
2. To investigate the difference of platelets oxLDL status between stable CAD and ACS patients.
3. To investigate interrelations between platelet oxLDL and plasma lipid profile (total cholesterol, LDL- and HDL-cholesterol and triglycerides)
4. To investigate interrelations between platelet oxLDL and the SDF-1/CXCR4/CXCR7 axis in a large patient cohort based on *in vitro* findings.

6. Materials and Methods

In this study, we investigated 152 patients and 15 healthy subjects as a control group for the platelet oxLDL status and CXCR4 and CXCR7 platelet surface expression. The samples were prepared and treated with respective antibodies against oxLDL, CXCR4, CXCR7, CXCL12, and the platelet-specific marker CD42b and then analyzed using flow cytometry.

6.1. Patient characteristics and blood samples:

We performed a cohort study from October 2014 till June 2015 on 152 patients with stable CAD (n= 94), patients with ACS (n= 58) and healthy subjects (n= 15) as a control group. All subjects gave written informed consent. This study was conducted in the department of cardiology at the university hospital of Tübingen. The blood samples were collected from patients during PCI and subsequently was analyzed using flow cytometry within one hour after collection of the blood samples. (170) The patient groups were categorized into stable patients with a known CAD, who were scheduled for elective coronary intervention with or without stent implantation, and ACS patients; the ACS group was subdivided into three categories of patients with unstable angina, NSTEMI, and STEMI. The diagnosis was based on cardiac biomarkers and ECG, Echocardiography and in some cases of detection of intracoronary thrombus using angiography. The cardiac biomarker values (Troponin) had to show at least one value above the 99th percentile of the upper reference limit. Signs of myocardial ischemia in ECG were the presence of ST-segment elevation, pathological Q or new left bundle branch block and detection of regional wall motion abnormality or a new loss of viable myocardium using imaging techniques like echocardiography. The study was approved by the institutional ethics committee (270/2011BO1) and complies with the declaration of Helsinki and the good clinical practice guidelines. (170)

6.2. Plasma/serum levels of lipid biomarkers (LDL, LDL-C, triglycerides, HDL) using ADVIA 1800 clinical chemistry analyzer:

We analyzed plasma samples from all patients using ADVIA 1800 clinical chemistry analyzer by Siemens. The blood was collected from patients in Lithium Heparin blood tubes and then sent to the central laboratory, where the circulating plasma lipid levels

were detected. Subsequently, we investigated the correlation between the levels of circulating plasma lipids in these patients and the mean value of oxLDL detected in platelets.

6.3. Intracellular oxLDL content in platelets from CAD patients measured by flow cytometry

Blood was drawn from patients during PCI and then put in CPDA anticoagulant. The blood was diluted with PBS; 980 µl PBS was added to an Eppendorf tube, and 20µl blood was added to achieve a ratio of 1:50; then, the blood was mixed carefully; after which, 90 µl from diluted blood was added to FACS tubes and fixed with 90 µl of 2% paraformaldehyde. This was followed by 20 minutes incubation time. The next step was permeabilization with 20 µl of 0.3% Triton-X-100, which was incubated for 10 minutes. Next, the sample was washed with 5 ml PBS and then centrifuged for 5 minutes with 3000 rpm speed with breaks. After this step, the platelets pellet was resuspended in 40 µl PBS, and then the sample was stained with 5 µl anti-human oxLDL-FITC (Rabbit Polyclonal antibody, Catalog number: Orb 16127, Biorbyt company) and the platelets were marked using 5 µl CD-42-PE platelet-specific marker for GPIbα (Anti-human CD42b PE Mouse, Cat: 555473, Lot: 4177907, BD Bioscience company) to be identified and differentiated from other cells during FACS measurements. At last, the reaction was stopped by adding 300 µl PBS to FACS tubes. The sample was acquired and analyzed immediately using flow cytometry gating for the platelet-specific CD42b-PE positive population. (Figure 5)

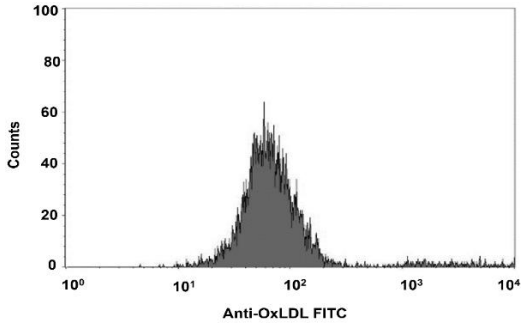

Figure 5: Histogram showing platelets oxLDL status using flow cytometry

6.4. Surface expression of CXCR4-CXCR7 on circulating platelets from CAD patients measured by flow cytometry

This measurement was carried out in whole blood. We diluted the blood, which was collected in CPDA tube at a ratio of 1:50 (980 µl PBS Ca^+ was added to an Eppendorf

cup with 20 µl blood). The FACS tubes were prepared, and then the antibodies were added. 5 µl CD42 FITC (PN IM06480, Beckman Coulter Company) as a specific marker for platelets GPIbα, along with 5 µl CXCR4 antibody (anti-human CXCR4 PE; Mouse, Catalog number: FAB 1701, Lot: LDV0611071, RD company) in the first sample and in the other FACS tube with 5 µl CXCR7 antibody (anti-human CXCR7 PE, Mouse, Catalog number: FAB 422711, Lot: AAEL0312121, RD company). After adding the antibodies, 40 µl diluted blood was added to each FACS tube. Then the prepared samples were incubated in the dark at room temperature for 30 minutes. After that, the reaction was stopped, and the samples were fixed with 300 µl of 0.5% Paraformaldehyde (PFA). The samples were measured immediately. Then the samples were acquired and analyzed using flow cytometry for the surface expression of CXCR4, CXCR7 gating for the platelet-specific marker GPIbα. (Figure 6, 7)

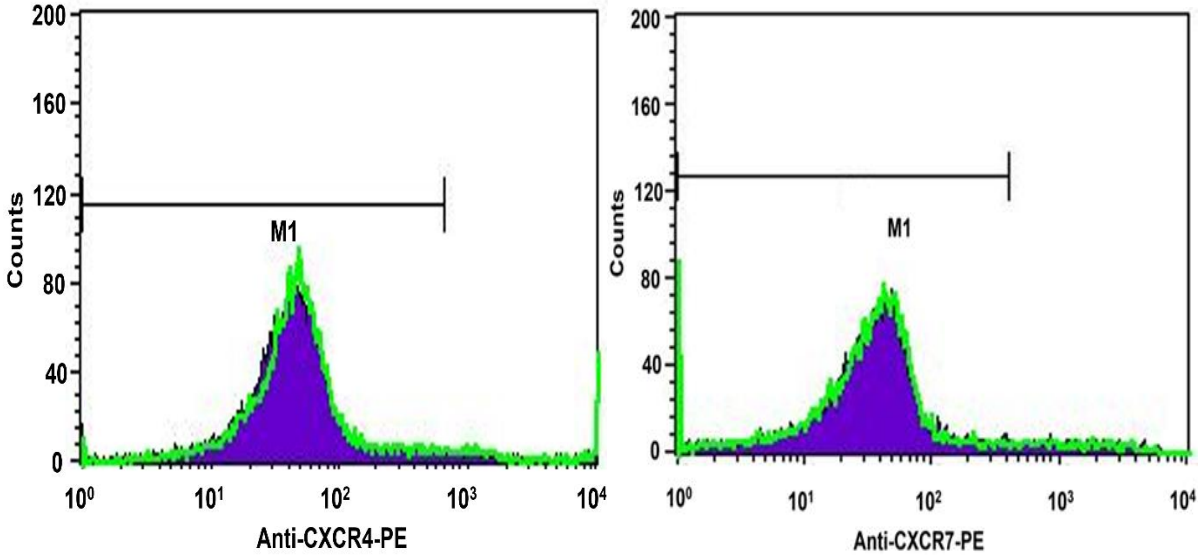

Figure 6: Histogram showing CXCR4 surface expression on platelets using CD42 FITC platelets marker on flow cytometry. **Figure 7:** Histogram showing CXCR7 surface expression on platelets using CD42 PE platelets marker on flow cytometry

