Dissertation

Anti-inflammatory and anti-aggregatory properties of secretory phospholipase A_2 modified high-density lipoprotein

submitted by

Sanja CURCIC

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Assoc.-Prof. Dr. Gunther Marsche

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, ---------- ----------

ACKNOWLEDGEMENTS

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First of all, I wish to express my sincere gratitude to the members of the institute for their help with my adaptation process at the institute, scientific research and their great encouragement during my learning of German. In particular, I am deeply grateful to my inspiring supervisor Dr. Gunther Marche, for his invaluable scientific help, optimism and endless patience. I owe many thanks to the members of the Marsche group Mihael Holzer, Liza Pasterk, Markus Trieb, Marilena Appierto and Sabine Kern for their generous help and advice at different stages of my research. I also wish to thank my friends and colleagues Jovana and Angela, for their generous help and moral support during my PhD studies.

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ABBREVIATIONS

ABCA1	ATP-binding cassette, sub-family A, member 1
ACAT	acyl-CoA:cholesterol acyltransferase
ADP	adenosine diphosphate
apo	apolipoprotein
BLT-1	block lipid transport-1
BSA	bovine serum albumin
C5a	complement component 5a
Ca^{2+}	calcium
CAD	cardiovascular disease
cAMP	cyclic adenosine monophosphate
CCL5 (RANTES)	C-C motif chemokine ligand 5/regulated on activation normal T cell expressed and secreted
CD	cluster of differentiation
CETP	cholesteryl-ester transfer protein
CRP	C-reactive protein
Cu^{2+}	copper
CVD	cardiovascular disease
DIC	disseminated intravascular coagulation
FBS	fetal bovine serum
FFA	free fatty acid
FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine

GP	glycoprotein
GPCR	G protein-coupled receptor
HDL	high-density lipoprotein
ICAM-1	intercellular adhesion molecule-1
IFN-ɣ	interferon-ɣ
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
JAMs	junctional adhesion molecules
KBr	potassium bromide
kDa	kilo Dalton
LDL	low-density lipoprotein
LFA-1 (CD11a/CD18)	lymphocyte function-associated antigen-1
LPC	lysophosphatidylcholine
Lp-PLA$_2$ (PAF-AH)	lipoprotein-associated phospholipase A$_2$/platelet-activating factor acetylhydrolase
LPS	lipopolysaccharide
LTB4	leukotriene B4
MAC-1 (CD11b/CD18)	macrophage-1 antigen
MCP-1	monocyte chemotactic protein-1
MI	myocardial infarction
MMP	metalloproteinase
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate

NET	neutrophil extracellular trap
NO	nitric oxide
oxHDL	oxidized high-density lipoprotein
oxLDL	oxidized low-density lipoprotein
PAF	platelet-activating factor
PBS	phosphate buffered saline
PC	phosphatidylcholine
PF4	platelet factor 4
PKC	protein kinase C
PLA_2	phospholipase A_2
PMNL	polymorphonuclear leukocytes
PSGL-1	P-selectin glycoprotein ligand-1
RCT	reverse cholesterol transport
rHDL	reconstituted high-density lipoprotein
ROS	reactive oxygen species
RT	room temperature
SAA	serum amyloid A
SMC	smooth muscle cell
$sPLA_2$	secretory phospholipse A_2
$sPLA_2$-HDL	secretory phospholipase A_2-treated HDL
$sPLA_2$-LDL	secretory phospholipase A_2-treated LDL
SR-BI	scavenger receptor, class B, type 1
TGF-β	transforming growth factor-β

TNF-α	tumor necrosis factor-α
T_XA_2	thromboxane A_2
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein
vWF	von Willebrand Factor

SUMMARY

Secretory phospholipases A_2 ($sPLA_2$s) are enzymes which catalyze the hydrolysis of glycerophospholipids to lysophospholipids and free fatty acids at the sn-2 position. Various chronic inflammatory diseases are accompanied by elevated $sPLA_2$ concentrations. Moreover, $sPLA_2$ levels in plasma can increase hundred-folds under acute inflammatory conditions, such as sepsis and septic shock. However, the role of $sPLA_2$ elevated levels in these conditions is not clear. Numerous studies implicated the possible involvement of several $sPLA_2$ types in the development of cardiovascular disease given that $sPLA_2$ mass and activity have been shown to correlate with increased cardiovascular risk in patients with coronary artery disease. Surprisingly, drugs inhibiting $sPLA_2$ activity had disappointing performance in patients with acute coronary syndrome leading to even increased rate of myocardial infarction and stroke.

Under inflammatory conditions $sPLA_2$ is mainly associated with high-density lipoproteins, which are the main plasma carriers of phospholipids. Given that high-density lipoprotein (HDL) is the major substrate for $sPLA_2$ in plasma, within the first part of the thesis we investigated the effects of $sPLA_2$-mediated modification of HDL ($sPLA_2$-HDL) on neutrophil function, an essential arm of the innate immune response and atherosclerosis.

Treatment of human neutrophils with $sPLA_2$-HDL rapidly prevented agonist-induced activation including cell shape change, CD11b activation, adhesion to intercellular adhesion molecule-1 (ICAM-1) under flow conditions, chemotaxis as well as neutrophil extracellular trap formation. We found that cholesterol-mobilizing activity of $sPLA_2$-HDL was highly increased when compared to native HDL, promoting disruption of cholesterol-rich signaling microdomains which are known to have crucial role in cellular signaling pathways. Moreover, $sPLA_2$-HDL effectively suppressed agonist-induced rise in intracellular Ca^{2+} levels. Removing $sPLA_2$-generated lysophospholipids from $sPLA_2$-HDL abolished all anti-inflammatory activities. We observed that lysophosphatidylcholine (LPC) 16:0, which was the most abundant LPC in $sPLA_2$-HDL, mimicked $sPLA_2$-HDL effects on neutrophils.

In the second part of the thesis we focused on the effects of $sPLA_2$-HDL on platelet functional responses as it is well established that platelets have an essential role in complications of atherosclerosis such as arterial thrombosis. Interestingly, we found that $sPLA_2$-HDL showed potent anti-aggregatory properties, inhibiting platelet aggregation induced by several agonists such as ADP, collagen and thrombin. In addition, $sPLA_2$-HDL suppressed P-selectin expression and GPIIb/IIIa integrin activation in platelets as well as reactive oxygen species production and Ca^{2+} flux. Furthermore, LPC 16:0 was the most effective when enriched in HDL. However, other LPC species such as LPC 18:1 and LPC 18:2 also inhibited platelet activation.

Of crucial importance is our finding that $sPLA_2$-HDL had highly increased ability to promote cholesterol efflux from platelets, which is in line with previous studies showing that cholesterol depletion from cell membrane diminishes platelet activation.

Summing up, our results provide novel evidence that under inflammatory conditions increased $sPLA_2$ levels generate HDL particle with potent anti-inflammatory activity suggesting that $sPLA_2$ inhibition might not be a useful strategy to reduce adverse cardiovascular outcomes.

ZUSAMMENFASSUNG

Sekretierte Phospholipasen A_2 ($sPLA_2$) katalysieren die Hydrolyse von Glycerophospholipiden zu Lysophospholipiden unter Freisetzung von freien Fettsäuren an der sn-2 Position. Die Plasmaspiegel dieser Enzyme sind bei unterschiedlichsten entzündlichen Erkrankungen erhöht und können bei Sepsis oder septischen Schock mehr als hundert-fach ansteigen. Interressanterweise ist die physiologische und pathophysiologische Rolle dieser Enzyme noch nicht geklärt. Zahlreiche Studien postulierten eine pro-atherogene Funktion von $sPLA_2$, da erhöhte $sPLA_2$ Plasmaspiegel mit einem verstärkten kardiovaskulären Risiko einhergehen. Überraschenderweise zeigten $sPLA_2$ hemmende Wirkstoffe in klinischen Untersuchungen keine Reduktion kardiovaskulärer Ereignisse, ja im Gegenteil, es wurde sogar eine Erhöhung der Myokardinfarkte und Schlaganfälle festgestellt.

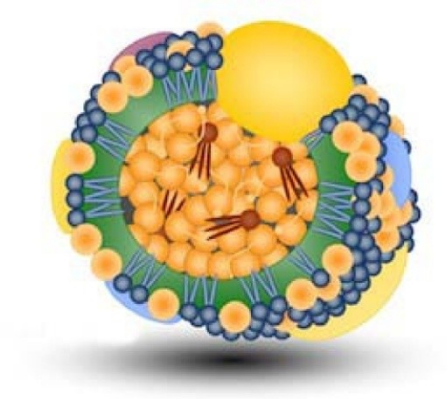
Im Plasma assoziiert $sPLA_2$ vorallem mit Lipoproteinen hoher Dichte (HDL), die den Großteil der Phospholipide transportieren. Nachdem HDL-assoziierte Phospholipide ein Haupttarget der $sPLA_2$ sind, haben wir im ersten Teil dieser Doktorarbeit untersucht, ob $sPLA_2$-modifiziertes HDL die Aktivität von Neutrophilen beeinflussen kann. Das ist möglicherweise von großer Bedeutung, da Neutrophile eine Hauptrolle bei der angeborenen Immunantwort spielen und ihnen eine entscheidente Rolle bei chronisch entzündlichen Erkrankungen, wie der Atherosklerose, zugeschrieben wird.

Überraschenderweise zeigte sich, dass eine Vorinkubation von Neutrophilen mit $sPLA_2$-HDL die Aktivierung von Neutrophilen komplett unterdrückte. Dieser potente anti-inflammatorische Effekt trat rapide und bei allen getesteten Neutrophil-stimulierenden Agonisten auf. $sPLA_2$-HDL unterdrückte den sogenannten „neutrophil shape change“, die Anstiege von intracellulären Ca^{2+} Konzentrationen, die CD11b Aktivierung, die Adhäsion unter „flow“, Chemotaxis und Neutrophil „extracellular trap“ Formation. Der zugrunde liegende Mechanismus scheint zu sein, dass die Cholesterin Efflux Kapazität nach $sPLA_2$ Modifikation von HDL beträchtlich zunimmt und $sPLA_2$-HDL cholesterinreiche “lipid rafts” delipidiert. Lipid rafts sind fundamentale Plattformen für die effektive Signalweiterleitung von G-Protein gekoppelten Rezeptoren, die eine

entscheidende Rolle bei Entzündungsprozessen spielen. Diese $sPLA_2$-HDL mediierten Effekte scheinen von HDL assoziierten Lysophospholipiden getriggert zu werden, wobei das am stärksten angereicherte Lysophosphatidylcholin (LPC) 16:0 auch die stärkste anti-inflammatorische Aktivität zeigte.

Im zweiten Teil der Dissertation untersuchten wir, ob $sPLA_2$-HDL auch die Aktivität von Thrombozyten moduliert. Interessanterweise zeigte sich, dass $sPLA_2$-HDL effektiv die Plättchenaggregation unterdrückt, unabhängig davon wie die Plättchen aktiviert wurden. $sPLA_2$-HDL reduziert die P-Selectin Expression, die GPIIb/IIIa Integrin Aktivierung, die Bildung reaktiver Sauerstoffverbindungen und den Ca^{2+} Flux von Plättchen. Ähnlich wie bei Neutrophilen scheinen HDL assoziierte Lysophospholipide diese anti-aggregatorischen Aktivitäten zu triggern und $sPLA_2$-HDL zeigt im Vergleich zum Kontroll-HDL eine markant gesteigerte Cholesterin Efflux Kapazität. Wir vermuten, dass die $sPLA_2$-HDL induzierte Reduktion von Membrancholesterin stark anti-aggregatorisch wirkt.

Zusammenfassend kann man sagen, dass unter inflammatorischen Bedingungen $sPLA_2$ die anti-aggregatorischen und anti-inflammatorischen Eigenschaften von HDL markant erhöht. Unsere Daten legen nahe, dass eine pharmakologische Inhibierung von $sPLA_2$ kein geeignetes “drug target” darstellt um kardiovaskuläre Ereignisse zu reduzieren.



I. CHAPTER:

General introduction

1. Phospholipases A_2

1.1 Classification of PLA_2s

Phospholipases A_2 (PLA_2) are enzymes that hydrolyze the ester bond of glycerophosphlipids at the sn-2 position, releasing free fatty acids (FFA) (principally arachidonic acid) and lysophospholipids ***(Figure I-1)***. This results in generation of numerous downstream bioactive molecules, such as prostaglandins, leukotriens, lysophospholipids, platelet activating factor (PAF) and oxidized lipids (1).

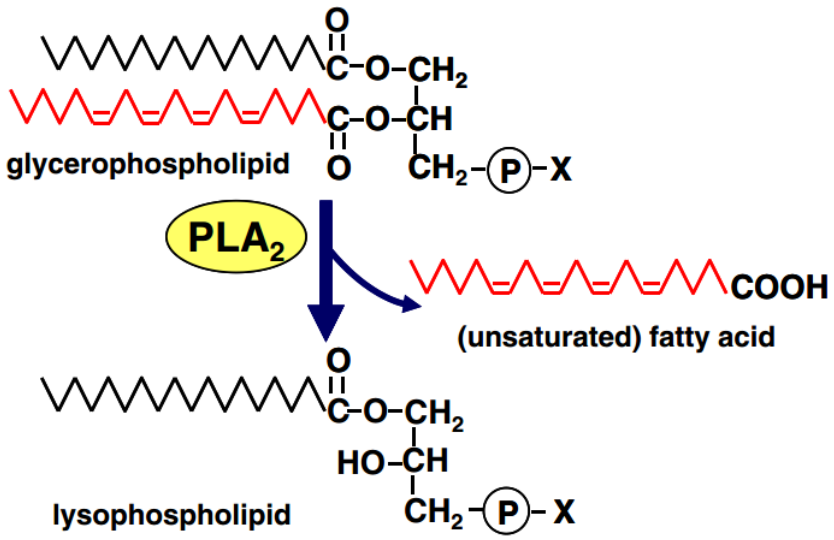


Figure I-1. *Phospholipases A_2 are enzymes which catalyze the hydrolysis of glycerophospholipids at the sn-2 position producing free fatty acids (mainly unsaturated) and lysophospholipids. Figure taken from (2).*

PLA_2 can be divided into two groups:

1) Cytosolic PLA_2 which comprise Ca^{2+}-dependent ($cPLA_2$), Ca^{2+}-independent ($iPLA_2$) and specific for PAF (intracellular PAF-acetylhydrolase)

2) Extracellular PLA_2 which can be lipoprotein-bound (Lp-PLA_2 or secreted PAF-acetylhydrolase) or secreted ($sPLA_2$) (1).

Lp-PLA_2 is a Ca^{2+}-independent enzyme with molecular mass of 45-kDa. It is produced by macrophages, T-lymphocytes and mast cells. Lp-PLA_2 is mostly associated with LDL, and to a much smaller extent to HDL. Lp-PLA_2 also shows PLA_1 and lipase activities and it is able to hydrolyze PAF, short-chain and oxidized phospholipids (1).

1.2. Secretory phospholipase A_2

$sPLA_2$ family comprises 12 mammalian low molecular mass (14-19 kDa), Ca^{2+}-dependent enzymes with His-Asp catalytic dyad. $sPLA_2$s (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA, XIIB and otoconin-95) are divided into group I/II/V/X structural collection and two atypical structural collections (III and XII) (3). Individual $sPLA_2$s differ regarding their structural characteristics, enzymatic properties, tissue localization and gene regulation. $sPLA_2$-III is localized in the kidney, liver, heart and skeletal muscles. $sPLA_2$-V is found in different tissues, with the highest concentration in the heart. $sPLA_2$-X is expressed in several immune and digestive organs as well as in the testis. $sPLA_2$-III, -V, and -X are present in plasma in low concentrations and they mainly act on lipoproteins in the intima where their concentration is high enough (1). Levels of $sPLA_2$-IIA and to a lesser extent $sPLA_2$-V highly increase under inflammatory conditions, which is induced by pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (4). Their expression is downregulated by anti-inflammatory cytokines and glucocorticoids (5). Vascular smooth muscle cells and hepatocytes are main producers of $sPLA_2$-IIA in response to cytokines under conditions of acute inflammation. Furthermore, $sPLA_2$-IIA is produced by a number of cell types such as platelets, neutrophils, macrophages and mast cells, where it is stored in secretory granules (6).

$sPLA_2$s differ in their affinity toward different phosphatidylcholine (PC) species. For example, $sPLA_2$-X has higher affinity toward arachidonate- and linoleate-containing PC species, $sPLA_2$-V preferentially hydrolyzes oleoyl- and linoleate-PC whereas $sPLA_2$-IIA

hydrolyzes all diacyl molecular species (7). $sPLA_2$-IIA activity on lipoproteins is weak in comparison to other $sPLA_2$ subtypes. However, $sPLA_2$-IIA can hydrolyze acute phase HDL 2 to 3 fold more efficiently than normal HDL (8). In addition, it is present in plasma and in the arterial wall in the high amounts and there it represents the predominant $sPLA_2$ isoform; hence, its high concentration might compensate for the low enzymatic activity (1).

1.3. Potential pro-atherogenic effects of $sPLA_2$

There are many studies confirming pro-atherogenic activity of different $sPLA_2$ subtypes. Groups IIA, III, V and X were detected in both human and mouse atherosclerotic lesions (1, 6). $sPLA_2$-IIA transgenic mice on the high-fat diet had elevated susceptibility to atherosclerosis (9). Similarly, $sPLA_2$-V-deficient $LDLR^{-/-}$ mice had reduced atherosclerotic lesion formation (10).

Most $sPLA_2$ subtypes can hydrolyze low-density lipoprotein (LDL), forming smaller and denser poratherogenic particles (11, 12) whose binding to LDL receptor is impaired prolonging their half-life in the circulation (13). In the subendothelial space $sPLA_2$ modified LDL binds to matrix proteoglycans with increased affinity (11) and forms aggregates which are taken up by macrophages (14). In this way, $sPLA_2$ modified LDL promotes foam cell formation ***(Figure I-2)***. $sPLA_2$ modified LDL was reported to induce upregulation of adhesion molecules in endothelial cells (15). Furthermore, $sPLA_2$ acts as pro-atherogenic mediator via formation of pro-atherogenic lipid products. Lipoprotein hydrolysis by $sPLA_2$ gives rise to lysophosphatidylcholines (LPCs) and FFAs. It has been shown that they can have pro-atherogenic and pro-inflammatory activities on endothelium, macrophages and smooth muscle cells via modulation of expression of cytokines, chemokines, growth factors, adhesion molecules, COX-2 and NO synthase (7). Apart from pro-atherogenic effects related to enzymatic activity of $sPLA_2$, it was reported that $sPLA_2$ can promote atherosclerosis and inflammation via binding to membrane receptors such as M-type receptor which is present on neutrophils, monocytes and macrophages (1). However, a recent study reported increased atherosclerosis in group X $sPLA_2$-deficient mice, raising the possibility that certain

$sPLA_2$ subtypes may be atheroprotective (16). This assumption is supported by the fact that inhibition of $sPLA_2$ did not translate into reduced cardiovascular risk (see below).

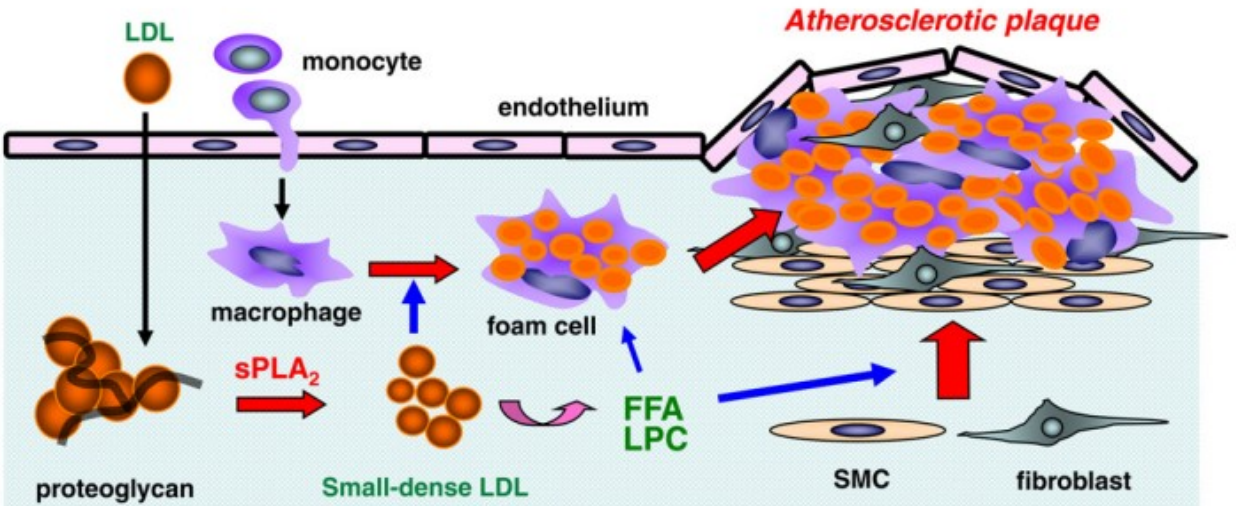


Figure I-2. Suggested role of $sPLA_2$ in the development of atherosclerosis*. In the arterial wall, where multiple $sPLA_2$ are present, LDL captured by proteoglycans is hydrolyzed by proteoglycan-bound (IIA and V) or -unbound (III and X) $sPLA_2$s and transformed into small dense LDL, which promotes foam cell formation and development of atherosclerosis. LPC, FFA and their metabolites, which are formed as a result of $sPLA_2$ activity on LDL, can induce activation of macrophages and smooth muscle cells leading to collagen deposition within the atherosclerotic plaque. LDL, low density lipoprotein; FFA, free fatty acid; LPC, lysophosphatidylcholine; SMC, smooth muscle cell. Figure modified from (2).*

1.4. $sPLA_2$ inhibition as a therapeutic strategy

Several epidemiologic studies showed association between circulating levels of $sPLA_2$ and cardiovascular risk. In EPIC-Norfolk study $sPLA_2$-IIA mass and total $sPLA_2$ activity were predictive of future risk of incident coronary heart disease amongst apparently healthy individuals (17). The largest study that addressed the prognostic utility of $sPLA_2$

in patients with stable coronary artery disease (CAD) was the PEACE study (18), in which $sPLA_2$ activity was found to be associated with an increased risk of adverse outcome in stable CAD patients. Furthermore, studies in patients with acute coronary syndrome (ACS) showed that elevated $sPLA_2$-IIA levels were associated with the higher risk of death and myocardial infarction (MI) (19). In addition, $sPLA_2$ increased levels have also been found to be associated with other diseases such as rheumatoid arthritis and cancer (3).

Animal studies showed that pharmacological inhibition of $sPLA_2$ is beneficial in atherosclerosis. The $sPLA_2$ inhibitor varespladib, which inhibits $sPLA_2$-IIA, V and X, but has no affinity for $sPLA_2$-III (20), reduced atherosclerosis by up to 75% in apoE knock-out mice on a high-fat diet (21). In addition, varespladib decreased total cholesterol level in both animal studies (21) and in coronary artery disease patients in PLASMA (Phospholipase Levels And Serological Markers of Atherosclerosis), a phase II clinical study (22). Hence, decreased cholesterol levels could also contribute to its atheroprotective effects. On the other hand, although varespladib showed anti-atherogenic effects in animal models, phase III VISTA-16 clinical trial failed to prove beneficial effects of this drug in patients with coronary artery disease. Moreover, the mortality in these patients was even increased (23). In line with the reported inefficacy of varespladib is the recent animal study showing that LDLR $^{-/-}$ mice reconstituted with bone marrow from $sPLA_2$ group X-deficient mice surprisingly had a doubling of plaque size in comparison to control mice (16).

Although numerous studies proved association between increased levels of different $sPLA_2$ groups and cardiovascular risk, it is not clear if $sPLA_2$ represents a biomarker of the disease severity or it is directly involved in the pathogenesis and complications of the disease. This can be elucidated by the application of Mendelian randomization which uses genetic variants of the enzyme to determine if gene variants associated with higher levels of the enzyme are also associated with increased adverse clinical outcomes (1). Recent large-scale Mendelian randomization study showed that $sPLA_2$-IIA is not likely to be a valid therapeutic target for the treatment of cardiovascular disease (24).

1.5. Role of $sPLA_2$ in inflammation

There are several studies confirming that, in addition to its potential pro-inflammatory activities in animal studies, $sPLA_2$ also has protective functions. $sPLA_2$-IIA has been reported to have bactericidal activity against *Staphylococcus aureus* and other Gram-positive bacteria (25). These effects were also confirmed *in vivo* in $sPLA_2$-IIA transgenic mice which showed higher resistance to both Gram-positive (26) and Gram-negative bacterial infections (27).

Additionally, selective inhibition of $sPLA_2$-IIA did not show favorable effects in patients with severe sepsis and even a negative trend in the 28-day-all-cause mortality was observed (28). $sPLA_2$-IIA transgenic mice, despite increased $sPLA_2$-IIA levels in plasma and most organs, had no detectable inflammation (29) and were found to be more susceptible to LPS-induced sepsis (30). Interestingly, several *in vitro* studies showed that statins, although known to have anti-inflammatory properties, promote IFN-γ-mediated $sPLA_2$-IIA induction instead of reduction (31). Furthermore, a retrospective review of 388 patients with bacteremic infections showed that the rate of mortality among patients taking statins was significantly lower (32). This beneficial effects of statins in sepsis could partially be explained by the fact that there is a synergism between statins and pro-inflammatory cytokines on the $sPLA_2$-IIA expression associated with efficient bactericidal protection (30). Besides not having beneficial effects in septic patients, $sPLA_2$ inhibition also proved to be inefficient in treatment of rheumatoid arthritis (33).

Along with its bactericidal properties, $sPLA_2$-IIA was described to have anti-thrombotic effects, which seems to be independent of its enzymatic activity. It inhibits formation of Xa-Va complex and subsequently thrombin synthesis. Furthermore, $sPLA_2$-IIA was shown to increase clearance of oxidatively modified lipoproteins by the liver and adrenals (30).

2. High density lipoprotein (HDL)

2.1 Composition of HDL

HDL is a macromolecule composed of numerous lipids and proteins. Mature HDL consists of hydrophobic core mainly containing cholesteryl-esters (CEs) and triacylglycerols, and the surface monolayer which is composed of phospholipids, unesterified cholesterol and apolipoproteins. HDL comprises a heterogeneous family of particles which differ in their apoliporotein and lipid distribution, density, size, shape and electrophobic mobility, forming various HDL subfractions. Ultracentrifugation separates HDL based on density into two subfractions, larger and less dense HDL_2 (1.063-1.125 g/mL) and smaller and denser HDL_3 (1.125-1.21 g/mL). According to size 5 HDL subclasses can be distinguished: HDL_{2b} (~10.6 nm), HDL_{2a} (~9.2 nm), HDL_{3a} (~8.4 nm), HDL_{3b} (~8.0 nm) and HDL_{3c} (~7.6 nm). Agarose gel electrophoresis separates HDL depending on its surface charge into major fraction called α-HDL and a small fraction, pre-β-HDL (34).

Proteomic studies have characterized up to 89 proteins associated with HDL (35). Apolipoproteins account for one-third to one-half of the HDL mass. The main HDL apolipoprotein is apoA-I which accounts for 70% of the HDL protein mass. HDL also contains apoA-II, which represents 15-20% of the protein content of HDL (36). Minor HDL associated proteins are apolipoprotiens apoA-IV, A-V, C-I, C-II, C-III, D, E, J, M, serum amyloid A (SAA), transferrin, ceruloplasmin and enzymes and lipid transfer proteins such as lecithin:cholesterol acyl transferase (LCAT), CE transfer protein (CETP) and paraoxonase-1 (PON1). Interestingly, analyses of HDL proteome identified not only proteins with well-known role in lipid metabolism of HDL, but also numerous proteins implicated in the acute phase response, complement regulation and proteinase inhibition (35).

Besides proteins, numerous lipid species are present in HDL particle. In comparison with other plasma lipoproteins, HDL is enriched in phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine and phosphatidylethanolamine-based plasmalogens. FFA, mono-, di-, and triacylglycerols and sphingolipids (ceramide,

sphingomyelins and sphingosine-1-phosphate) are also part of HDL lipidome (37). Interestingly, it has been confirmed that HDL-associated phospholipids represent an important determinant of cholesterol efflux capacity (38). Additionally, it has been demonstrated that phosphatidylcholine species mediate HDL´s anti-inflammatory effects (39). All these data imply that, apart from proteome, composition of HDL lipid moiety might play important role in modulation of HDL functionality.

Low HDL-cholesterol levels represent a strong risk factor for the development of cardiovascular disease (40). Mechanisms of the anti-atherogenic effects of HDL have been investigated and confirmed in both *in vitro* and *in vivo*. Besides its well established atheroprotective activity via promoting reverse cholesterol transport from peripheral cells and macrophages back to the liver, it has been demonstrated that HDL has a wide range of other RCT-independent atheroprotective effects, such as anti-inflammatory, anti-oxidant, anti-apoptotic, anti-thrombotic, anti-infectious and endothelium protective effects (41).

2.2 Anti-inflammatory activity of HDL

Several studies showed that HDL has various anti-inflammatory effects which comprise inhibition of cytokine-induced upregulation of ICAM-1, VCAM-1 and E-selectin in endothelial cells (42, 43), suppression of MCP-1 (44) and IL-6 (45) production by endothelial cells, inhibition of monocyte adhesion and transmigration (46) and neutrophil activation (47). Anti-inflammatory effects of reconstituted HDL (rHDL) have been confirmed in animal models (48). The observed anti-inflammatory properties of HDL were shown to be dependent on both protein moiety (apoA-I, apoA-II, apoA-IV) as well as on distinct phospholipid molecular species (39, 49, 50).

3. Aims of the thesis

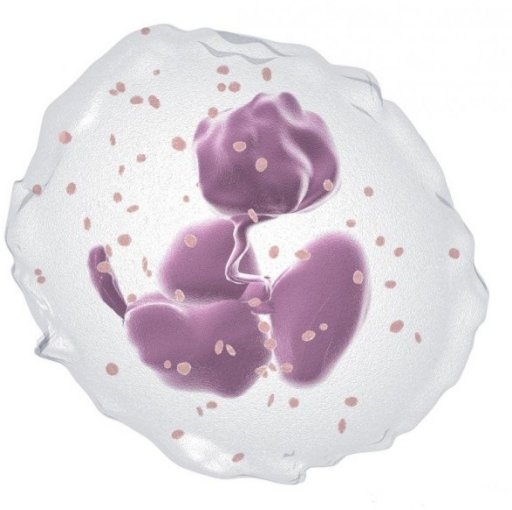
Elevated $sPLA_2$ concentrations have been reported in various chronic and acute inflammatory diseases (51-53). However, $sPLA_2$ inhibition as a treatment strategy of cardiovascular as well as sepsis patients did not show any beneficial effects (23, 28). This called into question the potential involvement of $sPLA_2$ in the pathophysiology of these diseases.

HDL is the principal carrier of phospholipids in plasma and a major substrate for $sPLA_2$ (54, 55). Furthermore, accumulating evidence suggests that HDL, apart from its well recognized atheroprotective role, also has important functions in regulation of innate and adaptive immune responses (56). However, direct effects of $sPLA_2$ modified HDL on immune cells and platelets have not been investigated yet.

