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Lipidomic Profile of Platelets as a Peripheral Biomarker in Patients with Coronary Artery Disease

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1. Contents

2. List of abbreviations

AA	Arachidonic acid
ACD	Acid-citrate-dextrose
ACEI	Angiotensin converting enzyme inhibitor
AMI	Acute myocardial infarction
ARB	Angiotensin II receptor blocker
ASA	Acetylsalicylic acid
AUC	Area under the curve
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CCL	CC chemokine ligand
CD40	Cluster of differentiation 40
CK-MB	Creatine kinase MB isoenzyme
COX	Cyclooxygenase
CRP	C-reactive protein
CXCL	CXC chemokine ligand
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
ESI	Electrospray-ionisation
FA	Fatty acid
FDA	Food and drug administration
FDR	False discovery rate
g	Gravity-force
GC	Gas chromatography
GFR-MDRD	Glomerular filtration rate (Modification of Diet in Renal Disease)
GP	Glycoprotein
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HETE	Hydroxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography
IR	Infrared

LC	Liquid chromatography
LDL	Low-density lipoprotein
LOX	Lipoxygenase
Lp(a)	Lipoprotein(a)
LPA-R	Lysophosphatidic receptor
LVEF	Left ventricular ejection fraction
lysoPC	Lysophosphatidylcholine
lysoPE	Lysophosphatidylethanolamine
lysoPL	Lysophospholipid
MAPK	Mitogen-activated protein kinase
m/z	Mass to charge ratio
mo.	Month
MPO	Myeloperoxidase
MS	Mass spectrometry
NADP	Nicotinamide adenine dinucleotide phosphate
NMS	Nanospray mass spectrometry
no.	Number
non-HDL-C	Non-high-density lipoprotein cholesterol
NOS	Nitric oxide synthase
NOX	NADPH oxidase
OPLS-DA	Orthogonal partial least squares discriminant analysis
oxLDL	Oxidized low-density lipoprotein
PBS	Phosphate buffered saline
PC	Phosphatidylcholin
PCA	Principal component analysis
PCI	Percutaneous coronary intervention
PCSK9	Proprotein convertase subtilisin/kexin type 9
PDGF	Platelet derived growth factor
PE	Phosphatidylethanolamine
PECAM-1	Platelet endothelial cell adhesion molecule 1
PG	Prostaglandin

PI	Phosphatidylinositide/phosphatidylinositol
PKC	Protein kinase C
PL	Phospholipid
PLA2	Phospholipase A2
PLS	Partial least squares
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Phosphatidylserine
psi	Pound-force per square inch
PUFA	Polyunsaturated fatty acid
QC	Quality control
QTOF	Quadrupole time-of-flight
RANTES	Regulated on activation, normal T cell expressed and secreted
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAP	Stable angina pectoris
SM	Sphingomyelin
TG	Triglyceride
TGFβ	Transforming growth factor beta 1
TLC	Thin layer chromatography
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
V	Volt
VLDL	Very low-density lipoprotein
vWF	von Willebrand factor
XO	Xanthine oxidase

3. Introduction

3.1. Atherosclerosis

Cardiovascular disease, especially atherosclerosis, is the leading cause of global death and premature disability in developed countries, accounting for more than 17.3 million deaths per year. The number is estimated to reach more than 23.6 million by 2030 (1). Atherosclerosis is a systemic disease induced by the accumulation of inflammatory cells in the intima layers of arteries (2). The pathogenesis of atherosclerosis is based on the building of atheromatous plaques in arteries, called atherogenesis. Depending on the damaged arteries atherosclerosis may manifest as a coronary artery disease, transient cerebral ischemia, renal artery stenosis, mesenteric ischemia or peripheral artery disease (3).

3.1.1. Contribution of platelets to atheroprogression

It has been proven that platelets not only take part in thromboembolic complications in advanced stages of atherosclerosis but also initiate the formation of atherosclerotic plaque and contribute to the development of the disease. There are two main mechanisms by which platelets contribute to the development of atherosclerotic lesions. Firstly, activated platelets adhere to the activated or inflamed endothelium based on a receptor/ligand interaction involving GP Ib/IX/V complex, vWF. Platelets can also interact with exposed subendothelial matrix components like collagen which leads to their activation (4,5). Platelets can also adhere to intact and/or inflamed endothelial monolayers and initiate endothelium activation. *In vivo* studies showed that platelet adherence to the endothelium of carotid arteries in apoE-deficient mice is initiated even without endothelial denudation or manifestation of atherosclerotic lesions (6). This interaction occurs in three steps: platelet tethering; platelet rolling ensured by selectin/endothelium communication, e. g. P-, E-selectins (4,7); and firm platelet adhesion ensured by integrins, e. g. GPIb, GPIIb-IIIa (these receptors contribute significantly to cell-cell interaction and platelet

aggregation) (5,8). These interactions result in receptor-specific activation signals in both platelets and endothelial cells.

P-selectin contributes not only to platelet-endothelium communication, but its soluble form in plasma, generated by activated platelets and endothelial cells leads to a pro-coagulant state and contributes to the progression of atheromatous lesions (9).

The second major mechanism attributing to atherogenesis is the production of pro-inflammatory chemokines by activated platelets (e. g. CCL3, RANTES (CCL5), CCL 7, CCL17, CXCL1, CXCL5, CXCL8 or CXCL12 as well as a precursor for CXCL7, such as β-thromboglobulin) and the inflamed endothelium. Their secretion is induced either by thrombin or by oxidized low-density lipoproteins (oxLDL) which activate platelets. Chemokines induce pro-inflammatory changes in endothelial and other inflammatory immune cells like monocytes, macrophages, lymphocytes; also promote the adhesion of platelet aggregates to the arterial wall. This, in turn, stimulates atherogenic monocyte recruitment and their subsequent differentiation into macrophages and foam cells (10,11).

3.1.2. Development of atheromatous plaque

Atherosclerotic plaques are defined as lipid-containing lesions on the intima of the wall of an artery. Hypercholesterolemia causes lipoprotein accumulation and binding to the extracellular matrix in the intima of the arteries which results in “fatty streaks” seen macroscopically (3). Plasma lipids, in particular native low-density lipoproteins (LDL), are taken up by endothelial cells via LDL receptor-mediated endocytosis (12). This facilitates oxidative modifications of lipoproteins whereby hydroperoxides, lysophospholipids, oxysterols, and aldehydic breakdown products of fatty acids and phospholipids are produced (3). OxLDL exhibit some new features compared to native LDL which enhances the proatherogenic process. Production of oxLDL and the chemoattractants, e.g. macrophage chemoattractant protein-1 produced by endothelial cells leads to an inflammatory response and triggers monocyte recruitment to subendothelial space (13). Certain cytokines (e. g. IL-1β, IFN- γ, tumor necrosis

factor α (TNF-α) induce the expression of leukocyte adhesion molecules on endothelial cells (13). These three pathways lead to monocyte accumulation in the arterial wall and their differentiation into macrophages.

Once inhabiting the arterial intima monocyte-derived macrophages become able to express scavenger receptors for modified lipoproteins which lead to receptor-mediated endocytosis of modified lipoproteins and development of foam cells (14). Thus the “fatty streak”, a precursor of atherosclerotic plaque, is formed.

The arterial lesion advances further as the smooth muscle cells proliferate and migrate into the intima the former being induced by transforming growth factor beta 1 (TGFβ) which changes the contractile phenotype of the smooth muscle cells to a proliferative one (15). TGFβ also markedly stimulates the smooth muscle cells to produce collagen 1 (16). The buildup of fibrous tissue leads to advancement and complication of atheromatous plaque. These processes are induced by various growth factors, e.g. platelet-derived growth factor (PDGF) or fibroblast growth factor, the production of which is driven by IL-1β or TNF-α (17).

After the advanced plaque erodes and ruptures, platelets are activated and aggregate on the atherosclerotic plaque surface (18). Thrombotic occlusion of the artery leads to the manifestation of atherosclerosis with thrombo-ischemic events.

3.1.3. Platelet and lipoprotein interplay

Dysregulation in plasma lipoproteins is one of the most firmly established risk factors for atherosclerosis, most important ones being elevated LDL, very low-density lipoprotein (VLDL) and triglyceride (TG) levels or decreased high-density lipoprotein (HDL) levels as detected conventionally in plasma. In patients with hypercholesterolemia, platelets exhibit an increased activity and are hypersensitive to agonists, change their membrane phospholipid and cell lipid composition, as a consequence of platelet-lipid interaction (19).

Previously it has been shown that presence of oxLDL in the surrounding microenvironment of platelets enhances their aggregation. This response is substantiated by NADPH oxidase (NOX2)-derived reactive oxygen species (ROS) through a scavenger receptor CD36/protein kinase C (PKC) mediated pathway. Generation of ROS promote platelet hyperactivity in the means of modulating cGMP signaling (20). Activated platelet derived ROS potentiates oxidation of LDL to more atherogenic oxLDL in the external microenvironment (19). Recently it has also been shown that both LDL and oxLDL can trigger generation of mitochondrial superoxide and ROS generation in platelets which further propagates intraplatelet oxidative and peroxidative modifications to lipids, besides prompting their functional responses (21).

Altered platelet functions are associated with LDL, VLDL, and especially oxLDL, which all contain apoprotein B-100 and are atherogenic lipoproteins. LDL stimulate platelets through a single receptor for lipoproteins called ApoE-R2' (22–24). After binding LDL through ApoE-R2' several intracellular cascades in platelets are initiated (Figure 1):

- p38MAPK (mitogen-activated protein kinase) pathway activates phospholipase A2 (PLA2) which leads to enhanced production of prostaglandin endoperoxides by cyclooxygenase 1 (COX1) and thromboxane A_2 by thromboxane synthase (Tx synthase).
- PECAM-1 (platelet endothelial cell adhesion molecule 1) pathway inhibits the functions of the p38MAPK.
- A third route leads to activation of focal adhesion kinase (FAK) which integrates different signaling pathways.
- A fourth route leads to activation of a small GTPase Rap1b which stimulates activation of the integrin αIIbβ3 on the platelet surface to support fibrinogen binding and platelet aggregation (24).