6.5. Flow Cytometry

Flow Cytometry (FACS-Calibur flow cytometer Becton-Dickinson) is used for evaluating the physiological and functional characteristic of particles in a fluid stream as they pass through laser. (171) The flow cytometry is used to obtain information about physical properties based on the physical interaction of the particle with laser light, measured in

the same wavelength as a laser (for example relative size, relative granularity or internal complexity, which are often a characteristic profile for a given particle). (172) Moreover, we used the flow cytometry to obtain functional prosperities based on signals from reagents that interact with the laser light (relative fluorescence intensity, FL1, FL2, etc., or FITC, PE, etc.). Flow cytometry is a technology, which plays a crucial role in deeper understanding and studying cells' life cycle, different types of cells population, how cells work, cell division and cell death. (173) It also helps the researcher to further investigate human diseases and cells. It is a fundamental method in cellular biology, equally important for the advanced and parallel discovery of monoclonal antibodies, that allows researchers to detect and label specific cell populations. (174) It analyses a population of cells on a cell-by-cell basis; it can analyze up to 2000 cells per second–an incredible advancement over historical methods of visually examining and counting cells. (173) A flow cytometer is made up of three main systems: fluidics, optics, and electronics. A sample of cells (for example, human blood) is then funneled using the fluidic system into a single stream, and the cells are passed one-by-one through a laser beam. As the cells pass, it scatters light and may emit fluorescent light. (175) The optic system of the machine collects these light signals and directs them to several detectors. (176) The electronic system of the machine then converts these light signals received by the detectors into numerical values, which can be later analyzed to study various characteristics of the cells using special software. (176)

Flow cytometry can both sort and analyzes cell population. The parameters are set in flow cytometry so that it can identify cells of interest with the help of the mentioned systems before and labeled antibodies. As the cell passes through a laser beam, the light is scattered in different directions. (177) This scattered light helps the researcher to determine both the size and complexity of a cell; it also differentiates in two types forward scatter (FSC) small angle and side scatter (SSC) wide angle. (178) In FSC, the diffracted light is related to the particle's surface area and refractive index, detected along the axis of incident light in the forward direction. On the other hand, the SSC refracted and reflected light measure cell granularity and complexity. (172) (Figure 8)

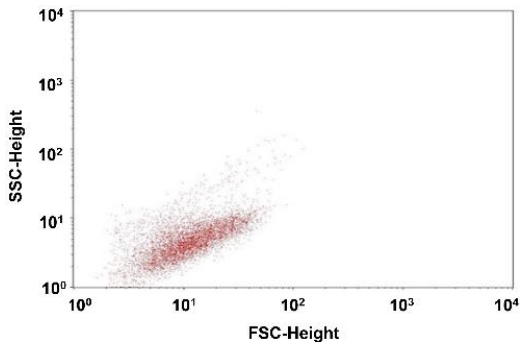

Figure 8: Dot plot diagram with the focus on platelets using flow cytometry

To identify or determine the type of cells by immunophenotyping, we use relative fluorescence intensity, which is usually generated using fluorescent labels that either directly bind to elements of particle or are bound to antibodies that bind to the particle. Then, all collected information is visualized in a type of graph called a dot plot. (179) In this study, we used CD42 FITC (PN IM06480, Beckman Coulter Company) as a specific marker for platelets GPIbα to be gated by flow cytometry, plus CXCR4 (anti-human CXCR4 PE; Mouse, Catalog number: FAB 1701, Lot: LDV0611071, RD company) in the first sample and in the other FACS tube, CD42 PE, was used as a specific marker for platelets GPIbα, plus CXCR7 (anti-human CXCR7 PE, Mouse, Catalog number: FAB 422711, Lot: AAEL0312121, RD company) to measure the surface expression of these receptors. (108) These samples were measured by the flow cytometry software (BD Bioscience).

6.6. Statistical analysis

We analyzed the entire data of clinical cohort SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA). As represented in figure legends below, the data is shown as Mean±S.D for the clinical cohort. For the comparison of non-normally distributed data, for example, mean fluorescence intensities (MFIs), we used the Mann–Whitney U test. Using Spearman's rank correlation coefficient (ρ), we determined the correlations of non-normally distributed data. On the other hand, for normally distributed data, the correlations were assessed using Pearson's correlation coefficient (r). The MFIs are determined as median values with 25th and 75th percentiles. To study the difference in oxLDL levels in stable CAD and the different stages of ACS (unstable CAD, NSTEMI, STEMI), we used the analysis of variance (ANOVA).

7. Results

7.1. Baseline characteristics of the CAD patient cohort

Characteristics of the total population (n=152) and patients presenting with a stable CAD, NSTEMI and STEMI are shown in Table 1. We included several cardiovascular risk factors (e.g. hypertension, smoking, hyperlipidemia, obesity and diabetes mellitus type II), basic characteristic (age, BMI, male gender) and medication (e.g. ASA, clopidogrel, prasugrel, Ticagrelor and ACE inhibitors, angiotensin converting enzyme inhibitors, Beta-blocker) and also lipid profile (e.g. LDL, HDL, cholesterol, Triglycerides). The mean age of the whole population was 70.1 years. Most of our study population were males 67,8%. The body mass index mean was 28.3. Regarding risk factors 87.5% were hypertensive, 61.8% were hypercholesterolemic, 36.8 % were diabetic, 13.8% were smokers and 28.9% were obese. Concerning medications 65.8% were taking Beta-Blocker medication and 57.9% of the patients were on statin medication. All patients showed a high lipid profile including LDL, Triglycerides and HDL. (Table 1)

Table 1: Baseline characteristics of the patient cohort for oxLDL analysis

	All (n=152)
Male, n (%)	103 (67.8%)
Age, years (mean ± SD)	70.1 (±12.3)
Body mass index (mean ± SD)	28.3 (±5.3)
Cardiovascular risk factors	
Arterial hypertension, n (%)	133 (87.5%)
Hyperlipidemia, n (%)	94 (61.8%)
Diabetes mellitus, n (%)	56 (36.8%)

Smoking, n (%)	21 (13.8%)
Obesity, n (%)	44 (28.9%)
Renal function (GFR) (mean ± SD)	71.4 (±33.1)
Medication of admission	
Acetylsalicylic acid, n (%)	77 (50.7%)
Clopidogrel, n (%)	29 (19.1%)
Prasugrel, n (%)	11 (7.2%)
Ticagrelor, n (%)	14 (9.2%)
Oral anticoagulants, n (%)	32 (21.1%)
Angiotensin-converting enzyme inhibitors, n (%)	74 (48.7%)
Angiotensin II receptor antagonists, n (%)	32 (21.1%)
Beta-blockers, n (%)	100 (65.8%)
Statins, n (%)	88 (57.9%)
Lipid profile parameters	
LDL-cholesterol (mg/dL)	95.3 (±41.4)
HDL-cholesterol (mg/dL)	45.9 (±12.1)
Triglycerides (mg/dL)	152.7 (±77)
Total cholesterol (mg/mL)	166.5 (38.4)
Leucocyte count (1/µg)	8445.3 (±2943.9)
Hemoglobin (g/dl)	13.3 (±1.9)

Platelet count (x1000/µl)	224.9 (±71.3)
INR	1.2 (±0.4)
PTT (sec)	40.1 (±36.4)
Creatinine (mg/dl)	1.2 (±0.8)
Bilirubin (mg/dl)	0.7 (±0.4)
CRP (mg/dl)	1.4 (±2.7)
Troponin I (µg/l)	13.9 (±61.3)
Glucose (mg/dl)	143.9 (±57. 3)
CK (U/l)	294.1 (±814.6)
GOT (U/l)	103.5 (±149)
GPT (U/l)	31.6 (±19)
LDH (U/l)	235.1 (±89.7)

Table 2: Baseline characteristics of subgroups for oxLDL analysis

We included 152 coronary artery disease patients in our study. These were divided in 3 subgroups including patients presenting with stable CAD group (n=94), NSTEMI group (n=46) and STEMI group (n=12). Characteristics of subgroups were shown in Table 2. More patients in subgroups were males (p=0.807). The cardiovascular risk factors did not differ significantly between subgroups except for smoking (p=0.004). The 3 subgroups were characterized by a high lipid profile without significant difference. Moreover, we noticed no significant interrelations regarding medical therapy except for ASA and statin in treatment of stable CAD vs. NSTEMI vs STEMI on platelets oxLDL levels shown Table 2. (108)