This doctoral thesis is divided into two parts:

In **PART I** we investigated the effects of **$sPLA_2$-mediated modification of HDL** on human peripheral blood **neutrophils**, as neutrophils represent an essential part of the innate immune system and are considered to have an important role in atherosclerosis and sepsis.

Given that platelets are important immune mediators and that they substantially contribute to both initiation and complications of atherosclerosis, we assessed in **PART II** whether **$sPLA_2$ modified HDL** alters **platelet reactivity**.



II. CHAPTER:

Neutrophil effector responses are suppressed by secretory phospholipase A_2 modified HDL

1. Role of Neutrophils in Inflammation

1.1 Neutrophils

Neutrophils are polymorphonuclear leukocytes with a segmented nucleus and an average size of 7-10 μm (57). In humans neutrophil counts range from 50 to 70 % of all circulating leukocytes (in contrast to only 10-25 % in mice) (58). They represent the first line of host defense against various pathogens. 1×10^{11} -2×10^{11} neutrophils per day are generated in adult human (59). Mature neutrophils reside in bone marrow, spleen, liver and lung, and these organs represent neutrophil reservoirs from which neutrophils can be quickly recruited to the sites of inflammation as a response to host- and pathogen-derived chemotactic factors (60).

The average half-life of mature neutrophils in the circulation is short, about 6-8 h. In the absence of inflammation and infection neutrophils die by spontaneous apoptosis and are taken up and cleared by the liver, spleen and bone marrow (60). However, when they are primed and activated by bacterial products, different cytokines and growth factors at the site of inflammation, neutrophil life span increases several fold (60) and they can survive up to two days (61, 62).

1.2 Neutrophil recruitment

Neutrophil recruitment into infected or injured tissues includes several steps, as shown in ***Figure II-1***. These are tethering, rolling, firm arrest with accompanying cell spreading, crawling and transmigration (63). Neutrophil recruitment is promoted by pathogen molecules or host-derived inflammatory mediators produced by tissue-resident cells (such as histamine, leukotriene B4 (LTB4), interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8)). One of the strongest host-derived chemoattractants for neutrophils is chemokine IL-8, released by monocytes, macrophages, mast cells, epithelial cells, keratinocytes, fibroblasts, endothelial cells and neutrophils (64). Pathogens also produce various molecules which trigger neutrophil recruitment (such as N-formylated peptides) (65). Neutrophil recruitment is

mediated by binding of neutrophil receptors to their respective ligands on the surface of the activated endothelial cells, such as P-selectin and E-selectin which interact with P-selectin glycoprotein ligand-1 (PSGL-1) constitutively present on neutrophils (66). These law affinity interactions result in the neutrophil tethering on the endothelial surface and facilitate subsequent rolling along the vessel (67). Host-derived chemokines or pathogen molecules immobilized on endothelial proteoglycans activate G protein-coupled receptors (GPCRs) on neutrophils. This induces conformational changes of the β1 and β2 cell surface integrins and their clustering, which is known as inside-out activation (68). Inside-out activation increases integrin binding affinity for endothelial adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and intercellular adhesion molecule-2 (ICAM-2) which bind neutrophil integrins lymphocyte function-associated antigen-1 (LFA-1 or CD11a/CD18) and macrophage-1 antigen (MAC-1 or CD11b/CD18). Interaction of neutrophil integrins with their respective ligands induces signaling pathways, which is known as outside-in signaling, finally leading to firm adhesion and subsequent transmigration through the endothelium into the tissues (69).

Transmigration is preceded by neutrophil crawling inside the blood vessel along the endothelium, which mediated by MAC-1-ICAM-1 interactions. While crawling, neutrophils search for preferred places for crossing the wall of capillaries. During the transmigration step (diapedesis) neutrophils have first to pass across the endothelium and then to cross basement membrane (69, 70). This process involves integrins and CAMs (ICAM-1, ICAM-2 and vascular cell adhesion molecule-1 (VCAM-1)) as well as additional adhesion molecules such as platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD31), CD99, junctional adhesion molecules (JAMs), epithelial cell adhesion molecule (ECAM) and some other endothelial cell molecules such as poliovirus receptor (PVR, CD155), ectoenzymes (VAP-1 and CD 157) and leukocyte-specific protein-1 (LSP-1) (69-71). Neutrophils can cross endothelium either via paracellular (between endothelial cells) or transcellular pathway (through endothelial cells), but they mainly use paracellular route. In order to pass the basement membrane neutrophils release proteases, such as metalloproteinases (MMPs) and neutrphil elastase which act on extracellular matrix proteins (collagen, laminins) (69).

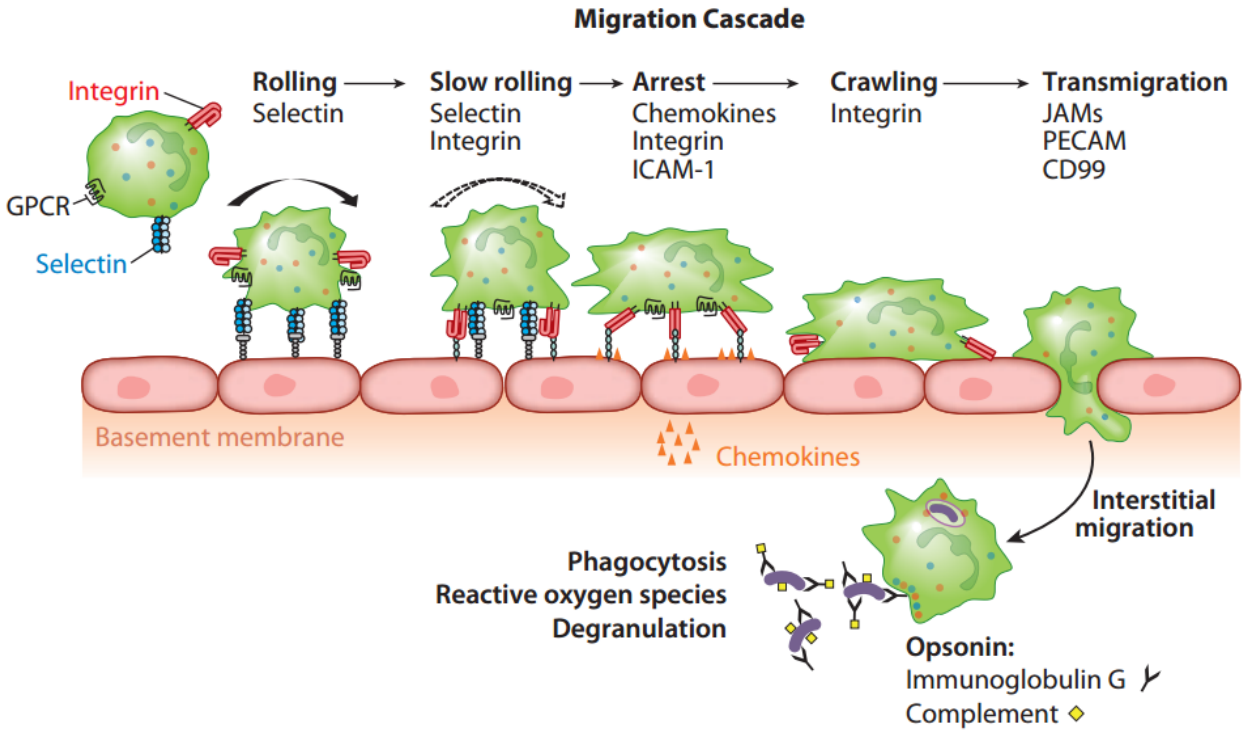


Figure II-1. Neutrophil recruitment cascade. *Pro-inflammatory signals lead to up-regulation of adhesion molecules on endothelial cells. Initial capture and rolling of neutrophils on the endothelium are mediated by selectins. These loose bonds decelerate the flowing neutrophils which get activated by host-derived chemokines or pathogen molecules. Neutrophil activation leads to conformational changes of neutrophil surface integrins and their binding to vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells. Integrin-adhesion molecule interactions secure neutrophil firm arrest and adhesion. Subsequently transmigration of adherent neutrophils across the endothelial barrier into the underlying tissue is mainly mediated by platelet endothelial cellular adhesion molecule-1 (PECAM-1) and junctional adhesion molecules (JAMs). Figure adapted from (61).*

Next step is neutrophil chemotaxis toward the site of infection/inflammation, when they are guided by chemotactic gradients in the interstitial space formed by chemoattractant

molecules such as bacteria-derived N-formyl-methionyl-leucyl-phenylalanine (fMLP) or complement component 5a (C5a). Following the entrance into the inflamed tissue, neutrophils become fully activated and release granule contents, acquire phagocytic ability and extrude neutrophil extracellular traps (NETs) (61, 69)

Neutrophils can eliminate pathogens in several ways ***(Figure II-2)***. Pathogens and other particles are recognized by neutrophils via pattern recognition receptors (PRRs) or via antibody and complement receptors for opsonized particles. Subsequently pathogens are engulfed in a phagosome and killed by reactive oxygen species (ROS) production and by antibacterial proteins (cathepsins, defensins, lactoferrin and lysozyme) which are released into phagosome (69, 72).

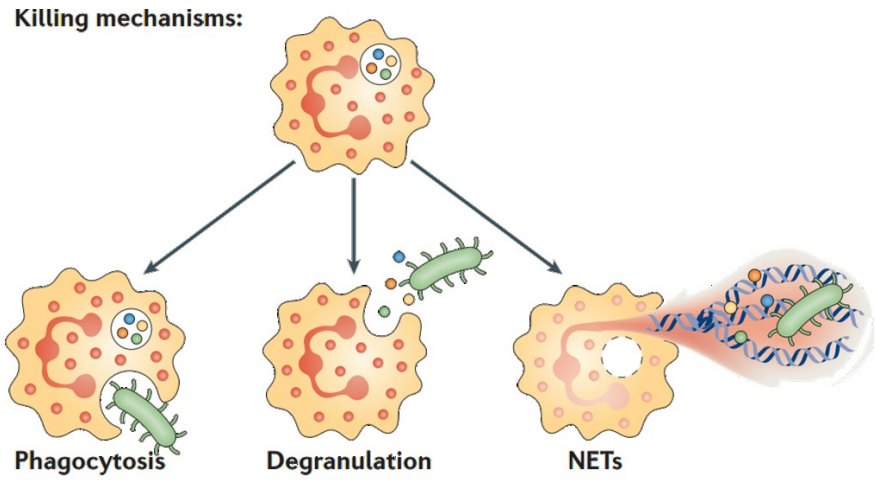


Figure II-2. How neutrophils eliminate pathogens. *Neutrophils are able to eradicate pathogens through several different mechanisms, which include phagocytosis, release of antimicrobial peptides and proteins (degranulation), production of reactive oxygen species (oxidative burst) and generation of neutrophil extracellular traps (NETs). Figure adapted from (69).*

Neutrophils can also act on microbes outside the cell, by releasing granule contents (degranulation) and expelling NETs. During degranulation, neutrophil granules fuse with the cell membrane releasing enzymes, antimicrobial and other molecules in

extracellular space. These molecules are myeloperoxidase (MPO), proteases, lysozyme (degrades the bacterial cell wall), bactericidal/permeability-increasing protein (BPI), cathelicidins and defensins (59). Besides beneficial effect on pathogen eradication, both released granule contents and NETs can have harmful effects on the host (73, 74).

1.3 Pro-inflammatory role of neutrophil extracellular traps

NETs are web-like structures, which consist of a DNA backbone with attached histones, cytoplasimic proteins (such as lactoferrin and cathepsins) and enzymes (MPO and neutrophil elastase) (75). NETs can capture and immobilize microbes facilitating their pagocytosis, but they have also been shown to kill pathogens by antimicrobial histones and proteases (76). NET formation (also known as NET*osis*) has been shown to be NADPH oxidase- and MPO-dependent (77, 78). Furthermore, recent studies revealed that platelet-neutrophil interactions have an important role in NET formation (79, 80).

Besides being beneficial in preventing dissemination of pathogens, extensive NET formation can be detrimental to the host (73). NETs have been reported to be involved in the pathogenesis of sepsis (81), thrombosis (79) and several chronic inflammatory diseases such as atherosclerosis (82), autoimmune diseases (for example systemic lupus erythematosus (73)) and vasculitis (83). NETs contain numerous pro-inflammatory molecules and autoantigens (84). In line with this, autoimmune diseases and vasculitis are characterized by the production of antibodies against some NET components (83).

Furthermore, NETs have been reported to promote platelet pro-coagulant activity which can result in thrombosis and vascular injury (85). Extracellular histone proteins can induce thrombin formation and platelet activation, which might lead to microaggregation and thrombocytopenia (86, 87).

Under septic conditions neutrophils are trapped in liver sinusoides and lung capillaries, where they are actively involved in lung and liver dysfunction and failure (88). A recent study reported that NETs could be major contributor in neutrophil-mediated liver damage observed in sepsis. Additionally, the same study revealed key role of platelet-

neutrophil interaction in promoting NET release and hepatotoxicity under septic conditions (81).

1.4 Role of neutrophils in atherosclerosis

Role of monocytes and monocyte-derived macrophages in atherosclerosis is well established and investigated. In addition, the involvement of other immune cells such as T-lymphocytes, mast cells, dendritic cells and platelets which invade atherosclerotic lesions has been confirmed (89). Neutrophils have long been considered to have a marginal relevance in cardiovascular disease. However, accumulating evidence suggests that neutrophils are involved in the pathogenesis of atherosclerosis and its complications (82) ***(Figure II-3)***. Neutrophils have been detected in atherosclerotic plaques in both mice and humans (90, 91) and their presence was associated with the severity of coronary artery disease (92). Increased activation of neutrophils in patients with unstable angina and myocardial infarction has been shown in clinical studies (93, 94). In addition, association between increased neutrophil count and cardiovascular risk has been reported (95).

Neutrophil recruitment to atherosclerotic lesion is mediated via P-selectin and E-selectin, expressed by dysfunctional endothelium (96). Neutrophils become activated and firmly adhere when they come in contact with chemotactic agents present on inflamed endothelium, such as C-C motif chemokine ligand 5 (CCL5 or RANTES) (97), which is deposited by platelets. C5a and LTB4 might also be involved in neutrophil recruitment to atherosclerotic lesions (98, 99). Adherent activated neutrophils release secretory vesicle and granule proteins such as MPO, azurocidin and proteinase-3. This further enhances endothelial dysfunction via promoting adhesion molecule expression and catalyzing reactions that consume vascular nitric oxide and decrease its bioavailability (82, 100). Neutrophil granule proteins such as cathelicidins, azurocidin, α-defensins and cathepisn G have been shown to be chemotactic for monocytes and T-lymphocytes promoting their recruitment and adhesion (101-103) as well as macrophage polarization towards M1 phenotype and upregulation of scavenger

receptors (82, 104). α-defensins and MPO induce foam cell formation via enhancement of LDL oxidation and lipid accumulation in atherosclerotic lesions (105, 106). In addition, necrotic and apoptotic neutrophils can contribute to the formation of necrotic core (82).

Neutrophils can also promote destabilization and disruption of the atherosclerotic plaque. MPO and formed ROS induce apoptosis in endothelial cells (107) whereas protinase-3 and MMPs (such as MMP-2 and MMP-9) released from activated neutrophils trigger matrix degradation, which subsequently promotes weakening of the fibrous cap and plaque rupture (82).

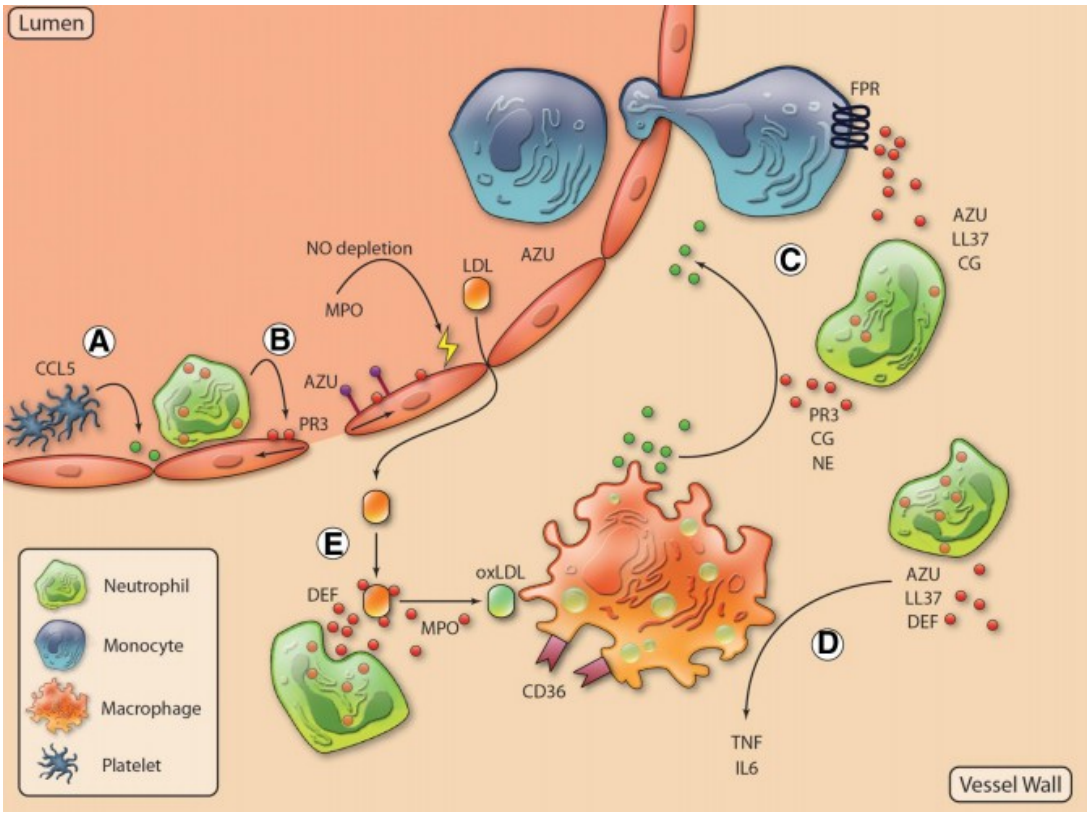


Figure II-3. Neutrophils feed mechanisms of atherogenesis. (A) *Neutrophil recruitment to the atheromatous lesion is partially platelet-dependent. Platelets deposit C-C motif chemokine ligand 5 (CCL5), which promotes neutrophil activation and subsequent firm adhesion.* ***(B)*** *Activated neutrophils release granule contents such as*

myeloperoxidase, azurocidin and proteinase-3, which induce upregulation of endothelial adhesion molecules, increase permeability of endothelium and deplete nitric oxide. All this exacerbates endothelial dysfunction. ***(C)*** *Released granule proteins deposited on the endothelium and secreted at the site of inflammation facilitate monocyte recruitment.* ***(D)*** *Neutrophil granule contents support macrophage shift toward a pro-inflammatory M1 phenotype and enhance expression of scavenger receptors (eg. CD36).* ***(E)*** *Binding of α-defensins to low-density lipoprotein (LDL) captures LDL in the arterial wall. LDL gets oxidized through MPO-dependent mechanisms which promotes macrophage foam cell formation. AZU, azurocidin; CG, cathepsin G; DEF, α-defensins; FPR, formyl-peptide receptor; MPO, myeloperoxidase; NE, neutrophil elastase; PR3, proteinase-3. Figure taken from (82).*

1.5 Cholesterol-rich microdomains (lipid rafts) in neutrophil activation

Cholesterol-reach microdomains (lipid rafts) are highly organized plasma membrane microdomains ***(Figure II-4)*** rich in cholesterol, gangliosides and sphingolipids (108). A high content of phospholipids with long saturated fatty acyl chains makes lipid raft domains more tightly packed than the surrounding non-raft phase of the membrane (109). Lipid rafts are highly dynamic and their lipid and protein composition is changing constantly which facilitates transmission of the signals from the cell surface membrane to the intracellular organelles and nucleus (110).

The main lipid component of lipid rafts is cholesterol, whose presence is crucial for the formation and maintenance of the raft structure. It maintains lipid rafts in a liquid-ordered phase, in contrast to the rest of the cell membrane which is in a liquid-disordered phase and has phosphatidylethanolamine and phosphatidylcholine as main lipid components (111).

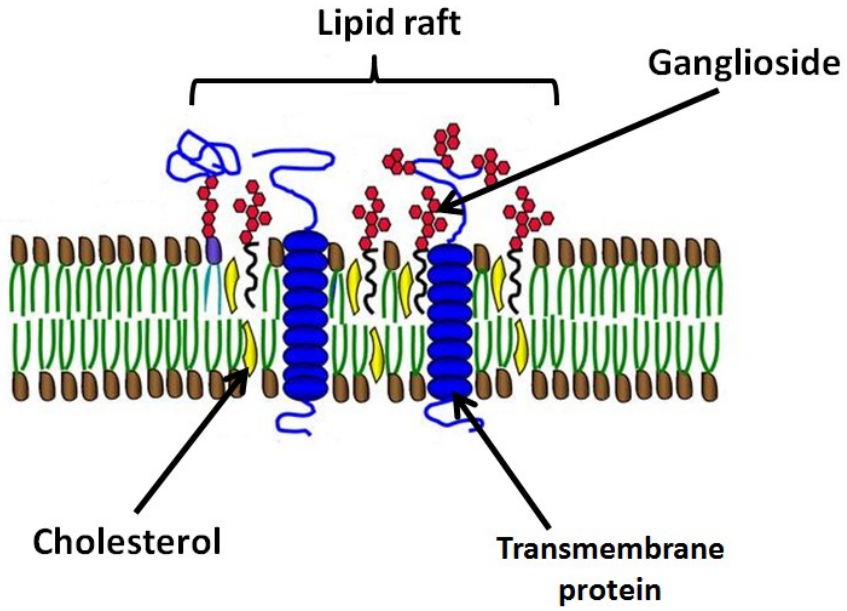


Figure II-4. Lipid raft structure. *Lipid rafts are specialized membrane microdomains with high content of cholesterol, sphingomyelin and gangliosides. They are also rich in phospholipids with saturated hydrocarbon chains which make them more tightly packed than surrounding membrane bilayer. Adapted from (112).*

Lipid rafts are recognized as specialized membrane domains for cell signaling (108) and they are thought to have a key role in immune cell function modulating many types of cellular responses such as cell adhesion, polarization, macrophinocytosis and lipoprotein uptake (113). In resting neutrophils lipid rafts are distributed uniformly through the cell membrane, but upon stimulation they tend to reorganize and aggregate (114, 115). Numerous membrane receptors and intracellular signaling proteins localize in lipid rafts. Lipid rafts are known to be enriched in various proteins important for neutrophil functional responses such as Toll-like receptors (TLR), chemokine receptors (CXCR1, CXCR4, CCR5), signaling molecules (Lyn and Src kinases, PI3K, Rho GTPases, G proteins) as well as cytoskeletal proteins.

The integrity of cholesterol-reach membrane microdomains has been reported to be essential for neutrophil actin polymerization, polarization and chemokinesis (116, 117).

In addition, lipid rafts are important for neutrophil trafficking and recruitment. This was confirmed in the study showing that P- and E-selectin dependent rolling of neutrophils on endothelium requires PSGL-1 presence in lipid rafts and its association with spleen tyrosine kinase (Syk) (118).

Furthermore, it was found that lipid rafts have important role in Ca^{2+} signaling given that their disruption via depletion of membrane cholesterol inhibited chemoattractant-induced Ca^{2+} influx in neutrophils (119).

In addition, lipid rafts play an important role in microbicidal functions of neutrophils, such as ROS production. Several studies showed that oxidative burst in neutrophils requires mobilization of NADPH oxidase components into lipid rafts (120, 121). It was reported that membrane-bound NADPH oxidase components are present in lipid rafts whereas cytosolic subunits and protein kinase C (PKC) are mobilized to the rafts following neutrophil activation (122).

2. Introduction

Levels of $sPLA_2$ have been reported to be significantly elevated in various chronic inflammatory diseases (51, 52). $sPLA_2$ plasma concentrations can increase hundred-folds under acute inflammatory conditions, such as sepsis and septic shock (53). However, the role of elevated levels of $sPLA_2$ under these conditions is not clear. As described above, numerous studies implicated the possible involvement of several $sPLA_2$ subtypes in the development of cardiovascular disease and $sPLA_2$ mass and activity have been shown to correlate with increased cardiovascular risk in patients with coronary artery disease (123). In addition, the presence of $sPLA_2$ in atherosclerotic lesions was confirmed in several studies (124). Surprisingly, drugs inhibiting $sPLA_2$ activity showed disappointing performance in patients with acute coronary syndrom leading to even increased rate of myocardial infarction and stroke (23). Furthermore, $sPLA_2$ inhibition showed no survival benefit in patients with severe sepsis (28, 125). Failure of the mentioned studies casts doubt on the proposed pathophysiologic role of $sPLA_2$.

Most $sPLA_2$ isoforms have the unique ability to hydrolyze lipoproteins, producing bioactive lipid mediators and altering the lipoprotein particles. In the acute phase of inflammatory processes, $sPLA_2$ is mainly associated with high-density lipoproteins (54), which are the principal plasma carriers of phospholipids (55). Moreover, it was shown in transgenic mice overexpressing apoA-I and $sPLA_2$ that almost all plasma $sPLA_2$ was associated with HDL fraction, which significantly influenced HDL particle size and composition leading to the shift toward smaller particles (126).

Increasing evidence suggests that HDL, apart from having key role in cardiovascular disease, also has important function in immune response and inflammation (35, 127). Surprisingly, the interaction between $sPLA_2$ modified HDL and innate immune cells has not been investigated so far.

Although neutrophils are major pathogen-killing immune cells, recent studies have revealed that neutrophils are involved in pathophysiology and can have harmful effects

in various inflammatory diseases such as pulmonary diseases (128), autoimmune diseases (61, 129), sepsis (130) and atherosclerosis (82, 131). Neutrophils were shown to promote endothelial dysfunction and to activate macrophages inducing foam cell formation and weakening of the fibrous cap (82).

Therefore, in this study we assessed whether $sPLA_2$-treated HDL modulates neutrophil functional responses. In order to assess neutrophil function, we measured neutrophil shape change in response to various agonists, CD11b activation as well as neutrophil adhesion, migration and Ca^{2+} flux.

3. Materials and Methods

3.1 Materials

Reagents	
Name	**Company**
[^{3}H]-cholesterol	Hartman Analytic
Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz 58-035	Sigma-Aldrich
Annexin V:PE Apoptosis Detection Kit	BD Biosciences
Block lipid transport-1 (BLT-1)	Sigma-Aldrich
Bovine serum albumin (BSA)	PAA
CellFix	BD Bioscience
Complement component 5a (C5a)	Sigma-Aldrich
cpt-cAMP	Sigma-Aldrich
DMEM medium	Sigma-Aldrich
F-127	Sigma-Aldrich
FACS-Flow	BD Bioscience
Fetal bovine serum (FBS)	PAA
Filipin	Sigma-Aldrich
FITC-cholera toxin B	Sigma-Aldrich
Fluo-3-AM	Life Technologies
Forskolin	Sigma-Aldrich
Free fatty acid 16:0 (FFA 16:0)	Sigma-Aldrich
Free fatty acid 18:1 (FFA 18:1)	Sigma-Aldrich
Free fatty acid 18:2 (FFA 18:2)	Sigma-Aldrich
Free fatty acid 20:4 (FFA 20:4)	Sigma-Aldrich
HEPES	PAA
Histopaque	Sigma-Aldrich
Human AB serum	Sigma-Aldrich
Human IL-8 ELISA kit	Peprotech
Human recombinant $sPLA_2$ type V	Cayman Europe
Interleukin-8 (IL-8)	Peprotech
Intracellular adhesion molecule-1 (ICAM-1)	Peprotech
Intracellular cAMP enzyme-linked immunosorbent assay	GE Healthcare
Lipopolysaccharide (LPS)	Sigma-Aldrich
Lysophosphatidylcholine (LPC) assay Kit	ALFRESA
Lysophosphatidylcholine 16:0 (LPC 16:0)	Avanti Polar Lipids
Lysophosphatidylcholine 18:1 (LPC 18:1)	Avanti Polar Lipids
Lysophosphatidylcholine 18:2 (LPC 18:2)	Avanti Polar Lipids
Lysophosphatidylcholine 20:4 (LPC 20:4)	Avanti Polar Lipids
Lysophosphatidylserine 16:0 (Lyso-PS 16:0)	Avanti Polar Lipids

NEFA (Non-Esterified Fatty Acid) FS Kit	Diasys
N-formyl-methionyl-leucyl-phenylalanin (fMLP)	Sigma-Aldrich
PD-10 columns	GE Healthcare
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Phosphate buffered saline (PBS) with or without Ca^{2+} and Mg^{2+}	PAA
Potassium bromide	Sigma-Aldrich
Probucol	Sigma-Aldrich
RPMI medium	Pasching
$sPLA_2$ type III from bee venom	Cayman Europe
SYTOX green	Life Technologies
Varespladib	Eubio
Vectashield mounting medium	Szabo Scandic
Vena8 biochips	Cellix Ltd
Antibodies	
Anti-CD11b-FITC	Biozym
Anti-CD16-PE	Biozym

3.2 Isolation of HDL

HDL was isolated by density gradient ultracentrifugation method as described (132-134). Plasma was obtained by centrifugation of the whole blood at 400 x g for 15 min. Plasma density was adjusted with potassium bromide (KBr) to 1.24 g/mL and a two-layer density gradient was generated in centrifuge tubes (16 x 76 mm, Beckman) by layering the density-adjusted plasma underneath a KBr-density solution (1.063 g/mL). Tubes were sealed and centrifuged at 90.000 rpm for 4 hours in a 90Ti fixed angle rotor (Beckman Instruments, Krefeld, Germany). After centrifugation, the HDL-containing band was collected by puncturing the centrifugation tube with a syringe. Isolated HDL was desalted using PD10 columns and immediately used for further experiments.

3.3 $sPLA_2$ treatment of HDL

In order to hydrolyse HDL-associated phospholipids, isolated HDL was treated with 200 ng/mL $sPLA_2$ type III from bee venom or 400 ng/mL human recombinant type V $sPLA_2$ in PBS with Ca^{2+} and Mg^{2+}, overnight at 37°C

3.4 Lysophosphatidylcholine (LPC), free-fatty acid (FFA) and lysophosphatidylserine enrichment/depletion of HDL

In order to generate LPC-, FFA-, or lysophosphatidylserine-enriched HDL, 1 mg/mL HDL was incubated with 0.6 mmol/L 16:0, 18:1, 18:2 or 20:4 FFA, with 0.6 mmol/L 16:0, 18:1, 18:2 or 20:4 LPC or with 0.6 mmol/L lysophosphatidylserine 16:0 for 2 h at 37°C. Unbound LPCs and FFAs were removed by gel filtration using PD10 columns. HDL-bound LPC and FFA contents were determined as described below. In some experiments, in order to remove LPCs and FFAs, $sPLA_2$-HDL was treated with 50 mg/mL BSA (1 h, 37°C) and re-isolated by density gradient ultracentrifugation. After the depletion, LPC and FFA concentrations were measured as described below.