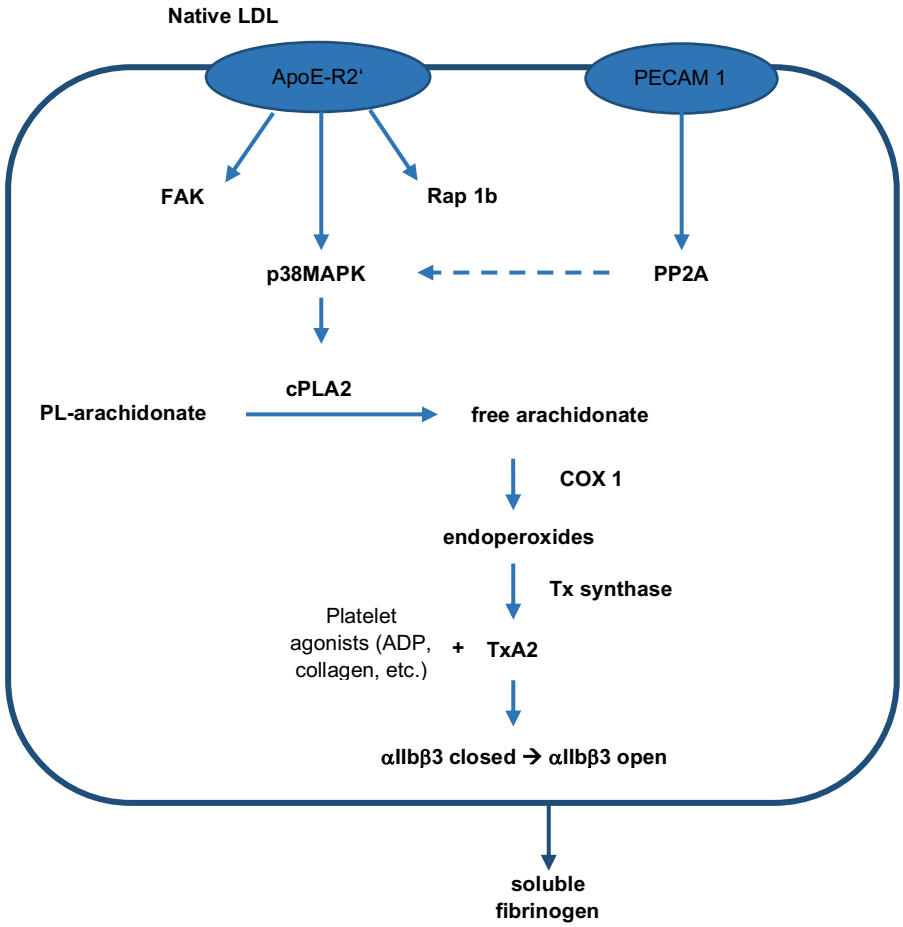

Figure 1. Platelet activation by native LDL. Binding to ApoE-R2' signals to p38MAPK, FAL and GTPase Rap 1b. Activation of cPLA2 leads to arachidonate release from membrane PL. Arachidonate is converted into endoperoxides by COX1 and the latter to TxA2 by Tx synthase. TxA2 acts synergistically with platelet agonists (ADP, thrombin, collagen). Integrin αIIbβ3 is activated and binds soluble fibrinogen. Platelet aggregation is initiated. PECAM1 pathway inhibits the signaling through p38MAPK (modified from 24).

HDL is a heterogeneous lipoprotein and there exist two HDL subclasses (HDL_2 and HDL_3). HDL_2 fraction exhibits antiatherogenic effects by inhibiting platelet functions (thrombin-induced platelet aggregation, platelet shape change, Ca^{2+} mobilization, inositol phospholipids production, and reduction of NA-synthase expression induced by oxLDL) (25).

As already mentioned above modified LDL uptake through scavenger receptors in macrophages leads to foam cell formation. Previous studies have shown that platelets also exhibit the ability to express scavenger receptors.

- Class B scavenger receptor CD36 which binds to oxLDL is found on resting and activated platelets (26).
- Class E scavenger receptor lipoxygenase-1 (LOX-1) was detected only on activated platelets and also exhibits affinity to oxLDL (27).
- Class B scavenger receptor B1 binds the anti-atherogenic HDL and is inversely associated with cholesteryl ester content of platelets (28).

Scavenger receptors also serve in platelet recognition by macrophages which then phagocyte platelets and accumulate their lipids resulting in foam cell formation (29).

OxLDL enhances the above mentioned four intraplatelet pathways even stronger than naive LDL as oxLDL can affect platelets not only through the usual ApoE-R2' receptors but also through scavenger receptors – CD36 and lysophosphatidic receptors (LPA-R) (24).

Platelets also contribute to the progression of atheromatous plaques by being able to oxidize LDL extracellularly by producing ROS in their immediate microenvironment (19).

3.2. Lipids

Lipids are small hydrophobic or amphipathic molecules which originate entirely or in part by carbanion-based condensation of thioesters (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides) and/or by carbonation-based condensation of isoprene units (prenol lipids and sterol lipids) (30). In 2005 the International Lipid Classification and Nomenclature Committee developed a comprehensive lipid classification which organized lipids into eight categories based on biochemical and chemical principles: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides (generated by condensation of ketoacyl subunits); and sterol lipids and prenol lipids (generated by condensation of isoprene subunits) (Table 1) (31).

Table 1. Current lipid classification (modified from 31).

Category	Structures in database
Fatty acyls	2678
Glycerolipids	3009
Glycerophospholipids	1970
Sphingolipids	620
Sterol lipids	1744
Prenol lipids	610
Saccharolipids	11
Polyketides	32

The total number of lipid molecular species is determined by intrinsic possible covalent moieties and molecular variants that result from enzymatic or non-enzymatic oxidation, nitrosylation, and other environmental factors (32).

Lipids are either produced endogenously or incorporated into cells from dietary sources (33). Lipids possess a variety of functions which include storing energy, acting as structural components of cell membranes, and serving as second messengers of signal transduction (34).

3.2.1. Phospholipids and glycerophospholipids in platelets

The main structural lipids in platelets as in all mammalian cells are phospholipids. They consist of hydrophobic fatty acid oriented to the core of membrane and a polar headgroup facing the aqueous phase. Phospholipids serve as substrates for certain active species, including 1,2-diacylglycerol (DAG), fatty acids (FA), eicosanoids/prostaglandins (PG), phosphatidylinositides (PI), lysophospholipids (lysoPL), and lysophosphatidic acid. Indirect oxidation of PLs by LOX and cyclooxygenases (COX) leads to the formation of PL-esterified eicosanoids and PG. Lipid rafts containing certain amounts of sphingomyelins and free cholesterol are located in platelet plasma membranes. Platelets also contain neutral lipids, including 1,2-diacylglycerol, triglycerides and cholesterol esters (33).

The most abundant phospholipids in platelets are phosphatidylcholine (PC) (40%) and phosphatidylethanolamine (PE) (28%). Sphingomyelin (SM) and phosphatidylserine (PS) are less abundant (18% and 10% of total phospholipids). PE and PS face the cytosol and are used for intracellular signaling whereas PC and SM are distributed on the outer layer of the cell membrane. PS is externalized during platelet stimulation leading to procoagulant activity (33). Normally this PL asymmetry is ensured by ATP-dependent enzyme flippase which keeps the PS internally in the plasma membrane of the platelet. An opposing enzyme scramblase initiates a substantial bidirectional trans-bilayer movement of PE as well as PS in thrombin-stimulated or apoptotic platelets (35,36). The externalized PS creates a negatively-charged pro-coagulant surface which enables calcium ions to form bridges with certain domains of coagulation factors. Factor Xa-Va complex enhances the activation of the prothrombinase complex and thrombin production from prothrombin is initiated (37).

Impaired calcium-dependent surface exposure of PS on platelets and other cells as well as or deficiency of scramblase result in Scott syndrome, a congenital bleeding disorder. According to the latest research, a defective transmembrane protein anoctamin-6 also known as a calcium-dependent ion channel for chloride ions and cations was reported to be resulting in the phenotype of Scott syndrome platelets (38).

3.2.2. Oxidized phospholipids

Phospholipid-bound polyunsaturated fatty acids (PUFA) are the main target for non-enzymatic or enzymatic oxidation during which several biologically active products, such as unesterified oxidized fatty acids (e.g., hydroperoxides and isoprostanes) and lysoPL are generated. Non-enzymatic oxidation of PL-esterified PUFA is induced by nonradical ROS (singlet oxygen), or by free radicals. The latter originate from non-enzymatic oxidation in the environment (e.g. air-pollution, radiation), or from endogenous oxidation, mediated by various enzymes, such as NOX, myeloperoxidase (MPO), nitric oxide synthase (NOS), xanthine oxidase (XO), or respiratory chain in mitochondria. The

produced peroxyl radical intermediates are further converted into different products through various subsequent reactions, e.g. oxidation, cyclization, fragmentation, oxygen atom transfer. (39)

Enzymatic oxidation of PL-PUFA is only available by 12/15 lipoxygenases which insert dioxygen directly producing hydroperoxides (39). To date, three main families of oxidized PL have been described, two from 12-LOX (10 total lipids) and four families of sixteen esterified PG generated via COX-1 (total 26 lipids).

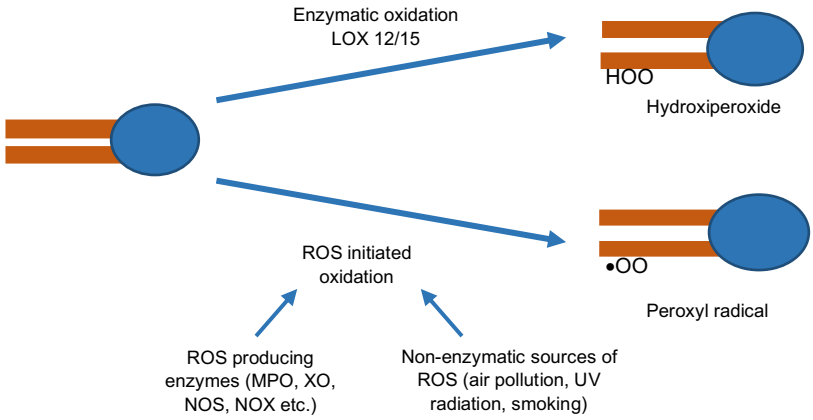

Figure 2. Phospholipid oxidation via enzymatic or non-enzymatic oxidation, producing various oxidized lipid species (modified from 39).

Phospholipid oxidation was thought to be an accidental consequence of tissue damaging inflammation although it was recently proved to be a regulated receptor/agonist dependent process during activation of platelets (e. g. with thrombin, collagen or Ca2+ ionophore) (40).

Arachidonic acid (AA) is one of the sources for a family of six 12-hydroxyeicosatetraenoic acid (12-HETE) containing PL, comprising four PE and two PC (40). In vitro, HETE-PL, found in physiological amounts in liposomes, significantly enhance tissue factor-dependent thrombin generation in plasma and therefore exhibit a pro-coagulant function (40).

Oxidation of DHA (docosahexaenoic acid)-containing PL in thrombin-activated platelets by 12-LOX results in production of four PE (18:0p/14-HDOHE-PE, 18:0a/14-HDOHE-PE, 16:0a/14-HDOHE-PE and 16:0p/14-HDOHE-PE) and

one unidentified species at mass to charge ratio (m/z) 818.6. These PE were only observed in activated platelets and supposedly exhibit antiplatelet activity by antagonizing the HETE-PL actions although the hypothesis is still being tested (41).