	Stable CAD (n=94)	NSTEMI (n=46)	STEMI (n=12)	p-Value
Male, n (%)	64 (68.1%)	30 (65.2%)	9 (75%)	0.807
Age, years (mean ± SD)	69.7 (±11.5)	71.7 (±13.9)	62.2 (±12.)	0.054
Body mass index (mean ± SD)	28.3 (±12.3)	27.6 (±6.3)	32.6 (±6.9)	0.322
Cardiovascular risk factors				
Arterial hypertension, n (%)	88 (93.6%)	38 (82.6%)	7 (58.3%)	0.013
Hyperlipidemia, n (%)	63 (67.0%)	27 (58.7%)	4 (33.3%)	0.101
Diabetes mellitus, n (%)	36 (38.3%)	17 (37.0%)	3 (25.0%)	0.827
smoking, n (%)	12 (12.8%)	4 (8.7%)	5 (41.7%)	0.004
Obesity, n (%)	27 (28.7)	15 (32.6%)	2 (16.7%)	0.156
Renal function (GFR) (mean ± SD)	70.7 (±28.4)	67.6 (±35.2)	97.0 (±44.0)	0.019
Medication of admission				
Acetylsalicylic acid, n (%)	58 (61.7%)	19 (41.3%)	0 (0.0%)	0.003
Clopidogrel, n (%)	22 (23.4%)	7 (15.2%)	0 (0.0%)	0.215
Prasugrel, n (%)	7 (7.4%)	4 (8.7%)	0 (0.0%)	0.786
Ticagrelor, n (%)	9 (9.6%)	5 (10.9%)	0 (0.0%)	0.737

Oral anticoagulants, n (%)	16 (17.0%)	16 (34.8%)	0 (0.0%)	0.040
Angiotensin-converting enzyme inhibitors, n (%)	52 (55.3%)	21 (45.7%)	1 (8.3%)	0.123
Angiotensin II receptor antagonists, n (%)	23 (24.4%)	8 (17.4%)	1 (8.3%)	0.529
Beta-blockers, n (%)	66 (69.3%)	33 (71.7%)	1 (8.3%)	0.024
Statins,n (%)	60 (63.8%)	28 (60.9%)	0 (0.0%)	0.007
Lipid profile parameters				
LDL-cholesterol (mg/dL)	96.6 (±41.6)	94.1 (±36.6)	91.4 (±48.6)	0.939
HDL-cholesterol (mg/dL)	47.0 (±12.8)	43.6 (±9.5)	40.4 (±7.4)	0.154

7.2. Platelet-oxLDL status in circulating platelets

To determine the levels of oxLDL in patients, we analyzed a consecutive cohort of 152 patients with symptomatic CAD (stable angina n=94, ACS n=58) and healthy controls (n=15), using an anti-oxLDL antibody, for detection of intracellular and surface-associated oxLDL in platelets by flow cytometry. Patients with symptomatic CAD showed enhanced levels of oxLDL in circulating platelets. (Figure 9) Patient demographics of the clinical cohort are shown in Table 1. We found that the level of oxLDL in circulating platelets was significantly enhanced in patients with CAD as compared to healthy subjects [median MFI 23.3 (25th; 75th percentile 18.0; 29.2) vs. median MFI 20.6 (25th; 75th percentile 17.8; 21.8), p=0.044]. (108) (Figure 10)

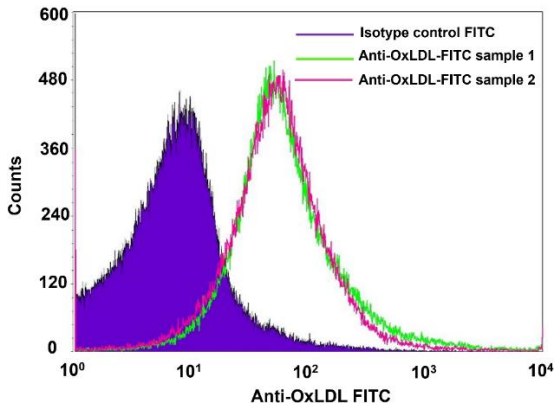

Figure 9: Flow cytometry histogram overlay showing oxLDL response with respect to Isotype control in two representative samples from CAD patients.

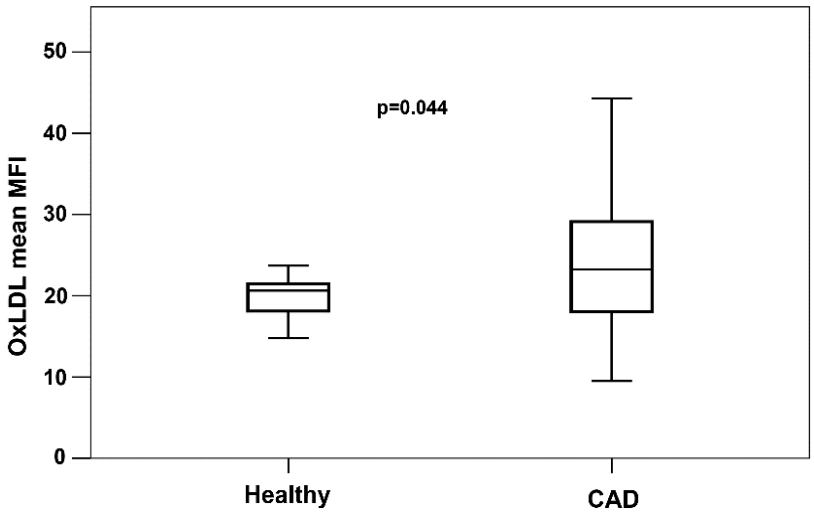

Figure 10: OxLDL levels in circulating platelets are enhanced in patients with symptomatic coronary artery disease. Modified after Chatterjee et al. (108)

7.3. Platelets-oxLDL status in Healthy, stable CAD and ACS patients

The next objective was to verify if intraplatelet oxLDL status changed with disease severity in the different clinically defined subgroups within the entire CAD cohort. However, intraplatelet OxLDL status did not differ significantly between healthy, stable CAD and ACS patients when considered individually as each group i.e., unstable angina, STEMI and NSTEMI (ρ=0.162, $p<0.001$). (108) (Figure 11)

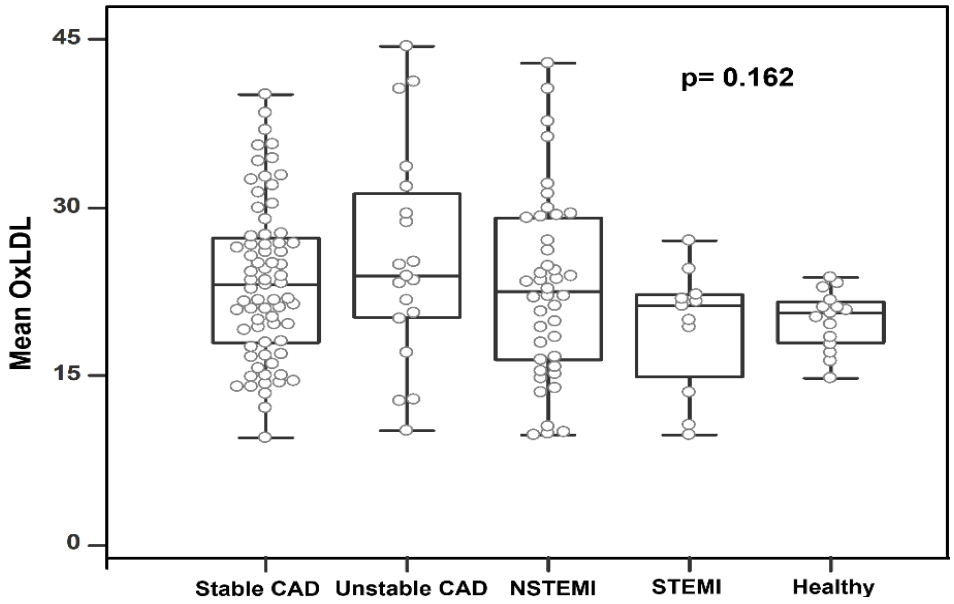

Figure 11: OxLDL status does not differ significantly between healthy, stable CAD and ACS subgroups. Modified after Chatterjee et al. (108)