3.5 Determination of FFAs and LPCs

HDL-associated lipids were extracted using method developed by Bligh and Dyer (135) and dried under a nitrogen stream. Dried extracted lipids were then dissolved in 200 µL $CHCl_3$/MeOH (1:1, v/v) containing 1 pmol/µL of LPC 17:1 serving as internal standard. Chromatographic separation of lipids was performed by an Accela HPLC (Thermo Scientific) on a Thermo Hypersil GOLD C18, 100 x 1 mm, 1.9 µm column. Solvent A was a water solution of 1% ammonium acetate (v/v) and 0.1% formic acid (v/v) and solvent B was acetonitrile/2-propanol (5:2, v/v) supplemented with 1% ammonium acetate (v/v) and 0.1% formic acid (v/v), respectively. The gradient was run from 35% to 70% B for 4 min, then to 100% B in additional 16 min with subsequent hold at 100% for 10 min. The flow rate was 250 µL/min. Phospholipid species were determined by a TSQ Quantum ultra (Thermo Scientific) triple quadrupole instrument in positive ESI mode. The spray voltage was set to 4500 V and capillary voltage to 35 V. The LPC species were detected in a precursor ion scan on m/z 184 at 34 eV. Peak areas were calculated by QuanBrowser for all lipid species and the calculated peak areas for each species were expressed as a % of internal standard. Results are shown as nmol/mg HDL

protein. FFA content was determined enzymatically using a NEFA FS Kit. In some experiments, LPC content of $sPLA_2$-HDL was measured with Azwell LPC Assay Kit.

3.6 Isolation and preparation of human neutrophils

Human polymorphonuclear leukocytes (PMNL) containing neutrophils and eosinophils were isolated as previously described (136, 137). Peripheral blood was sampled from healthy human donors according to the protocol approved by the Ethics Committee of the Medical University of Graz. Prior to collecting blood all volunteers signed an informed consent form. Whole blood in the presence of 3.8% sodium citrate was first centrifuged in order to separate platelet rich plasma from blood cells. Erythrocytes were sedimented using 6 % of dextran (30 min, room temperature). The upper phase, containing leukocytes, was placed on Histopaque solution and centrifuged (400 x g, 20 min). Peripheral blood mononuclear cells (PBMC), saline and Histopaque were removed and PMNL pallet was washed with PBS without Ca^{2+} and Mg^{2+} supplemented with 0.1 % BSA, 10 mmol/L HEPES and 10 mmol/L glucose pH 7.4. Any remaining erythrocyte contamination of the PMNL pellet was removed by hypotonic shock lysis with 0.2 % saline. The purity and viability of neutrophil preparation was greater than 95%. All functional assays with neutrophils were done in assay buffer (PBS with Ca^{2+} and Mg^{2+}, HEPES 10 mmol/L, glucose 10 mmol/L, bovine serum albumin 0.1%, pH 7.4).

3.7 Neutrophil shape change assay

Neutrophil shape change was performed as described before (138, 139). Isolated PMNL were resuspended in assay buffer and aliquots of cells (about 3 x 10^5 cells per sample) were pretreated with HDL preparations and then stimulated with interleukin-8 (IL-8), N-formyl-methionyl-leucyl-phenylalanin (fMLP) or complement component 5a (C5a) (4 min, at 37°C, in shaking water bath), with lipopolysaccharide (LPS) in the presence of 2 % human AB serum (90 min, 37°C) or with *Escherichia coli* bacteria (60 min, 37°C) at a final volume of 100 µL. After the treatment, cells were immediately transferred to ice in order to stop the reaction and fixed with 150 µL of ice-cold fixative solution. To measure shape change responses, samples were analyzed on a

FACScalibur flow cytometer (BD Biosciences). Eosinophils were distinguished from neutrophils according to their granularity (side scatter) and autofluorescence in the FL-2 channel. Shape change was calculated as the increase in the forward scatter property of the cell compared with unstimulated control.

3.8 CD11b activation

In order to measure activation of CD11b integrin, PMNL preparations were incubated with HDL samples and stimulated with IL-8 (3 nmol/L), fMLP (5 nmol/L) or C5a (30 nmol/L) for 4 min at 37°C in shaking water bath in the presence of FITC-conjugated Ab (dilution 1:25) which recognizes only the activated conformation of CD11b. After the treatment, cells were placed on ice, fixed with ice-cold fixative solution and immediately analyzed by flow cytometry (140).

3.9 Neutrophil adhesion under flow conditions

Neutrophil adhesion under flow conditions was performed using Vena8 biochips (Cellix Ltd, Dublin, Ireland). The chips were first coated with 10 µg/mL intracellular adhesion molecule-1 (ICAM-1) at 4°C overnight in a humidified box. On the next day, the chips were rinsed twice with distilled water, blocked with 0.1 % bovine serum albumin for 30 minutes and washed again with distilled water. Isolated PMNL in assay buffer with Ca^{2+} and Mg^{2+} were incubated with either vehicle, HDL or $sPLA_2$-HDL for 15 min at 37°C. Cells (3 x 10^6/mL) were then perfused over the ICAM-1 coated channels at constant shear stress of 0.5 dyne cm^{-2} using the Mirus nanopump (Cellix). Neutrophil adhesion was recorded on an Olympus IX70 fluorescence microscope and an Olympus UPlanFI-X20/0.40 lens, using a Hamamatsu ORCA-ER digital camera and the Olympus CellP software. Images were taken 5 minutes after the start of perfusion and adherent neutrophils were counted using ImageJ software (National Institutes of Health) as previously described (141).

3.10 Migration assay

Neutrophil migration in response to IL-8 was assessed using 96-well transwell plates with 8 µm pore size (Corning) as described before (142). Neutrophils were preincubated with vehicle, $sPLA_2$-HDL, HDL or $sPLA_2$ for 15 minutes at 37°C and 1.5 x 10^5 cells were added to the upper wells. Cells were let to migrate (1 h at 37°C in humidified incubator) toward IL-8, which was used as chemoattractant in the bottom wells. Neutrophils that had migrated to the lower compartment were counted by flow cytometric counting for 30 s. Spontaneous migration was determined in wells containing only assay buffer in lower compartment. Chemotactic index was expressed as the number of cells which migrated in response to IL-8 divided by the number of spontaneously migrated cells.

3.11 Ca^{2+} flux assay in neutrophils

Intracellular Ca^{2+} flux in neutrophils was assessed by flow cytometry as described previously (143). PMNL were treated with the cell membrane permeable Ca^{2+}-sensitive dye Fluo-3-AM (2 µmol/L) in the presence of 0.02 % F-127 pluronic acid for 60 min at RT. Neutrophils were washed once and stained with anti-CD16-PE antibody (10 min, room temperature). Cells were washed and resuspended in assay buffer. Changes in intracellular Ca^{2+} levels were detected by flow cytometry as an increase in fluorescence intensity in the FL-1 channel.

3.12 Neutrophil extracellular traps (NET)

NET formation was assessed as previously described (144). PMNL (5 x 10^4 cells per well) placed in 96-well black plates were incubated with HDL preparations in a final volume of 200 µL in the presence of 10 nmol/L PMA, which is a strong NET inducer. SYTOX green (5 µmol/L), a cell-impermeable nucleic acid dye was added in order to monitor the kinetics of NET formation. NET formation was measured as mean fluorescence (Ex 488 nm, Em 523 nm) every 10 min for 5 h at 37°C (FlexStation II; Molecular Devices).

3.13 Cholesterol-rich microdomain (lipid raft) assessment

Isolated human neutrophils were treated with different HDL preparations for 5 to 120 min at 37°C. Cells were washed once with PBS without Ca^{2+} and Mg^{2+} and stained with 1 µg/mL FITC-cholera toxin B for 1 h at RT. Samples were fixed and lipid rafts were analyzed by flow cytometry. In order to assess lipid rafts by fluorescence microscopy, cells were spun on glass coverslips using a Cytospin 3 centrifuge (Shandon), mounted with Vectashield mounting medium including DAPI and images were taken using OLYMPUS fluorescence microscope equipped with a Hamamatsu ORCA CCD camera.

3.14 Cholesterol efflux measurement

Isolated neutrophils were loaded for 3 h with [^{3}H]-cholesterol (0.5 µCi/mL) at 37°C in a total volume of 400 µL in humidified incubator in the presence of 2 µg/mL acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz 58-035. After labelling with cholesterol, cells were washed three times by centrifugation with PBS supplemented with 1 mg/mL BSA and treated with HDL for 1 h at 37°C in a total volume of 400 µL. After the efflux period, cells were pelleted, supernatants collected and cell pellets were lysed with 0.1% SDS in 0.3N NaOH in order to asses cell associated [^{3}H]-cholesterol. The aliquots of the supernatants or the complete cell lysates were transferred to the scintillation vial and scintillation cocktail was added. The radioactivity (cpm) in both cells and supernatants was assessed and cholesterol efflux (%) was calculated as the radioactivity in the supernatant relative to total radioactivity in cells and supernatant.

3.15 Filipin staining of unesterified cholesterol

Isolated human neutrophils were treated with HDL samples for 30 min at 37ºC. Subsequently, cells were washed with PBS, placed on the ice and fixed for 20 min, washed again with PBS and free cholesterol in the cell membrane was stained with filipin (100 µg/mL) for 1 h at room temperature. Samples were washed once more and analyzed by flow cytometry (LSR II, BD Biosciences).

3.16 Apoptosis assay

After the treatment with HDL preparations, neutrophils were washed once with PBS by centrifugation and the cell pellet was resuspended in 50 µL binding buffer containing 2.5 µL fluorescein isothiocyanate (FITC)-conjugated annexin V and 2.5 µL propidium iodide (PI). Samples were incubated 15 min at RT in the dark, 200 µL binding buffer was added to the samples and apoptosis was immediately assessed by flow cytometry, as reported (145). Cells treated with 3.7 % formaldehyde were used as a positive control for apoptosis. Annexin V-positive/PI-negative cells were regarded as early apoptotic cells, Annexin V-positive/PI-positive cells as late apoptotic and Annexin V-negative/PI-positive cells as necrotic cells.

3.17 IL-8 production by neutrophils

PMNL were resuspended (2 x 10^6/mL) in RPMI medium containing 5 % FBS. Cell aliquots were placed in polypropylene tubes and treated with 100 ng/mL LPS for 4 hours at 37°C. Subsequently, cells were centrifuged (800 x g, 10 min, 20°C) and supernatants were collected for IL-8 analysis. Supernatants were stored at –80°C and thawed only once for IL-8 detection. IL-8 concentrations in the supernatants were assessed using commercial enzyme-linked immunosorbent assay (ELISA).

3.18 cAMP assay

Intracellular cAMP was determined using a competitive enzyme-linked immunosorbent assay, as described before (146).

3.19 Cholesterol efflux in J774.2 macrophages

Cholesterol efflux in J774.2 macrophages was assessed as described before (147). Briefly, J774.2 cells cultured in DMEM medium supplemented with 10% FBS were seeded on 48-well plates (3 x 10^5 cells per well). After 24 hours, cells were then loaded with [^{3}H]-cholesterol (0.5 µCi/mL) in medium containing 5 % FBS for 24 hours in the presence of 2 µg/mL ACAT inhibitor and in the presence or absence of 0.3 mmol/L Cpt-

cAMP in order to induce ABCA1 expression, as previously described (146). Labeled cells were washed, equilibrated in serum free medium in the presence of 0.2 % BSA for 2 h and washed again. To assess [^{3}H]-cholesterol efflux cells were incubated with 50 µg/mL HDL-protein for 3 hours at 37°C. Supernatants were collected and cells were lysed in order to determine cell-associated radioactivity. Supernatant aliquots and complete cell lysates were placed into the scintillation tubes and scintillation cocktail was added. Radioactivity was measured by liquid scintillation counting. Cholesterol efflux is expressed as radioactivity in the medium relative to total radioactivity in medium and cells.

3.20 Statistical analysis

All data are shown as mean ± SD for n separate experiments. Experiments were repeated three to six times using neutrophils isolated from different donors. Statistical analyses were performed with GraphPad Prism Version 4.03. 2-tailed student's t-test was used for experiments in which two groups were compared and one-way ANOVA with Tukey multiple-comparison post hoc test was used for experiments comparing three or more groups. Significances were accepted at *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

4. Results

4.1 sPLA$_2$ modification of HDL generates particles with potent shape change inhibitory activity

First step in locomotion of activated neutrophils toward the chemoattractant source is the shape change (neutrophil polarization). Chemotactic factors stimulate neutrophils to rapidly rearrange their cytoskeleton and change their shape (148). These chemoattractant-induced changes in neutrophil morphology can be assessed by flow cytometry as an increase in forward scatter property of the cell ***(Figure II-5)***.

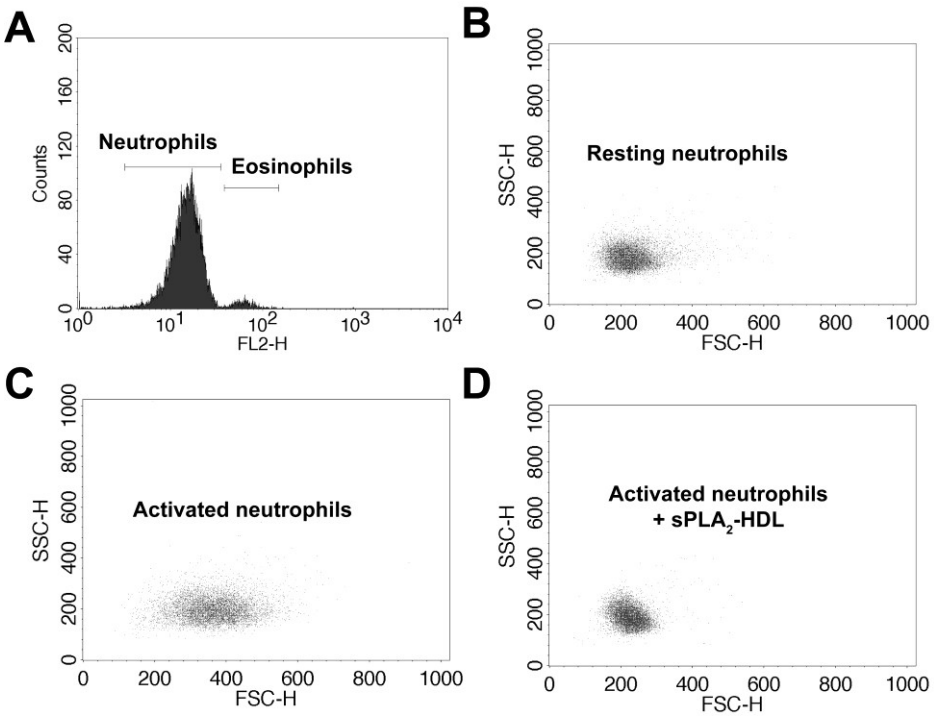


Figure II-5. sPLA$_2$-treated HDL inhibits neutrophil shape change. *Stimulation of neutrophils with chemoattractants results in rapid reorganization of the cytoskeleton and shape change, which can be detected by flow cytometry.* ***(A)*** *Eosinophils were distinguished from neutrophils by their autofluorescence in the FL-2 channel. Neutrophil shape change was determined as the increase of the forward scatter property of the* ***(C)*** *activated cells in comparison with* ***(B)*** *vehicle stimulation (resting cells).* ***(D)***

Pretreatment of neutrophils with $sPLA_2$-HDL inhibited neutrophil shape change. The figure depicts plots from one typical experiment in which neutrophil shape change was induced with IL-8.

In order to investigate the effects of $sPLA_2$ on chemoattractant-iduced neutrophil shape change responses, human isolated neutrophils were preincubated with 20% of $sPLA_2$ type III-treated serum and subsequently stimulated with IL-8. To our surprise, in comparison to control serum, $sPLA_2$-treated serum almost completely inhibited IL-8-induced shape change ***(Figure II-6A)***. The HDL surface is surrounded by phospholipids, among them the most abundant is phosphatidylcholine (PC), which is a very good target for several, if not for all $sPLA_2$ isoforms (7). Considering that HDL represents the major carrier of PC in plasma and the major supstrate for $sPLA_2$ (54, 126), we supposed that HDL might be responsible for the observed effects of $sPLA_2$-treated plasma. Thereby, we isolated HDL-containing fraction from $sPLA_2$-treated plasma by ultracentrifugation and used it to treat neutrophils. As we expected, HDL isolated from $sPLA_2$-treated plasma potently reversed neutrophil shape change ***(Figure II-6B)***, while HDL from control plasma showed no effect.

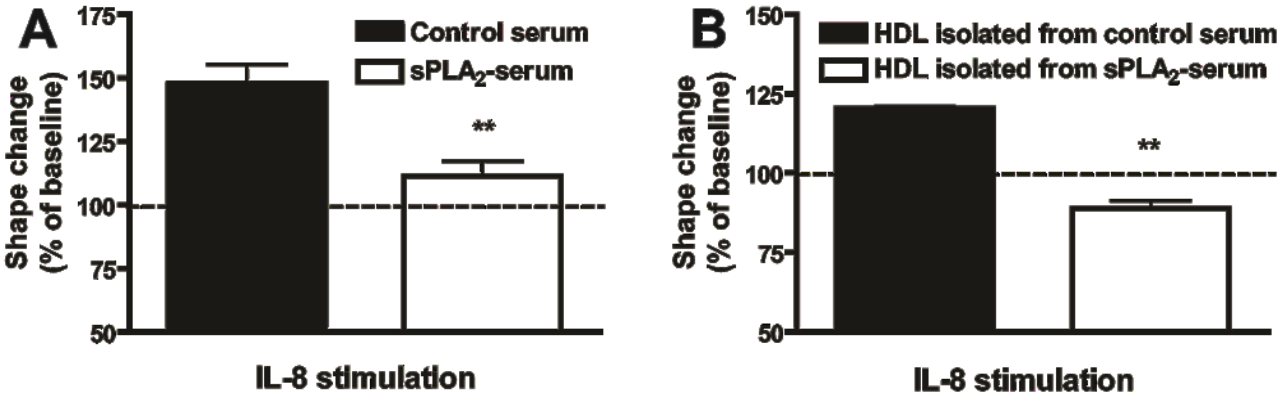


Figure II-6. $sPLA_2$-treated serum inhibits neutrophil activation. *Freshly isolated human serum was incubated in the absence or presence of $sPLA_2$ type III for 24 hours at 37°C.* ***(A)*** *Treated and untreated serum (20 % v/v) was subsequently added to neutrophils followed by stimulation with IL-8 (3 nmol/L) for 4 min.* ***(B)*** *HDL was isolated from $sPLA_2$-treated serum and control serum and subsequently added to human*

*neutrophils (125 µg HDL-protein/mL, 15 min at 37°C) followed by stimulation with IL-8 (3 nmol/L) for 4 min. Neutrophil shape change was assessed by flow cytometry. Values are expressed as % of baseline. The results are shown as mean ± SD of three separate experiments using PMNL from different healthy donors. Statistical analysis was performed using Student's t-test. *p < 0.05, **p < 0.01 versus control.*

In the following experiments we assessed whether direct modification of HDL with $sPLA_2$ produces the particles with inhibitory effects on neutrophil activation. The lysophosphatidylcholine (LPC) content of $sPLA_2$-HDL and control HDL is shown in ***Table 1***.

Table 1. Analysis of LPC species present in $sPLA_2$-HDL

LPC	nmol/mg protein (±SD)	
	HDL	**$sPLA_2$-HDL**
16:0	10.82 (9.07)	149.23 (23.19)
16:1	0.20 (0.21)	2.67 (0.21)
18:0	0.00 (0.00)	0.17 (0.02)
18:1	3.48 (2.81)	23.79 (6.34)
18:2	6.99 (5.64)	6.10 (1.06)
Total	21.49 (17.72)	181.96 (30.07)

LPC contents of native and $sPLA_2$-treated HDL were analyzed by mass spectrometry, as described in Materials and Methods. Results are shown as mean ± SD of HDL obtained from three different donors.

We found that $sPLA_2$-HDL potently inhibited neutrophil shape change induced by wide range of agonists including IL-8, LPS, fMLP, C5a and *Escherichia coli* ***(Figure II-7A to II-7E)*** However, neither native freshly isolated HDL nor $sPLA_2$ alone were able to abolish neutrophil activation. The inhibitory effect of $sPLA_2$-HDL on neutrophil activation was concentration-dependent ***(Figure II-7F)***. Notably, $sPLA_2$-HDL reversed the shape change of already activated neutrophils ***(Figure II-8)***.

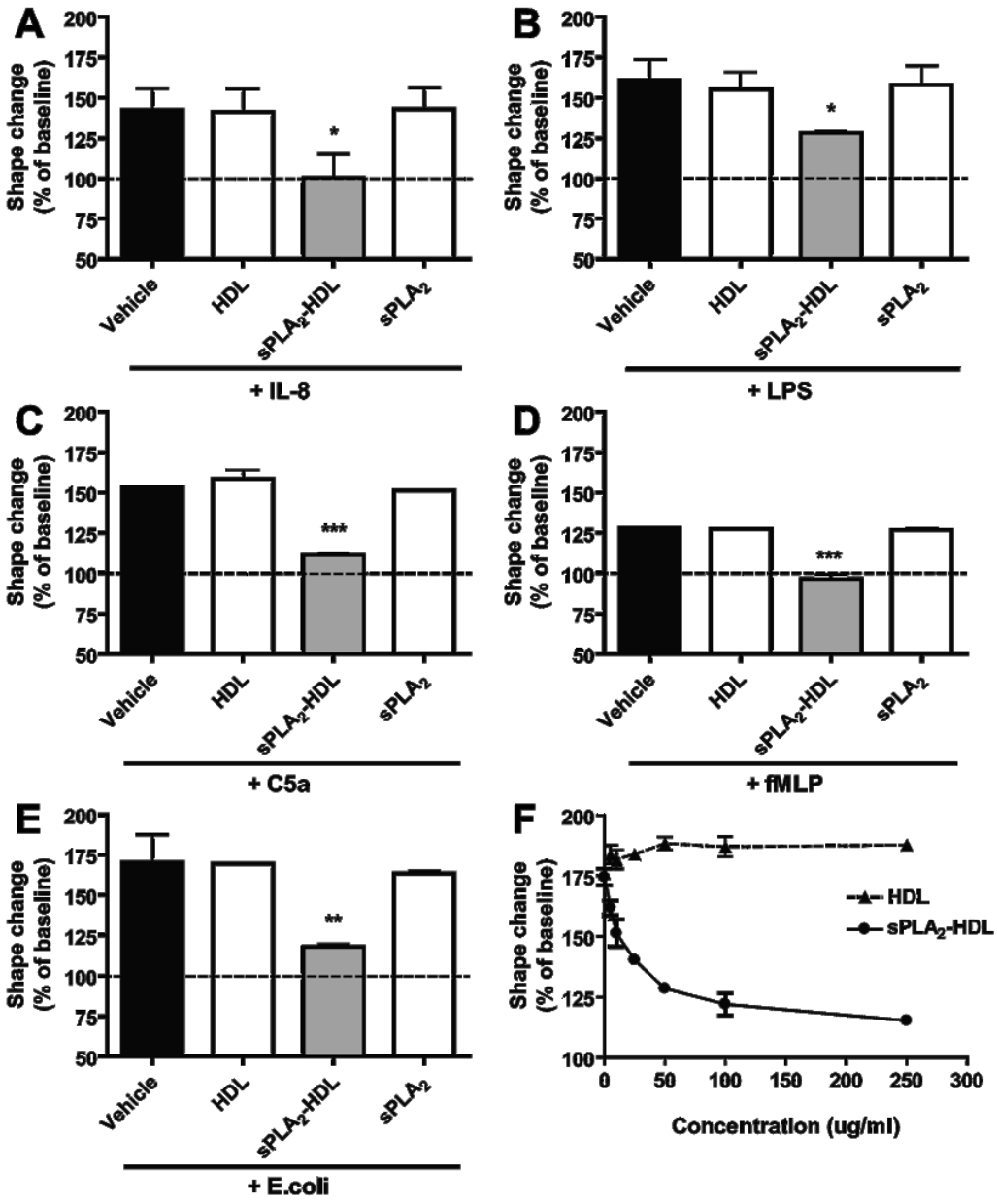


Figure II-7. $sPLA_2$-treated HDL inhibits IL-8, LPS, C5a, fMLP and E. coli-induced neutrophil activation. *Isolated neutrophils were incubated with vehicle, native HDL (100 µg protein/mL), $sPLA_2$-treated HDL (100 µg protein/mL) or $sPLA_2$ alone for 15 min at 37°C, and stimulated with* ***(A)*** *IL-8 (3 nmol/L, 4 min, 37°C)* ***(B)*** *1 ng/mL LPS in the presence of 2 % serum (90 min, 37°C)* ***(C)*** *C5a (30 nmol/L, 4 min, 37°C)* ***(D)*** *fMLP (5 nmol/L, 4 min, 37°C) or* ***(E)*** *E. coli bacteria (1 h, 37°C, the ratio of neutrophils and bacteria was 1:5). Neutrophil shape change was measured by flow cytometry. Values*

*are expressed as % of baseline. Results shown are mean ± SD. The data represent the results from three to five experiments done using PMNL from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.* ***(F)*** *Concentration-response curve with $sPLA_2$-treated HDL. Neutrophils were incubated with increasing concentrations of native HDL or $sPLA_2$-treated HDL (0 up to 250 µg/mL) for 15 min at 37°C and stimulated with 1 ng/mL LPS (90 min, 37°C). Neutrophil shape change was measured by flow cytometry and results are shown as mean ± SD (n=3).*

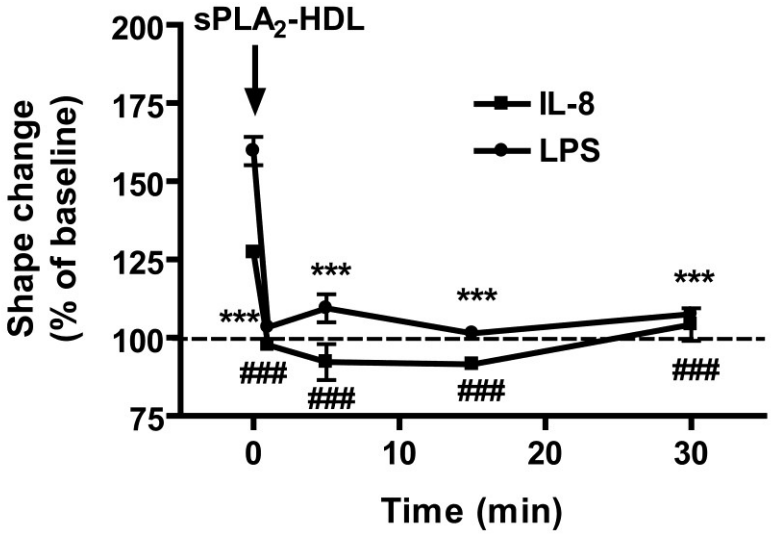


Figure II-8. $sPLA_2$-treated HDL reverses IL-8 and LPS-induced activation of neutrophils. *Neutrophils were stimulated with either IL-8 (3 nmol/L, 4 min, 37°C) or 1 ng/mL LPS in 2 % serum for 90 min and subsequently incubated with $sPLA_2$-HDL (100 µg protein/mL) for 1 to 30 min. Neutrophil shape change was measured by flow cytometry. Values are expressed as % of baseline. Results represent means ± SD (n=3). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treated and IL-8-stimulated cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus vehicle-treated and LPS-stimulated cells.*

Of great importance is the fact that $sPLA_2$-HDL effects on neutrophil shape change were observed in neutrophils isolated from all donors. Moreover, different HDLs isolated from several donors showed very similar inhibitory effects after modification with $sPLA_2$. Importantly, neutrophil viability was not altered upon treatment with $sPLA_2$-HDL ***(Figure II-9)***.

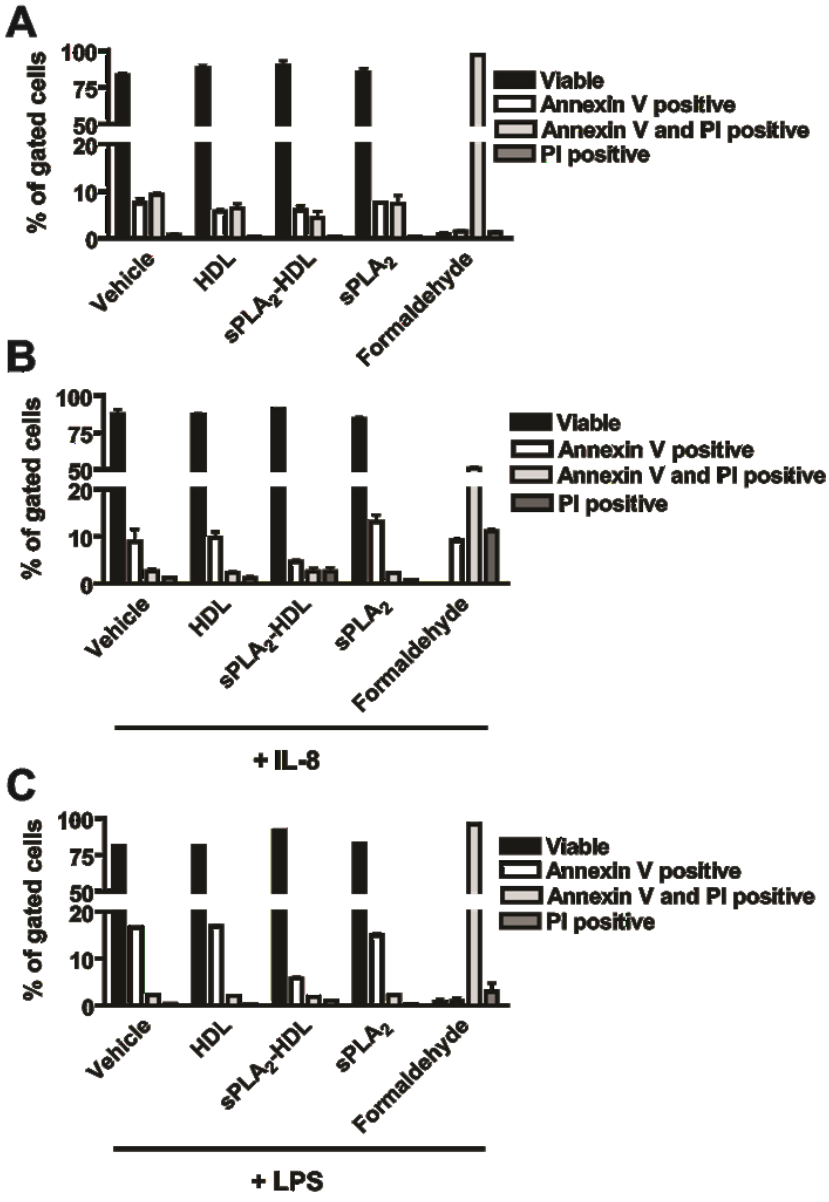


Figure II-9. Cell viability assay*. **(A)** Neutrophils were incubated with vehicle (assay buffer), native HDL (100 µg protein/mL), $sPLA_2$-treated HDL (100 µg protein/mL) or $sPLA_2$ alone for 3 hours at 37°C or cells were preincubated with different HDL*

*preparations for 15 min at 37°C and stimulated with **(B)** IL-8 (3 nmol/L, 4 min, 37°C) or **(C)** 1 ng/mL LPS in the presence of 2 % serum (90 min, 37°C). Cell viability was assessed by Annexin V/Propidium iodide apoptosis assay by flow cytometry and formaldehyde-treated cells were used as a positive control. Viable neutrophils are detected as both Annexin V- and PI-negative cells. Early apoptotic cells are Annexin V-positive and PI-negative, late apoptotic are both Annexin V- and PI-positive, whereas necrotic cells are detected as Annexin V-negative and PI-positive. Results represent means ± SD of 3 separate experiments using PMNL from different healthy donors.*

Interestigly, also $sPLA_2$ modified low density lipoprotein inhibited agonist induced neutrophil shape change ***(Figure II-10)***.