Free radical-induced oxidation of 1-palmityl-2-arachidonyl-sn-glycero-3-phosphocholine (PAPC) generates various polyunsaturated phospholipid peroxyl radicals which are transformed into oxidized phospholipids after undergoing cyclisation and fragmentation reactions. These oxPLs promote adhesion of monocytes to endothelium which is a similar property to that of low-density lipoproteins minimally modified by mild oxidation (MM-LDL) (42).

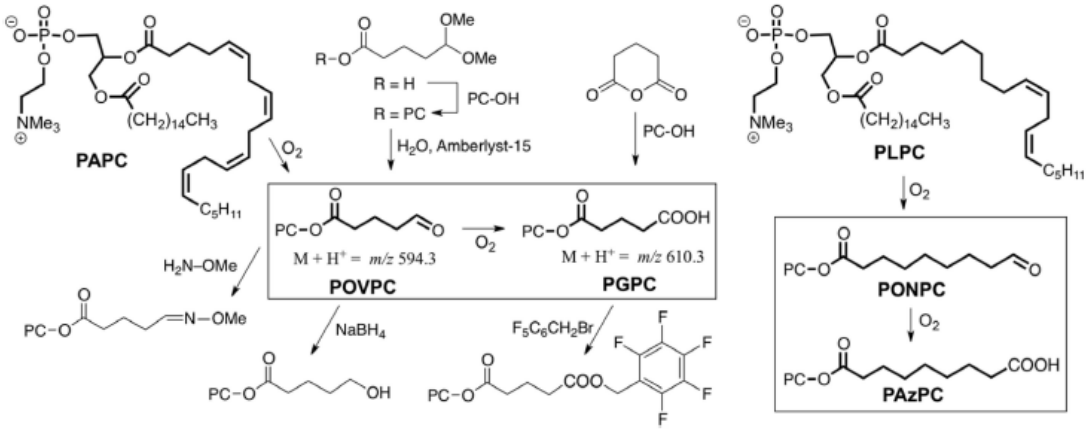

Figure 3. Overview of products of lipid oxidative fragmentation. Oxidative cleavage of PAPC generates POVPC. Similarly oxidation of PLPC generates PONPC (42).

1-palmityl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (POVPC) increases monocyte binding to endothelial cells whereas 1-palmityl-2-(5-glutaryl)-sn-glycero-3-phosphocholine (PGPC) induces both monocyte and neutrophil binding by promoted E-selectin and vascular cell adhesion molecule (VCAM) 1 expression. POVPC can be converted into PGPC under prolonged oxidative conditions whereas the biological activities of PGPC overtake those of POVPC (42). Moreover, PGPC promotes monocyte maturation and uptake of oxLDL whereby foam cells are formed. Both POVPC and PGPC induce apoptosis by increased expression of caspase. Increased apoptosis of macrophages as well

as the proliferation of smooth muscle cells (induced by POVPC) lead to progression of atherosclerosis.

3.2.3. Sphingophospholipids (SP) and ceramides

SPs together with glycerophospholipids and sterols are one of the most important cell membrane lipids. Sphingolipids consist of a sphingoid long chain base (normally sphingosine, sphinganine or phytosphingosine) and FA attached to it. The simplest of SP is ceramide which takes part in cell signaling and acts as a precursor of more complex SP (43). SP act as constituents of cell membranes which protect them against harmful environmental factors. SP also function as intracellular signaling messengers (33,43).

Most abundant ceramides in human plasma are C24:0 and C24:1 and their concentration was showed to positively correlate with concentrations of total cholesterol (TC) and TG. Therefore ceramides might be potential risk factors at early stages of atherosclerosis (44). Certain ceramides were significantly associated with mortality from CVD and could improve the predictive potential of GRACE and Marschner scores. Furthermore, research suggests that ceramides of different chain-length have a different effect on atherosclerosis development. E.g. long-chain species (d18:1/16:0 and d18:1/18:0) were observed to be more harmful than very-long-chain (d18:1/24:0) species (45).

Sphingosine-1-phosphate is generated after sphingosine is phosphorylated by sphingosine kinases and released from the granules during platelet activation (33). S1P regulates cell proliferation and migration, immune regulation, vascular development, brain inflammation and has shown to exhibit a role in thrombopoiesis (33,46). Activated platelets release huge amounts of S1P during blood clotting (47). It was also shown that intracellular and extracellular pools of S1P exist. The extracellular S1P is a platelet activator which induces platelet shape changes and platelet aggregation (48).

3.2.4. Lysophospholipids (lysoPL) and lysophosphatidic acid (LPA)

LysoPL and LPA are generated from membrane phospholipids and sphingolipids (SL) by phospholipases, e.g. PLA2. They act as extracellular mediators by activating G-protein coupled receptors (GPCR) and regulate diverse cellular responses (49). Lysophosphatydilcholine (lysoPC) activates endothelial cells and recruits phagocytic cells during apoptosis (50). It is also a major component of oxLDL and contributes to its atherogenic effects (51). Activated platelets produce various lysoPL because of actions of PLA2 (52). The oxidation products of PLA2 become substrates for a higher demand of energy in activated platelets which links the lipid oxidation with energetic processes in mitochondria. Fatty acids and various eicosanoids, generated via PLA2, are acutely used as a source for mitochondrial beta-oxidation generating energy supplies needed for keeping the asymmetry of the plasma membrane, assuring the production of oxidized PL in activated platelets etc. (53).

3.3. Lipidomics

Lipidomics is one of the branches of metabolomics, analyzing different lipid species and their multiple functions in the living system (54).

In general analysis of lipids consists of three main steps:

- Preparation and extraction of lipids from biological materials e.g. serum/plasma, cellular extracts;
- Lipid fragmentation by the means of enzymatic or non-enzymatic reactions and subsequent analysis of the fragmentation products;
- Analysis of prepared, non-fragmented lipids by means of elementary analysis, UV- and IR-spectroscopy, mass spectrometry (MS) or NMS-spectrometry (34).

Traditional techniques for studying lipids include thin layer chromatography (TLC), gas chromatography/mass spectrometry (GC/MS), and high-performance liquid chromatography (HPLC) coupled to radioactive, ultraviolet or fluorescence detection (33).

The major disadvantages of older approaches over liquid chromatography-mass spectrometry (LC/MS) are (i) low sensitivity and selectivity (TLC, HPLC), (ii) time-consuming derivatization methods (e.g. for GC/MS), and (iii) health issues associated with the usage of radioisotopes (33). These disadvantages subsequently led to the development of more sophisticated techniques with higher sensitivity and specificity, leading to better qualitative and quantitative accuracy of results.

Lipidomics investigations use the two major methods, which include a highly sensitive and quantitative liquid chromatography-tandem mass spectrometry (LC/MS/MS), and "shotgun" lipidomics approach, based on direct infusion of complex mixtures into a spectrometer without former separation, which detects only the most abundant species (32).

A newer quantitative approach is a combination of LC with a high-resolution MS/MS on rapid scanning Fourier transform (FT) or Time-of-flight (ToF) instruments, termed multidimensional MS (MDMS). In Fourier transform mass spectrometry, mass is measured by detecting the image current generated by ions in a magnetic field. ToF machines calculate mass based on the time (*m/z* ratio) it takes for molecules in an electric field to reach a detector at a certain distance. It takes longer for heavier molecules to reach the detector (55).

Lipidomics approach is fundamental for understanding of lipid biology. It can also be applied in the clinical setting. Certain novel lipids can help detect cardiovascular diseases in early stages by the means of potential lipids being used as biomarkers with high-risk assessment values of prognostic impact. Moreover, it can aid in deciphering new targets for therapeutic interventions (53).

3.4. Lipids in human blood stream

Cholesterol is the main sterol of all higher animals, found in body tissues, especially the brain and spinal cord, and is a major constituent of animal fats and oils. Cholesterol is transported in the blood as particles containing both lipid and proteins – lipoproteins. Three major classes of lipoproteins are found in the plasma of a fasting individual: LDL, VLDL and HDL cholesterols. LDL is the

major carrier for cholesterol. It contains a single apolipoprotein, namely apoB-100 (24,56). LDL cholesterol is the main atherogenic cholesterol and is directly associated with cardiovascular disease (57,58). Therefore LDL cholesterol is the primary target of cholesterol-lowering therapies as well as a marker for the efficacy of the initiated treatment. In contrast, HDL exhibits an anti-atherogenic effect.

In order to estimate cardiovascular risk using various scores (e. g. Framingham risk calculator, ESC SCORE, PROCAM) baseline lipid profile of TC, TGs, HDL and LDL cholesterols is determined in fasting plasma. In patients with hypertriglyceridemia values of lipids may be inaccurate leading to underestimation of actual lipid status (59). That is why additional plasma lipid markers may be considered, e. g. lipoprotein A, apoB:apoA1 ratio or nonHDL-C:HDL-C ratio.

Potential markers may be distinct ceramide species which showed to predict cardiovascular death significantly better, compared to the currently used lipid markers (45). Moreover, some ceramide species not only were significantly associated with mortality in CAD but also were superior in predicting mortality in CAD to currently used standard LDL-C measurement (60). Further studies identified lysoPC and SM associated with CAD. LPC16:0 or LPC20:4 were associated with a decreased risk of developing CVD over the 12-year follow-up period whereas SM38:2 was associated with increased odds of future CVD (61).

Another factor promoting research of novel non-invasive atherosclerosis biomarkers is disease detection at its earliest. Establishing new atherosclerosis markers may as well lead to the development of novel treatment options. Recent studies identified six classes of ceramide synthases which might potentially be novel therapeutic targets in the diseases in which the ceramide acyl chain length is altered (62). The novel PCSK-9 inhibitors which are already applied in clinical practice were shown to decrease the plasma levels not only of LDL-C but also of CVD associated ceramides (60).

3.5. Lipid biomarkers in the context of CAD

TC and LDL-C are proven independent CVD risk factors and have been established as lipid biomarkers for estimating the risk of CVD as well as following the clinical course of the disease (63). At last but not the least they are the main therapeutic targets of the current lipid-lowering therapy.

HDL-C is an anti-atherogenic LPL as it is able to remove cholesterol from cells, inhibit LDL-C oxidation and exhibits anti-inflammatory features (64). Low HDL-C level is independently associated with higher CVD risk (65).

Hypertriglyceridemia is also an independent CVD risk biomarker although its association with CVD is weaker than that of LDL-C (66).

Total amounts of cholesterol in LDL, intermediate-density lipoprotein, remnant, and VLDL, comprise another calculated parameter called a non-high-density lipoprotein cholesterol (non-HDL-C). This combination of all the pro-atherogenic lipids can estimate CVD risk even more accurately than LDL-C (67).

Several other biomarkers are gaining its way into clinical practice. Apoprotein B (apoB) is the main apoprotein of atherogenic lipoproteins. Higher plasma levels of apoB are also associated with a higher CVD risk (65). Lipoprotein(a) [Lp(a)] constitutes of an LDL attached to an additional protein called apolipoprotein(a). High levels of Lp(a) are associated with increased risk of CVD (65).