7.4. Platelets-oxLDL status between stable CAD and ACS patients

The next objective was to determine possible difference between platelet oxLDL status among stable CAD patients and ACS patients (unstable angina, NSTEMI and STEMI patients taken together). However, OxLDL status did not differ significantly between stable CAD and ACS patients ($\rho=0.162$, $p<0.001$). (108) (Figure 12)

But intraplatelet oxLDL status significantly differs between ACS patients with angiographic evidence of intracoronary thrombus as compared to those without any thrombotic occlusion or burden (Chatterjee et al., Eur Heart J 2017). (108) Parallel studies detected significant oxLDL deposition in platelet enriched areas of intracoronary thrombus sections (Chatterjee et al., Eur Heart J 2017) by immunofluorescence detection. (108) This suggests that platelets can be considered as critical oxLDL or lipid depository in thrombus and may deliver atherogenic lipid to the site of vascular erosion or inflammation and contribute to the initiation of atherosclerosis. (108)

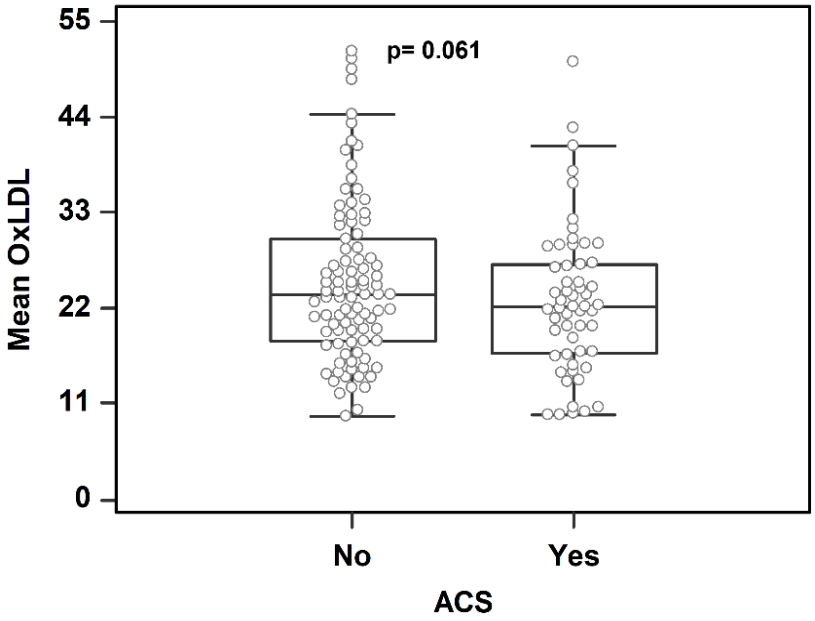

Figure 12: OxLDL status does not differ significantly between stable CAD and ACS. Modified after Chatterjee et al. (108)

7.5. Correlation between platelet oxLDL and surface expression of CXCR4

Platelet lipid interaction is greatly influenced by inflammatory state and therefore might be influenced by the presence of chemokines or pro-inflammatory cytokines in the surrounding microenvironment. Parallel ongoing experimental studies suggested that recombinant chemokine CXCL12 can enhance lipid uptake in platelets through the involvement of the chemokine receptors CXCR4 and CXCR7 (Chatterjee et al., Eur Heart J 2017). (108) Lately CXCR7 has also been associated with lipid uptake and lipase activity in adipose tissue in atherosclerosis prone *ApoE*$^{-/-}$ mice. (162) Experimental studies in our laboratory have further shown that lipids like LDL and oxLDL can also modify the activation status of platelets, enhance surface expression of CXCL12 and its receptor CXCR7 whereas decrease the surface expression of CXCR4 on platelets (Chatterjee et al., Eur Heart J 2017). (108) Therefore, we decided to validate a possible association between these two chemokine receptors CXCR4-CXCR7 and oxLDL status in platelets from CAD patients. We investigated the level of CXCR4 surface expression on platelets in healthy subjects and CAD patients. We noticed that the CXCR4 surface expression on platelets is decreased in CAD patients as compared to healthy subjects (p=

0.19). (Figure 13 A) In our analysis of clinical samples, we also observed that levels of platelet-oxLDL correlated inversely with the surface expression of CXCR4 (ρ=0.395, p<0.001) on platelets which corresponded with the experimental observation. (108) (Figure 13 B)

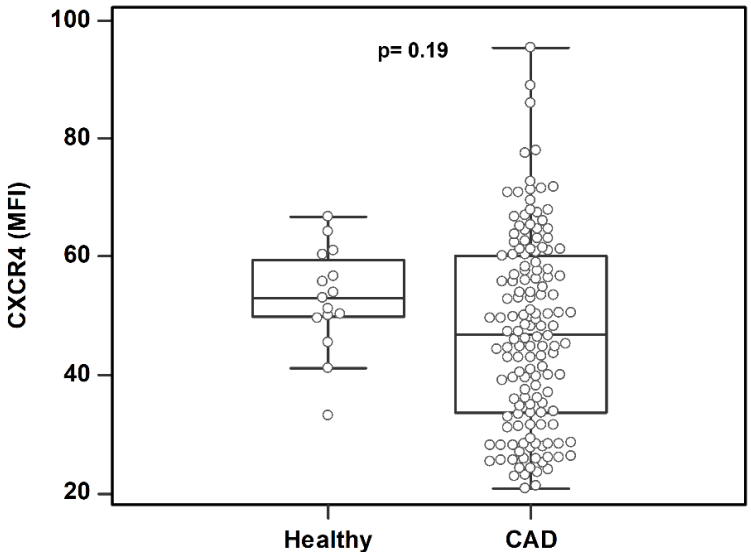

Figure 13 A: This diagram shows the comparison between the levels of CXCR4 surface expression on platelets in healthy subjects and CAD patients. The levels of CXCR4 surface expression on platelets is decreased in CAD patients as compared to healthy subjects.

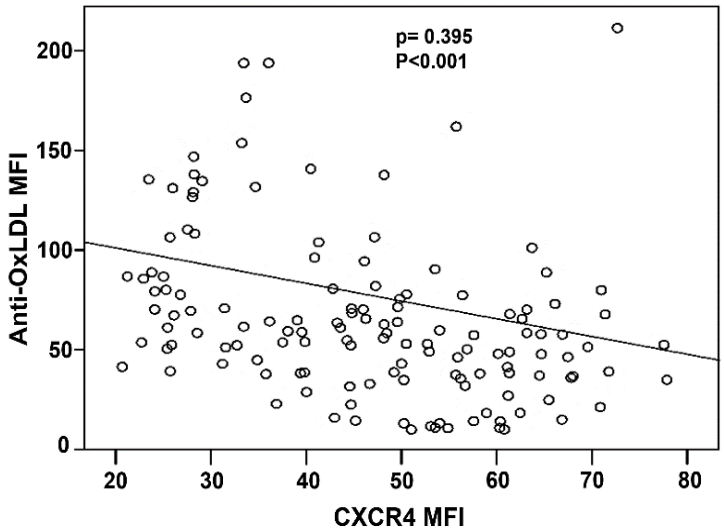

Figure 13 B: Correlation of platelet–oxLDL with platelet CXCR4 surface expression among CAD patients. Elevated platelet–oxLDL levels in CAD correlate inversely with expression of CXCR4. Modified after Chatterjee et al. (108)

7.6. Correlation between platelet oxLDL and surface expression of CXCR7

We investigated the level of CXCR7 surface expression on platelets in healthy subjects and CAD patients. We observed an elevated level of CXCR7 surface expression on platelets in CAD patients as compared to healthy subjects without clinical significance (p=0.39). (Figure 14 A)

In addition, levels of platelet-oxLDL correlated positively to a significant extent with the surface expression of CXCR7 on platelets (ρ=0.354, p<0.001) (Figure 14 B). This data also corroborates with experimental findings that in presence of LDL or oxLDL surface expression of CXCR7 on platelets is increased whereas that of CXCR4 is down-regulated (Chatterjee et al., Eur Heart J 2017). (108)