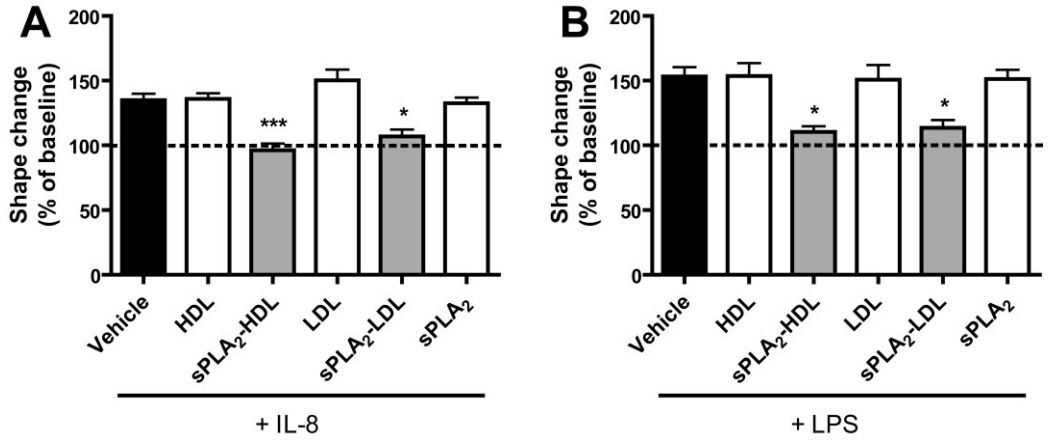


Figure II-10. $sPLA_2$-treated LDL inhibits neutrophil activation. *Isolated neutrophils were incubated with vehicle, native LDL (100 µg protein/mL), $sPLA_2$-treated LDL (100 µg protein/mL), HDL (100 µg protein/mL), $sPLA_2$-HDL (100 µg protein/mL) or $sPLA_2$ alone for 15 min at 37°C and stimulated with **(A)** IL-8 (3 nmol/L, 4 min, 37°C) or **(B)** 1 ng/mL LPS in the presence of 2 % serum (90 min, 37°C). Neutrophil shape change was measured by flow cytometry. Values are expressed as % of baseline. Results shown are mean ± SD. The data represent the results from five experiments done using PMNL from different healthy donors. Statistical significance was assessed by one-way ANOVA*

*with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.*

4.2 $sPLA_2$-generated lysophosphatidylcholines are the active moiety in $sPLA_2$-HDL

In order to confirm that inhibition of neutrophil shape change was the result of enzymatic activity of $sPLA_2$ on HDL, we added varespladib (an inhibitor of $sPLA_2$ types IIA, V and X, but not $sPLA_2$ type III) during incubation of $sPLA_2$ with HDL. In line with results above, varespladib abolished the ability of $sPLA_2$ type V-treated HDL to suppress agonist-induced neutrophil shape change ***(Figure II-11)***.

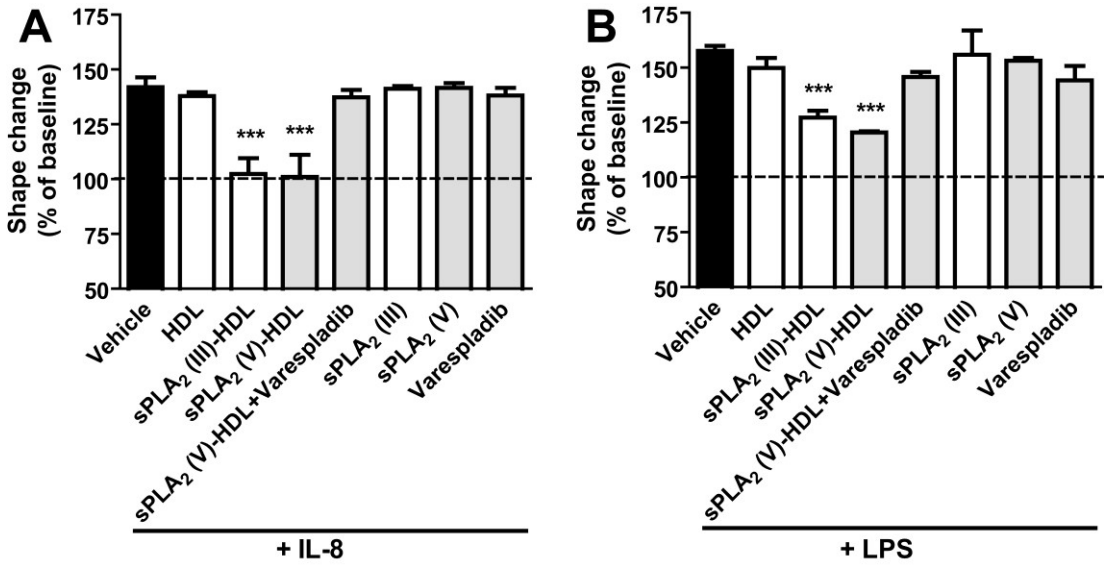


Figure II-11. The $sPLA_2$ inhibitor varespladib inhibits the effects of $sPLA_2$-treated HDL on neutrophils. *HDL was incubated with 200 ng/mL $sPLA_2$ type III or 400 ng/mL $sPLA_2$ type V in the presence or absence of varespladib (1 µmol/L), overnight, at 37°C. These samples were used for neutrophil treatment. Isolated neutrophils were preincubated (15 min at 37°C) with vehicle (assay buffer), HDL (100 µg protein/mL), $sPLA_2$ (III)-HDL (100 µg protein/mL), $sPLA_2$ (V)-HDL, $sPLA_2$ (V)-HDL + varespladib, varespadib, $sPLA_2$ (III) or $sPLA_2$ (V) alone. Neutrophils were then stimulated with **(A)** IL-*

*8 (3 nmol/L, 4 min, 37°C) or **(B)** 1 ng/mL LPS in the presence of 2 % serum (90 min, 37°C) and neutrophil shape change was assessed by flow cytometry. Values are expressed as % of baseline. Results represent means ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.*

Next, we investigated if the potent effects of $sPLA_2$-HDL on neutrophils rise from the structural alteration of HDL particle or rather depend on phospholipid hydrolysis products that are formed and enriched in $sPLA_2$-HDL. In order to assess this, we removed lysophospholipids and free fatty acids from $sPLA_2$-HDL by incubation with fatty-acid free albumin and subsequent re-isolation using density gradient ultracentrifugation. Albumin addition to $sPLA_2$-HDL almost completely removed LPCs and FFAs as shown in the ***Figure II-12***.

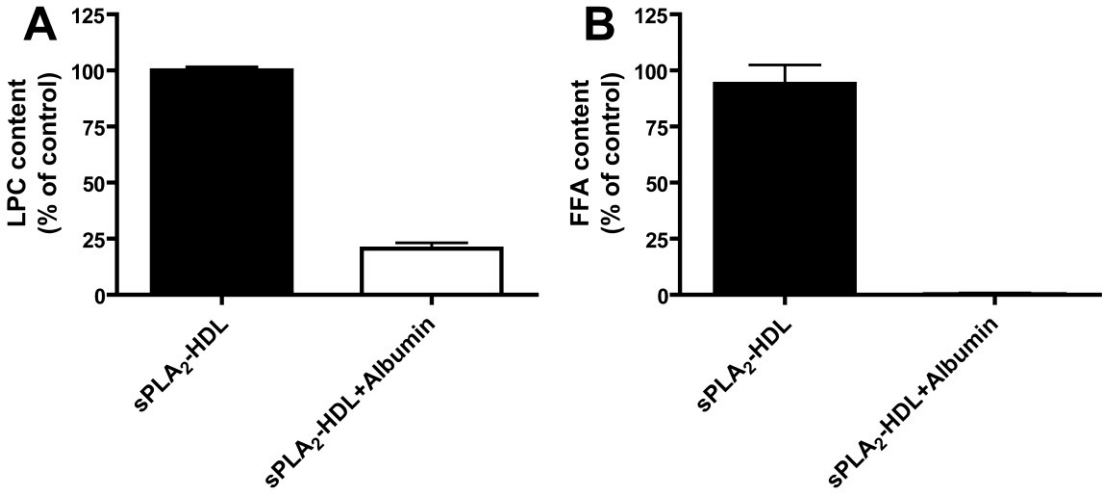


***Figure II-12. LPC and FFA content in albumin-treated $sPLA_2$-HDL**. $sPLA_2$-treated HDL (1 mg protein/mL) was incubated in the presence or absence of 50 mg/mL albumin (1 h, 37°C) and $sPLA_2$-HDL was re-isolated by density gradient ultracentrifugation. LPC **(A)** and FFA **(B)** contents were assessed using commercially available kits.*

When LPCs and FFAs were depleted from $sPLA_2$-HDL, its activity on neutrophils was abolished, showing that the presence of phospholipid hydrolysis products is responsible

for the observed effects and not the reduced size or other structural alterations of HDL particle ***(Figure II-13A and II-13B)***. Next, we wanted to assess whether specific LPC and FFA species in $sPLA_2$-HDL mediate the inhibitory effects on neutrophil activation. We enriched native HDL with different LPCs and FFAs and subsequently assessed neutrophil shape change. Interestingly, we found that LPC 16:0 which is the most abundant LPC species present in $sPLA_2$-HDL as shown in ***Table 1***, was also the most effective when enriched in HDL. However, other LPC species or FFAs showed week or had no suppressing effects on neutrophil activation. Apart from LPC 16:0, we observed that HDL enriched with Lyso-PS 16:0 also showed potent inhibitory effects ***(Figure II-13C and II-13D)***.

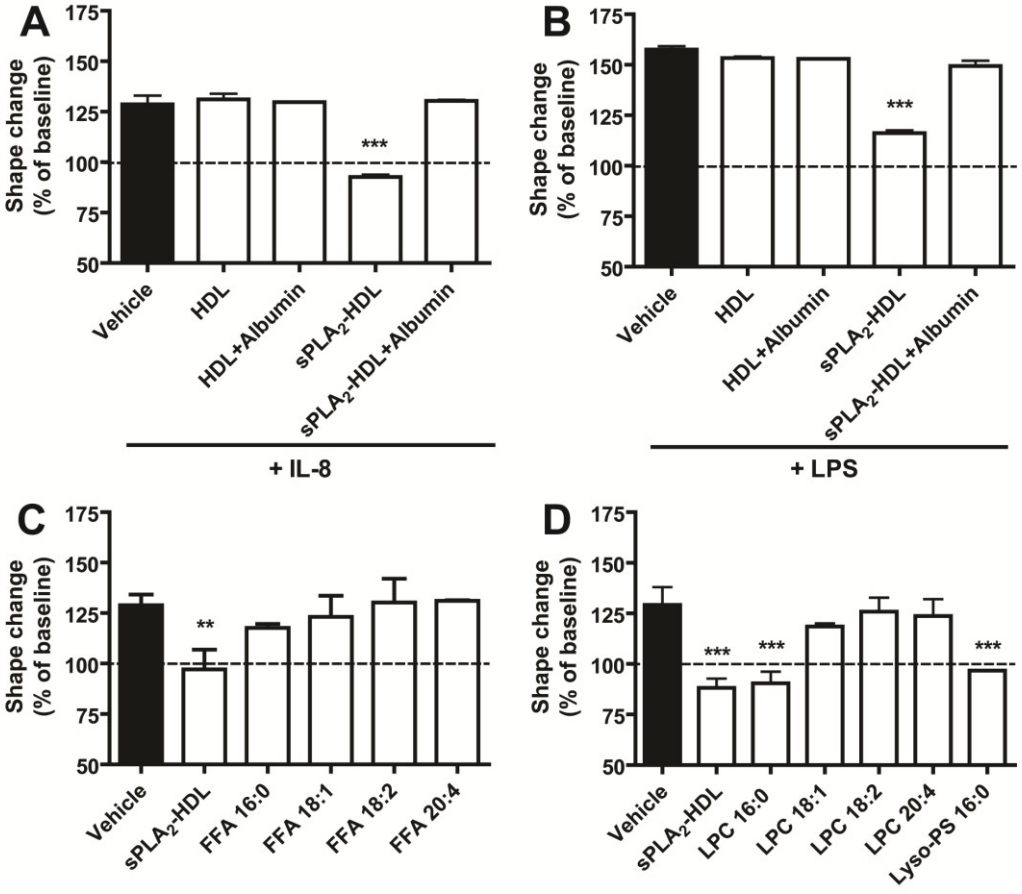


Figure II-13. Presence of LPC on $sPLA_2$-treated HDL is necessary for the inhibition of neutrophil shape change. *Native or $sPLA_2$-treated HDL were incubated in the presence or absence of 50 mg/mL albumin (1 h, 37°C) and HDL was re-isolated by density gradient ultracentrifugation to remove FFAs and lysophospholipids as described*

*in Methods. 100 µg/mL HDL protein was used in the shape change assay with **(A)** IL-8 (3 nmol/L, 4 min) or **(B)** 1 ng/mL LPS (90 min, 37°C). **Enrichment of HDL with FFAs and LPCs.** Neutrophils were preincubated (15 min, 37°C) with sPLA$_2$-HDL (100 µg/mL protein) and **(C)** HDL enriched with FFA (16:0, 18:1, 18:2, 20:4) or **(D)** HDL enriched with LPC (16:0, 18:1, 18:2, 20:4) and Lyso-PS 16:0. Neutrophils were then stimulated with IL-8 (3 nmol/L, 4 min, 37°C). Neutrophil shape change was measured by flow cytometry. Values are expressed as % of baseline. Results shown are mean ± SD of three separate experiments using cells from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.*

4.3 sPLA$_2$-HDL suppresses CD11b activation, adhesion, migration and extracellular trap (NET) formation of neutrophils

In order to further assess the effects of sPLA$_2$-HDL on neutrophil function, we measured activation of CD11b integrin as well as neutrophil adhesion, chemotaxis and migration. Neutrophil stimulation leads to conformational change and subsequent activation of CD11b, which is an integrin essential for neutrophil firm adhesion to the endothelium and subsequent transmigration through endothelial layer. Importantly, sPLA$_2$-HDL potently suppressed CD11b activation induced by several agonists including IL-8, fMLP and C5a. Inhibition of CD11b activation was also inhibited by sPLA$_2$ inhibitor varespladib ***(Figure II-14)***.

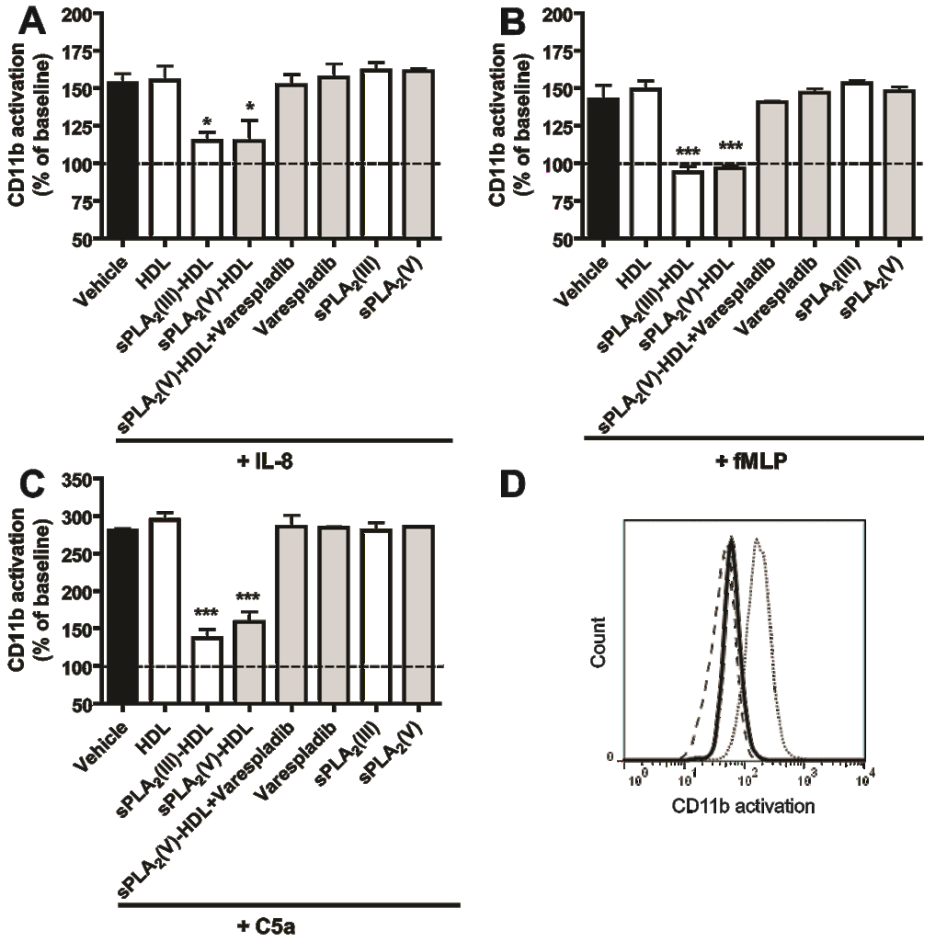


Figure II-14. $sPLA_2$-treated HDL inhibits CD11b activation in neutrophils. *HDL was incubated with 200 ng/mL $sPLA_2$ type III or 400 ng/mL $sPLA_2$ type V in the presence or absence of varespladib (1 µmol/L), overnight, at 37°C. These samples were used for neutrophil treatment. Isolated neutrophils were preincubated (15 min at 37°C) with vehicle (assay buffer), HDL (100 µg protein/mL), $sPLA_2$ (III)-HDL (100 µg protein/mL), $sPLA_2$ (V)-HDL (FFA release was measured and HDL concentration was adjusted according to FFA content), $sPLA_2$ (V)-HDL + varespladib, varespadib, $sPLA_2$ (III) or $sPLA_2$ (V) alone in the presence of antibody to the active epitope of CD11b and were then stimulated with* ***(A)*** *IL-8 (3 nmol/L)* ***(B)*** *fMLP (10 nmol/L) or* ***(C)*** *C5a (30 nmol/L) for 4 min at 37°C. CD11b activation was measured by flow cytometry. Vehicle-treated (unstimulated) control was set as baseline and values are expressed as % of baseline. Results represent means ± SD (n=3). Statistical significance was assessed by one-way*

*ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.* ***(D)*** *Histogram of CD11b activation of neutrophils stimulated with fMLP (dotted), sPLA$_2$-HDL+ fMLP (solid) and unstimulated cells (dashed).*

Next, we assessed if sPLA$_2$-HDL-mediated inhibition of CD11b activation translates to the alterations in neutrophil adhesion and migration. Notably, in line with inhibition of integrin activation, sPLA$_2$-HDL significantly suppressed neutrophil adhesion to ICAM-1 coated surface under flow conditions ***(Figure II-15A and II-15B)***. In addition, sPLA$_2$-HDL-treated neutrophils showed significantly lower migratory response toward IL-8 ***(Figure II-15C)***.

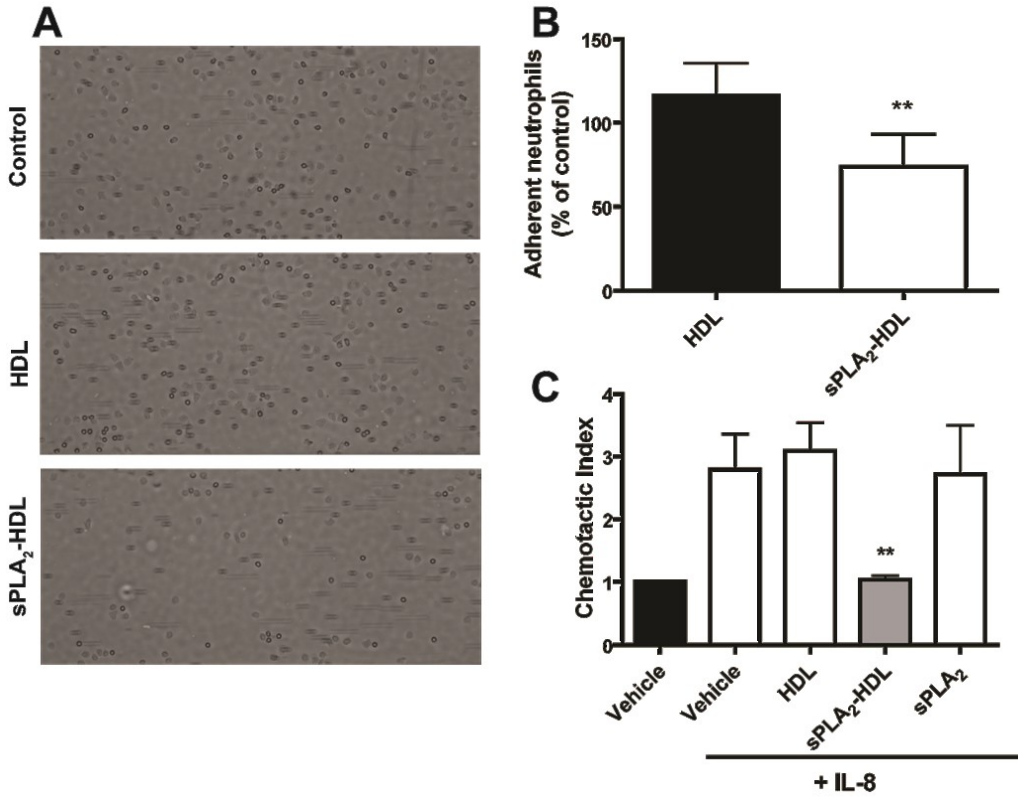


Figure II-15. sPLA$_2$-HDL inhibits neutrophil adhesion under flow conditions and neutrophil migration. *Isolated human neutrophils were treated with vehicle (assay buffer), HDL (100 µg protein/mL) or sPLA$_2$-HDL (100 µg protein/mL) for 15 minutes at 37°C before perfusion over ICAM-1 coated microchannels.* ***(A)*** *Representative images*

were taken 5 min after the start of the perfusion with neutrophils. ***(B)*** *Images were quantified by computerized image analysis. Values are expressed as % of vehicle treated control. Data are shown as mean ± SD of 6 experiments with neutrophils from different donors. Statistical analysis was performed using Student's t-test. *p < 0.05, **p < 0.01 versus HDL.* ***(C)*** *Neutrophils were preincubated with vehicle, HDL (100 µg protein/mL), $sPLA_2$-HDL (100 µg protein/mL) or $sPLA_2$ for 15 minutes at 37°C and seeded into the upper wells. Cells were allowed to migrate towards IL-8 (10 nmol/L) for 1 hour at 37°C. Cells that had migrated to the lower chamber were enumerated by flow cytometry. The results are shown as mean ± SD of 3 separate experiments using PMNL from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01 versus vehicle.*

Several lines of evidence showed that during infiltration of neutrophils into inflammatory sites and atherosclerotic lesions they extrude neutrophil extracellular traps (149, 150), which are pro-inflammatory antimicrobial structures composed of extracelluar DNA backbone with bound granular and cytoplasmic proteins including MPO and neutrophil elastase. Furthermore, given that excessive NET formation has been shown to play role in pathophysiology of atherosclerosis, we investigated neutrophil ability to produce NET in response to protein kinase C activator phorbol 12-myristate 13-acetate (PMA). Interestingly, pretreatment of neutrophils with $sPLA_2$-HDL significantly abolished PMA-induced formation of NETs **(Figure II-16)**.

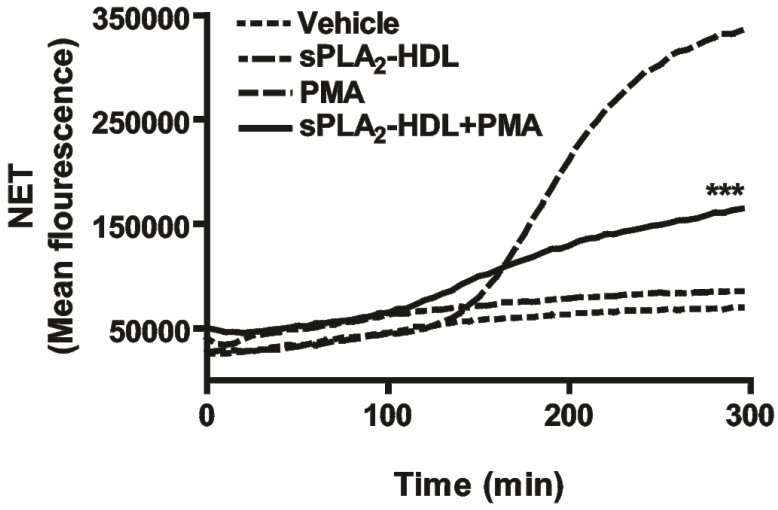


Figure II-16. *Isolated neutrophils were treated with vehicle or $sPLA_2$-HDL (100 µg protein/mL) in the presence or absence of 10 nmol/L PMA and NET formation was measured as an increase in fluorescence of the SYTOX Green dye every 10 min for 5 hours at 37°C. Results show a representative experiment of three independent experiments. Values are expressed as mean fluorescence. *p < 0.05, **p < 0.01, ***p < 0.001 versus PMA at 5 h time point.*

Interestingly, IL-8 production by LPS-stimulated neutrophils was not inhibited by $sPLA_2$-HDL **(Figure II-17)**.

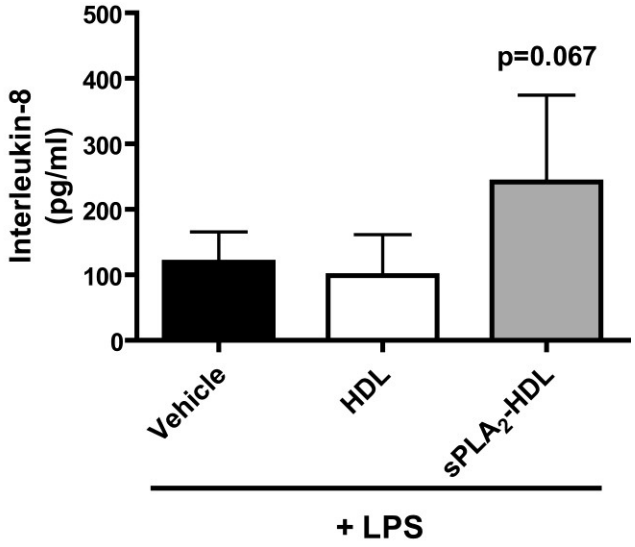


Figure II-17. $sPLA_2$-HDL does not inhibit IL-8 production of LPS stimulated neutrophils. *Isolated human neutrophils were incubated with vehicle, HDL (100 µg protein/mL) or $sPLA_2$-HDL (100 µg protein/mL) in the presence of 100 ng/mL LPS for 4 h at 37°C. IL-8 in the supernatants was measured by ELISA. Values are expressed as pg/mL supernatant. Results shown are mean ± SD. The data represent the results from five experiments done using PMNL from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.*

4.4 $sPLA_2$-HDL suppresses agonist induced Ca^{2+} flux and reduces lipid raft assembly

Since increase in intracellular cAMP levels is known to result in suppression of neutrophil activation and there are studies showing that lysophospholipids can promote increase in intracellular cAMP in neutrophils (151), we investigated if this mechanism was responsible for the observed effects of $sPLA_2$-HDL. However, $sPLA_2$-HDL had no impact on cAMP levels in neutrophils ***(Figure II-18)***.

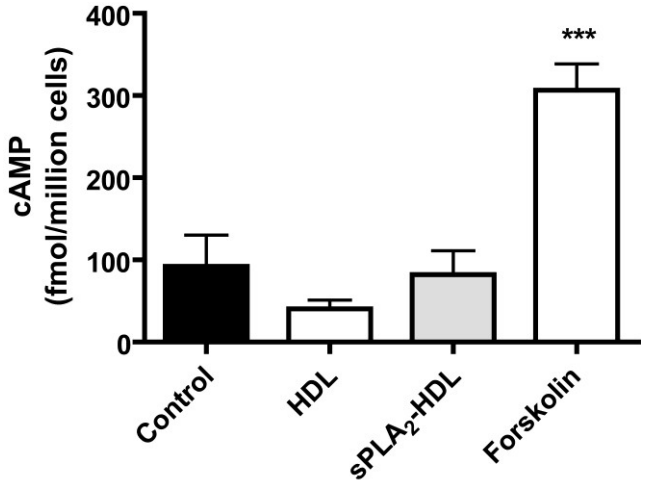


Figure II-18. cAMP is not involved in the effects of sPLA$_2$-HDL on neutrophils. *Human neutrophils were incubated with vehicle, HDL (100 µg protein/mL), sPLA$_2$-HDL (100 µg protein/mL) or forskolin (100 µmol/L) for 15 min, at 37°C in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.5 mmol/L). The cells were lysed, and cAMP was assayed by competitive immunoassay. Vehicle treated control was set at 100 % and values are expressed as the amount of cAMP per one million cells. Results represent means ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.*

Changes in intracellular Ca^{2+} levels in neutrophils play a key role in a number of pathways which result in neutrophil activation. Upon IL-8 stimulation, intracellular Ca^{2+} levels in neutrophils highly increase, which was potently inhibited by sPLA$_2$-HDL in comparison with native HDL. Interestingly, sPLA$_2$-HDL itself moderately induced Ca^{2+} flux in unstimulated resting neutrophils ***(Figure II-19A)***. As main HDL function to promote cholesterol efflux from the peripheral cells has been shown to be related to its anti-inflammatory activities, we assessed if sPLA$_2$-HDL had altered cholesterol efflux capability. Interestingly, sPLA$_2$-HDL showed increased ability to promote cholesterol efflux from neutrophils loaded with radioactively labeled cholesterol ***(Figure II-19B)***, reflected by significantly decreased content of unesterified cholesterol in neutrophil

membrane as measured by filipin staining ***(Figure II-19C)***. Considering that cholesterol-rich lipid rafts are of key importance in leukocyte activation, we assessed whether increased ability of $sPLA_2$-HDL to deplete cholesterol in neutrophils leads to alterations of lipid raft abundance. In order to visualize lipid rafts in neutrophils and assess its abundance, we used FITC-labeled cholera toxin B which binds to the lipid raft marker ganglioside GM1. Indeed, $sPLA_2$-HDL rapidly disrupted lipid rafts in neutrophils, as shown in figure ***Figure II-19D***.