3.6. Current lipid-lowering therapies

Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), a rate-limiting enzyme in cholesterol synthesis. Decreased hepatic cholesterol concentrations lead to up-regulated synthesis of LDL receptors so that the clearance of LDL-C from the blood into hepatic cells increases and the pro-atherogenic LDL-C levels in the blood are reduced. (56) Lowering LDL-C levels in patients with ACS showed significantly lower mortality rates in the PROVE-IT clinical trial (68). Nevertheless, statins exhibit a variety of pleiotropic functions such as anti-inflammatory effects, mediated by both direct (modulation of the immune-response) and indirect (inhibition of platelet functions) mechanisms (69).

Another LDL-C lowering agent is ezetimibe – one of the agent of azetidinone cholesterol absorption inhibitors (56). Ezetimibe used in combination with a statin showed to significantly lower LDL-C levels and improve cardiovascular outcomes in ACS patients (70).

Fibrates are fibric acid derivatives which reduce VLDL secretion from hepatocytes. Fibrates reduce levels of circulating VLDL, therefore lowering TAG levels with a modest (approximately 10%) reduction in LDL-C (56).

Cholestyramine is one of the bile acid sequestrants which also lowers the levels of LDL-C. Binding the bile acids enhances their synthesis in liver from cholesterol which causes clearance and reduction of the levels of LDL-C from the circulation (71).

Nicotinic acid raises HDL-C and reduces LDL-C levels. Nicotinic acid also uniquely lowers Lp(a) levels (72).

The most novel lipid-lowering agents are monoclonal antibodies inhibiting the proprotein convertase subtilisin/kexin type 9 (PCSK9), which is involved in the degradation of LDL receptors in the liver. Inhibition of PCSK9 results in increased numbers of LDL receptors and enhanced hepatic LDL clearance (73). One of the PCSK9 inhibitors, evolocumab, was showed to significantly reduce LDL-C levels and the risk for cardiovascular events, in combination with standard therapy (statin), as compared with standard therapy (statin) alone. Levels of the Lp(a) could also be significantly reduced (74).

Moreover, loss-of-function mutations in PCSK9 resulted in reduced plasma LDL-C levels. Inhibiting the PCSK9 synthesis by a small interfering RNA (ALN-PCS) showed a mean 40% reduction of LDL-C compared to placebo (75). Clinical application of the new approach by means of RNA interference needs to be further investigated.

3.7. Current guidelines on lipid-lowering therapies

The guidelines on cardiovascular disease prevention used in the clinical practice focus mainly on a statin-based primary or secondary prevention. The most recent guidelines from 2016 published by the European Society of

Cardiology (ESC) or the guideline from 2013 from the American Heart Association (AHA) both recommend estimating the 10-year risk of a first fatal atherosclerotic event, either using the European SCORE (Systemic Coronary Risk Estimation) system or the American 10-year ASCVD (atherosclerotic cardiovascular disease) risk calculator. After determining the estimated 10-year risk patients are categorized in different groups (low, moderate, high, very high) depending on the estimated CVD risk, based upon which different dosages of statins are prescribed. The European guidelines focus on reaching a certain LDL-C level under the statin-therapy. On the contrary, the American guideline does not suggest an absolute target level of LDL-C and recommends initiating a certain intensity statin-treatment to reduce the LDL-C levels by ≥50 % or 30–50 % in high or moderate risk patients (65,76). Ezetimibe is recommended as a supplement to the statin-therapy when the levels of LDL-C cannot be reduced under the highest-dose statin monotherapy. Fibrates are also combined with statins, especially in cases of severe hypertriglyceridemia (65). PCSK9 inhibitors are still gaining their way into guidelines on CVD prevention as the impact of PCSK9 inhibitors on cardiovascular outcomes is still being investigated and the first results have been recently published. Additional indications for a therapy with PCSK9 inhibitors include familial hypercholesterolemias whereas only in combination with other lipid-lowering agents.

3.8. Aims of the study

After reviewing the literature on the lipid biology, platelet and lipid interactions as well as the pathogenesis of atherosclerosis the following aims of this study where formulated:

1. Demonstrate the effect of oxLDL on platelet aggregation
2. Examine the platelet lipidome in healthy subjects compared to STEMI and SAP patients
3. Extract the most significantly abundant lipid species in platelets of SAP and STEMI patients

4. Materials and methods

4.1. Materials

4.1.1. Chemicals

Chemical	Source
1-heptadecenoyl-2-eicosatetraenoyl-sn-glycero-3-phosphocholine (PC (17:0/20:4))	Avanti Polar Lipids, Inc. (Alabaster, AL, USA)
1-heptadecenoyl-sn-glycero-3-phosphocholine (LysoPC 17:1)	Avanti Polar Lipids, Inc. (Alabaster, AL, USA)
1-Palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC)	Avanti Polar Lipids, Inc. (Alabaster, AL, USA)
1-palmitoyl-2-(9-oxononanoyl)-sn-glycero-3-phosphocholine (PONPC)	Avanti Polar Lipids, Inc. (Alabaster, AL, USA)
1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC)	Avanti Polar Lipids, Inc. (Alabaster, AL, USA)
1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC)	Avanti Polar Lipids, Inc. (Alabaster, AL, USA)
Acetonitrile	Sigma-Aldrich Chemie GmbH, München, Germany
Ammonium acetate	Sigma-Aldrich Chemie GmbH, München, Germany
Citric acid	Sigma-Aldrich Chemie GmbH, München, Germany
D-(+)-Glucose	Sigma-Aldrich Chemie GmbH, München, Germany
Ethanol, 75%	Carl Roth, Karlsruhe, Germany
Hydrochloride acid	Carl Roth, Karlsruhe, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Magnesium chloride hexahydrate	Merck KGaA, Darmstadt, Germany
Potassium chloride	Carl Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate	Merck KGaA, Darmstadt, Germany

Sodium chloride *BioChemica*	AppliChem GmbH, Darmstadt, Germany
Sodium hydrogen carbonate	Merck KGaA, Darmstadt, Germany
tri-Sodium citrate dihydrate	Merck KGaA, Darmstadt, Germany
Trizma® base	Sigma-Aldrich Chemie GmbH, München, Germany

4.1.2. Solutions/buffers

Solution	Components
6 mM hydrochloride acid	
ACD buffer	7 mM citric acid 22 mM tri-Sodium citrate dihydrate 25 mM glucose 6 mM hydrochloride acid
JNL buffer	JNL A 5 ml JNL B 5 ml JNL D 5 ml JNL E 0,5 ml ACD pH 7,2 Distilled water
JNL A	60 mM glucose Distilled water
JNL B	1,3 M sodium chloride 90 mM sodium hydrogen carbonate 100 mM tri-Sodium citrate dihydrate 100 mM Trizma® base 30 mM potassium chloride Distilled water
JNL D	8,1 mM potassium dihydrogen phosphate Distilled water
JNL E	90 mM magnesium chloride

	hexahydrate

4.1.3. Laboratory materials

Material	Source
10 ml pipette	Corning Incorporated, NY, USA
15 ml Falcon tube	Greiner Bio One, Frickenhausen, Germany
20 ml syringe	B. Braun, Melsungen, Germany
50 ml Falcon tube	Greiner Bio One, Frickenhausen, Germany
Eppendorf Safe-Lock Tubes 1.5 ml	Eppendorf AG, Hamburg, Germany
Pasteur pipette	RatioLab GmbH, Dreieich, Germany

4.1.4. Devices

Product name	Source
Agilent 1290 UHPLC instrument	Agilent Technologies, Waldbronn, Germany
Eppendorf centrifuge 5417	Eppendorf AG, Hamburg, Germany
Hettich® ROTANTA 460/460R centrifuge	Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany
Multiplate® Analyzer	Roche Deutschland Holding GmbH
Phenomenex Kinetex C8 column	Phenomenex, Inc., CA, USA
PIPETBOY acu 2	INTEGRA Biosciences AG
Sciex TripleTOF 5600+ hybrid mass spectrometer	Agilent Technologies, Waldbronn, Germany
Sysmex KX-21N™ Automated Hematology Analyzer	Sysmex Deutschland GmbH, Nordertstedt, Germany
Tisch-pH/Ionen-Messgerät SevenCompactTM S220, pH-Meter SevenCompactTM S220-basic	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ultra-sonication bath	BANDELIN electronic GmbH & Co. KG, Berlin, Germany

Vortex mixer	IKA Labortechnik, Staufen, Germany

4.2. Methods

4.2.1. Study design

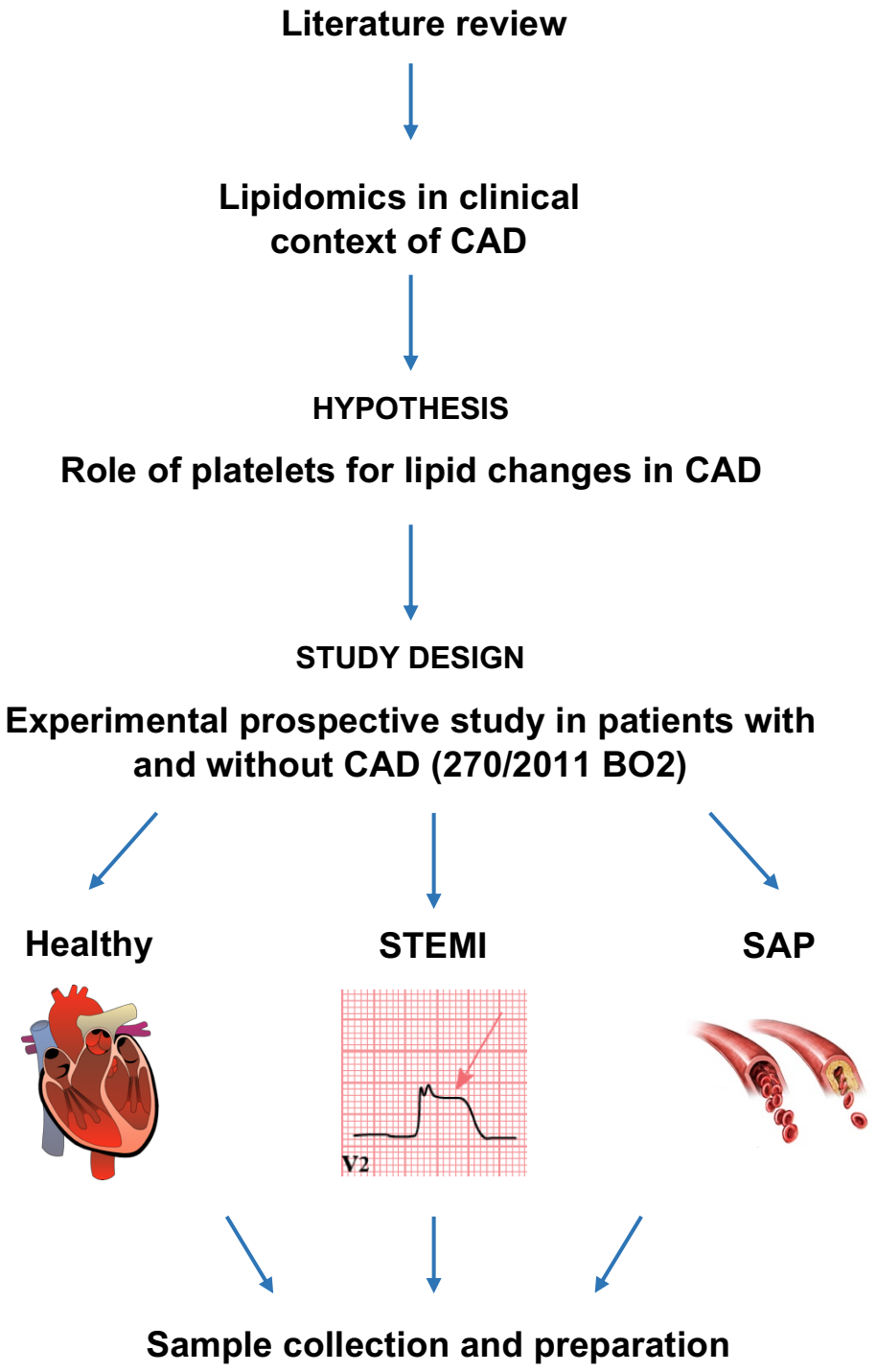

Figure 4. Hypothesis and patient selection.