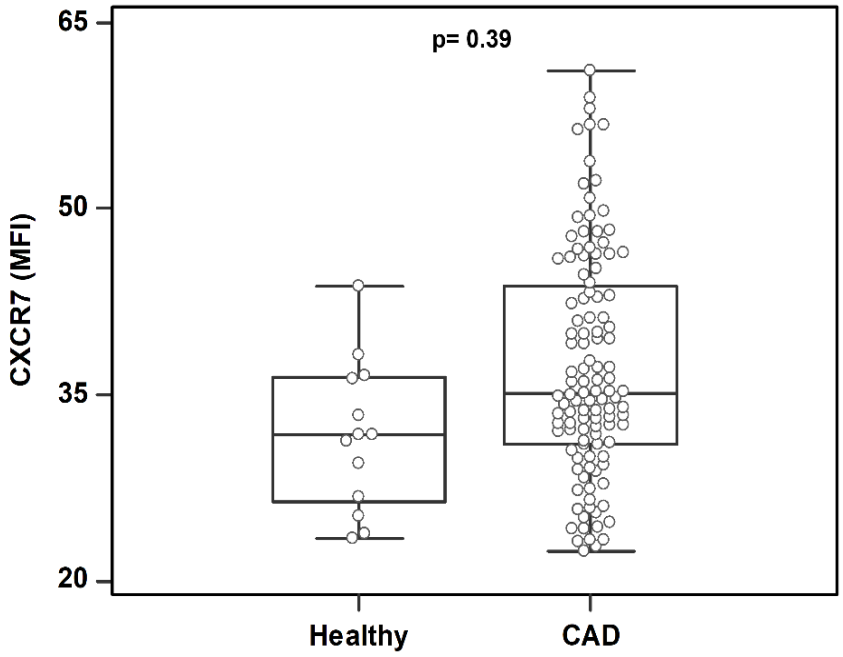

Figure 14 A: The diagram shows the comparison between the level of CXCR7 surface expression on platelets in healthy subjects and CAD patients. This showed higher level of CXCR7 surface expression on platelets in CAD patients as compared to healthy subjects.

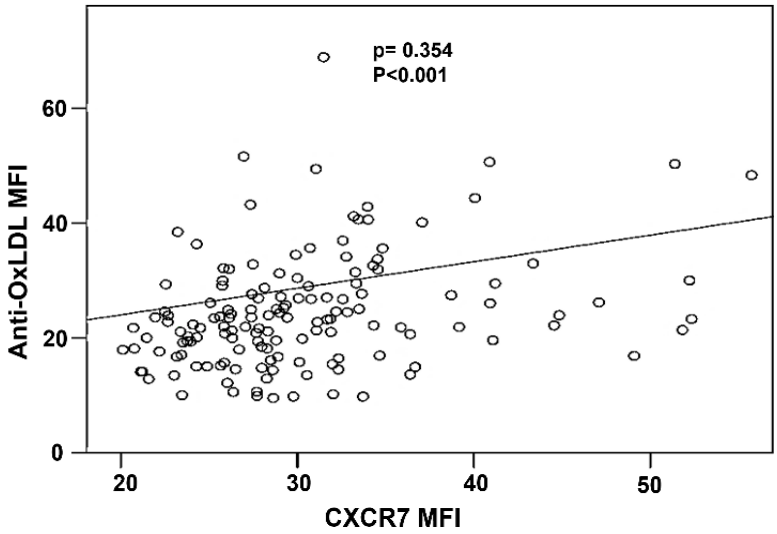

Figure 14 B: Elevated platelet–oxLDL levels in CAD correlate significantly with surface expression of CXCR7 on platelets. Modified after Chatterjee et al. (108)

7.7. Correlation between plasma lipids and platelet oxLDL status in CAD patients

Platelets are in a dynamic association with plasma lipids. Equipped with several scavenger receptors like CD36, LOX-1, ApoER2, (180) platelets can take up plasma lipids which might influence the composition of lipids within the intracellular compartment of these cells. Besides, being storage for plasms lipids, platelets can also intracellularly modify these lipids into atherogenic lipids with deleterious pathological consequences. Parallel experimental studies showed that activation of platelets with physiological agonists like collagen related peptide or uptake of LDL leading to platelet activation can trigger intraplatelet oxidation of LDL to oxLDL which is a more harmful metabolite than native LDL (Chatterjee et al., Eur Heart J 2017). (108) Therefore we checked the association of platelet oxLDL with plasma lipid profile measured as LDL, HDL, triglyceride.

7.7.1. Correlation between plasma levels of HDL and platelet oxLDL status in CAD patients

We observed that platelet oxLDL levels correlate positively with plasma levels of HDL (HDL: $r=0.220$, $p<0.05$). (108) (Figure 15). Although HDL has been shown to have some anti-thrombotic effects uptake of HDL by platelets may lead to further

intracellular metabolism or modifications into oxidized metabolites like oxLDL. Although sustained levels of HDL in plasma is clinically diagnosed as beneficial, one might take into consideration the interaction of platelets with plasma lipids and potential modifications within the platelets which is not detectable by conventional plasma lipid profile routinely done in clinics.

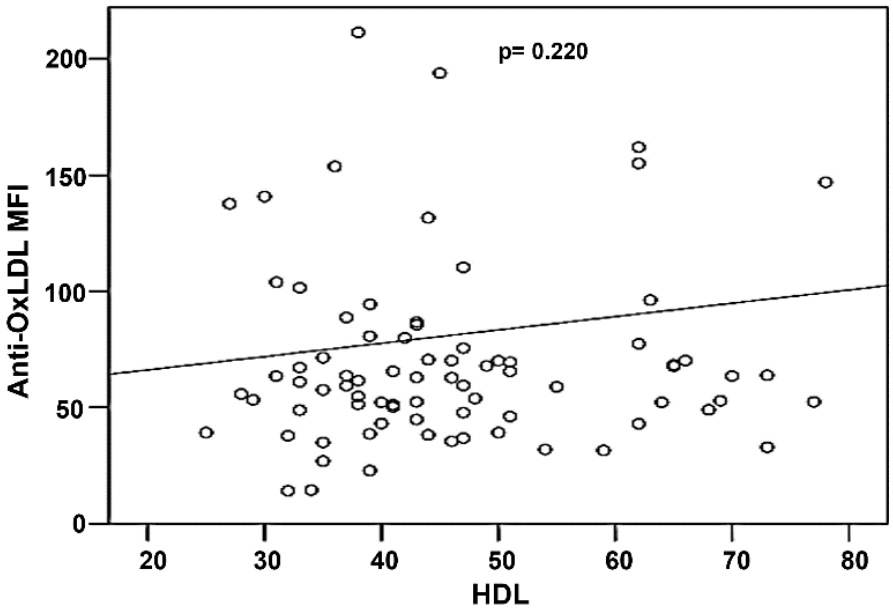

Figure 15: Correlation between HDL and platelet oxLDL status in CAD patients. Platelet oxLDL levels correlate positively with plasma levels of HDL. Modified after Chatterjee et al. (108)

7.7.2. Correlation between plasma levels of triglycerides and platelet oxLDL status in CAD patients

Further, we noticed that platelet oxLDL levels correlated positively with plasma levels of triglycerides (TG: $r=0.223$, $p<0.05$). (Figure 16)

Triglycerides can be considered as an energy source for blood cells including platelets therefore actively taken up by these cells and stored in their lipid depository or lipid droplets, Parallel lipidomic analysis of the platelet lipidome from CAD patients revealed elevated levels of triglyceride and cholesteryl esters in CAD patients as compared to control age matched healthy subjects (Chatterjee et al., Eur Heart J 2017). (108) A positive correlation between plasma triglyceride and platelet oxLDL status suggests that

platelets may actively take up plasma triglycerides and further metabolize it into oxidized lipid metabolites.