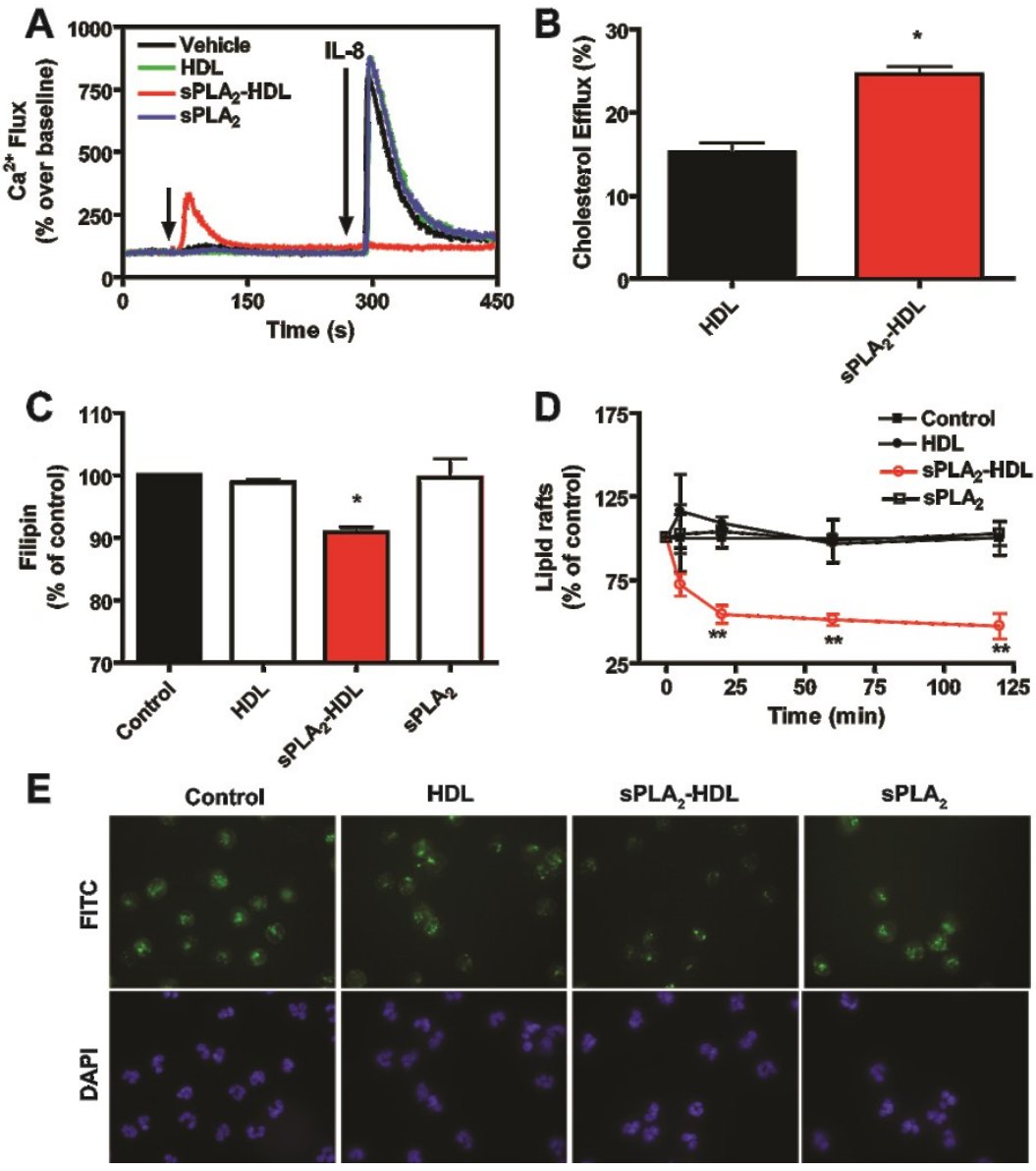


Figure II-19. sPLA$_2$-HDL abolishes agonist-induced Ca^{2+} flux and efficiently removes unesterified cholesterol from neutrophils. (A) *Baseline Ca^{2+} levels were measured for 1 min and then neutrophils were treated with vehicle, HDL (100 µg protein/mL), sPLA$_2$-HDL (100 µg protein/mL) or sPLA$_2$ for 4 min and Ca^{2+} flux was induced with IL-8 (10 nmol/L). Ca^{2+} flux was detected at room temperature by flow-cytometry as an increase in fluorescence intensity of the Ca^{2+}-sensitive dye Fluo-3-AM in the FL-1 channel. Results show a representative of 4 independent experiments done with cells from different donors. Values are expressed as % over baseline.* ***(B)*** *Isolated neutrophils were preloaded with [^{3}H]-cholesterol for 3 hours and incubated with HDL (100 µg protein/mL) or sPLA$_2$-HDL (100 µg protein/mL protein) for 60 minutes, and percentage of cholesterol efflux was assessed. Cholesterol efflux is expressed as the radioactivity in the medium relative to total radioactivity in medium and cells. The results are shown as mean ± SD of three separate experiments using PMNL from different healthy donors. Statistical analysis was performed using Student's t-test. *p<0.05, **p<0.01, ***p <0.001 versus HDL.* ***(C)*** *Neutrophils were incubated with vehicle, HDL (100 µg protein/mL), sPLA$_2$-HDL (100 µg protein/mL), sPLA$_2$ alone for 30 min at 37°C and stained with Filipin. The amount of unesterified cholesterol was assessed by flow-cytometry. Vehicle treated control was set at 100 % and values are expressed as % of control. Results are shown as mean ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus control.* ***(D)*** *Neutrophils were incubated with vehicle, HDL (100 µg protein/mL), sPLA$_2$-HDL (100 µg protein/mL) or sPLA$_2$ alone for 5 up to 120 min at 37°C. Lipid raft abundance was determined by assessing FITC-cholera toxin B stained cholesterol-containing microdomains by flow cytometry. Control was set at 100 % and values are expressed as % of vehicle-treated control. Results are shown as mean ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01 versus control.* ***(E)*** *Fluorescence microscopy analysis of lipid rafts (green staining). DAPI (blue staining) was used to visualize the nuclei. Original magnification X60 was used. Representative images from 3 independent experiments with neutrophils from different donors are shown.*

ABCA1 and SR-BI, HDL-cholesterol transporters, have been shown to localize in lipid rafts (152, 153). However, inhibition of these receptors with probucol or BLT-1, which are ABCA-1 and SR-BI inhibitors respectively (154, 155), had no impact on cholesterol efflux mediated by both $sPLA_2$-HDL and native HDL showing that increased cholesterol efflux capability of $sPLA_2$-HDL does not depend on these receptors ***(Figure II-20)***.

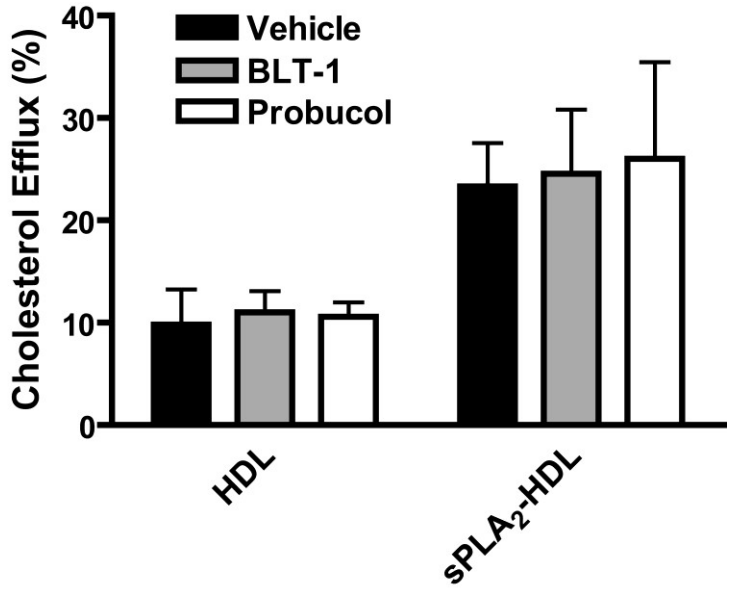


Figure II-20. Increased $sPLA_2$-HDL cholesterol efflux capability in neutrophils is independent of SR-BI and ABCA-1 receptors. *[^{3}H]-cholesterol-loaded neutrophils were incubated for 1h at 37°C in the presence or absence of the SR-BI inhibitor BLT-1 (10 µmol/L) or the ABCA-1 inhibitor probucol (20 µmol/L). To determine [^{3}H]-cholesterol efflux cells were incubated with HDL (100 µg protein/mL) or $sPLA_2$-HDL (100 µg protein/mL) for 1 hour at 37°C. Cholesterol efflux is expressed as the radioactivity in the medium relative to total radioactivity in medium and cells. The results are shown as mean ± SD of three separate experiments using PMNL from different healthy donors. Results represent means ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treated control.*

Interestingly, cholesterol efflux of $sPLA_2$-HDL from J774 macrophages did not differ in comparison to native HDL, suggesting that cholesterol efflux ability of $sPLA_2$-HDL substantially varies between different cell types ***(Figure II-21).***

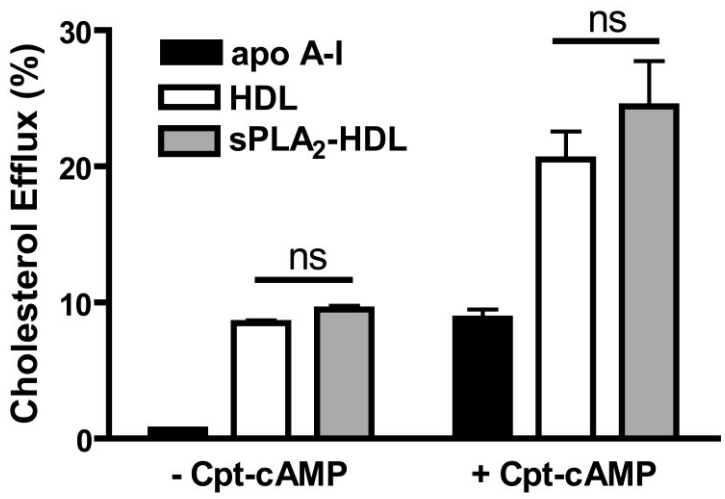


Figure II-21. $sPLA_2$-HDL cholesterol efflux from J774.2 macrophages. *Cells were labeled with [^{3}H]-cholesterol (0.5 µCi/mL) in medium containing 5 % FBS for 24 h in the presence of 2 µg/mL ACAT inhibitor and in the presence or absence of 0.3 mmol/L Cpt-cAMP to induce ABCA1 expression. [^{3}H]-cholesterol-labeled J774.2 macrophages were incubated with apoA-I (20 µg/mL), HDL (50 µg protein/mL) or $sPLA_2$-HDL (50 µg protein/mL) for 3 h at 37ºC. Cholesterol efflux is expressed as radioactivity in the medium relative to total radioactivity in medium and cells. The values shown represent the mean ± SD of three different experiments. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.*

5. Discussion

Our data show that $sPLA_2$ mediates conversion of HDL to anti-inflammatory particle capable of effectively modulating neutrophil functional responses. Interestingly, we observed that $sPLA_2$-HDL, in contrast to native freshly isolated HDL, reduced cholesterol content in neutrophils and inhibited agonist-induced elevation in intracellular Ca^{2+} levels (156). Numerous studies confirmed that alterations in membrane cholesterol levels are essential for the modulation of cellular signaling responses via acting on cholesterol-rich microdomains, also known as lipid rafts (157). Several lines of evidence strongly suggest that HDL is able to regulate the membrane cholesterol levels in various cell types, via depleting cholesterol and other lipid species from cells and thereby diminishing plasma membrane receptor signaling (158). $sPLA_2$ modification of HDL generates particle with strong ability to mobilize cholesterol from neutrophils as well as to suppress rise in intracellular Ca^{2+}. Our data suggest that these are likely mechanisms that act against agonist-induced neutrophil activation which leads to potent inhibition of neutrophil activation, including neutrophil shape change, CD11b activation and NET formation (156).

Of particular interest is our observation that $sPLA_2$-HDL effectively inhibited neutrophil functions important for cell recruitment to the inflammatory site, such as adhesion to ICAM-1 and chemotactic response toward IL-8 (156). Furthermore, $sPLA_2$-HDL strongly abolished formation of NET (156), which are, apart from being beneficial in preventing dissemination and killing various pathogens, implicated in pathogenesis of several inflammatory diseases such as autoimmune diseases and cardiovascular complications (149, 150). Given that NETs can promote excessive inflammation leading to endothelial damage, further infiltration of neutrophils into atherosclerotic lesions, thrombus formation and autoimmunity, NET elimination in non-infectious conditions may be beneficial.

Interestingly, we observed that LPC 16:0, the major $sPLA_2$-HDL-associated lysophospholipid, is mediating the $sPLA_2$-HDL effects on neutrophils, whereas other

LPC species and FFAs showed only moderate or no effects on neutrophil activation (156). Considering that $sPLA_2$ subtypes differ in their affinity for different phosphatidylcholine species (159), under inflammatory conditions this might lead to formation of HDLs particles with various activities on neutrophils.

Interestingly, neutrophil function was only minimally affected by control native HDL, even though HDL showed the ability to efflux cholesterol from neutrophils. This might be explained by the fact that the net movement of cholesterol from neutrophil membrane following HDL treatment is minimal as shown by filipin staining of free cholesterol in neutrophil membrane. This could be a consequence of bidirectional lipid flux (efflux of cholesterol is accompanied by influx of HDL cholesteryl-ester) mediated by native HDL. We suppose that increase in lysophospholipid content in HDL is responsible for its enhanced ability to remove cholesterol from neutrophils (156). Our results support previous findings that LPC can rapidly enter and incorporate into the membrane lipid rafts (160) thereby promoting cholesterol efflux (161), which is a possible explanation for the increased ability of $sPLA_2$-HDL to mobilize cell cholesterol. In line with this, $sPLA_2$-HDL potently and rapidly disrupted lipid rafts, as shown by staining of GM1 with cholera toxin. Furthermore, increased cholesterol efflux property of $sPLA_2$-HDL seems not to be mediated by ABCA1 and SR-BI, receptors known to be involved in cholesterol efflux in neutrophils (47). Interestingly, improved ability of $sPLA_2$-HDL to mobilize cholesterol seems to be cell-type dependent, given that cholesterol efflux from J774 macrophages was not altered in comparison to native HDL (156).

According to several studies, reconstituted HDL (rHDL) has potent anti-inflammatory effects. Interestingly, in recent study it was observed that following parenteral administration of rHDL its phospholipid moiety is rapidly hydrolyzed by $sPLA_2$. This leads to marked increase in plasma LPC levels, which can reach up to 300 μM 4 hours after rHDL administration (162, 163). This raises the possibility that the potent anti-inflammatory activity of administered rHDL may - in part - be mediated by LPC.

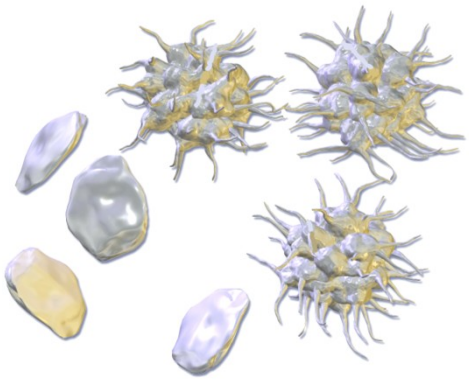
Previous studies in both animal models and in humans affirmed important role of certain $sPLA_2$ isoforms in the pathogenesis of cardiovascular disease (CVD), from initiation of atherosclerosis to cardiovascular complications (1). These studies have stimulated the

great interest in the potential application of $sPLA_2$ inhibitors in the treatment of cardiovascular disease. Initial clinical studies in patients with stable coronary disease and acute coronary syndrome showed that varespladib, potently inhibited $sPLA_2$-IIA activity by about 90 procent. In addition, it decreased LDL cholesterol levels and CRP. However, $sPLA_2$ inhibition in the phase III randomized clinical trial (VISTA-16) did not reduce cardiovascular ischemic complications and unexpectedly resulted in an excess rate of myocardial infarction and stroke in patients with acute coronary syndromes (23). In agreement with the disastrous outcome of this clinical trial is the recent meta-analysis of 19 general populations studies which failed to show associations between $sPLA_2$ enzyme activity and major vascular events, providing evidence that increased $sPLA_2$ levels are a consequence rather than a cause of cardiovascular disease (24). Furthermore, when tested in septic patients, $sPLA_2$ inhibitor did not have any beneficial effects on survival (125). Interestingly, also a large phase III clinical study which tested an inhibitor of lipoprotein-associated phospholipase A_2 darapladib has failed, as darapladib was unable to lower the risk of cardiovascular events in patients with acute coronary syndrome (164) providing further evidence that the physiologic and pathophysiologic roles of phospholipases are not well understood.

Potential limitation of this study might be the fact that we investigated isolated impact of $sPLA_2$ modification of HDL. However, in *in vivo* conditions HDL particles can be influenced by wide range of compositional alterations and remodeled by numerous factors acting together. $sPLA_2$-mediated hydrolysis of HDL-associated phospholipids leads to structural and compositional alterations of HDL particle ultimately resulting in its increased catabolism. Animal studies have shown that the LPS administration to CETP transgenic mice led to substantial decrease in CETP activity. This had major effect on HDL levels and it is considered to be an adaptive response in order to preserve or increase HDL levels (165). In addition, other factors can also play role in HDL remodeling under inflammatory conditions. SAA, an acute phase proteins, which concentrations are markedly elevated in inflammation, mainly associates with HDL altering its composition and function (166). Interestingly, SAA overexpression in mice in the absence of acute phase response did not result in reduced HDL cholesterol or apoA-I levels (167), suggesting that there are other components of acute phase

response which influence HDL metabolism. On the other hand, overexpression of $sPLA_2$ alone caused alterations of HDL metabolism even in the absence of inflammation (168). Even though increased $sPLA_2$ and decreased CETP activities might be advantageous during acute inflammation, displacement of HDL-apolipoproteins by SAA could contribute to increased cardiovascular risk.

Summing up, our results provide novel evidence that under inflammatory conditions increased $sPLA_2$ levels promote or at least may contribute to compositional and functional alterations of HDL, generating a particle which modulates neutrophil trafficking and effector responses. Increased cholesterol-mobilizing activity and inhibition of agonist-induced rise in intracellular Ca^{2+} levels are likely underlying mechanisms which mediate strong anti-inflammatory effects of $sPLA_2$-HDL on neutrophils (156). Given that there is a lot of evidence for the importance of neutrophils in atherosclerosis and their activation and accumulation during atherosclerotic lesion formation (82), $sPLA_2$ inhibition may be rather harmful than a useful strategy to reduce adverse cardiovascular outcomes.



III. CHAPTER:

Phospholipase A_2 modified HDL potently inhibits platelet activation

1. Role of Platelets in Inflammation

1.1 Platelets

Platelets are disc-shaped anucleate cell fragments derived from large precursors, known as megakaryocytes, which reside in the bone marrow (169). Platelets are the smallest blood cells, with the diameter 2 to 5 μm and 0.6 μm thickness. In adult person about 100 billion platelets per day are produced. Platelet lifespan in the bloodstream is 7-10 days, during which they constantly circulate through vascular system, survey the structural integrity of the blood vessel wall and are finally eliminated by the spleen (170, 171).

1.2 Platelets in thrombosis and hemostasis

A severe vascular injury results in deendothelization and exposure of subendothelial extracellular matrix proteins such as von Willebrand factor (vWF) and collagen (172, 173). Platelet integrin complex GPIb-IX-V, which is constitutively expressed on platelets, binds to the vWF whereas GPVI binds to collagen (174). vWF is produced by the endothelium or adsorbed from plasma and it is essential for platelet adhesion. Thrombin is also formed at the vascular injury site as a product of coagulation cascade, and potently stimulates platelets to secrete granule contents. Adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2) released from granules additionally activate platelets. This leads to conformational activation of platelet integrins GPIIb/IIIa and GPIa/IIab (GPIIb/IIIa is receptor for fibrinogen and GPIa/IIa is collagen receptor), which then mediate firm platelet adhesion required to promote thrombus growth. Adherent activated platelets then recruit and activate additional platelets from circulation promoting thrombus growth. During thrombus growth important step is cross-linking of platelets, their aggregation and thrombus stabilization. This is mediated by fibronectin binding to GPIIb/IIIa integrins, which play the most important role in platelet aggregation and stabilization (175).

The most severe complications of atherosclerosis such as myocardial infarction and stroke come from the sudden thrombotic occlusion of an artery (176). Atherosclerotic plaque disruption is the main cause of thrombotic occlusions. Activated immune cells, mainly macrophages, T cells and mast cells play important role in processes which weaken fibrous cap. Interferon-γ (IFN-γ) released from T helper type 1 (Th1) cells inhibits production of extracellular matrix collagen by SMC. In addition, activated macrophages in the lipid core produce proteases which degrade collagenous cap and tissue factor, which is strong pro-coagulant. Upon plaque rupture, procoagulants such as tissue factor and exposed subendothelial matrix induce platelet aggregation and thrombosis (176) ***(Figure III-1)***.

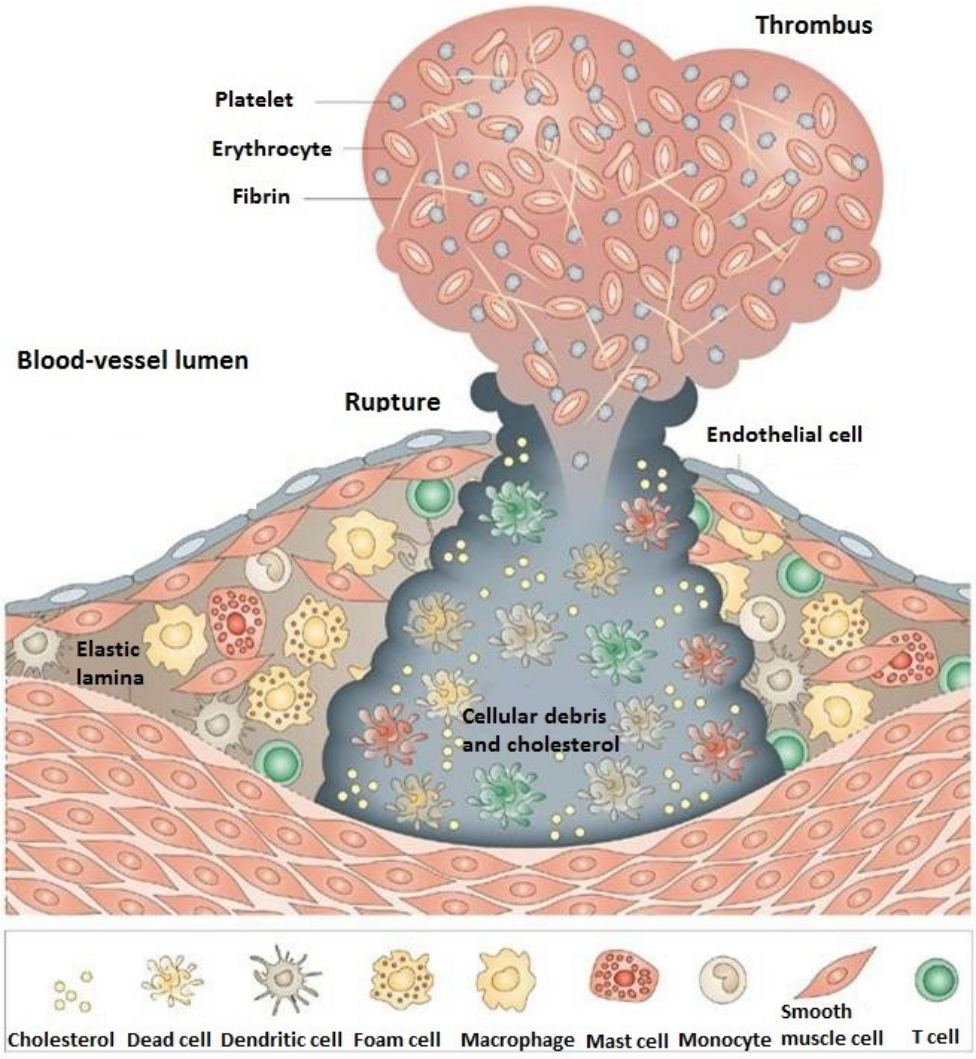


Figure III-1. Atherosclerotic plaque rupture and thrombosis. *Activated immune cells, mainly macrophages, T cells and mast cells, release pro-inflammatory mediators which promote collagen synthesis and tissue factor expression. Immune cells also release proteases which degrade and weaken collagenous cap. As the result of the forces of arterial blood pressure the weakend plaque might fissure, which leads to the exposure of subendothelial matrix and procoagulants such as tissue factor. This induces platelet aggregation and thrombosis. A thrombus forms and occludes the blood vessel lumen resulting in acute ischaemia. Figure taken from (176).*

1.3. Role of platelets in the systemic inflammatory response

Systemic inflammatory responses comprise a diverse group of diseases which can be caused by ischemia, inflammation, multiple trauma and tissue injury, infection or by several insults combined (177). It is well known that systemic infections are accompanied by increased activation of platelets in the circulation which can lead to conditions with a high mortality rate such as disseminated intravascular coagulation (DIC), thrombocytopenia and subsequent multiple organ failure (178). During systemic inflammatory response, such as found in sepsis, platelet-activating factor (PAF) and thrombin are produced by host cells (179). They activate platelets to release granule contents and express P-selectin on their surface ***(Figure III-2)***. Furthermore, PAF and thrombin induce GPIIb/IIIa activation, which then binds fibrinogen and facilitates platelet aggregation leading to thrombosis and DIC (178). In addition, recent studies with $TLR4^{-/-}$ mice revealed that LPS-mediated activation of platelet Toll-like receptor 4 (TLR4) is involved in pathogenesis of thrombocytopenia present in sepsis and related disorders (180).

Moreover, in the systemic inflammatory response activated platelets can modulate leukocyte functions via expression of P-selectin, which mediates platelet binding to monocytes and neutrophils (181). In line with this, number of platelet complexes with leukocytes in circulation was shown to be highly increased in septic patients (182). It has been demonstrated that platelets are responsible for neutrophil activation, trafficking and sequestration during acute lung injury (183). Studies in mouse sepsis

models indicate that platelets promote neutrophil release of NETs in sepsis (81). In addition, platelet-monocyte aggregates have been shown to induce the production of pro-inflammatory mediators such as MCP-1, IL-1β and IL-8 (184).

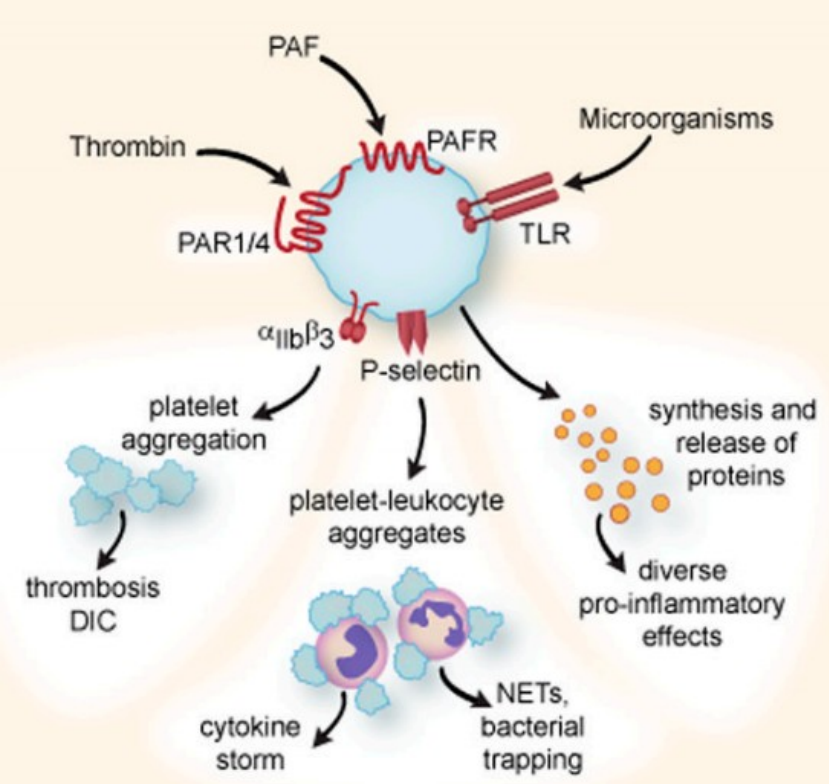


Figure III-2. Platelets play important role in pathology of systemic inflammatory responses. *Thrombin, PAF and pathogen products can induce platelet activation and subsequent formation of platelet-platelet and platelet-leukocyte aggregates which contribute to DIC, the formation of NETs as well as to the production of pro-inflammatory cytokines. These responses can be important for the host defence, but on the other hand, they can significantly contribute to the pathology of the disease. DIC, disseminated intravascular coagulation; NETs, neutrophil extracellular traps; PAF, platelet activating factor; PAFR, PAF receptor; PAR, protease activated receptor; TLR, Toll-like receptor. Figure taken from (178).*

1.4 Role of platelets in the development of atherosclerosis

When it comes to the role of platelets in atherosclerosis, it is well known that atherosclerotic plaque rupture induces rapid platelet activation and aggregation with subsequent thrombotic vascular occlusion and ischemia in various organs. However, there is substantial body of evidence showing that platelets also contribute to atherosclerotic lesion development ***(Figure III-3)***.

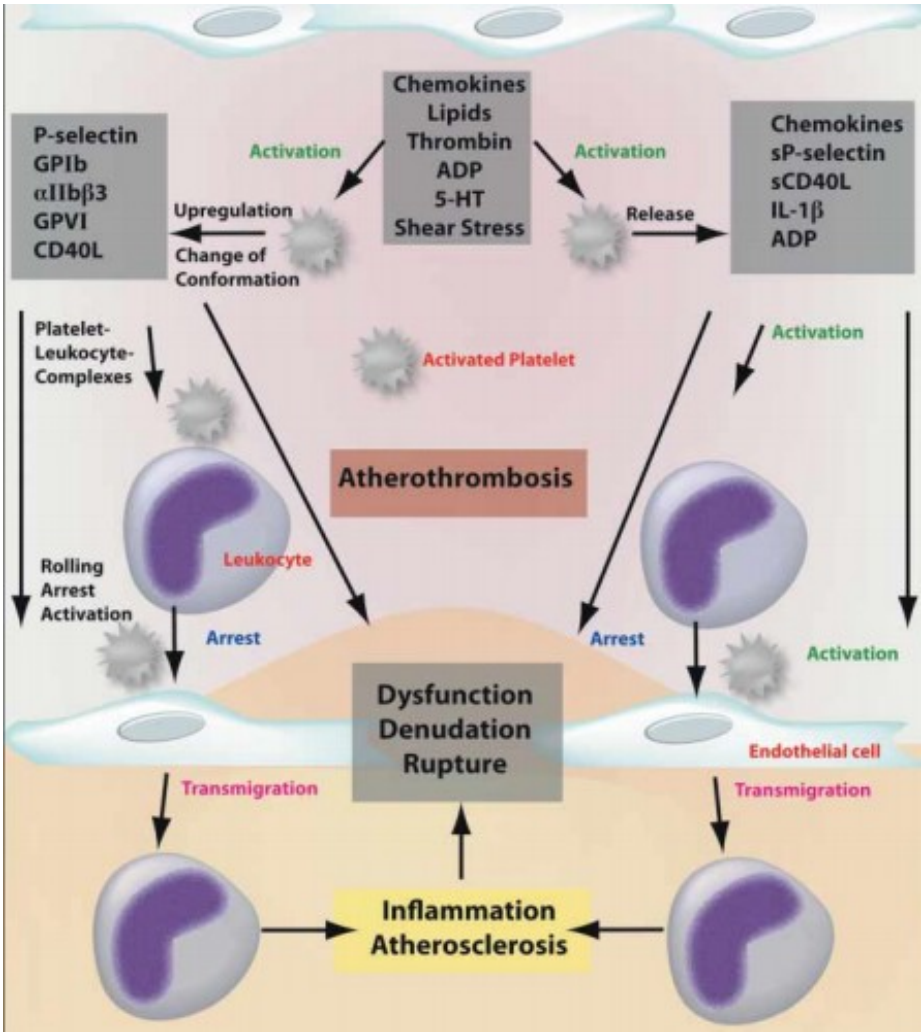


Figure III-3. Platelets in atherosclerosis. *Platelets get activated by soluble mediators and shear stress. This leads to expression of adhesion molecules and integrin activation*

on the platelet surface, as well as release of platelet granule contents. Activated platelets promote leukocyte recruitment to the arterial wall which leads to formation of atherosclerotic infiltrate. Leukocyte recruitment is facilitated by direct leukocyte activation through platelet-released soluble mediators, by formation of platelet-leukocyte complexes and by activation of endothelial cells. Upon lesion denudation and plaque rupture, platelets rapidly aggregate and form thrombi which may occlude the arterial lumen. Figure taken from (175).