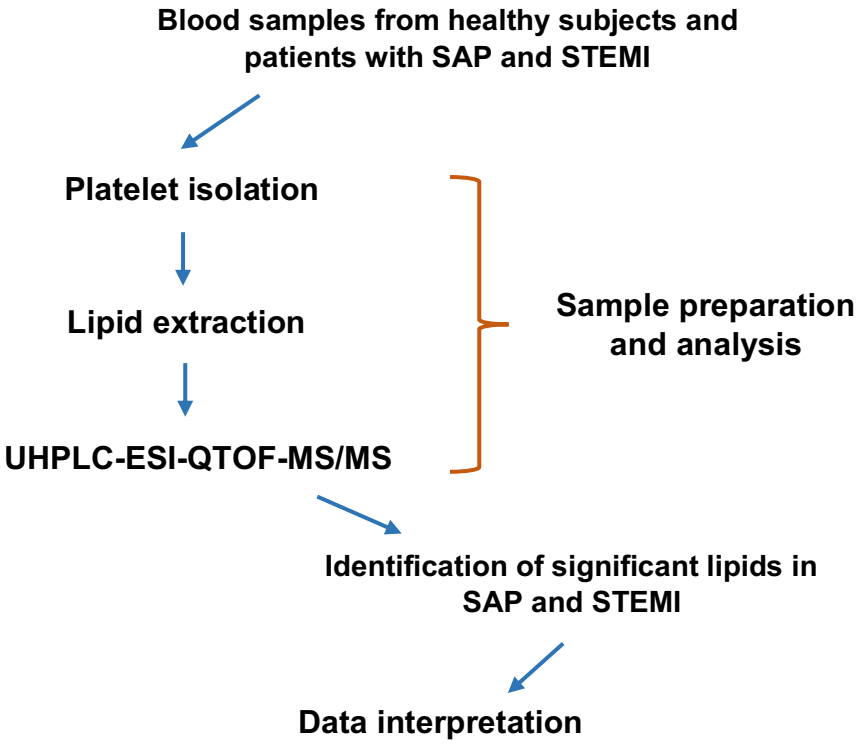

Figure 5. Sample acquisition and workflow.

4.2.2. oxLDL effect on platelet aggregation

In order to test the hypothesis that platelet aggregation is enhanced by oxidized lipids, which plays a crucial role in the development of atherosclerosis, effect of oxLDL on platelet aggregation was examined. Peripheral blood was collected from 7 healthy subjects in hirudin anticoagulant. 270 μl of whole blood was incubated with oxLDL (1 mg/ml). In another setting 30 μl of phosphate buffered saline (PBS) Ca^{2+} was added to 270 μl of blood as a control set. Blood samples were incubated in room temperature for 30 minutes and afterwards analyzed with Multiplate® analyzer. The aggregation was induced with ADP (6,5 μM). The aggregation curves were registered for 6 minutes. Platelet aggregation data were represented as area under the curve (AUC).

4.2.3. Study population

Blood samples from patients presenting with a STEMI (n=13) were collected in less than two hours after onset of symptoms before the percutaneous coronary intervention (PCI). Patients with stable angina pectoris (SAP) (n=10) were also included in the study. 10 healthy subjects served as a control group to compare all the data with STEMI and SAP patients. Blood samples from SAP patients were collected before the PCI during the admission at the hospital. All patients were admitted to the Department of Cardiology at the University Clinic of Tübingen, Germany. The study was approved by the institutional ethics committee (270/2011BO2) and complies with the declaration of Helsinki and good clinical practice guidelines. All subjects gave written informed consent.

STEMI was diagnosed by a rise and/or fall of cardiac troponin and symptoms of ischemia, and/or typical changes in electrocardiogram, and/or signs of new ischemia in cardiac imaging, and/or an intracoronary thrombus detected by coronary angiography (2). Stable coronary artery disease was characterized by transient chest pain (angina pectoris) episodes caused by reversible ischemia or hypoxia in myocardium, occurring under physical or emotional stress (74). Healthy volunteers presented with no obvious symptoms of disease and were free of medication at the time of donating blood (n=10, age (mean ± SD) 30 ± 7 years).

4.2.4. Platelet isolation

40 ml of whole blood was collected into two 20 ml syringes each filled with 3 ml ACD anticoagulant and distributed into four 15 ml Falcon tubes afterward. The blood was centrifuged at 200 x g for 10 minutes with no brakes applied, at room temperature. The upper two thirds of platelet rich plasma (PRP) were transferred into a 50 ml Falcon tube containing Thyroid buffer (composition) pH 6.4 using a Pasteur pipette. The PRP samples were centrifuged at 150 x g for 7 minutes with brake level 2 under maximum speed at room temperature to separate platelets. The centrifuged platelet pellet was transferred into a 50 ml Falcon tube using a Pasteur pipette and resuspended in 500 µl of acidic

ethanol. Resting platelet suspension (1 ml) containing $300x10^3$/µl platelets was transferred into a 1.5 ml Eppendorf tube, which was placed in an ultra-sonication water bath with ice for cooling for 20 minutes. After the ultra-sonication, the platelet suspension was placed on a vortex mixer for 10 s and then centrifuged at 2100 rpm for 5 minutes under 4°C. 500 µl of the platelet supernatant were transferred into an Eppendorf tube and stored at -20°C temperature.

Measurements described further on (4.2.5. – 4.2.8.) were performed in cooperation with Prof. Dr. Michael Lämmerhofer and Jörg Schlotterbeck (Institute of Pharmaceutical Sciences, Universität Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany). Data analysis was done by Prof. Dr. Michael Lämmerhofer, Jörg Schlotterbeck and Dr. Madhumita Chatterjee (Department of Cardiology and Cardiovascular Sciences, Internal Medicine III, Universitätsklinikum Tübingen).

4.2.5. Lipid extraction

Supernatants after such extraction were dried under a gentle stream of nitrogen and reconstituted in 500µL of methanol. Samples were centrifuged, and the supernatant was carefully removed without disturbing the pellet. The entire sample preparation was carried out under light protection. The collected supernatants were spiked with internal standards (LPC(17:1), PC(17:0/20:4)) at a final concentration of 40 ng/mL and subjected to UHPLC-ESI-QTOF-MS/MS analysis (21).

4.2.6. UHPLC-ESI-QTOF-MS/MS

LC-MS/MS analysis of the lipid extract was carried out on an Agilent 1290 UHPLC instrument hyphenated to a Sciex TripleTOF 5600+ hybrid mass spectrometer. A Phenomenex Kinetex C8 column (150 x 2.1 mm; 2.6 µm) was used for chromatography. Solvents were aqueous 10 mM ammonium acetate (A) and a mixture of acetonitrile, isopropanol and water (55:40:5 v/v) containing 10 mM ammonium acetate (B). The gradient was 10% B to 40% B in 2 minutes and 100% B in 20 minutes followed by a cleaning step of 10 minutes 100% B

and 2 minutes equilibration with 10% B at a flow rate of 400µL/min and 50°C oven temperature. Injection volume was 2µL.

The MS platform was operated in ESI positive mode at 5000V and 500°C. Nebulizer, heater and curtain gas were used at 50, 40, 30 psi respectively. Declustering potential was set to 100 V. Data-independent MS/MS acquisition was carried out using SWATH 2.0 covering a range of 30-1000 m/z with optimized Q1 windows using a quality control (QC) sample and swathTUNER. 31 SWATH windows were used with a minimum size of 5Da in high sensitivity mode.

The collision energy was 35V for each window. Data processing was done with commercial software PeakView, MasterView and MarkerView (Sciex) and freely available MS-DIAL5.

Data quality was evaluated by a QC sample which was embedded throughout the analysis batch consisting of 44 injections in total with 11 injections of the QC sample (3 x before analysis of the first sample, 3 x after the last sample and the other injections of the QC sample distributed within the sample sequence after every 5th injection of a sample). It is a commonly accepted quality attribute for untargeted profiling methods in lipidomics and metabolomics if the QC samples injected across the entire sequence are closely grouped together and largely superimposed upon each other in the score plot of multivariate statistical procedures like PCA, PLS or OPLS-DA. In the present study, the detected molecular features (ca. 7,500 in the QC sample in the positive mode) were subjected to MS-DIAL for peak finding, LOWESS normalization and identification via spectral matching. Logarithmic transformation to the base e and autoscaling was done with MarkerView software. Subsequently, these responses were subjected to supervised principal component analysis-discriminant analysis (PCA-DA). The results are illustrated in Figure 7 which clearly documents a clustering of the distinct sample classes (QC, healthy control R, STEMI and SAP) and most importantly the QC samples are totally superimposed upon each other meaning that the assay has been robust and thus the results have been highly consistent throughout the analysis batch. This

documents the stable performance of the LC-MS assay during the analysis sequence and high precision of retention times, mass accuracies, and responses throughout the assay. CV values of retention time and intensity of the internal standards were 0.4 % and 12 % for LPC(17:1) and 0.2 % and 10 % for PC(17:0/20:4) respectively. These data clearly document the reliability of the measurements (21).