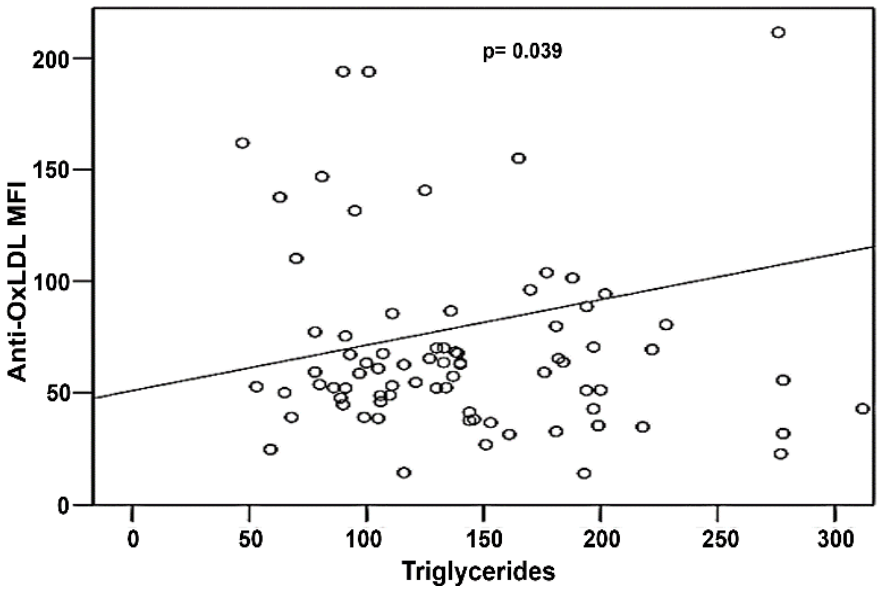

Figure 16: Correlation between triglycerides and platelet oxLDL status in CAD patients. Platelet oxLDL levels correlate positively with plasma levels of triglycerides. Modified after Chatterjee et al. (108)

7.7.3. Correlation between plasma levels of LDL and platelet oxLDL status in CAD patients

Platelet oxLDL levels correlate inversely with circulating levels of LDL ($r=-0.238$, $p<0.05$), which further emphasizes the potential of circulating platelets for lipid turnover and a dynamic interaction between plasma and platelet lipids. (108) (Figure 17). Parallel experimental data shows that platelets may take up LDL and intracellularly convert it into oxidized LDL involving reactive oxygen species (ROS) like superoxide generated in activated platelets. Therefore, incubation of platelets with LDL increases intraplatelet oxLDL levels in a dose and time dependent manner. This is counteracted in presence of a superoxide scavenger suggesting a ROS mediated process (Chatterjee et al., Eur Heart J 2017). (108)

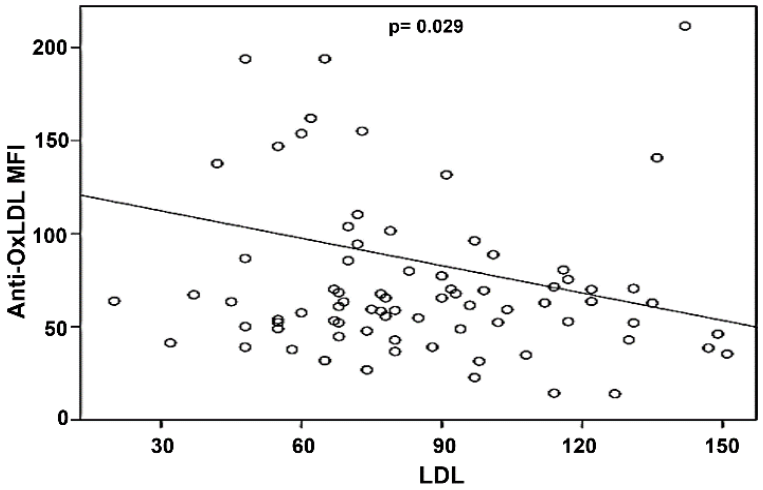

Figure 17: Correlation between LDL and platelet oxLDL status in CAD patients. Platelet oxLDL levels correlate inversely with circulating levels of LDL. Modified after Chatterjee et al. (108)

7.8. Correlation between platelets oxLDL and angiographic severity of CAD using Gensini score

The objective was to determine possible correlation between platelets oxLDL and severity of CAD. Gensini score is one of the commonly used scores to assess the severity of CAD. This score depends on the anatomy of stenosed vessel, the degree of stenosis in this vessel and the number of the affected vessels. (181) This was assessed using coronary angiography. The sums of these scores for each lesion lead to the value for Gensini score of this lesion. (181) Platelet oxLDL status was not associated with the angiographic severity of CAD using the Gensini score (ρ=0.627, $p<0.001$). (Figure 18) We also investigated the relation of oxLDL with different Gensini score quantiles (ρ=0.364, $p<0.001$), these also showed no correlation. (Figures 19) Taken together these evidence suggest that platelet oxLDL status although a potential risk factor for thrombotic, atherosclerotic disposition and contribute to the development of thromboischemic complications but cannot influence disease severity which is chiefly determined by the extent of myocardial damage and degree of recovery.

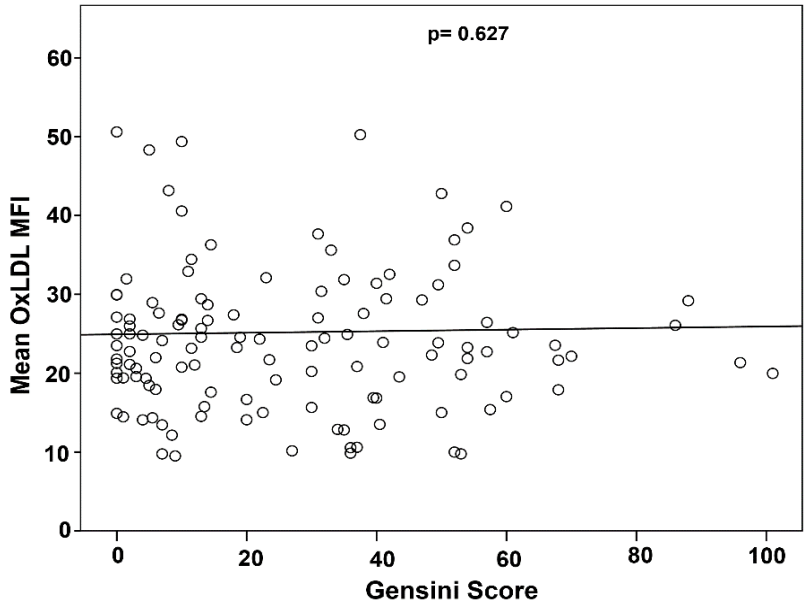

Figure 18: OxLDL status was not associated with the angiographic severity of CAD using the Gensini score.

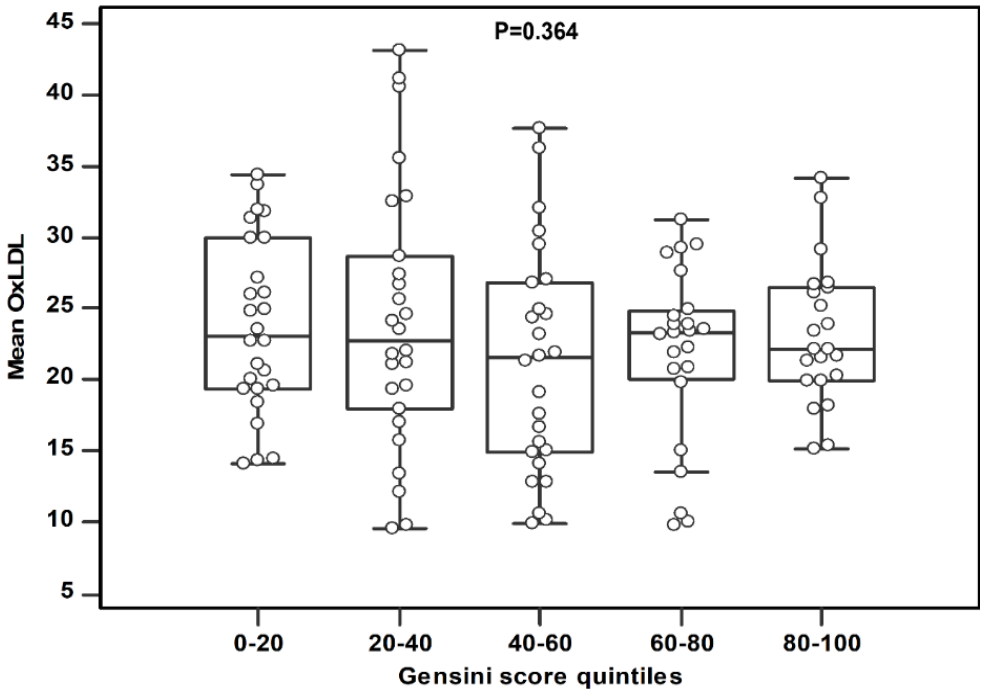

Figure 19: OxLDL status was not associated with the angiographic severity of CAD using different quintiles of Gensini score.