Several studies showed that absence of P-selectin reduces development of atherosclerotic lesions in $LDLR^{-/-}$ (185) or $apoE^{-/-}$ (186) mice. Apart from endothelial P-selectin, platelet P-selectin was found to mediate development of atherosclerosis in $apoE^{-/-}$ mice (187). Massberg at al (188) observed in hypercholesterolemic $apoE^{-/-}$ mice that platelets accumulate on the carotid endothelium in the initial phases of atherosclerosis development. There are also studies showing that platelets have role in the development of atherosclerosis in humans. Platelets in patients with various atherosclerotic diseases were found to be hyperreactive (189, 190). Moreover, platelet activation correlated with increased wall thickness of the carotid artery (189, 191).

Under inflammatory conditions intact endothelium can become adhesive for platelets. In endothelial cells, inflammatory stimuli induce rapid P-selectin transfer from Weibel-Palade bodies to the plasma membrane. First loose contacts of platelets with endothelium trigger their tethering and rolling. These processes are mediated by interactions between P-selectin and its lignads (GPIb/IX/V complex and PSGL-1) expressed on platelets. Firm adhesion to extracellular matrix and endothelium is mediated by platelet integrins, mainly GPIIb/IIIa. Rolling and firm adherent platelets get activated and release potent inflammatory mediators initiating series of events which promote the development of atherosclerosis (192).

IL-1β and CD40L released from activated platelets further inflame the endothelium and induce expression of adhesion molecules (ICAM-1, VCAM-1, P-selectin and E-selectin) as well as secretion of MCP-1 and IL-8, which are potent chemoattractants for neutrophils and monocytes (174, 192-196). All these events recruit monocytes and

neutrophils to the inflamed sites, support their transmigration into the intima, transformation of monocytes into macrophages and consequently initiate atherosclerotic lesion formation. Platelets also induce neutrophil recruitment by secretion of PAF and macrophage inflammatory protein-1α (MIP-1α) (192). In addition, RANTES and platelet factor 4 (PF4), which are secreted and deposited on the endothelium by activated platelets, can stimulate monocytes and further attract them to the atherosclerotic lesion (197, 198). PF4 is the most abundant protein released by platelets and it is known to stimulate differentiation of monocytes into macrophages (199). Furthermore, PF4 supports the uptake of oxidized LDL (oxLDL) by macrophages and their transformation into the foam cells (200). Platelets stimulate lesion development and progression by secretion of transforming growth factor-β (TGF-β) and subsequent stimulation of smooth muscle cell and fibroblast proliferation (201). Additionally, activated platelets are involved in extracellular matrix degradation via secretion of matrix metalloproteinases, such as MMP-2 which is released during platelet aggregation (202).

In addition, interactions between platelets and leukocytes have important role in the development of atherosclerosis. Activated platelets bind to monocytes and neutrophils in the circulation via P-selectin-mediated interactions. This facilitates leukocyte recruitment to the vessel wall through platelet interaction with endothelium. Furthermore, circulating leukocytes can bind platelets already adhered to the endothelium, and this is promoted by expression of P-selectin, GPIα, JAM-3, ICAM-3 on platelets (192).

Apart from leukocytes, platelets can promote recruitment of progenitor cells and stimulate their differentiation to foam cells (203).

1.5 Platelet-lipoprotein interaction

There is a lot of evidence that both platelets in the circulation and those attached to the endothelium, can come in contact with lipoproteins ant that these platelet-lipoprotein interactions can modulate platelet function. LDL, VLDL and oxLDL have been reported to activate platelets, whereas HDL attenuates platelet activation (204, 205). Platelets from patients with hypercholesterolemia were found to be hyperreactive, while lipid-lowering medication reduced platelet activation (206, 207).

There are studies showing that LDL and HDL can bind to platelets (208) leading to transfer of lipids between platelets and lipoproteins (209). However, it is not completely clear via which receptors they act. Some studies suggest that LDL receptor related protein-8 might be involved in platelet responses to LDL (210). In addition, ApoER2 was suggested as LDL receptor on platelets (211). Upon contact with native LDL, phospholipid composition of platelet membrane is being remodeled, which results in increased platelet sensitivity to physiological agonists (210). Apart from native LDL, oxLDL can also efficiently bind to platelets inducing fast platelet activation and subsequent pro-atherothrombotic effects (212).

Studies about direct effects of native HDL on platelets *in vitro* are not consistent. Some studies reported inhibitory effects on platelet activation including inhibition of platelet aggregation, release of granule contents and TxA_2 (213-215) while others showed no effect or even increase in agonist-induced activation (216-218). These discrepancies can be due to different HDL preparations used (total HDL, diverse subclasses, rHDL) or different agonist concentrations (219). In addition, there is one study reporting inhibitory effects of oxidized (oxHDL) on platelet activation (216).

Several studies showed that HDL has antithrombotic effects *in vivo* and *ex vivo* (219-221). Infusion of reconstituted HDL (rHDL) attenuated agonist-induced platelet activation *ex vivo* (222). Furthermore, inverse correlation was found between HDL cholesterol and apoA-I levels and the incidence of recurrent venous thromboembolism (219, 223, 224).

HDL can also exhibit antithrombotic effects on platelets indirectly via acting on endothelial cells and inducing NO (225) and prostacyclin (226) production.

It is not clear which receptor/s are involved in the interaction between platelets and HDL. Some studies indicated the involvement of LDL receptor family. ApoER2/LRP8 is expressed on platelets and can be activated by apoE present on HDL (211). Recent studies identified SR-BI as HDL receptor which mediates HDL inhibitory effects on platelet function (227). It was found that rHDL interferes with platelet lipid rafts and depletes cholesterol from the cell membrane, which leads to inhibition of platelet activation (222). Hence, the mechanism of action of native HDL and rHDL might be different, with native HDL acting via receptors and stimulating intracellular signaling and rHDL promoting cholesterol efflux and reduction of free cholesterol in the membrane (219).

When it comes to oxHDL, there are also discrepancies. There are studies showing induction of platelet aggregation by Cu^{2+} or hypochlorous acid oxidized HDL, which was dependent on CD36 receptor (228). On the other hand, one study reported inhibition of agonist-induced platelet activation mediated by SR-BI receptor (216). The reason for these conflicting findings might be the fact that mild oxidation by Cu^{2+} leads to formation of oxidized phospholipids with subsequent increase in negative charge and higher affinity for SR-BI receptor, whereas strong oxidation by hypochlorite oxidizes HDL apolipoproteins, which are ligands for CD36 (219).

1.6 Lipid rafts in platelets

Recent studies confirmed the existence of lipid rafts in platelet membrane and their importance in platelet activation (229). Signaling via GPCRs in platelets seems to be lipid raft-dependent, as it was found that following thrombin stimulation alpha subunit of G proteins comes in contact with cholesterol-rich microdomains, which leads to formation of PI3P and other signaling molecules in lipid rafts and subsequent platelet activation (230). There are also observations that lipid rafts play important role in GPVI

and GPIb-IX-V-dependent platelet activation. For example, GPVI is not found in the lipid raft parts of the membrane in non-activated platelets. However, upon stimulation it is rapidly transferred to these domains (231). In addition, cholesterol relocation within platelet plasma membrane was observed upon collagen-induced activation (232). In line with this observation, treatment of platelets with cholesterol depletion agents prevented lipid raft aggregation and cell activation (230).

2. Introduction

Numerous clinical studies have firmly established the key role of platelets in arterial thrombosis, which is the most common cause of myocardial infarction and stroke (174). In addition, platelets have been reported to be actively involved in the induction of inflammation (233) and atherosclerosis (175).

Furthermore, besides their well recognized role in hemostasis, accumulating evidence suggests that platelets are linked to inflammatory response. Increased platelet activation in the bloodstream is found in patients with systemic inflammation and sepsis (234) and it is well known that platelets substantially contribute to sepsis complications such as disseminated intravascular coagulation, thrombotic microangiopathy and multiple organ failure via their haemostatic and thrombotic potential (235).

It is considered that conventional platelet functions such as adherence, aggregation and release of soluble mediators contribute to systemic inflammatory responses (178). However, these anucleate cell fragments are also able to interact with other immune cells and to modulate their function via direct contacts and released soluble mediators (236). In line with this, platelet-leukocyte complexes were found to be markedly increased in the circulation of septic patients (182).

As described in detail in the General Introduction Chapter, levels of $sPLA_2$ enzymes are markedly increased in various chronic and acute pathologic conditions (51-53), which led to conclusions that $sPLA_2$ might have important role in pathophysiology of these diseases. However, clinical trials testing $sPLA_2$ inhibitors in both septic and cardiovascular patients failed (23, 28, 125). Moreover, $sPLA_2$ inhibition in patients with acute coronary syndrome had opposite outcome and led to increased rate of myocardial infarction (23).

It was reported that HDL exhibits anti-thrombotic activities both indirectly via acting on endothelial cells (225, 226) or via direct contacts with platelets (213-215). Given that numerous studies showed that platelets can actively interact with HDL, which results in modulation of platelet function (204, 205, 209), we aimed to investigate the effects of

$sPLA_2$ modified HDL on platelet reactivity. In order to elucidate this, we performed several functional assays such as platelet aggregation assay, P-selectin expression, GPIIb/IIIa activation, production of reactive oxygen species and Ca^{2+} flux. Interestingly, we found that $sPLA_2$-HDL has potent anti-aggregatory properties and might be an important regulator of platelet functional responses under inflammatory conditions.

3. Materials and Methods

3.1 Materials

Reagents	
Name	**Company**
[^{3}H]-cholesterol	Hartman Analytic
Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz 58-035	Sigma-Aldrich
Adenosine diphosphate (ADP)	Sigma-Aldrich
Bovine serum albumin (BSA)	PAA
CellFix	BD Biosciences
Collagen	Chronopar
Cytochalasin B	Sigma-Aldrich
FACS-Flow	BD Bioscience
Fibrinogen	Sigma-Aldrich
Fluo-3-AM	Life Technologies
HEPES	PAA
Human recombinant $sPLA_2$ type V	Cayman Europe
Lysophosphatidylcholine (LPC) assay Kit	ALFRESA
Lysophosphatidylcholine 16:0 (LPC 16:0)	Avanti Polar Lipids
Lysophosphatidylcholine 18:1 (LPC 18:1)	Avanti Polar Lipids
Lysophosphatidylcholine 18:2 (LPC 18:2)	Avanti Polar Lipids
Lysophosphatidylcholine 20:4 (LPC 20:4)	Avanti Polar Lipids
Lysophosphatidylserine 16:0 (Lyso-PS 16:0)	Avanti Polar Lipids
PD-10 columns	GE Healthcare
Phosphate buffered saline (PBS) with or without Ca^{2+} and Mg^{2+}	PAA
Potassium bromide	Sigma-Aldrich
Probenecid	Sigma-Aldrich
$sPLA_2$ type III from bee venom	Cayman Europe
Thrombin	Chronopar
Total ROS and superoxide detection kit	Enzo Life Sciences
Varespladib	Eubio
Antibodies	
PAC-1 (GPIIb/IIIa) antibody-FITC	BD Biosciences
CD62P (P-selectin) antibody-FITC	BD Biosciences

3.2 Blood collection and platelet isolation

The study was approved by the Ethics Committee of the Medical University of Graz. All volunteers signed an informed consent form. Blood was collected into tubes with 3.8 % (w/v) sodium citrate and platelet rich plasma (PRP) was obtained by centrifugation at 400 x g for 20 min at room temperature (RT). Platelets were washed twice with a low pH platelet wash buffer (140 mmol/L NaCl, 10 mmol/L $NaHCO_3$, 2.5 mmol/L KCl, 0.9 mmol/L Na_2HPO_4, 2.1 mmol/L $MgCl_2$, 22 mmol/L $C_6H_5Na_3O_7$, 0.055 mmol/L glucose monohydrate and 0.35 % bovine serum albumin) by centrifugation at 1000 x g for 15 min at RT. The final platelet preparation was resuspended in Tyrode's buffer (10 mmol/L HEPES, 134 mmol/L NaCl, 1 mmol/L $CaCl_2$, 12 mmol/L $NaHCO_3$, 2.9 mmol/L KCl, 0.34 mmol/L Na_2HPO4, 1 mmol/L $MgCl_2$ and 0.055 mmol/L glucose) used for functional platelet assays.

3.3 Platelet aggregation

Platelet aggregation was performed at 37°C with constant stirring (1000 rpm) using the four-channel platelet aggregometer APACT4004 (LABiTec, Ahrensburg, Germany), which works on the principle of light transmission, as previously described (237, 238). Aggregation was induced with ADP (5-20 µmol/L) in the presence of 1 µg/mL fibrinogen, collagen (2 µg/mL) or thrombin (0.025 U/mL) and measured for 5 min. Data were expressed as percentage of maximum light transmission, with non-stimulated washed platelets being 0 % and Tyrode's buffer 100 %.

3.4 P-selectin (CD62P) expression

Isolated washed platelets were resuspended in Tyrode's buffer. To analyze P-selectin expression platelets were stimulated with ADP (3 µmol/L) in the presence of cytochalasin B (5 µg/mL) for 30 min at RT in the presence of anti-CD62P-FITC conjugated antibody. The samples were washed once with ice cold PBS without Ca^{2+} and Mg^{2+} by centrifugation (400 x g, 7 min), fixed and P-selectin upregulation was detected by flow cytometry (238, 239).

3.5 GPIIb/IIIa (PAC-1) activation

Platelet reach plasma was suspended in Tyrode's buffer and pretreated with different HDL preparations. The activation of glycoprotein receptor GPIIb/IIIa was induced with 3 µmol/L ADP for 30 min at RT in the presence of the FITC-conjugated anti-PAC-1 antibody that recognizes a conformation-dependent determinant on the GPIIb/IIIa complex. The samples were washed once with ice cold PBS without Ca^{2+} and Mg^{2+} by centrifugation (400 x g, 7 min), fixed and analyzed by flow-cytometry (238, 240).

3.6 Ca^{2+} Flux in platelets

Platelet reach plasma was loaded with the cell membrane permeable Ca^{2+}-sensitive dye Fluo-3-AM (5 µmol/L) in the presence of 2.5 mmol/L probenecid for 30 min at 37°C and resuspended in Tyrode's buffer (1.5 µL of PRP/500 µL buffer). Changes in intracellular Ca^{2+} levels in platelets were measured by flow cytometry as an increase in fluorescence intensity of Fluo-3-AM in FL-1 channel (238).

3.7 ROS and superoxide production

Washed platelets (1 x 10^7 per sample) resuspended in Tyrode's buffer were pretreated with different HDL preparations and stimulated with 8 µg/mL collagen for 20 min, at 37°C. Total ROS and superoxide production was detected with Total ROS/Superoxide Detection Kit according to manufacturer's protocol. Shortly, after the treatment platelets were washed once, resuspended in Total ROS or superoxide detection reagent and incubated for 30 min at 37°C in the dark. Total ROS or superoxide production was measured immediately by flow cytometry.

3.8 Cholesterol efflux in platelets

Cholesterol efflux in platelets was assessed as described (222). Washed platelets were loaded with [^{3}H]-cholesterol (0.5 µCi/mL) at 37°C in the presence of 2 µg/mL acyl-

CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz 58-035 for 2 h at 37°C. Cells were washed by centrifugation and incubated with the respective cholesterol acceptors for 1 h at 37°C in a total volume of 300 µL. After the efflux period, platelets were pelleted, supernatant was removed and cells were lysed. Radioactivity was measured in the supernatant and in the cells and the efflux was expressed as the radioactivity in the supernatant relative to total radioactivity (in the supernatant and cells).

3.9 Statistical analysis

All data are shown as mean ± SD for n separate experiments. Experiments were repeated three to six times using platelets from different donors. Statistical analyses were performed with GraphPad Prism Version 4.03. 2-tailed student's t-test was used for experiments in which two groups were compared and one-way ANOVA with Tukey multiple-comparison post hoc test was used for experiments comparing three or more groups. Significances were accepted at *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

4. Results

4.1 $sPLA_2$ modified HDL potently inhibits platelet aggregation

A role of platelets in complications of atherosclerosis and thromboemoblic events is well known and accepted (174). Furthermore, platelets have been recognized to be involved in the early phases of atherosclerosis development (175) as well as in the various inflammatory conditions (178, 233). In order to assess the effects of $sPLA_2$ on platelet function, we treated isolated platelets with $sPLA_2$-treated plasma. Surprisingly, in comparison to native plasma, already short treatment of plasma with $sPLA_2$ potently inhibited platelet aggregation ***(Figure III-4)***.

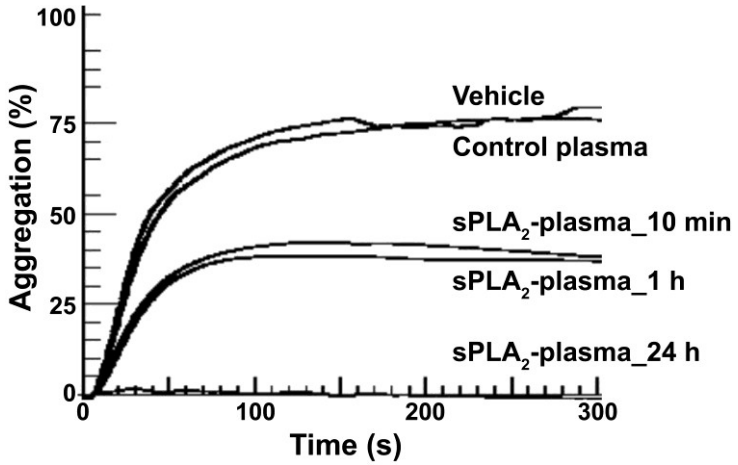


Figure III-4. $sPLA_2$-treated plasma inhibits platelet aggregation. *Freshly isolated human plasma was incubated in the absence (control) or presence of $sPLA_2$ type III for 10 min, 1 h or 24 h at 37°C. Washed platelets were pretreated with $sPLA_2$-plasma for 10 min at 37°C and ADP-induced aggregation was recorded for 5 min. One representative out of three independent experiments is shown. Values are expressed as % of maximal platelet aggregation. *p < 0.05, **p < 0.01 versus control.*

$sPLA_2$-HDL showed similar effects as $sPLA_2$-plasma, completely inhibiting platelet aggregation induced by several agonist, including ADP, thrombin and collagen ***(Figure III-5A to III-5C)***. The effects of $sPLA_2$-HDL on platelets were concentration dependent

(Figure III-5D). Importantly, inhibition of platelet aggregation was rapid, as $sPLA_2$-HDL showed effects already after one minute preincubation with platelets ***(Figure III-5E)***.

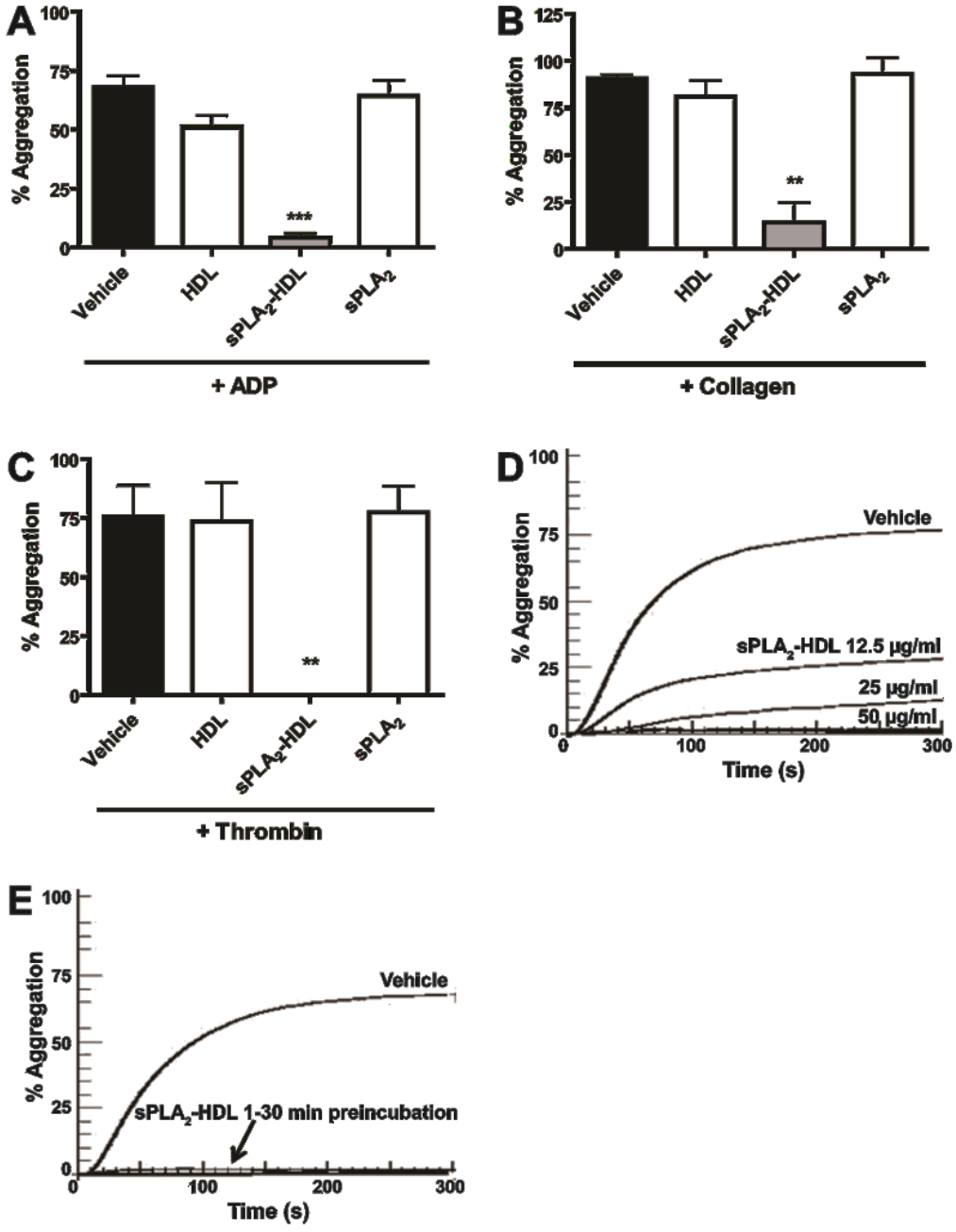


Figure III-5. $sPLA_2$-treated HDL rapidly inhibits ADP, thrombin and collagen-induced platelet activation. *Washed platelets were preincubated with vehicle, HDL (50 µg protein/mL), $sPLA_2$-HDL (50 µg protein/mL) or $sPLA_2$ alone for 10 min at 37°C.*

*Subsequently, platelet aggregation was induced with **(A)** ADP (5-20 µmol/L) **(B)** 2 µg/mL collagen or **(C)** thrombin (0.025 U/mL). Aggregation was recorded for 5 min. Values are expressed as % of maximal platelet aggregation. Results shown are mean ± SD. The data represent the results from three to five experiments done using platelets from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle. **Concentration and time dependence of sPLA$_2$-HDL-mediated inhibition of platelet aggregation. (E)** Washed platelets were pretreated with increasing concentration of sPLA$_2$-HDL (0 up to 50 µg/mL) for 10 min at 37°C. **(F)** Platelets were preincubated with sPLA$_2$-HDL for increasing periods of time (0 up to 30 min) at 37°C. Aggregation was induced with ADP (5-20 µmol/L) and values are expressed as % of maximal platelet aggregation. One representative out of three independent experiments is shown.*

4.2 sPLA$_2$-HDL-associated LPCs mediate effects of sPLA$_2$-HDL on platelets

To test whether the observed effects of sPLA$_2$-HDL on platelets depend on enzymatic activity of sPLA$_2$, we used the sPLA$_2$ inhibitor varespladib (inhibits sPLA$_2$ types IIA, V and X but has no effect on sPLA$_2$-III) during preparation of sPLA$_2$-HDL. Addition of varespladib completely abolished the effects of sPLA$_2$-HDL on platelet aggregation as shown in ***Figure III-6***. Varespladib inhibited the effects of sPLA$_2$-HDL on platelet aggregation, demonstrating that the observed effects on platelets depend on sPLA$_2$ enzymatic activity.

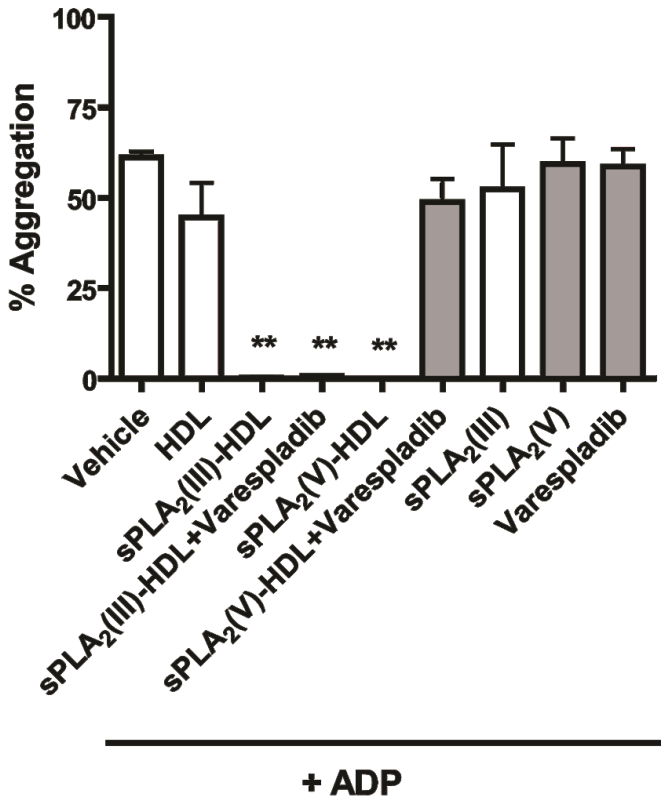


Figure III-6. The $sPLA_2$ inhibitor varespladib inhibits the effects of $sPLA_2$-treated HDL on platelets. *HDL was incubated with 200 ng/mL $sPLA_2$ type III or 400 ng/mL $sPLA_2$ type V in the presence or absence of varespladib (1 µmol/L), overnight, at 37°C. These samples were subsequently used for platelet treatment. Platelets were preincubated (10 min at 37°C) with vehicle (assay buffer), HDL (50 µg protein/mL), $sPLA_2$ (III)-HDL (50 µg protein/mL), $sPLA_2$ (III)-HDL + varespladib, $sPLA_2$ (V)-HDL, $sPLA_2$ (V)-HDL + varespladib, varespadib, $sPLA_2$ (III) or $sPLA_2$ (V) alone. Aggregation was induced with ADP (5-20 µmol/L). Values are expressed as % of maximal aggregation. Results represent means ± SD (n=3). Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.*

When albumin was added to remove LPCs and FFAs from $sPLA_2$-HDL and HDL was subsequently reisolated by ultracentrifugation (to remove albumin), the effects of $sPLA_2$-HDL on platelets were completely abolished ***(Figure III-7A)***. When HDL was enriched with different LPCs, we observed that LPC 16:0 showed the most potent anti-aggregatory effect on platelets **(Figure III-7B)**.

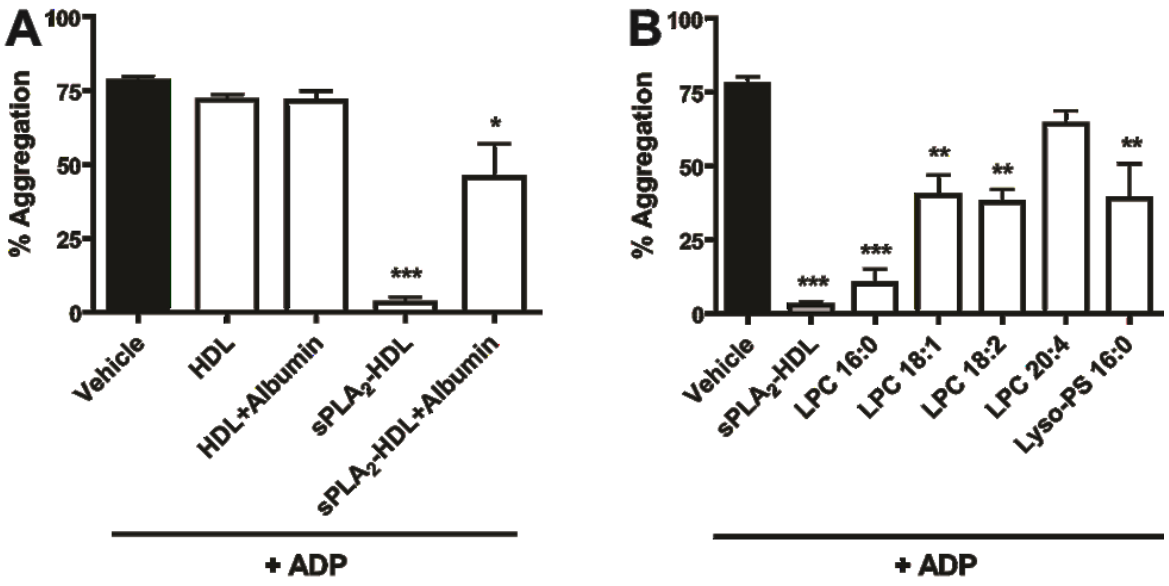


Figure III-7. The LPC content in $sPLA_2$-treated HDL is required for the anti-aggregatory effects. (A) *Native or $sPLA_2$-treated HDL were incubated in the presence or absence of 50 mg/mL albumin (1 h, 37°C) and HDL was re-isolated by density gradient ultracentrifugation to remove FFA and LPCs, as described in Materials and Methods. 50 µg/mL HDL protein was used in the aggregation assay.* ***(B) Enrichment of HDL with LPC.*** *Platelets were preincubated (10 min, 37°C) with $sPLA_2$-HDL (50 µg/mL protein) and HDL enriched with LPC (16:0, 18:1, 18:2, 20:4) or lysophosphatidyserine (Lyso-PS) 16:0. Aggregation was induced with ADP. Results shown are mean ± SD of three separate experiments using cells from three different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.*

4.3 $sPLA_2$-HDL abolishes P-selectin expression and GPIIb/IIIa actvation in platelets

Next, we investigated important molecules involved in platelet contact with endothelium and firm arrest, such as P-selectin and GPIIb/IIIa. P-selectin expression induced by ADP in the presence of cytochalasin B was completely inhibited ***(Figure III-8A)*** as well as activation of GPIIb/IIIa integrin ***(Figure III-8B)***.