4.2.7. Data analysis

From thousands of molecular features detected by UHPLC-ESI-QTOF-MS/MS, distinct marker lipids from healthy and CAD patients were extracted by t-tests and volcano plots after structural annotation by MS-DIAL and LipidBlast database search. Annotated lipids with P-value ≤0.05 in the comparison between CAD (STEMI or SAP) and the healthy group were used to construct a heat map (Figure 8) in order to visualize the differences in lipid abundances among the distinct groups. Of the 3054 molecular features extracted from ESI(+) mode data, 695 molecular features exhibited significantly different abundance in lipid extracts of healthy and STEMI platelets. 172 of them could be identified by manual revision of spectrometric data in MS-DIAL and LipidBlast database as shown in the heat map (Figure 8) indicating different lipid classes. Free radical–induced oxidative cleavage of 1-palmityl-2-arachidonyl-sn-glycero-3-phosphocholine (PAPC) generates POVPC that is further oxidized to PGPC. POVPC is the aldehydic fragmentation product from PAPC, while PGPC is the carboxylic acid product following further oxidation. Oxidative cleavage of 1-palmityl-2-linoleyl-sn-glygero-3-phosphocholine (PLPC) generates PONPC that is further oxidized to PAzPC. For these oxidized lipids, standards were available, and their structures could, therefore, be manually annotated to their respective peaks in the chromatograms (21).

4.2.8. Statistical analysis

Samples for the lipidomics study comprised lipid extracts from healthy controls (n=10), SAP (n=10) and STEMI (n=13) patients. Raw data from the UHPLC-ESI-QTOF-MS/MS analysis (Sciex wiff files) were imported into MS-DIAL and

pre-processed before statistical analysis (peak alignment, noise filtering, LOWESS normalization, structure annotation). In compliance with FDA guidelines on bioanalytical method validation, the intra-assay precision of raw signal intensities was < 15%, as estimated by internal standards as surrogate variables. Limits of detection are substance-dependent and typically in the low ng/ml-range. Preprocessed data (normalized peak areas) were exported from MS-DIAL and used for univariate and multivariate statistical analysis. Normalized peak areas were log-transformed and subjected to unpaired Student *t*-test to identify peaks varying significantly ($p \leq 0.05$) between distinct groups. Only molecular features with $p \leq 0.05$ (uncorrected) were then further processed and considered as putative biomarkers of potential interest. Responses for these lipids are presented as a heat map (Figure 8). To account for the problem of multiple hypotheses testing a false discovery rate (FDR) controlling procedure was further adopted to correct significance levels for FDR ≤5% according to Benjamini and Hochberg. Multivariate statistical data analysis was performed using MarkerView (Sciex). Normalized peak areas were log-transformed and autoscaled before they were subjected to principal component analysis–discriminant analysis (PCA-DA) (21).

5. Results

5.1. Baseline characteristics of the patient cohort

Characteristics	STEMI (n=13)	SAP (n=10)
Male	11 (84.6%)	8 (80%)
Female	2 (15.4%)	2 (20%)
Age (mean ± SD)	71.5 (± 11.8)	64.8 (±13.7)
BMI (mean ± SD)	25.23 (±2.59)	29.23 (±4.1)
CVRF		
Arterial Hypertension	11 (84.6%)	8 (80.0%)
Hyperlipidemia	5 (38.5%)	3 (30.0%)
Diabetes mellitus	3 (23.1%)	3 (30.0%)
Smoking	2 (15.4%)	3 (30.0%)
Ex/Smoking (>6 mo.)	1 (7.7%)	2 (20.0%)
Atrial fibrillation	1 (7.7%)	1 (10.0%)
Positive family history	2 (15.4%)	1 (10.0%)
Obesity	1 (7.7%)	3 (30.0%)
LV Function (%) (Mean ± SD)	48.08 (±10.71)	54.80 (±8.26)
LVEF normal	3 (23.08%)	7 (70.0%)
LVEF mild impairment	5 (38.46%)	2 (20.0%)
LVEF moderate impairment	3 (23.08%)	0 (00.0%)
LVEF severe impairment	2 (15.38%)	1 (10.0%)
Chronic kidney disease	1 (7.7%)	2 (20.0%)
Renal function (GFR) (Mean ± SD)	80.87 (±26.84)	77.51 (±17.95)
Medication on admission		
Acetylsalicylic acid	2 (15.4%)	5 (50.0%)
Clopidogrel	0 (00.0%)	2 (20.0%)
Prasugrel	0 (00.0%)	1 (10.0%)
Ticagrelor	0 (00.0%)	1 (10.0%)
Oral anticoagulants	0 (00.0%)	2 (20.0%)
Angiotensin-converting enzyme	1 (7.7%)	5 (50.0%)

inhibitors		
Angiotensin II receptor antagonists	2 (15.4%)	1 (10.0%)
Aldosterone antagonists	0 (0.00%)	1 (10.0%)
Diuretics	1 (7.7%)	5 (50.0%)
Calcium channel antagonists	2 (15.4%)	3 (30.0%)
Beta-blockers	3 (23.1%)	8 (80.0%)
Statins	1 (7.7%)	6 (60.0%)
Laboratory parameters		
Troponin I sensitive	19,96 (±25,10)	not measured
CK-MB	737,54 (±751,87)	119,30 (±83,29)
Platelet count	261,69 (±61,60)	197,0 (±66,12)
Cholesterol	169,00 (±37,02)	164,88 (±35,96)
LDL-cholesterol	106,86 (±39,10)	101,0 (±32,61)
HDL-cholesterol	39,29 (±21,20)	43,77 (±8,93)
Triglycerides	123,10 (±70,70)	155,5 (±51,73)
C-Reactive protein	2,40 (±3,12)	0,64 (±0,91)
Leukocyte count	11260,0 (±3955,66)	7483,33 (±2049,3)
Creatinine	0,96 (±0,31)	0,96 (±0,27)
Hemoglobin	12,74 (±2,34)	13,69 (±1,96)
INR	1,14 (±0,21)	1,23 (±0,59)
PTT	67,46 (±50,36)	37,0 (±38,87)
Bilirubin	0,73 (±0,39)	0,70 (±0,38)
Glucose	160,0 (±110,8)	116,0 (±34,66)
GOT	104,57 (±108,86)	26,88 (±6,71)
GPT	32,38 (±18,73)	26,33 (±11,15)
LDH	308,23 (±164,12)	203,22 (±37,91)

5.2. oxLDL influenced platelet aggregation response to ADP

In order to test the effect of oxidized lipids on platelet reactivity blood samples from 7 healthy subjects were examined. Platelet aggregation was induced with ADP in two settings – untreated whole blood vs. whole blood treated with

oxLDL. Measurements could show that oxLDL enhanced platelet aggregation (Figure 6). The average AUC in whole blood incubated with oxLDL was higher than in untreated whole blood samples.

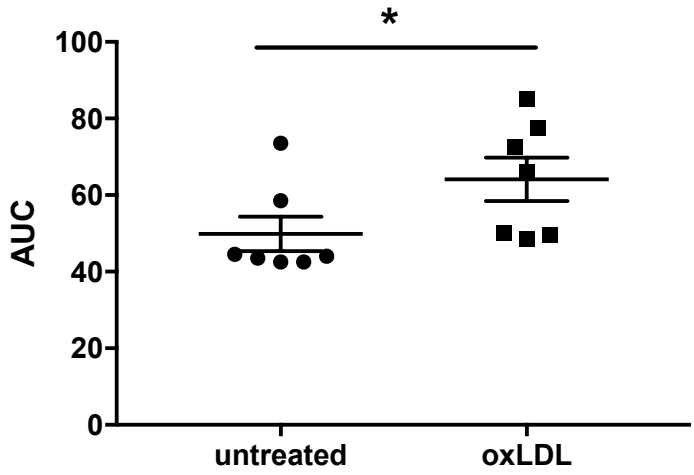

Figure 6. Platelet aggregation in whole blood (untreated vs. oxLDL), p=0,0156 (*, p<0,05).

5.3. Lipid profile of circulating platelets

To characterize the lipid profile of circulating platelets in STEMI and SAP groups blood samples were analyzed by lipidomics profiling using liquid chromatography hyphenated to high-resolution mass spectrometry (UHPLC-ESI-QTOF-MS/MS) using data-independent acquisition with SWATH (77,78). The following measurements were performed by Prof. Michael Lämmerhofer and Jörg Schlotterbeck (Institute of Pharmaceutical Sciences, Universität Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany).

The lipid profiles of 13 STEMI and 10 SAP patients were compared to 10 healthy controls.

The initial UHPLC-ESI-QTOF-MS/MS analysis detected over 7500 of molecular features in the samples of the healthy and CAD subjects (21).

The data was normalized and aligned as well as subsequently subjected to principal component analysis (PCA-DA). The score plot clearly illustrates how samples of the healthy controls (n=10), SAP (n=10) and STEMI (n=13) patients with similar lipid profiles are clustered closely together. The same plot also depicts how platelet lipid profiles differ in healthy subjects compared to CAD patients as well as among the SAP and STEMI patients (Figure 7) (21).

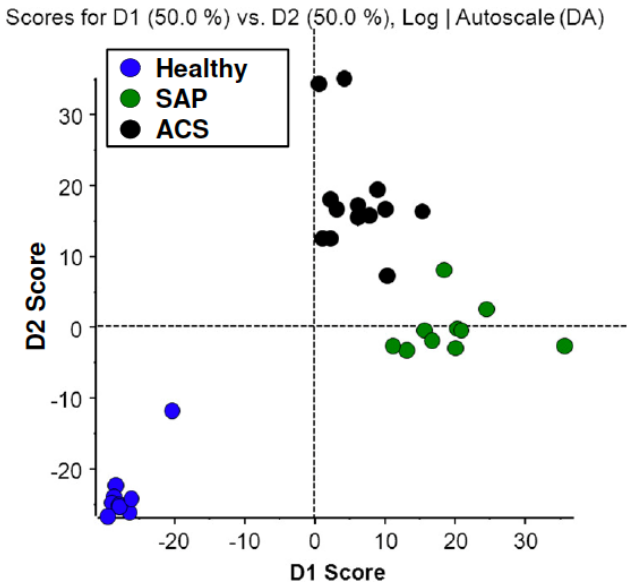

Figure 7. Samples of healthy, SAP and STEMI patients clustering in different groups depending on lipid profile (21)**. In cooperation with Prof. Michael Lämmerhofer and Jörg Schlotterbeck.**

Out of more than 7500 primary molecular species distinct lipids were further extracted by t-tests and volcano plots which led to reduced number of molecular features which significantly differed in abundance among the three target groups (21).

Out of 664 molecular features exhibiting significantly different abundance in lipid extracts of healthy and CAD platelets, 172 of them were identified by manual revision of spectrometric data in MS-DIAL and LipidBlast tandem mass spectrometry databases for lipid identification. Database search revealed 172 known molecular structures with p-values ≤0.05 which were eventually classified according to the lipid class they belong to. The following heat map presents the significantly different expression of separate lipid classes among

STEMI, SAP and healthy subject groups (Figure 8) red color meaning the highest abundance and blue depicting the low abundant species (21).