Future perspective from current observations: Current ongoing investigations in our laboratory have reported altered lipidomic profile of platelets from CAD patients as compared to

age matched control subjects (Chatterjee et al., Eur Heart J 2017). (108) Levels of several lipid classes are altered within the platelet lipidome including ceramides, sphinomyelins, triglycerides, cholesteryl esters, diacylglycerol, and also oxidized phospholipids. Altered platelet lipidome could be a result of the hyperactive status of circulating platelets besides their dynamic interaction with plasma lipids. OxLDL status in platelets is a marker of both platelet hyper-reactivity and altered redox status. Previously we have shown that oxLDL binding to platelets in ACS patients correlates with the activation status of platelets. (169) In the current study we have established that not only binding of oxLDL but intraplatelet levels of oxLDL is elevated in CAD patients as compared to healthy subjects. Further investigations in larger cohorts in future are required to see the impact of anti-platelet therapies on oxLDL status of platelets. Although current results show that oxLDL status in platelets does not change with disease severity, platelets can deposit atherogenic lipids like oxLDL in thrombus and possibly at atherosclerotic lesions. Therefore, a potential prognostic impact of platelet oxLDL cannot be ruled out and needs to be validated in follow up studies.

8. Discussion

This major findings of the present study are: 1) Platelet-oxLDL is significantly elevated in patients with CAD as compared to healthy subjects; 2) Platelet-oxLDL correlates with the surface expression of CXCR7 but inversely with that of CXCR4; 3) Platelet-oxLDL correlates with plasma levels of HDL, triglycerides, but inversely with LDL levels in CAD patients; however 4) platelet oxLDL does not correlate with the angiographic severity of CAD.

Platelets-lipid interactions play a major role in atheroprogression and consequently the development of CAD. The CXCL12/CXCR4-CXCR7 axis is associated with the regulation of platelet functions, their regenerative contribution in cardiovascular pathophysiologies and also contributes to lipid uptake in platelets. (108, 126, 141) The potential influence of the CXCL12/CXCR4-CXCR7 axis on the platelet lipidome might be of pathophysiological relevance in CAD patients and is therefore explored in this project. (108)

In this study, the platelet-oxLDL levels were significantly elevated in patients with CAD in comparison to healthy subjects; however, oxLDL levels did not differ between stable CAD and ACS. This confirms the results of previous studies, where no significant difference was observed between the binding of oxLDL to platelets in patients with STEMI and NSTEMI. (169) Previous studies showed that oxLDL initiates and contributes to atheroprogression and endothelial dysfunction and triggers inflammatory response which leads to plaque destabilization. (182, 183) Ischemic occlusion and atherothrombosis are triggered by lipid-enriched plaque rupture. (91) OxLDL is composed of a series of metabolites that result from the oxidative alteration of its protein and lipid components, which yield different immunogenic oxidation-specific epitopes. (184)

Blood platelets and oxidized low-density lipoprotein (oxLDL) are fundamentally associated with atherogenesis and ACS. (87, 185-187). OxLDL plays a pivotal role in CAD and especially in ACS by inducing coronary atherosclerotic plaques instability. (185, 188) OxLDL enhances the generation of foam cell, activates macrophages, proliferates smooth muscle cells and reduces nitric oxide endothelial production. Moreover, oxLDL induces thrombogenicity by enhancement of the tissue factor liberation but, more significantly, by promoting activation of platelets and their endothelium adhesion. (169)

Despite the fact that the interaction between platelet and inflammatory cells lead to differentiation of macrophages and foam cells, actively dispose to vascular inflammation and thrombotic complications, as suggested by multiple experimental studies, its clinical relevance remained undefined. (189, 190) Through five scavenger receptors, which are expressed on platelets surface, oxLDL binds to platelets. These receptors include scavenger receptors that bind phosphatidylserine and oxidized lipoprotein/chemokine (C-X-C motif) ligand 16 (CXCL16-SR), CD36, lectin-like oxidized LDL receptor-1, class B scavenger receptor I, and class A scavenger receptor. (189, 191) Binding of oxLDL on platelets leads to platelets' activation and phagocytosis of activated -apoptotic platelets by macrophages and their subsequent differentiation into foam cells. (186, 190, 191) Thus, platelet-oxLDL may critically contribute to atheroprogression and initiation, but its clinical importance has not been clarified until now. Platelet-oxLDL surface exposure differs depending on relative levels and availability of LDL, HDL, VLDL and oxLDL concentration in blood (circulatory lipids) or at sites of unstable lesions where after plaque rupture, oxLDL is exposed. (192) Previous *in vitro* studies showed that through oxLDL binding to platelet CD36, platelet activation is promoted. (193) On the other hand activation status of platelets modulate intraplatelet lipid metabolism and their oxidation. (108) Moreover, an increment in oxLDL platelet binding among ACS patients has a correlation with the platelet activation level among patients with CAD. (169) The relationship between oxLDL and platelets enhance platelets to be more reactive and adhesive. It also enhances coronary thrombosis among patients with ACS. (169) The interaction of lipoproteins and platelet might be a potential target of therapy within atherothrombotic disease patients. (169) This study showed that platelets play a critical role in CAD lipid metabolism. (108) The profile of intraplatelet lipid can be changed by platelets through enzymatic and intracellular oxidative alterations. This results in endothelial dysfunction, monocyte/macrophage differentiation, instability and growth of plaques and ischemic complications which are stimulated by pro-inflammatory oxidized lipids. (91, 182, 183) However, the influence and mechanism of intraplatelet oxidized lipid metabolites on the severity of CAD is still unclear. Recently we have shown that lipid-induced changes in redox status and thrombotic functions can be influenced by the CXCL12-CXCR4-CXCR7 axis through experimental studies. These evidence promoted

us to parallel explore the potential influence of this chemokine receptor axis on intraplatelet lipid status in CAD patients. (108)

Currently, we found a significant positive correlation between platelet-oxLDL and CXCR7 surface expression and an inverse correlation with CXCR4 among patients with CAD. (108) Increasing evidence proposes that CXCL12 plays an important role in tissue healing, progenitor cells trafficking, and myocardial recovery after myocardial infarction. (163, 194) Platelets are a significant source of CXCL12. (137) In ACS patients, CXCL12 platelet surface expression levels are upregulated. (165) CXCR4 and CXCR7 were identified as receptors for CXCL12. (151, 130) The biological function and expression of CXCR4 and CXCR7 differ between various cell types. CXCR4 is important for mediation of progenitor cell migration. In contrast, CXCR7 is crucial for adhesion of progenitor cells and CXCL12 induced cell survival. (152) Any change in CXCL12 gradients might result in various CXCR4 signaling. (195, 196) CXCL12 treatment influences CXCR4 and CXCR7 differently. CXCR4 is internalized, (197) whereas CXCR7 undergoes a dynamic externalization. (198) Previous studies demonstrated that CXCR7 platelet surface expression is elevated in ACS patients. (157) Moreover, CXCR4 and CXCR7 correlate with CXCL12 surface expression. (157) Furthermore, platelets CXCR7 is associated with functional recovery after ACS and correlates significantly with platelet CXCL12. (167) Platelet-CXCL12 and its receptors CXCR4-CXCR7 might also influence prognosis in CAD. (126, 167) The potential correlation between cholesterol, left ventricular function, cardiovascular risk factors and therapy on plasma CXCL12 in SAP, STEMI and NSTEMI patients is not clearly understood. (163) Previous studies demonstrate an inverse correlation between cholesterol and CXCL12 in the whole population and STEMI patients. Moreover, it showed a decrease in SDF-1 levels in hyperlipidemia and STEMI patients, as well as a potential effect of statin therapy on CXCL12 levels in SAP and NSTEMI patients. (163) These findings propose a possible effect of cholesterol on CXCL12 expression and regulation in patients with CAD, which is consistent with recent findings reported in hypercholesterolemic mice. (199) Previously we have shown that the presence of CXCL12 promotes phagocytosis of lipid-laden platelets by monocytes and M1-M2 macrophages by acting through its receptors CXCR4 and CXCR7 receptors, a process which further substantiates their differentiation into foam cells. (96) It was shown

that, if platelets were stimulated with oxLDL, this would lead to the formation of platelet-monocyte aggregates (PMA) and phagocytosis of platelets as well as enhanced oxLDL uptake by monocytes. (84) This means that platelets can store and transfer a significant amount of oxLDL to atherosclerotic lesions, which show the significant role of platelets in atherogenesis. (97) On the other hand platelet lipid interactions might also influence the release of CXCL12 from platelets and the surface availability of its receptors. It was shown that oxLDL enhanced the surface expression of CXCL12, which decreased CXCR4's surface exposure. (108) This may be due to CXCR4 receptor internalization and CXCR7 externalization *in vitro*. (167)