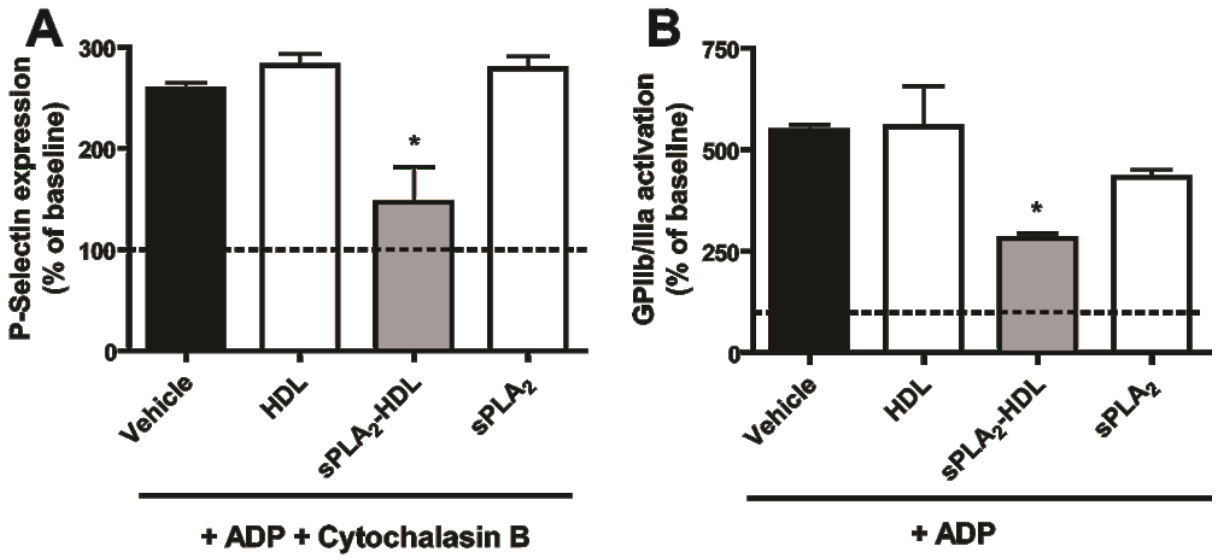


Figure III-8. $sPLA_2$-HDL inhibits P-selectin expression and GPIIb/IIIa activation. (A) *Washed platelets were pretreated with vehicle, HDL (50 µg protein/mL), $sPLA_2$-HDL (50 µg protein/mL) or $sPLA_2$ alone for 10 min at 37°C. Surface expression of P-selectin was induced with 3 µmol/L ADP and cytochalasin B (5 µg/mL) for 15 min at 37°C in the presence of anti-P-selectin antibody.* ***(B)*** *Platelet reach plasma was suspended in Tyrode's buffer and pretreated with vehicle, HDL (50 µg protein/mL), $sPLA_2$-HDL (50 µg protein/mL) or $sPLA_2$ alone for 10 min at 37°C. For GPIIb/IIIa activation, platelets were stimulated with 3 µmol/L ADP for 30 min at room temperature in the presence of the conformation-sensitive anti-PAC-1 antibody. P-selectin expression and GPIIb/IIIa activation were measured by flow cytometry. Vehicle treated (unstimulated) control was set as baseline and values are expressed as % over baseline. Results shown are mean*

*± SD of three separate experiments using cells from different healthy donors. Statistical significance was assessed by one-way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.*

4.4 $sPLA_2$-HDL inhibits production of reactive oxygen species (ROS) in platelets

Because ROS can influence platelet activation and aggregation, we thought to assess effects of $sPLA_2$-HDL on platelet production of reactive oxygen species, and specifically on superoxide anion generation. Of particular interest, $sPLA_2$-HDL strongly inhibited ROS and superoxide production in collagen-treated platelets ***(Figure III-9)***.

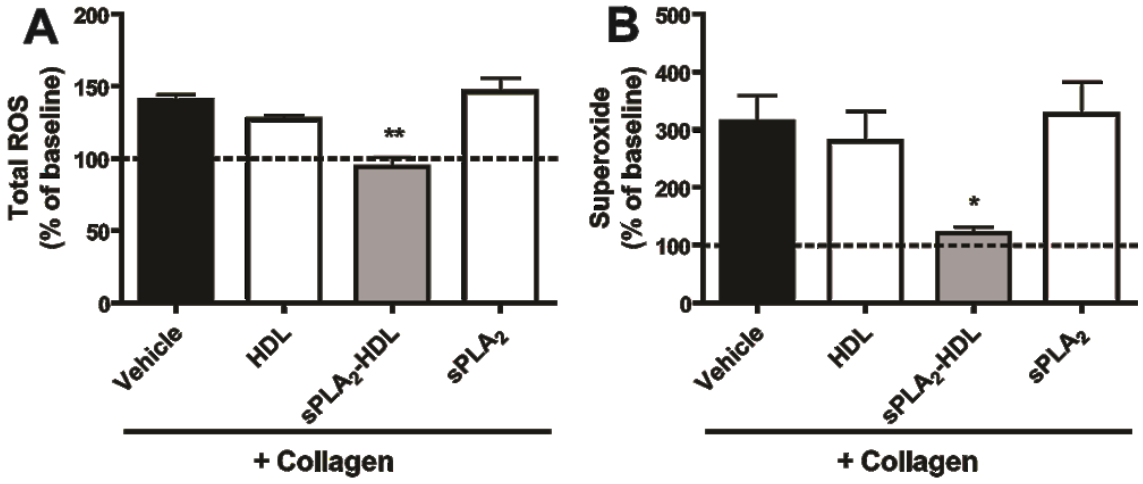


Figure III-9. $sPLA_2$-HDL inhibits ROS and superoxide production in platelets. *Washed human platelets were preincubated with vehicle, HDL (50 µg protein/mL), $sPLA_2$-HDL (50 µg protein/mL) or $sPLA_2$ alone for 10 min at 37°C. Cells were then stimulated with 6 µg/mL collagen (20 min, 37°C) and **(C)** total ROS and **(D)** superoxide production were detected as described in Materials and Methods. Vehicle treated (unstimulated) control was set as baseline and values are expressed as % over baseline. The results are shown as mean ± SD of 4 separate experiments using platelets from different healthy donors. Statistical significance was assessed by one-*

*way ANOVA with Tukey multiple-comparison post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle.*

4.5 $sPLA_2$-HDL suppresses agonist-induced Ca^{2+} flux and strongly promotes cholesterol efflux in platelets

In order to investigate the possible mechanisms involved in the $sPLA_2$-HDL effects on platelets, we first measured Ca^{2+} flux, as Ca^{2+} is essential for platelet activation and most of functional responses. Following incubation of platelets with $sPLA_2$-HDL, Ca^{2+} flux induced by ADP was abolished, very similar to results observed with neutrophils in Chapter II. However, we found that $sPLA_2$-HDL slightly induced intracellular Ca^{2+} flux in unstimulated (resting) platelets ***(Figure III-10A).*** Since recent investigations reported that cholesterol depletion leads to inhibition of platelet functional responses, we next tested the hypothesis whether improved cholesterol acceptor activity of $sPLA_2$-HDL (as seen with neutrophils in Chapter II) is linked to inhibitory effects. Indeed, $sPLA_2$-HDL showed a dramatically increased ability to promote cholesterol efflux from platelets. ***(Figure III-10B)***.

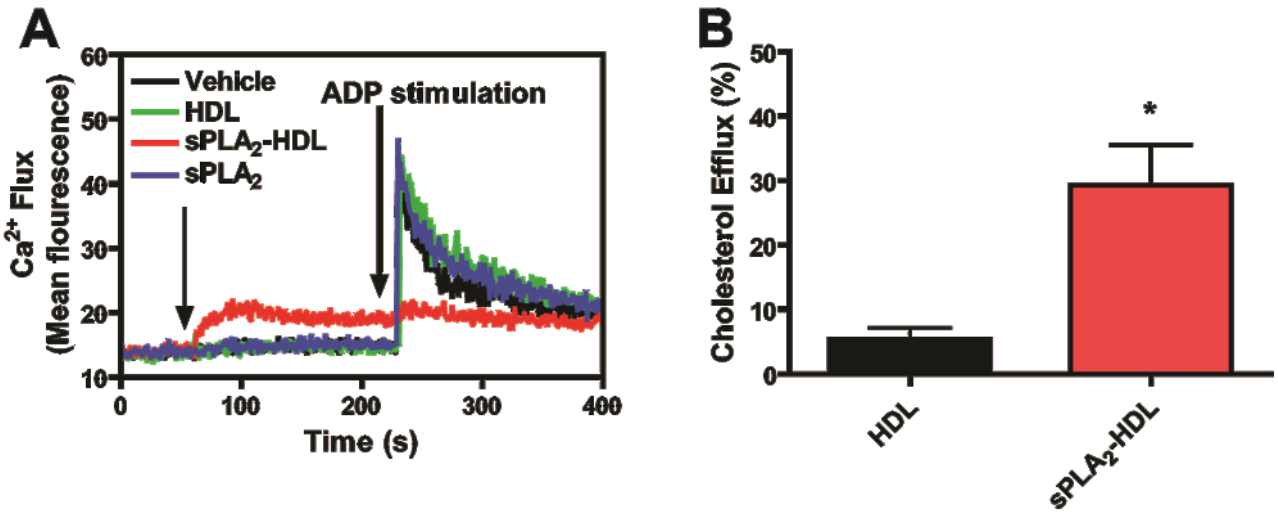


Figure III-10. $sPLA_2$-HDL inhibits Ca^{2+} flux and promotes cholesterol efflux in platelets. (A) *Baseline Ca^{2+} levels were measured for 1 min and then platelets were treated with vehicle, HDL (50 µg protein/mL), $sPLA_2$-HDL (50 µg protein/mL) or $sPLA_2$ for 3 min. Ca^{2+} flux was subsequently induced with ADP (10 µmol/L). Ca^{2+} flux was*

detected at RT by flow cytometry as an increase in fluorescence intensity of the Ca^{2+}-sensitive dye Fluo-3-AM in the FL-1 channel. Values are expressed as mean flourescence. Results show a representative of 3 independent experiments done with cells from different donors. ***(B)*** *Washed platelets were preloaded with [^{3}H]-cholesterol for 2 h at 37°C and incubated with HDL (50 µg protein/mL) or $sPLA_2$-HDL (50 µg protein/mL) for 60 minutes, and percentage of cholesterol efflux was assessed. Cholesterol efflux is calculated as the radioactivity in the medium relative to total radioactivity in medium and cells. The results are shown as mean ± SD of three separate experiments using platelets from different healthy donors. Statistical analysis was performed using Student's t-test. *p<0.05, **p<0.01, ***p <0.001 versus HDL.*

5. Discussion

A crucial role of platelet activation and aggregation in arterial thrombosis is well established. Apart from their main function in hemostasis and coagulation, recent studies clearly showed that platelets have important role not only in the complication of cardiovascular disease, but they are also key mediators in the initial and later steps of the development of atherosclerosis (175, 192, 202). Given that several studies showed that HDL can interact with platelets and regulate their functional responses (219), we focused on the effects of $sPLA_2$ modified HDL on platelet function in the second part of the thesis.

Notably, we found that $sPLA_2$-HDL strongly and rapidly inhibited platelet aggregation induced by several potent platelet agonists such as ADP, collagen and thrombin. Freshly isolated control HDL showed only a weak anti-aggregatory effect on platelets. These results suggest that upon hydrolysis by $sPLA_2$, HDL is transformed into a particle with potent anti-aggregatory properties. In order to investigate the effects of $sPLA_2$-HDL on other platelet functional responses, we assessed P-selectin expression and GPIIb/IIIa activation. Importantly, P-selectin expression, as well as activation of GPIIb/IIIa, which is the essential integrin required for platelet aggregation and thrombus growth and stabilization, were suppressed by $sPLA_2$-HDL. Furthermore, LPCs enriched in HDL mimicked the observed effects of $sPLA_2$-HDL on platelets. Interestingly, LPC 16:0, which is the major LPC present in $sPLA_2$-HDL, was the most effective when enriched in HDL. In addition, other LPCs such as LPC 18:1 and LPC 18:2 as well as lyso-PS 16:0 were effective. Whether some of the FFA, which are also formed as a result of $sPLA_2$ activity on HDL, contribute to $sPLA_2$-HDL effects on platelets still remains to be investigated in future experiments.

Recent studies demonstrated that ROS play important role in platelet functional responses via regulation of several steps in platelet recruitment, activation and aggregation (241). Furthermore, it was reported that superoxide anions released by platelets promote platelet activation and their recruitment to the thrombus formation sites (242). Importantly, $sPLA_2$-HDL suppressed collagen-induced production of ROS by

platelets. In addition, we investigated changes in intracellular Ca^{2+} levels in platelets upon treatment with $sPLA_2$-HDL. We observed that $sPLA_2$-HDL potently inhibited ADP-induced Ca^{2+} flux, which is of great importance since an increase in cytosolic Ca^{2+} levels is a main component of the signaling pathways which regulate platelet activation during thrombosis and hemostasis (243).

Of crucial importance is our finding that $sPLA_2$-HDL had highly increased ability to promote cholesterol efflux from platelets in comparison to control HDL. This finding is in agreement with previous studies demonstrating that cholesterol depletion diminishes platelet activation (222). Additional evidence for the importance of membrane cholesterol in platelet functional responses comes from human pathologic states such as familial hypercholesterolemia, in which platelets are found to be hyperreactive (244). Platelets are known to express scavenger receptors involved in HDL metabolism (such as SR-BI) and to interact with lipoproteins in circulation (219). Hence, it would be of interest to elucidate if increased capability of $sPLA_2$-HDL to promote cholesterol efflux from platelets is mediated via SR-BI.

In addition, similar to other cells, lipid rafts are known to play an essential role in platelet activation and function, facilitating the contact between receptors and signaling molecules (229). Various platelet glycoproteins and G proteins are located in lipid rafts. Moreover, receptors that are not found in lipid rafts of resting platelets move to these microdomains upon agonist stimulation (229, 231). Since cholesterol has been shown to play important role in platelet lipid raft function (222), we will investigate if $sPLA_2$-HDL-mediated mobilization of cholesterol is associated with depletion of platelet lipid rafts, similar to results obtained with neutrophils in Chapter II.

Our findings suggest that $sPLA_2$-mediated hydrolysis of HDL generates particle with potent anti-aggregatory properties. One of possible letal complications during severe sepsis and septic shock is microvascular thrombosis, which can lead to tissue ischemia and organ failure (178, 235). Inhibition of platelet activation and aggregation through $sPLA_2$-HDL might therefore be advantageous under septic conditions. In line with that assumption, a previous study reported that LPC content in serum is positively

associated with survival in sepsis (245). Moreover, administration of LPC significantly reduced septic mortality in mice (246).

In conclusion, our results suggest that elevated levels of $sPLA_2$ during the acute phase response appear to have favorable effects on platelets and neutrophils. Our study shows that HDL remodeling by $sPLA_2$ generates a particle with potent immunoregulatory ability which appears to be due to its increased capability to deplete cholesterol from the cells. Interestingly, $sPLA_2$-HDL seems to exert differential effects depending on the cell type, since in contrast to potent effects on platelets and neutrophils, $sPLA_2$-HDL did not show increased cholesterol efflux capability from macrophages when compared to control HDL. The reason for this surprising observation is not clear, but different lipid raft content and/or lipid raft composition of freshly isolated neutrophils compared with a macrophage cell line might be a likely explanation (156).

Recent studies have revealed the importance of platelet-neutrophil cooperation in inflammation and immunity as well as the involvement of interactions between these two cell types in several inflammatory diseases (79, 80, 236). Hence, it would be of interest to assess how $sPLA_2$-HDL influences and modulates the interplay between platelets and neutrophils.

REFERENCES

1. Mallat, Z., G. Lambeau, and A. Tedgui. 2010. Lipoprotein-associated and secreted phospholipases A(2) in cardiovascular disease: roles as biological effectors and biomarkers. *Circulation* 122: 2183-2200.

2. Murakami, M., Y. Taketomi, Y. Miki, H. Sato, T. Hirabayashi, and K. Yamamoto. 2011. Recent progress in phospholipase A(2) research: from cells to animals to humans. *Prog. Lipid Res.* 50: 152-192.

3. Lambeau, G. and M. H. Gelb. 2008. Biochemistry and physiology of mammalian secreted phospholipases A2. *Annu. Rev. Biochem.* 77: 495-520.

4. Crowl, R. M., T. J. Stoller, R. R. Conroy, and C. R. Stoner. 1991. Induction of phospholipase A2 gene expression in human hepatoma cells by mediators of the acute phase response. *J. Biol. Chem.* 266: 2647-2651.

5. Hamaguchi, K., H. Kuwata, K. Yoshihara, et al. 2003. Induction of distinct sets of secretory phospholipase A(2) in rodents during inflammation. *Biochim. Biophys. Acta* 1635: 37-47.

6. Rosenson, R. S. 2009. Future role for selective phospholipase A2 inhibitors in the prevention of atherosclerotic cardiovascular disease. *Cardiovasc. Drugs Ther.* 23: 93-101.

7. Yamamoto, K., Y. Isogai, H. Sato, Y. Taketomi, and M. Murakami. 2011. Secreted phospholipase A2, lipoprotein hydrolysis, and atherosclerosis: integration with lipidomics. *Anal. Bioanal Chem.* 400: 1829-1842.

8. Pruzanski, W., E. Stefanski, F. C. de Beer, et al. 1998. Lipoproteins are substrates for human secretory group IIA phospholipase A2: preferential hydrolysis of acute phase HDL. *J. Lipid Res.* 39: 2150-2160.

9. Ivandic, B., L. W. Castellani, X. P. Wang, et al. 1999. Role of group II secretory phospholipase A2 in atherosclerosis: 1. Increased atherogenesis and altered lipoproteins in transgenic mice expressing group IIa phospholipase A2. *Arterioscler. Thromb. Vasc. Biol.* 19: 1284-1290.

10. Boyanovsky, B., M. Zack, K. Forrest, and N. R. Webb. 2009. The capacity of group V sPLA2 to increase atherogenicity of ApoE-/- and LDLR-/- mouse LDL in vitro predicts its atherogenic role in vivo. *Arterioscler. Thromb. Vasc. Biol.* 29: 532-538.

11. Sartipy, P., G. Camejo, L. Svensson, and E. Hurt-Camejo. 1999. Phospholipase A(2) modification of low density lipoproteins forms small high density particles with increased affinity for proteoglycans and glycosaminoglycans. *J. Biol. Chem.* 274: 25913-25920.

12. Sartipy, P., G. Camejo, L. Svensson, and E. Hurt-Camejo. 2002. Phospholipase A2 modification of lipoproteins: potential effects on atherogenesis. *Adv. Exp. Med. Biol.* 507: 3-7.

13. Flood, C., M. Gustafsson, R. E. Pitas, L. Arnaboldi, R. L. Walzem, and J. Boren. 2004. Molecular mechanism for changes in proteoglycan binding on compositional changes of the core and the surface of low-density lipoprotein-containing human apolipoprotein B100. *Arterioscler. Thromb. Vasc. Biol.* 24: 564-570.

14. Wooton-Kee, C. R., B. B. Boyanovsky, M. S. Nasser, W. J. de Villiers, and N. R. Webb. 2004. Group V sPLA2 hydrolysis of low-density lipoprotein results in spontaneous particle aggregation and promotes macrophage foam cell formation. *Arterioscler. Thromb. Vasc. Biol.* 24: 762-767.

15. Karabina, S. A., I. Brocheriou, G. Le Naour, et al. 2006. Atherogenic properties of LDL particles modified by human group X secreted phospholipase A2 on human endothelial cell function. *FASEB J.* 20: 2547-2549.

16. Ait-Oufella, H., O. Herbin, C. Lahoute, et al. 2013. Group X secreted phospholipase A2 limits the development of atherosclerosis in LDL receptor-null mice. *Arterioscler. Thromb. Vasc. Biol.* 33: 466-473.

17. Boekholdt, S. M., T. T. Keller, N. J. Wareham, et al. 2005. Serum levels of type II secretory phospholipase A2 and the risk of future coronary artery disease in apparently healthy men and women: the EPIC-Norfolk Prospective Population Study. *Arterioscler. Thromb. Vasc. Biol.* 25: 839-846.

18. O'Donoghue, M. L., Z. Mallat, D. A. Morrow, et al. 2011. Prognostic utility of secretory phospholipase A(2) in patients with stable coronary artery disease. *Clin. Chem.* 57: 1311-1317.

19. Mallat, Z., P. G. Steg, J. Benessiano, et al. 2005. Circulating secretory phospholipase A2 activity predicts recurrent events in patients with severe acute coronary syndromes. *J. Am. Coll. Cardiol.* 46: 1249-1257.

20. Oslund, R. C., N. Cermak, and M. H. Gelb. 2008. Highly specific and broadly potent inhibitors of mammalian secreted phospholipases A2. *J. Med. Chem.* 51: 4708-4714.

21. Fraser, H., C. Hislop, R. M. Christie, et al. 2009. Varespladib (A-002), a secretory phospholipase A2 inhibitor, reduces atherosclerosis and aneurysm formation in ApoE-/- mice. *J. Cardiovasc. Pharmacol.* 53: 60-65.

22. Rosenson, R. S., M. Elliott, Y. Stasiv, C. Hislop, and PLASMA II Investigators. 2011. Randomized trial of an inhibitor of secretory phospholipase A2 on atherogenic lipoprotein subclasses in statin-treated patients with coronary heart disease. *Eur. Heart J.* 32: 999-1005.

23. Nicholls, S. J., J. J. Kastelein, G. G. Schwartz, et al. 2014. Varespladib and cardiovascular events in patients with an acute coronary syndrome: the VISTA-16 randomized clinical trial. *JAMA* 311: 252-262.

24. Holmes, M. V., T. Simon, H. J. Exeter, et al. 2013. Secretory phospholipase A(2)-IIA and cardiovascular disease: a mendelian randomization study. *J. Am. Coll. Cardiol.* 62: 1966-1976.

25. Buckland, A. G. and D. C. Wilton. 2000. The antibacterial properties of secreted phospholipases A(2). *Biochim. Biophys. Acta* 1488: 71-82.

26. Laine, V. J., D. S. Grass, and T. J. Nevalainen. 1999. Protection by group II phospholipase A2 against Staphylococcus aureus. *J. Immunol.* 162: 7402-7408.

27. Laine, V. J., D. S. Grass, and T. J. Nevalainen. 2000. Resistance of transgenic mice expressing human group II phospholipase A2 to Escherichia coli infection. *Infect. Immun.* 68: 87-92.

28. Zeiher, B. G., J. Steingrub, P. F. Laterre, et al. 2005. LY315920NA/S-5920, a selective inhibitor of group IIA secretory phospholipase A2, fails to improve clinical outcome for patients with severe sepsis. *Crit. Care Med.* 33: 1741-1748.

29. Grass, D. S., R. H. Felkner, M. Y. Chiang, et al. 1996. Expression of human group II PLA2 in transgenic mice results in epidermal hyperplasia in the absence of inflammatory infiltrate. *J. Clin. Invest.* 97: 2233-2241.

30. Menschikowski, M., A. Hagelgans, and G. Siegert. 2006. Secretory phospholipase A2 of group IIA: is it an offensive or a defensive player during atherosclerosis and other inflammatory diseases? *Prostaglandins Other Lipid Mediat.* 79: 1-33.

31. Menschikowski, M., A. Hagelgans, B. Heyne, et al. 2005. Statins potentiate the IFN-gamma-induced upregulation of group IIA phospholipase A2 in human aortic smooth muscle cells and HepG2 hepatoma cells. *Biochim. Biophys. Acta* 1733: 157-171.

32. Liappis, A. P., V. L. Kan, C. G. Rochester, and G. L. Simon. 2001. The effect of statins on mortality in patients with bacteremia. *Clin. Infect. Dis.* 33: 1352-1357.

33. Bradley, J. D., A. A. Dmitrienko, A. J. Kivitz, et al. 2005. A randomized, double-blinded, placebo-controlled clinical trial of LY333013, a selective inhibitor of group II secretory phospholipase A2, in the treatment of rheumatoid arthritis. *J. Rheumatol.* 32: 417-423.

34. Rothblat, G. H. and M. C. Phillips. 2010. High-density lipoprotein heterogeneity and function in reverse cholesterol transport. *Curr. Opin. Lipidol.* 21: 229-238.

35. Birner-Gruenberger, R., M. Schittmayer, M. Holzer, and G. Marsche. 2014. Understanding high-density lipoprotein function in disease: Recent advances in proteomics unravel the complexity of its composition and biology. *Prog. Lipid Res.* 56C: 36-46.

36. Davidson, W. S., R. A. Silva, S. Chantepie, W. R. Lagor, M. J. Chapman, and A. Kontush. 2009. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. *Arterioscler. Thromb. Vasc. Biol.* 29: 870-876.

37. Sattler, K. and B. Levkau. 2009. Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. *Cardiovasc. Res.* 82: 201-211.

38. Fournier, N., J. L. Paul, V. Atger, et al. 1997. HDL phospholipid content and composition as a major factor determining cholesterol efflux capacity from Fu5AH cells to human serum. *Arterioscler. Thromb. Vasc. Biol.* 17: 2685-2691.

39. Baker, P. W., K. A. Rye, J. R. Gamble, M. A. Vadas, and P. J. Barter. 2000. Phospholipid composition of reconstituted high density lipoproteins influences their ability to inhibit endothelial cell adhesion molecule expression. *J. Lipid Res.* 41: 1261-1267.

40. Assmann, G., H. Schulte, A. von Eckardstein, and Y. Huang. 1996. High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM

experience and pathophysiological implications for reverse cholesterol transport. *Atherosclerosis* 124 Suppl: S11-20.

41. Mineo, C. and P. W. Shaul. 2012. Novel biological functions of high-density lipoprotein cholesterol. *Circ. Res.* 111: 1079-1090.

42. Cockerill, G. W., K. A. Rye, J. R. Gamble, M. A. Vadas, and P. J. Barter. 1995. High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules. *Arterioscler. Thromb. Vasc. Biol.* 15: 1987-1994.

43. Calabresi, L., G. Franceschini, C. R. Sirtori, et al. 1997. Inhibition of VCAM-1 expression in endothelial cells by reconstituted high density lipoproteins. *Biochem. Biophys. Res. Commun.* 238: 61-65.

44. Bursill, C. A., M. L. Castro, D. T. Beattie, et al. 2010. High-density lipoproteins suppress chemokines and chemokine receptors in vitro and in vivo. *Arterioscler. Thromb. Vasc. Biol.* 30: 1773-1778.

45. Gomaraschi, M., N. Basilico, F. Sisto, et al. 2005. High-density lipoproteins attenuate interleukin-6 production in endothelial cells exposed to pro-inflammatory stimuli. *Biochim. Biophys. Acta* 1736: 136-143.

46. Murphy, A. J., K. J. Woollard, A. Hoang, et al. 2008. High-density lipoprotein reduces the human monocyte inflammatory response. *Arterioscler. Thromb. Vasc. Biol.* 28: 2071-2077.

47. Murphy, A. J., K. J. Woollard, A. Suhartoyo, et al. 2011. Neutrophil activation is attenuated by high-density lipoprotein and apolipoprotein A-I in in vitro and in vivo models of inflammation. *Arterioscler. Thromb. Vasc. Biol.* 31: 1333-1341.

48. Nicholls, S. J., G. J. Dusting, B. Cutri, et al. 2005. Reconstituted high-density lipoproteins inhibit the acute pro-oxidant and proinflammatory vascular changes induced by a periarterial collar in normocholesterolemic rabbits. *Circulation* 111: 1543-1550.

49. Nofer, J. R. and G. Assmann. 2005. Atheroprotective effects of high-density lipoprotein-associated lysosphingolipids. *Trends Cardiovasc. Med.* 15: 265-271.

50. Recalde, D., M. A. Ostos, E. Badell, et al. 2004. Human apolipoprotein A-IV reduces secretion of proinflammatory cytokines and atherosclerotic effects of a chronic infection mimicked by lipopolysaccharide. *Arterioscler. Thromb. Vasc. Biol.* 24: 756-761.

51. Nevalainen, T. J. 1993. Serum phospholipases A2 in inflammatory diseases. *Clin. Chem.* 39: 2453-2459.

52. Lin, M. K., V. Farewell, P. Vadas, A. A. Bookman, E. C. Keystone, and W. Pruzanski. 1996. Secretory phospholipase A2 as an index of disease activity in rheumatoid arthritis. Prospective double blind study of 212 patients. *J. Rheumatol.* 23: 1162-1166.

53. Vadas, P. and W. Pruzanski. 1993. Induction of group II phospholipase A2 expression and pathogenesis of the sepsis syndrome. *Circ. Shock* 39: 160-167.

54. Gijon, M. A., C. Perez, E. Mendez, and M. Sanchez Crespo. 1995. Phospholipase A2 from plasma of patients with septic shock is associated with high-density lipoproteins and C3 anaphylatoxin: some implications for its functional role. *Biochem. J.* 306 (Pt 1): 167-175.

55. Dashti, M., W. Kulik, F. Hoek, E. C. Veerman, M. P. Peppelenbosch, and F. Rezaee. 2011. A phospholipidomic analysis of all defined human plasma lipoproteins. *Sci. Rep.* 1: 139.

56. Kaji, H. 2013. High-density lipoproteins and the immune system. *J. Lipids* 2013: 684903.

57. Borregaard, N. 2010. Neutrophils, from marrow to microbes. *Immunity* 33: 657-670.

58. Mestas, J. and C. C. Hughes. 2004. Of mice and not men: differences between mouse and human immunology. *J. Immunol.* 172: 2731-2738.

59. Bardoel, B. W., E. F. Kenny, G. Sollberger, and A. Zychlinsky. 2014. The balancing act of neutrophils. *Cell. Host Microbe* 15: 526-536.

60. Summers, C., S. M. Rankin, A. M. Condliffe, N. Singh, A. M. Peters, and E. R. Chilvers. 2010. Neutrophil kinetics in health and disease. *Trends Immunol.* 31: 318-324.

61. Mayadas, T. N., X. Cullere, and C. A. Lowell. 2014. The multifaceted functions of neutrophils. *Annu. Rev. Pathol.* 9: 181-218.

62. Mayadas, T. N. and X. Cullere. 2005. Neutrophil beta2 integrins: moderators of life or death decisions. *Trends Immunol.* 26: 388-395.

63. Schmidt, S., M. Moser, and M. Sperandio. 2013. The molecular basis of leukocyte recruitment and its deficiencies. *Mol. Immunol.* 55: 49-58.

64. Kobayashi, S. D., J. M. Voyich, C. Burlak, and F. R. DeLeo. 2005. Neutrophils in the innate immune response. *Arch. Immunol. Ther. Exp. (Warsz)* 53: 505-517.

65. Showell, H. J., R. J. Freer, S. H. Zigmond, et al. 1976. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal secretion for neutrophils. *J. Exp. Med.* 143: 1154-1169.