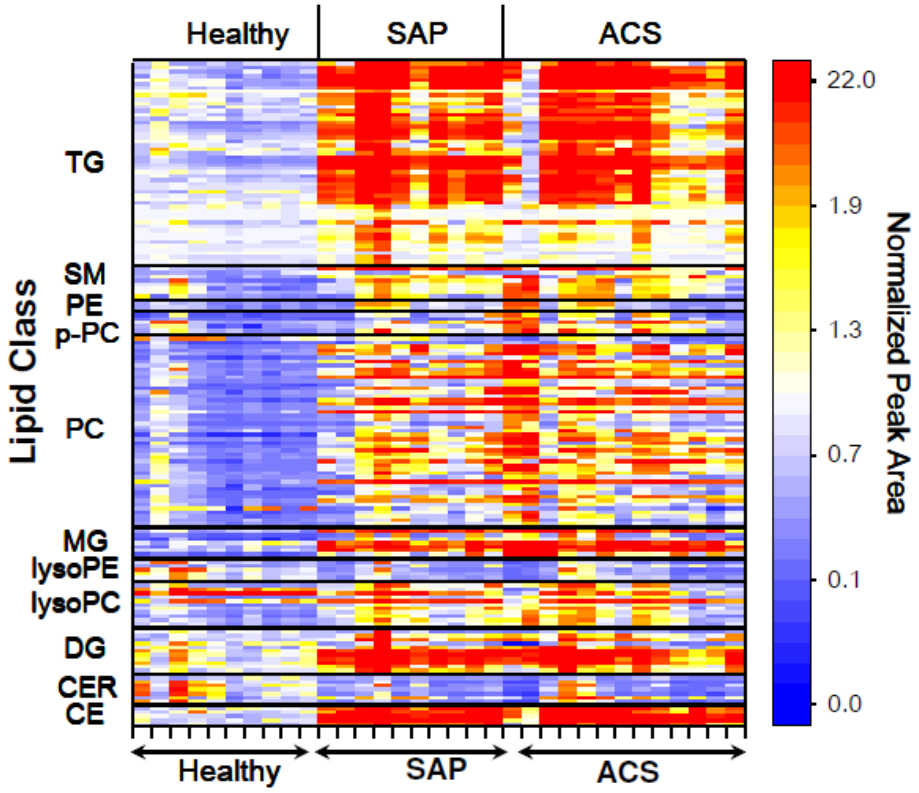

Figure 8. Heat map representing abundance of different lipid classes in healthy, SAP and ACS (STEMI) patients (21)**. TG – triglyceride, SM – sphingomyelin, PE – phosphatidylethanolamine, PC – phosphatidylcholine, MG – monoglyceride, DG – diglyceride, CER – ceramide, CE – cholesteryl ester. In cooperation with Prof. Michael Lämmerhofer and Jörg Schlotterbeck.**

Significantly higher levels of triglycerides (TG), phosphatidylcholin (PC), diacylglycerols (DG), monoacylglycerols (MG), plasmenyl-PCs (p-PC), sphingomyelin (SM) and ceramides (CE) were found in SAP patients as compared to healthy subjects (21).

Abundance of distinct lipid metabolites – oxPLs, ceramides, cholesteryl-esters was further compared between healthy, SAP and STEMI subgroups. However, oxPLs are not contained in LipidBlast database and were therefore not annotated by MS-DIAL. For some specific oxidized lipids, standards were available, and their structures could therefore be manually annotated to their respective peaks in the chromatograms. Several oxPLs e.g. POVPC (*m/z=594.3766*), PGPC (*m/z=610.3715*), PONPC (*m/z=650.4392*) and PAzPC

(*m/z=666.4341*) were detected in the samples of the subgroups. They were elevated in SAP and were significantly increased in STEMI patients when compared to healthy subjects (Figure 9) (21).

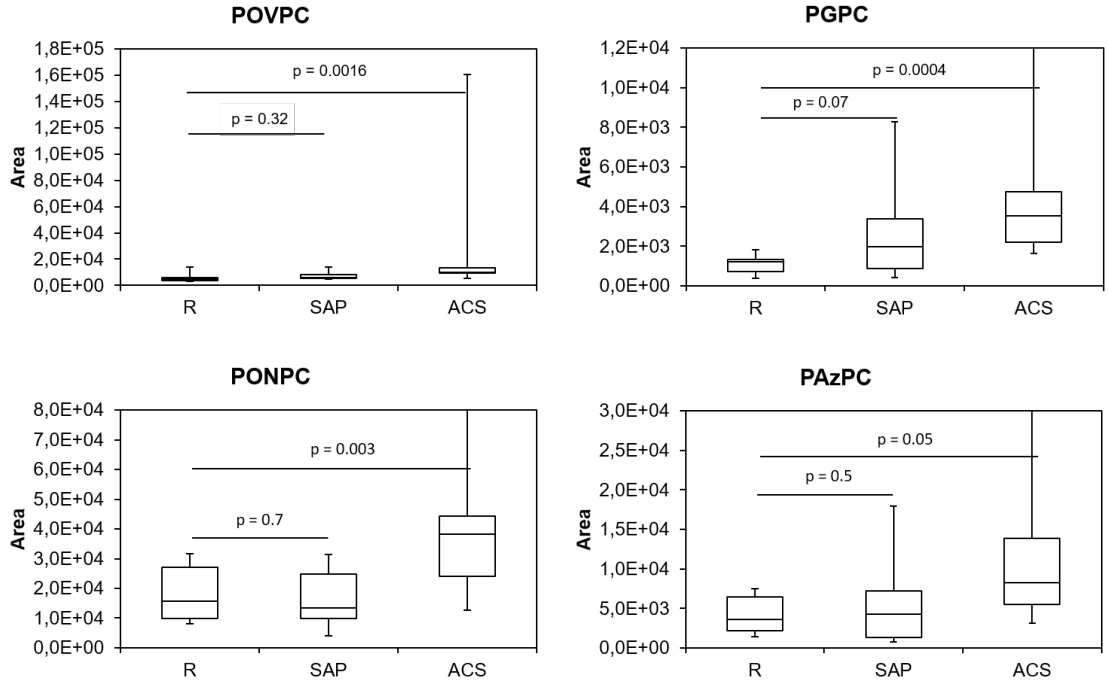

Figure 9. Expression of POVPC, PGPC, PONPC and PAzPC in the samples of the subgroups (21)**. In cooperation with Prof. Michael Lämmerhofer and Jörg Schlotterbeck.**

The same tendency was observed among other oxidized lipid metabolites. Several lysoPCs (18:1, 18:2, 22:6) were found to be elevated in SAP and STEMI patients compared to healthy controls (21).

Cholesteryl esters (CE) (18:1, 18:2, 20:4, 20:5, and 22:6) levels were also significantly elevated in SAP and STEMI patients compared to healthy controls (21).

Ceramide levels in platelets of SAP patients showed a different tendency in abundancy. Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:0) and Cer(d18:1/24:1) plasma levels were significantly downregulated in SAP compared to healthy controls. The levels of particular ceramides showed a significant downregulation between the subgroups. Cer 41:4;(d17:0/24:4) and Cer 42:5;(d18:1(4E)/24:4) were significantly downregulated in platelets from STEMI patients when compared to SAP (21).

6. Discussion

CVD is the major cause of worldwide mortality expected to reach the rate of 23.6 million deaths per year by 2030 (1). The major cause accounting for cardiovascular mortality is CAD resulting in AMI and exerting its lethal complications. The reason for ischemic events of the large arteries is atherosclerosis, the pathophysiological mechanism of which lies in a systemic inflammatory state caused by circulatory platelet hyper-reactiveness and monocyte/macrophage and leucocyte accumulation in the intima of arteries. Hyperlipidemia is one of the factors contributing to systemic inflammation and the pathogenesis of atherosclerosis and is, therefore, one of the prominent cardiovascular risk factors. Another important factor is platelets and especially platelet-lipid interactions leading to enhanced thrombus formation on atherosclerotic lesions which exert in ischemic events (AMI, stroke etc.). Incubating whole blood from healthy subjects with oxLDL showed an enhanced platelet aggregation response (Figure 6). Therefore lipid oxidation products are also important contributors to the development of atherosclerosis.

Lipids can contribute to platelet activation and ROS play a crucial role in this process along with other regulatory parameters which trigger the aggregation cascade (20). ROS production from phospholipids can be initiated not only by external triggers (e.g. UV radiation, smoking) but also be enzyme-induced during platelet aggregation. (39) Activation of platelets by different agonists also leads to the generation of a variety of intracellular lipid signaling intermediates and second messengers which contribute to platelet activation (21). Many lipid metabolites generated in the arachidonic acid pathway e.g. thromboxane, leukotrienes, oxPL can influence platelet activation acting as autocrine agonists or boost thromboinflammatory processes acting as paracrine inducers. Current experimental findings showing the synergistic effect of oxLDL on ADP induced platelet aggregation was one of the main reasons to further investigate other intraplatelet lipid metabolites which might influence platelet hyperreactivity in CAD patients and thereby contribute to atheroprogression. Moreover, a further aim was to compare the platelet lipid profiles between the healthy controls and

the subgroups of CAD, including the stable disease (SAP) and its advanced stage (STEMI).

For the purpose of the study liquid chromatography hyphenated to high-resolution mass spectrometry (UHPLC-ESI-QTOF-MS/MS) was applied. This is currently one of the leading lipid profiling method in lipidomics research providing highly sensitive and quantitative results. After global lipid profiling, specific lipid targets were investigated.

The final cohort for lipidomics included 13 STEMI and 10 SAP patients which platelet lipid profiles were compared to 10 healthy controls. This study was one of the first ones to investigate lipid profile in the platelets in the clinical context. The study showed significant changes in platelet lipid profiles among healthy subjects and CAD patients (21). Platelets of SAP and STEMI patients showed higher abundance of various lipids and their metabolites compared to healthy subjects (21). As already discussed changes in the platelet lipid profile of CAD patients could arise from oxidative/peroxidative and differential enzymatic lipid metabolism which is reflective of the activation state of platelets and may constitute a driving mechanism of atherosclerosis. Atherogenic lipid metabolites enhance the inflammation in the intima layers of arteries and interact with platelets leading to their hyperreactivity both of which contribute to plaque building and their instability.

Significant differences in platelet lipid profiles were also observed among the subgroups of CAD. Platelets of STEMI patients showed higher levels of most lipid metabolites compared to SAP patients (21). This confirms that AMI is the state of the most active platelet and lipid interaction where inflammatory and oxidation processes are the most active leading to the production and higher levels of oxidized lipid metabolites.

Overall, the study identified 172 lipid species which were significantly more abundant in platelets of CAD patients compared to platelets of healthy individuals. Lipid species belonging to the main lipid classes including triglycerides (TG), phospholipids (PC), diacylglycerols (DG), monoacylglycerols (MG), plasmenyl-PC (p-PC), and sphingomyelin (SM) were detected. (21)

Ceramides were recently confirmed to be significant predictors of CVD, independent of currently used lipid markers (45).