In this study, we observed an interaction between CXCR7 surface expression and elevated platelet-oxLDL levels. The CXCR7 receptor induces the uptake of cholesterol in adipose tissue to downregulate the levels of plasma cholesterol. (162) The correlation between plasma levels of CXCL12 and hypercholesterolemia was previously described. (163) Furthermore, circulating CXCL12 via CXCR4 and CXCR7 receptors may play an important role in the regulation of the lipid uptake (LDL/OxLDL) by platelets. (170) This suggests a possible effect of the CXCL12/CXCR4/CXCR7 axis on circulatory lipid turnover in platelets. (108, 163, 164) Additionally, CXCL12 enhances platelets' activation and thrombotic potential. (108) These novel findings suggest on a prothrombotic and prooxidative role of platelet-derived chemokines in hyperlipidemic conditions, proposing the CXCL12/CXCR4/CXCR7 to be a possible future therapeutic target in the modulation of the platelet-lipoprotein interaction in influencing predisposition to and progression of CAD. (108)

The platelet lipidome is associated with plasma lipids not only in CAD but also in healthy subjects. (200) In this study, we have shown a correlation between platelet-oxLDL levels and the plasma lipids. On the one hand, we demonstrated a positive correlation between platelet-oxLDL levels and plasma HDL and triglyceride levels in CAD patients; on the other hand, we demonstrated an inverse correlation between platelet-oxLDL levels and plasma-LDL levels. (108) Platelets play a significant role in lipid metabolism in CAD. The intraplatelet lipid profile may be altered by plasma lipid uptake by platelets and subsequent intercellular oxidative and enzymatic adjustment. (108) The interplay between platelets and lipoproteins was shown to lead to platelet activation and the

differentiation of macrophages and foam cells. (184) Several studies have shown the direct relation between the hemostatic function of platelets and plasma lipoproteins. (100, 201, 202) Lipoproteins may alter the aggregation response of platelets. Furthermore, HDL may reduce thrombin-induced platelet aggregation, while LDL might enhance thrombin-induced platelet aggregation. (100) Moreover, plasma LDL levels correlate with platelet aggregation and enhanced platelet sensitivity. We observed that LDL uptake by platelets significantly upregulated intracellular oxLDL status. (108) Further studies are needed to better understand the mechanisms regulating this process. Furthermore, the clinical relevance of platelet oxLDL is still to be investigated. (108)

Finally, we have examined the relationship between the angiographic severity of CAD and the levels of platelets oxLDL. (49) We found no relation between platelets oxLDL and the severity of CAD. These results suggest an important role of oxLDL in the interplay between platelets and lipoproteins regardless of the severity of the CAD, suggesting a more global function of oxLDL in CAD.

9. Conclusion

CAD constitutes the most prevalent cause of mortality globally. Recent evidence strongly proposes that CAD originates from vessel occlusion through lipid deposition as well as chronic inflammatory reaction towards injury or infection. Within the study, 152 symptomatic CAD patients alongside 15 healthy subjects who served as the control cohort for platelet oxLDL status and CXCR 4 and CXCR7 platelet surface expression were investigated using flow cytometry. The findings revealed that levels of platelet oxLDL increased among CAD patients than in healthy subjects. It also demonstrated that platelet oxLDL significantly correlated with surface expression of CXCR7 but negatively correlated with CXCR4 in CAD patients. From the study's results, CXCL12 circulation through receptors of CXCR4 and CXCR7 can be part of lipid uptake regulation by platelets. This promotes the platelets' thrombotic ability and activation.

Furthermore, a correlation between platelet-oxLDL levels and the plasma lipids was investigated in this study. A positive correlation between platelet-oxLDL levels and plasma HDL and triglyceride levels in CAD patients was demonstrated; on the other hand, we have shown an inverse correlation between platelet-oxLDL levels and plasma-LDL levels. Further studies are warranted to better understand the mechanisms regulating this process. However, the influence of intraplatelet-oxidized lipid metabolites on the severity of CAD is still unclear. Furthermore, the clinical relevance of platelet oxLDL warrants further investigation. The findings of this study suggest a potential prothrombotic and prooxidative role of platelet-derived chemokines in hyperlipidemic conditions, proposing the CXCL12/ CXCR4/ CXCR7 axis to be a possible future therapeutic target for modulation of the platelet-lipoprotein mediated progression of CAD.

10. Zusammenfassung

Die koronare Herzkrankheit ist die häufigste Todesursache in Industrienationen weltweit. In dieser Studie haben wir bei 152 Patienten mit symptomatischer koronarer Herzerkrankung (KHK) und 15 gesunden Probanden thrombozytäres oxLDL, CXCR4 sowie CXCR7 untersucht. In dieser Studie wurde die thrombozytäre oxLDL Oberflächenexpression bei Gesunden, Patienten mit stabiler KHK sowie bei Patienten mit akutem Koronarsyndrom (ACS) untersucht. Der thrombozytäre oxLDL Spiegel war bei Patienten mit KHK im Vergleich zu gesunden Probanden erhöht. In der aktuellen Studie wurde eine signifikante positive Korrelation zwischen der thrombozytären oxLDL Oberflächenexpression mit derjenigen von CXCR7 gezeigt. Des Weiteren stellten wir eine inverse Korrelation mit CXCR4 fest. Hieraus schließen wir auf den potentiellen Einfluss der CXCL12/CRCR4/CXCR7 Achse auf die Regulation der thrombozytären Lipidaufnahme sowie auf das thromboembolische Potential der Blutplättchen. Dies bestätigen auch die früheren Studienergebnisse, die die Bedeutung dieser Achse bei KHK-Patienten und ihre Beziehung zum Plasmalipidom betreffen. Darüber hinaus wurden in dieser Studie Assoziationen zwischen thrombozytären oxLDL Spiegeln und Plasma-Lipiden untersucht. Es wurde einerseits eine positive Korrelation zwischen oxLDL und HDL sowie Triglyzeridspiegeln festgestellt. Andererseits zeigten wir eine inverse Korrelation zwischen oxLDL und Plasma-LDL Konzentrationen. Weitere Studien sind notwendig, um die Mechanismen, die diesen Prozess steuern, besser zu verstehen. Aktuell ist der Einfluss von intrathrombozytären oxidierten Lipidmetaboliten auf den Schweregrad der KHK unklar. Darüber hinaus muss die klinische Relevanz von thrombozytärem oxLDL weiter untersucht werden. Die Ergebnisse diese Studie suggerieren eine pro-thrombotische und pro-oxidative Rolle thrombozytärer Chemokine bei hyperlipidämer Stoffwechsellage.

Die CXCL12/CXCR4/CXCR7 Achse könnte als mögliches therapeutisches Ziel zur Modulation der thrombozytären Lipide dienen, mit potentiell günstigen Auswirkungen auf die Progression der KHK.

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Ich versichere, das Manuskript selbständig nach Anleitung durch Dr.med. Dominik Rath und Dr.rer.nat. Madhumita Chatterjee verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben

13. Veröffentlichungen:

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