Another important aspect of the study was the platelet oxPL profile in CAD patients (21). Oxidized lipid metabolites, generated by ROS-mediated platelet oxidative conversion, including oxPL, lysoPC and CE, were found to be expressed in significantly higher amounts in platelets of CAD patients as compared to healthy controls. As already discussed POVPC or PGPC promote adhesion of monocytes to endothelium, oxLDL uptake, and formation of foam cells as well as apoptosis of macrophages and proliferation of smooth muscle cells. LysoPC are nonetheless responsible for the development of and were proven to independently predict the risk of CVD (79). This confirms the significance of inflammation and oxidative stress being as one of the primary factors in the pathogenesis of atherosclerosis (80). PL oxidation products accumulate in plasma, atheromatous plaques and are essential constituents of lipoproteins. Detection of oxPL in platelets suggests that platelets could be considered as an important source of oxPL.

Current guidelines to CVD prevention are based on LDL-C lowering therapies as LDL has been proven to be an independent CVD risk factor. Statins and some novel agents, e. g. ezetimibe or PCSK9 inhibitors mainly lower the levels of LDL-C exhibiting little effect on VLDL, chylomicrons or Lp(a) etc. This study detected various lipid entities which are associated with CAD and are significantly more abundant in SAP and STEMI patients (21). The detected lipid metabolites may be used as novel CVD markers in the clinical practice next to the already established ones (TC, TAG, LDL, HDL etc.). This opens new possibilities in detecting high-risk CAD patients earlier and improving the prevention of CVD and its lethal complications. Novel lipid metabolites may also be used as substrate for developing new lipid-lowering therapies in atherosclerosis.

7. Conclusion

Atherosclerosis is a systemic inflammatory disease caused by monocyte/macrophage and leucocyte accumulation in the intima of arteries, leading to the formation of atheromatous plaques. This can subsequently result in plaque rupture and thrombotic occlusion of arteries, manifesting in various ischemic events (myocardial infarction, cerebral stroke, mesenteric ischemia etc.). Platelets contribute to the development of atherosclerosis by the means of aggregation, activation of endothelium, production of inflammatory cytokines, and thrombus formation upon plaque rupture.

Lipids, especially oxidized lipid metabolites, also play a crucial role in the building of atheromatous plaques. Of crucial importance in the pathogenesis of atherosclerosis is the lipid-platelet interplay as oxidized lipids exhibit a characteristic feature to activate and enhance the functions of platelets. Platelets are therefore able to endocytose lipids and generate its atherogenic oxidation products.

Therefore, hyperlipidaemia is one of the main cardiovascular risk factors. The main atherogenic lipoprotein in human bloodstream is LDL-C and is the major target of cholesterol-lowering therapy. On the other hand, the number of potential novel lipid biomarkers may grow as oxidation processes in the development of atherosclerosis generate a variety of oxidized lipid metabolites which are currently under investigation. Phospholipid oxidation can be either induced enzymatically or non-enzymatically. The former oxidation is initiated by different LOX or COX whereas the latter one by ROS. Enzymatic PL-oxidation creates an enormous variety of new species of oxPLs which exhibit atherogenic features and contribute to the pathogenesis of CAD. E.g. 12-HETE containing oxPLs are known for initiating or triggering pro-coagulant functions. Another group of PAPC-derived oxPLs (e. g. POVPC or PGPC) promotes adhesion of monocytes to endothelium, oxLDL uptake, and formation of foam cells as well as apoptosis of macrophages and proliferation of smooth muscle cells. Another PAPC-oxidation product PEIPC exhibits the latter functions even more potently. Therefore, these oxidized lipid metabolites may serve as novel laboratory

markers and help diagnose hyperlipidemic states earlier especially in stratifying high risk patients and initiating an appropriate therapeutic strategy for prevention of CVD.

The findings of the current study suggest that the platelet lipid profile differs substantially among the healthy subjects and CAD patients. The abundance of detected oxidized lipid metabolites was found to be increased in acute stages of CAD, i.e. STEMI. The conventional concept of LDL-C being the most important factor in the pathogenesis of atherosclerosis might be challenged as these novel lipid metabolites also enhance atherosclerosis. Therefore, the detected metabolites may be gradually introduced as novel biomarkers to the conventional ones (TC, LDL-C etc.) into clinical practices and could improve the primary and secondary prevention of CVD. These oxidized lipid metabolites may also become novel therapeutic targets. Moreover, the findings of the study bring a better understanding of the platelet-related pathomechanisms of atherosclerosis. Finally, these findings contribute to the current knowledge of lipid metabolism and associated pathophysiology.

8. Deutsche Zusammenfassung

Kardiovaskuläre Erkrankungen sind eine der Hauptursachen für Mortalität in der industrialisierten Welt und werden bis zum Jahr 2030 für 23,6 Millionen der Todesfälle weltweit verantwortlich sein (1). Der akute Myokardinfarkt (AMI) und die damit einhergehenden Komplikationen stellen die schwerste Manifestation einer koronaren Herzerkrankung (KHK) und somit eine der häufigsten Todesursachen weltweit dar. Zur Entstehung von ischämischen Ereignissen wie z. B. AMI trägt die Atherosklerose maßgeblich bei. Hyperlipidämie ist ein entscheidender Faktor, der zur systemischen Inflammation und Pathogenese der Atherosklerose führt, und stellt somit einen kardiovaskulären Risikofaktor mit größter klinischer Bedeutung dar.

Ziel dieser Studie war die Identifizierung des thrombozytären Lipidprofils in gesunden Probanden sowie Patienten mit einer KHK. Darüber hinaus wurde das thrombozytäre Lipidprofil zwischen den gesunden Probanden, Patienten mit stabiler Angina pectoris (SAP) und ST-Hebungsinfarkt (STEMI) verglichen.

Das Thrombozytenlipidrofil wurde bei 13 STEMI-Patienten, 10 SAP-Patienten und 10 gesunden Probanden mittels einer Flüssigkeitschromatographie und einer gekoppelten high-resolution Massenspektrometrie (UHPLC-ESI-QTOF-MS/MS) untersucht und verglichen.

Die Analyse der Hauptkomponenten zeigte eine signifikante Verteilung der Thrombozytenlipidprofile in den zwei Gruppen, welche gesunde Probanden und Patienten mit einer KHK darstellen (Abbildung 7). Zudem wurden signifikante Unterschiede zwischen den Myokardinfarktpatienten und SAP-Patienten beobachtet. Somit wurden die größten Mengen der unterschiedlichen Lipide und Lipidmetabolite in der akutesten Phase der KHK, das heißt in STEMI-Patienten, nachgewiesen und zeigten somit einen signifikanten Unterschied im Vergleich mit SAP-Patienten und gesunden Probanden.

Insgesamt konnten 664 Molekulareinheiten beobachtet werden, welche signifikante Mengenunterschiede zwischen den KHK-Patienten und gesunden Probanden zeigten. 172 Molekulareinheiten konnten manuell identifiziert

werden und zeigten die gleiche Tendenz bezüglich des spezifischen Vorkommens. Die Ergebnisse sind in einer Heatmap dargestellt und zeigen, dass gewisse Lipide in Patienten mit einer KHK, im Vergleich mit den gesunden Probanden, vermehrt ($p>0,05$) ausgeschüttet werden (Abbildung 8). Die identifizierten Lipidklassen stellten in erster Linie Triglyzeride, Phospholipide, Diacylglycerine, Monoacylglycerine, plasmenyl-Phosphatidylcholine, Sphingomyeline und Ceramide dar.

Ein anderer wichtiger Aspekt dieser Studie war die Identifizierung des Profils der oxidierten Phospholipide in Thrombozyten von KHK-Patienten. Oxidierte Lipidenmetabolite, einschließlich oxidierter Phospholipide, Lysophosphatidylcholine und Ceramide, wurden in signifikant größeren Mengen in KHK-Patienten im Vergleich mit den gesunden Probanden sezerniert. Dies bestätigt die Signifikanz der entzündlichen und oxidativen Prozesse in der Pathogenese der Arteriosklerose (80). Phospholipidenoxidationsprodukte kummulieren sich in Plasma, atheromatösen Plaques und sind Grundbestandtteile der Lipoproteine. Somit sind die Thrombozyten wahrscheinlich eine der wichtigsten Quelle oxidierter Phospholipide im Kreislauf.

Zusammen mit Lipoproteinen können die oxidierten Phospholipide, Lysophosphatidylcholine und Ceramide somit als wichtige kardiovaskuläre Risikofaktoren betrachtet werden. Ceramide, die reichlich in KHK-Patienten ausgeschüttet werden wurden vor kurzem als signifikante Prädiktoren kardiovaskulärer Erkrankungen bezeichnet, unabhängig von aktuellen Lipidmarkern (45). Lysophosphatidylcholine beschleunigen die Entwicklung kardiovaskulärer Erkrankungen und können somit als unabhängige Prädiktoren angesehen werden (79). Die oben genannten Lipidmetabolite könnten eventuell als neue Biomarker im klinischen Alltag angewendet werden, um die Patienten mit hohem kardiovaskulärem Risiko zu identifizieren. Zudem könnten diese Lipidmetabolite zur Erfindung neuer Medikamente zur Lipidsenkung im Blut und somit zur kardiovaskulären Prävention angewendet werden. Die Studie trägt den aktuellen Kenntnissen in der Forschung der Atherosklerose bei und

vertiefet das Verstehen der pathophysiologischen Mechanismen in der Atherosklerose.

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10. Erklärung zum Eigenteil

Die Arbeit wurde in der Medizinischen Klinik III am Universitätsklinikum Tübingen unter Betreuung vom Prof. Dr. med. Meinrad Gawaz durchgeführt.

- Das Studiendesign, Identifikation und Rekrutierung von Patienten wurden von Frau Monika Zdanyte durchgeführt.
- Probenakquirierung für die Studienkohorte von gesunden Probanden, SAP und STEMI Patienten wurden von Frau Monika Zdanyte durchgeführt.
- Erhebung der Patientencharakteristika und Isolation von Probenmaterial (Thrombozytenisolation) wurden von Frau Monika Zdanyte durchgeführt.
- Lipidextraktion und UHPLC-ESI-QTOF-MS/MS, sowie Analyse und statistische Auswertung von Daten wurden durchgeführt in Kooperation mit Herrn Jörg Schlotterbeck (Kandidat Ph. D.), Herrn Prof. Dr. Michael Lämmerhofer (Ph.D.) (Pharmazeutisches Institut, Universität Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany) und Dr. Madhumita Chatterjee (Department of Cardiology and Cardiovascular Sciences, Internal Medicine III, Universitätsklinikum Tübingen).

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den

11. Veröffentlichungen

Teile der vorliegenden Dissertationsschrift wurden bereits in den folgenden Publikationen veröffentlicht:

Chatterjee M, Rath D, Schlotterbeck J, Rheinlaender J, Walker-Allgaier B, Alnaggar N, Zdanyte M, Müller I, Borst O, Geisler T, Schäffer TE, Lämmerhofer M, Gawaz M. Regulation of oxidized platelet lipidome: implications for coronary artery disease. Eur Heart J. England; 2017 Apr