66. Petri, B., M. Phillipson, and P. Kubes. 2008. The physiology of leukocyte recruitment: an in vivo perspective. *J. Immunol.* 180: 6439-6446.

67. Lawrence, M. B. and T. A. Springer. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 65: 859-873.

68. Constantin, G., M. Majeed, C. Giagulli, et al. 2000. Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* 13: 759-769.

69. Kolaczkowska, E. and P. Kubes. 2013. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13: 159-175.

70. Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7: 678-689.

71. Phillipson, M. and P. Kubes. 2011. The neutrophil in vascular inflammation. *Nat. Med.* 17: 1381-1390.

72. Hager, M., J. B. Cowland, and N. Borregaard. 2010. Neutrophil granules in health and disease. *J. Intern. Med.* 268: 25-34.

73. Hakkim, A., B. G. Furnrohr, K. Amann, et al. 2010. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc. Natl. Acad. Sci. U. S. A.* 107: 9813-9818.

74. Wright, H. L., R. J. Moots, R. C. Bucknall, and S. W. Edwards. 2010. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology (Oxford)* 49: 1618-1631.

75. Brinkmann, V., U. Reichard, C. Goosmann, et al. 2004. Neutrophil extracellular traps kill bacteria. *Science* 303: 1532-1535.

76. Papayannopoulos, V. and A. Zychlinsky. 2009. NETs: a new strategy for using old weapons. *Trends Immunol.* 30: 513-521.

77. Papayannopoulos, V., K. D. Metzler, A. Hakkim, and A. Zychlinsky. 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J. Cell Biol.* 191: 677-691.

78. Metzler, K. D., T. A. Fuchs, W. M. Nauseef, et al. 2011. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood* 117: 953-959.

79. von Bruhl, M. L., K. Stark, A. Steinhart, et al. 2012. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J. Exp. Med.* 209: 819-835.

80. Caudrillier, A., K. Kessenbrock, B. M. Gilliss, et al. 2012. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J. Clin. Invest.* 122: 2661-2671.

81. Clark, S. R., A. C. Ma, S. A. Tavener, et al. 2007. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat. Med.* 13: 463-469.

82. Soehnlein, O. 2012. Multiple roles for neutrophils in atherosclerosis. *Circ. Res.* 110: 875-888.

83. Kessenbrock, K., M. Krumbholz, U. Schonermarck, et al. 2009. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat. Med.* 15: 623-625.

84. Knight, J. S. and M. J. Kaplan. 2012. Lupus neutrophils: 'NET' gain in understanding lupus pathogenesis. *Curr. Opin. Rheumatol.* 24: 441-450.

85. Fuchs, T. A., A. Brill, D. Duerschmied, et al. 2010. Extracellular DNA traps promote thrombosis. *Proc. Natl. Acad. Sci. U. S. A.* 107: 15880-15885.

86. Fuchs, T. A., A. A. Bhandari, and D. D. Wagner. 2011. Histones induce rapid and profound thrombocytopenia in mice. *Blood* 118: 3708-3714.

87. Semeraro, F., C. T. Ammollo, J. H. Morrissey, et al. 2011. Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood* 118: 1952-1961.

88. Welbourn, C. R. and Y. Young. 1992. Endotoxin, septic shock and acute lung injury: neutrophils, macrophages and inflammatory mediators. *Br. J. Surg.* 79: 998-1003.

89. Galkina, E. and K. Ley. 2009. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu. Rev. Immunol.* 27: 165-197.

90. Rotzius, P., S. Thams, O. Soehnlein, et al. 2010. Distinct infiltration of neutrophils in lesion shoulders in ApoE-/- mice. *Am. J. Pathol.* 177: 493-500.

91. Naruko, T., M. Ueda, K. Haze, et al. 2002. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* 106: 2894-2900.

92. Ionita, M. G., P. van den Borne, L. M. Catanzariti, et al. 2010. High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions. *Arterioscler. Thromb. Vasc. Biol.* 30: 1842-1848.

93. Dinerman, J. L., J. L. Mehta, T. G. Saldeen, et al. 1990. Increased neutrophil elastase release in unstable angina pectoris and acute myocardial infarction. *J. Am. Coll. Cardiol.* 15: 1559-1563.

94. Takeshita, S., T. Isshiki, M. Ochiai, et al. 1997. Systemic inflammatory responses in acute coronary syndrome: increased activity observed in polymorphonuclear leukocytes but not T lymphocytes. *Atherosclerosis* 135: 187-192.

95. Avanzas, P., R. Arroyo-Espliguero, J. Cosin-Sales, J. Quiles, E. Zouridakis, and J. C. Kaski. 2004. Multiple complex stenoses, high neutrophil count and C-reactive protein levels in patients with chronic stable angina. *Atherosclerosis* 175: 151-157.

96. Eriksson, E. E., X. Xie, J. Werr, P. Thoren, and L. Lindbom. 2001. Direct viewing of atherosclerosis in vivo: plaque invasion by leukocytes is initiated by the endothelial selectins. *FASEB J.* 15: 1149-1157.

97. Drechsler, M., R. T. Megens, M. van Zandvoort, C. Weber, and O. Soehnlein. 2010. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation* 122: 1837-1845.

98. Heller, E. A., E. Liu, A. M. Tager, et al. 2005. Inhibition of atherogenesis in BLT1-deficient mice reveals a role for LTB4 and BLT1 in smooth muscle cell recruitment. *Circulation* 112: 578-586.

99. Manthey, H. D., A. C. Thomas, I. A. Shiels, et al. 2011. Complement C5a inhibition reduces atherosclerosis in ApoE-/- mice. *FASEB J.* 25: 2447-2455.

100. Nicholls, S. J. and S. L. Hazen. 2005. Myeloperoxidase and cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.* 25: 1102-1111.

101. Chertov, O., H. Ueda, L. L. Xu, et al. 1997. Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J. Exp. Med.* 186: 739-747.

102. Chertov, O., D. F. Michiel, L. Xu, et al. 1996. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J. Biol. Chem.* 271: 2935-2940.

103. De, Y., Q. Chen, A. P. Schmidt, et al. 2000. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med.* 192: 1069-1074.

104. Soehnlein, O., Y. Kai-Larsen, R. Frithiof, et al. 2008. Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages. *J. Clin. Invest.* 118: 3491-3502.

105. Bdeir, K., W. Cane, G. Canziani, et al. 1999. Defensin promotes the binding of lipoprotein(a) to vascular matrix. *Blood* 94: 2007-2019.

106. Carr, A. C., M. R. McCall, and B. Frei. 2000. Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. *Arterioscler. Thromb. Vasc. Biol.* 20: 1716-1723.

107. Sugiyama, S., K. Kugiyama, M. Aikawa, S. Nakamura, H. Ogawa, and P. Libby. 2004. Hypochlorous acid, a macrophage product, induces endothelial apoptosis and tissue factor expression: involvement of myeloperoxidase-mediated oxidant in plaque erosion and thrombogenesis. *Arterioscler. Thromb. Vasc. Biol.* 24: 1309-1314.

108. Simons, K. and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1: 31-39.

109. Simons, K. and W. L. Vaz. 2004. Model systems, lipid rafts, and cell membranes. *Annu. Rev. Biophys. Biomol. Struct.* 33: 269-295.

110. George, K. S. and S. Wu. 2012. Lipid raft: A floating island of death or survival. *Toxicol. Appl. Pharmacol.* 259: 311-319.

111. Rajendran, L. and K. Simons. 2005. Lipid rafts and membrane dynamics. *J. Cell. Sci.* 118: 1099-1102.

112. Malchiodi-Albedi, F., V. Contrusciere, C. Raggi, et al. 2010. Lipid raft disruption protects mature neurons against amyloid oligomer toxicity. *Biochim. Biophys. Acta* 1802: 406-415.

113. Nakayama, H., H. Ogawa, K. Takamori, and K. Iwabuchi. 2013. GSL-enriched membrane microdomains in innate immune responses. *Arch. Immunol. Ther. Exp. (Warsz)* 61: 217-228.

114. Gomez-Mouton, C., R. A. Lacalle, E. Mira, et al. 2004. Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. *J. Cell Biol.* 164: 759-768.

115. Kindzelskii, A. L., R. G. Sitrin, and H. R. Petty. 2004. Cutting edge: optical microspectrophotometry supports the existence of gel phase lipid rafts at the lamellipodium of neutrophils: apparent role in calcium signaling. *J. Immunol.* 172: 4681-4685.

116. Pierini, L. M., R. J. Eddy, M. Fuortes, S. Seveau, C. Casulo, and F. R. Maxfield. 2003. Membrane lipid organization is critical for human neutrophil polarization. *J. Biol. Chem.* 278: 10831-10841.

117. Niggli, V., A. V. Meszaros, C. Oppliger, and S. Tornay. 2004. Impact of cholesterol depletion on shape changes, actin reorganization, and signal transduction in neutrophil-like HL-60 cells. *Exp. Cell Res.* 296: 358-368.

118. Abbal, C., M. Lambelet, D. Bertaggia, et al. 2006. Lipid raft adhesion receptors and Syk regulate selectin-dependent rolling under flow conditions. *Blood* 108: 3352-3359.

119. Barabe, F., G. Pare, M. J. Fernandes, S. G. Bourgoin, and P. H. Naccache. 2002. Cholesterol-modulating agents selectively inhibit calcium influx induced by chemoattractants in human neutrophils. *J. Biol. Chem.* 277: 13473-13478.

120. Guichard, C., E. Pedruzzi, C. Dewas, et al. 2005. Interleukin-8-induced priming of neutrophil oxidative burst requires sequential recruitment of NADPH oxidase components into lipid rafts. *J. Biol. Chem.* 280: 37021-37032.

121. Vilhardt, F. and B. van Deurs. 2004. The phagocyte NADPH oxidase depends on cholesterol-enriched membrane microdomains for assembly. *EMBO J.* 23: 739-748.

122. Shao, D., A. W. Segal, and L. V. Dekker. 2003. Lipid rafts determine efficiency of NADPH oxidase activation in neutrophils. *FEBS Lett.* 550: 101-106.

123. Koenig, W., C. Y. Vossen, Z. Mallat, H. Brenner, J. Benessiano, and D. Rothenbacher. 2009. Association between type II secretory phospholipase A2 plasma concentrations and activity and cardiovascular events in patients with coronary heart disease. *Eur. Heart J.* 30: 2742-2748.

124. Rosenson, R. S. and E. Hurt-Camejo. 2012. Phospholipase A2 enzymes and the risk of atherosclerosis. *Eur. Heart J.* 33: 2899-2909.

125. Abraham, E., C. Naum, V. Bandi, et al. 2003. Efficacy and safety of LY315920Na/S-5920, a selective inhibitor of 14-kDa group IIA secretory phospholipase A2, in patients with suspected sepsis and organ failure. *Crit. Care Med.* 31: 718-728.

126. Tietge, U. J., C. Maugeais, S. Lund-Katz, D. Grass, F. C. deBeer, and D. J. Rader. 2002. Human secretory phospholipase A2 mediates decreased plasma levels of HDL cholesterol and apoA-I in response to inflammation in human apoA-I transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* 22: 1213-1218.

127. Catapano, A. L., A. Pirillo, F. Bonacina, and G. D. Norata. 2014. HDL in innate and adaptive immunity. *Cardiovasc. Res.* 103: 372-383.

128. Donnelly, L. E. and P. J. Barnes. 2012. Defective phagocytosis in airways disease. *Chest* 141: 1055-1062.

129. Xiao, H., P. Heeringa, Z. Liu, et al. 2005. The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am. J. Pathol.* 167: 39-45.

130. Medzhitov, R. and C. Janeway Jr. 2000. Innate immunity. *N. Engl. J. Med.* 343: 338-344.

131. Guasti, L., F. Dentali, L. Castiglioni, et al. 2011. Neutrophils and clinical outcomes in patients with acute coronary syndromes and/or cardiac revascularisation. A systematic review on more than 34,000 subjects. *Thromb. Haemost.* 106: 591-599.

132. Holzer, M., M. Trieb, V. Konya, C. Wadsack, A. Heinemann, and G. Marsche. 2013. Aging affects high-density lipoprotein composition and function. *Biochim. Biophys. Acta* 1831: 1442-1448.

133. Holzer, M., K. Zangger, D. El-Gamal, et al. 2012. Myeloperoxidase-derived chlorinating species induce protein carbamylation through decomposition of thiocyanate and urea: novel pathways generating dysfunctional high-density lipoprotein. *Antioxid. Redox Signal.* 17: 1043-1052.

134. Holzer, M., P. Wolf, M. Inzinger, et al. 2014. Anti-psoriatic therapy recovers high-density lipoprotein composition and function. *J. Invest. Dermatol.* 134: 635-642.

135. BLIGH, E. G. and W. J. DYER. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911-917.

136. Schratl, P., J. F. Royer, E. Kostenis, et al. 2007. The role of the prostaglandin D2 receptor, DP, in eosinophil trafficking. *J. Immunol.* 179: 4792-4799.

137. Balenga, N. A., E. Aflaki, J. Kargl, et al. 2011. GPR55 regulates cannabinoid 2 receptor-mediated responses in human neutrophils. *Cell Res.* 21: 1452-1469.

138. Luschnig-Schratl, P., E. M. Sturm, V. Konya, et al. 2011. EP4 receptor stimulation down-regulates human eosinophil function. *Cell Mol. Life Sci.* 68: 3573-3587.

139. Sabroe, I., A. Hartnell, L. A. Jopling, et al. 1999. Differential regulation of eosinophil chemokine signaling via CCR3 and non-CCR3 pathways. *J. Immunol.* 162: 2946-2955.

140. Konya, V., S. Blattermann, K. Jandl, et al. 2014. A Biased Non-Galphai OXE-R Antagonist Demonstrates That Galphai Protein Subunit Is Not Directly Involved in Neutrophil, Eosinophil, and Monocyte Activation by 5-Oxo-ETE. *J. Immunol.* 192: 4774-4782.

141. Konya, V., S. Philipose, Z. Balint, et al. 2011. Interaction of eosinophils with endothelial cells is modulated by prostaglandin EP4 receptors. *Eur. J. Immunol.* 41: 2379-2389.

142. Sturm, G. J., R. Schuligoi, E. M. Sturm, et al. 2005. 5-Oxo-6,8,11,14-eicosatetraenoic acid is a potent chemoattractant for human basophils. *J. Allergy Clin. Immunol.* 116: 1014-1019.

143. Heinemann, A., M. Ofner, R. Amann, and B. A. Peskar. 2003. A novel assay to measure the calcium flux in human basophils: effects of chemokines and nerve growth factor. *Pharmacology* 67: 49-54.

144. Kirchner, T., S. Moller, M. Klinger, W. Solbach, T. Laskay, and M. Behnen. 2012. The impact of various reactive oxygen species on the formation of neutrophil extracellular traps. *Mediators Inflamm.* 2012: 849136.

145. Heinemann, A., G. J. Sturm, M. Ofner, et al. 2005. Stem cell factor stimulates the chemotaxis, integrin upregulation, and survival of human basophils. *J. Allergy Clin. Immunol.* 116: 820-826.

146. Takahashi, Y., M. Miyata, P. Zheng, T. Imazato, A. Horwitz, and J. D. Smith. 2000. Identification of cAMP analogue inducible genes in RAW264 macrophages. *Biochim. Biophys. Acta* 1492: 385-394.

147. Holzer, M., P. Wolf, S. Curcic, et al. 2012. Psoriasis alters HDL composition and cholesterol efflux capacity. *J. Lipid Res.* 53: 1618-1624.

148. Schratl, P. and A. Heinemann. 2009. Differential involvement of Ca2+ and actin filament in leukocyte shape change. *Pharmacology* 83: 131-140.

149. Doring, Y., M. Drechsler, S. Wantha, et al. 2012. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ. Res.* 110: 1052-1056.

150. Doring, Y., H. D. Manthey, M. Drechsler, et al. 2012. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. *Circulation* 125: 1673-1683.

151. Welch, E. J., R. P. Naikawadi, Z. Li, et al. 2009. Opposing effects of platelet-activating factor and lyso-platelet-activating factor on neutrophil and platelet activation. *Mol. Pharmacol.* 75: 227-234.

152. Peng, Y., W. Akmentin, M. A. Connelly, S. Lund-Katz, M. C. Phillips, and D. L. Williams. 2004. Scavenger receptor BI (SR-BI) clustered on microvillar extensions suggests that this plasma membrane domain is a way station for cholesterol trafficking between cells and high-density lipoprotein. *Mol. Biol. Cell* 15: 384-396.

153. Drobnik, W., H. Borsukova, A. Bottcher, et al. 2002. Apo AI/ABCA1-dependent and HDL3-mediated lipid efflux from compositionally distinct cholesterol-based microdomains. *Traffic* 3: 268-278.

154. Favari, E., I. Zanotti, F. Zimetti, N. Ronda, F. Bernini, and G. H. Rothblat. 2004. Probucol inhibits ABCA1-mediated cellular lipid efflux. *Arterioscler. Thromb. Vasc. Biol.* 24: 2345-2350.

155. Nieland, T. J., M. Penman, L. Dori, M. Krieger, and T. Kirchhausen. 2002. Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI. *Proc. Natl. Acad. Sci. U. S. A.* 99: 15422-15427.

156. Curcic, S., M. Holzer, R. Frei, et al. 2014. Neutrophil effector responses are suppressed by secretory phospholipase A modified HDL. *Biochim. Biophys. Acta* 1851: 184-193.

157. Simons, K. and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature* 387: 569-572.

158. Feig, J. E., R. Shamir, and E. A. Fisher. 2008. Atheroprotective effects of HDL: beyond reverse cholesterol transport. *Curr. Drug Targets* 9: 196-203.

159. Pruzanski, W., L. Lambeau, M. Lazdunsky, W. Cho, J. Kopilov, and A. Kuksis. 2005. Differential hydrolysis of molecular species of lipoprotein phosphatidylcholine by groups IIA, V and X secretory phospholipases A2. *Biochim. Biophys. Acta* 1736: 38-50.

160. Tanaka, T., R. Sano, Y. Yamashita, and M. Yamazaki. 2004. Shape changes and vesicle fission of giant unilamellar vesicles of liquid-ordered phase membrane induced by lysophosphatidylcholine. *Langmuir* 20: 9526-9534.

161. Hara, S., T. Shike, N. Takasu, and T. Mizui. 1997. Lysophosphatidylcholine promotes cholesterol efflux from mouse macrophage foam cells. *Arterioscler. Thromb. Vasc. Biol.* 17: 1258-1266.

162. Levels, J. H., D. Pajkrt, M. Schultz, et al. 2007. Alterations in lipoprotein homeostasis during human experimental endotoxemia and clinical sepsis. *Biochim. Biophys. Acta* 1771: 1429-1438.

163. Drew, B. G., A. L. Carey, A. K. Natoli, et al. 2011. Reconstituted high-density lipoprotein infusion modulates fatty acid metabolism in patients with type 2 diabetes mellitus. *J. Lipid Res.* 52: 572-581.

164. O'Donoghue, M. L., E. Braunwald, H. D. White, et al. 2014. Effect of darapladib on major coronary events after an acute coronary syndrome: the SOLID-TIMI 52 randomized clinical trial. *JAMA* 312: 1006-1015.

165. Masucci-Magoulas, L., P. Moulin, X. C. Jiang, et al. 1995. Decreased cholesteryl ester transfer protein (CETP) mRNA and protein and increased high density lipoprotein following lipopolysaccharide administration in human CETP transgenic mice. *J. Clin. Invest.* 95: 1587-1594.

166. Artl, A., G. Marsche, S. Lestavel, W. Sattler, and E. Malle. 2000. Role of serum amyloid A during metabolism of acute-phase HDL by macrophages. *Arterioscler. Thromb. Vasc. Biol.* 20: 763-772.

167. Hosoai, H., N. R. Webb, J. M. Glick, et al. 1999. Expression of serum amyloid A protein in the absence of the acute phase response does not reduce HDL cholesterol or apoA-I levels in human apoA-I transgenic mice. *J. Lipid Res.* 40: 648-653.

168. Tietge, U. J., C. Maugeais, W. Cain, et al. 2000. Overexpression of secretory phospholipase A(2) causes rapid catabolism and altered tissue uptake of high density lipoprotein cholesteryl ester and apolipoprotein A-I. *J. Biol. Chem.* 275: 10077-10084.

169. PEASE, D. C. 1956. An electron microscopic study of red bone marrow. *Blood* 11: 501-526.

170. Machlus, K. R. and J. E. Italiano Jr. 2013. The incredible journey: From megakaryocyte development to platelet formation. *J. Cell Biol.* 201: 785-796.

171. Harker, L. A. and C. A. Finch. 1969. Thrombokinetics in man. *J. Clin. Invest.* 48: 963-974.

172. Ruggeri, Z. M. 2002. Platelets in atherothrombosis. *Nat. Med.* 8: 1227-1234.

173. Jackson, S. P., W. S. Nesbitt, and S. Kulkarni. 2003. Signaling events underlying thrombus formation. *J. Thromb. Haemost.* 1: 1602-1612.

174. Gawaz, M. 2004. Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. *Cardiovasc. Res.* 61: 498-511.

175. Lievens, D. and P. von Hundelshausen. 2011. Platelets in atherosclerosis. *Thromb. Haemost.* 106: 827-838.

176. Hansson, G. K. and P. Libby. 2006. The immune response in atherosclerosis: a double-edged sword. *Nat. Rev. Immunol.* 6: 508-519.

177. Bone, R. C., R. A. Balk, F. B. Cerra, et al. 2009. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. 1992. *Chest* 136: e28.

178. Smith, T. L. and A. S. Weyrich. 2011. Platelets as central mediators of systemic inflammatory responses. *Thromb. Res.* 127: 391-394.

179. Yost, C. C., A. S. Weyrich, and G. A. Zimmerman. 2010. The platelet activating factor (PAF) signaling cascade in systemic inflammatory responses. *Biochimie* 92: 692-697.

180. Andonegui, G., S. M. Kerfoot, K. McNagny, K. V. Ebbert, K. D. Patel, and P. Kubes. 2005. Platelets express functional Toll-like receptor-4. *Blood* 106: 2417-2423.

181. Zimmerman, G. A. 2001. Two by two: the pairings of P-selectin and P-selectin glycoprotein ligand 1. *Proc. Natl. Acad. Sci. U. S. A.* 98: 10023-10024.

182. Gawaz, M., S. Fateh-Moghadam, G. Pilz, H. J. Gurland, and K. Werdan. 1995. Platelet activation and interaction with leucocytes in patients with sepsis or multiple organ failure. *Eur. J. Clin. Invest.* 25: 843-851.

183. Zarbock, A., K. Singbartl, and K. Ley. 2006. Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. *J. Clin. Invest.* 116: 3211-3219.

184. Weyrich, A. S., M. R. Elstad, R. P. McEver, et al. 1996. Activated platelets signal chemokine synthesis by human monocytes. *J. Clin. Invest.* 97: 1525-1534.

185. Dong, Z. M., S. M. Chapman, A. A. Brown, P. S. Frenette, R. O. Hynes, and D. D. Wagner. 1998. The combined role of P- and E-selectins in atherosclerosis. *J. Clin. Invest.* 102: 145-152.

186. Dong, Z. M., A. A. Brown, and D. D. Wagner. 2000. Prominent role of P-selectin in the development of advanced atherosclerosis in ApoE-deficient mice. *Circulation* 101: 2290-2295.

187. Burger, P. C. and D. D. Wagner. 2003. Platelet P-selectin facilitates atherosclerotic lesion development. *Blood* 101: 2661-2666.

188. Massberg, S., K. Brand, S. Gruner, et al. 2002. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J. Exp. Med.* 196: 887-896.

189. Fateh-Moghadam, S., Z. Li, S. Ersel, et al. 2005. Platelet degranulation is associated with progression of intima-media thickness of the common carotid artery in patients with diabetes mellitus type 2. *Arterioscler. Thromb. Vasc. Biol.* 25: 1299-1303.

190. Fateh-Moghadam, S., W. Bocksch, A. Ruf, et al. 2000. Changes in surface expression of platelet membrane glycoproteins and progression of heart transplant vasculopathy. *Circulation* 102: 890-897.

191. Koyama, H., T. Maeno, S. Fukumoto, et al. 2003. Platelet P-selectin expression is associated with atherosclerotic wall thickness in carotid artery in humans. *Circulation* 108: 524-529.

192. Lindemann, S., B. Kramer, P. Seizer, and M. Gawaz. 2007. Platelets, inflammation and atherosclerosis. *J. Thromb. Haemost.* 5 Suppl 1: 203-211.

193. Gawaz, M., F. J. Neumann, T. Dickfeld, et al. 1998. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. *Circulation* 98: 1164-1171.

194. Gawaz, M., S. Page, S. Massberg, et al. 2002. Transient platelet interaction induces MCP-1 production by endothelial cells via I kappa B kinase complex activation. *Thromb. Haemost.* 88: 307-314.

195. Gawaz, M., K. Brand, T. Dickfeld, et al. 2000. Platelets induce alterations of chemotactic and adhesive properties of endothelial cells mediated through an interleukin-1-dependent mechanism. Implications for atherogenesis. *Atherosclerosis* 148: 75-85.

196. Henn, V., J. R. Slupsky, M. Grafe, et al. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391: 591-594.

197. von Hundelshausen, P., K. S. Weber, Y. Huo, et al. 2001. RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. *Circulation* 103: 1772-1777.

198. von Hundelshausen, P., R. R. Koenen, M. Sack, et al. 2005. Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood* 105: 924-930.

199. Scheuerer, B., M. Ernst, I. Durrbaum-Landmann, et al. 2000. The CXC-chemokine platelet factor 4 promotes monocyte survival and induces monocyte differentiation into macrophages. *Blood* 95: 1158-1166.

200. Nassar, T., B. S. Sachais, S. Akkawi, et al. 2003. Platelet factor 4 enhances the binding of oxidized low-density lipoprotein to vascular wall cells. *J. Biol. Chem.* 278: 6187-6193.

201. Wagner, D. D. and P. C. Burger. 2003. Platelets in inflammation and thrombosis. *Arterioscler. Thromb. Vasc. Biol.* 23: 2131-2137.

202. Gawaz, M., H. Langer, and A. E. May. 2005. Platelets in inflammation and atherogenesis. *J. Clin. Invest.* 115: 3378-3384.

203. Daub, K., H. Langer, P. Seizer, et al. 2006. Platelets induce differentiation of human CD34+ progenitor cells into foam cells and endothelial cells. *FASEB J.* 20: 2559-2561.

204. Surya, I. I. and J. W. Akkerman. 1993. The influence of lipoproteins on blood platelets. *Am. Heart J.* 125: 272-275.

205. Siegel-Axel, D., K. Daub, P. Seizer, S. Lindemann, and M. Gawaz. 2008. Platelet lipoprotein interplay: trigger of foam cell formation and driver of atherosclerosis. *Cardiovasc. Res.* 78: 8-17.

206. Betteridge, D. J., M. B. Cooper, E. D. Saggerson, et al. 1994. Platelet function in patients with hypercholesterolaemia. *Eur. J. Clin. Invest.* 24 Suppl 1: 30-33.

207. Aviram, M. and J. G. Brook. 1987. Platelet activation by plasma lipoproteins. *Prog. Cardiovasc. Dis.* 30: 61-72.

208. Koller, E., F. Koller, and W. Doleschel. 1982. Specific binding sites on human blood platelets for plasma lipoproteins. *Hoppe Seylers Z. Physiol. Chem.* 363: 395-405.

209. Curtiss, L. K. and E. F. Plow. 1984. Interaction of plasma lipoproteins with human platelets. *Blood* 64: 365-374.

210. Relou, I. A., C. M. Hackeng, J. W. Akkerman, and E. Malle. 2003. Low-density lipoprotein and its effect on human blood platelets. *Cell Mol. Life Sci.* 60: 961-971.

211. Korporaal, S. J., I. A. Relou, M. van Eck, et al. 2004. Binding of low density lipoprotein to platelet apolipoprotein E receptor 2' results in phosphorylation of p38MAPK. *J. Biol. Chem.* 279: 52526-52534.

212. Volf, I., T. Moeslinger, J. Cooper, W. Schmid, and E. Koller. 1999. Human platelets exclusively bind oxidized low density lipoprotein showing no specificity for acetylated low density lipoprotein. *FEBS Lett.* 449: 141-145.

213. Nofer, J. R., M. Walter, B. Kehrel, et al. 1998. HDL3-mediated inhibition of thrombin-induced platelet aggregation and fibrinogen binding occurs via decreased production of phosphoinositide-derived second messengers 1,2-diacylglycerol and inositol 1,4,5-tris-phosphate. *Arterioscler. Thromb. Vasc. Biol.* 18: 861-869.

214. Higashihara, M., M. Kinoshita, S. Kume, T. Teramoto, and K. Kurokawa. 1991. Inhibition of platelet function by high-density lipoprotein from a patient with apolipoprotein E deficiency. *Biochem. Biophys. Res. Commun.* 181: 1331-1336.

215. Fujimoto, Y., M. Tsunomori, E. Muta, et al. 1994. High density lipoprotein inhibits platelet 12-lipoxygenase activity. *Res. Commun. Mol. Pathol. Pharmacol.* 85: 355-358.

216. Valiyaveettil, M., N. Kar, M. Z. Ashraf, T. V. Byzova, M. Febbraio, and E. A. Podrez. 2008. Oxidized high-density lipoprotein inhibits platelet activation and aggregation via scavenger receptor BI. *Blood* 111: 1962-1971.

217. Beitz, J. and H. J. Mest. 1986. Thromboxane A2 (TXA2) formation by washed human platelets under the influence of low and high density lipoproteins from healthy donors. *Prostaglandins Leukot. Med.* 23: 303-309.

218. Hassall, D. G., J. S. Owen, and K. R. Bruckdorfer. 1983. The aggregation of isolated human platelets in the presence of lipoproteins and prostacyclin. *Biochem. J.* 216: 43-49.

219. Nofer, J. R., M. F. Brodde, and B. E. Kehrel. 2010. High-density lipoproteins, platelets and the pathogenesis of atherosclerosis. *Clin. Exp. Pharmacol. Physiol.* 37: 726-735.

220. Naqvi, T. Z., P. K. Shah, P. A. Ivey, et al. 1999. Evidence that high-density lipoprotein cholesterol is an independent predictor of acute platelet-dependent thrombus formation. *Am. J. Cardiol.* 84: 1011-1017.

221. Li, D., S. Weng, B. Yang, et al. 1999. Inhibition of arterial thrombus formation by ApoA1 Milano. *Arterioscler. Thromb. Vasc. Biol.* 19: 378-383.

222. Calkin, A. C., B. G. Drew, A. Ono, et al. 2009. Reconstituted high-density lipoprotein attenuates platelet function in individuals with type 2 diabetes mellitus by promoting cholesterol efflux. *Circulation* 120: 2095-2104.

223. Deguchi, H., N. M. Pecheniuk, D. J. Elias, P. M. Averell, and J. H. Griffin. 2005. High-density lipoprotein deficiency and dyslipoproteinemia associated with venous thrombosis in men. *Circulation* 112: 893-899.

224. Eichinger, S., N. M. Pecheniuk, G. Hron, et al. 2007. High-density lipoprotein and the risk of recurrent venous thromboembolism. *Circulation* 115: 1609-1614.

225. Uittenbogaard, A., P. W. Shaul, I. S. Yuhanna, A. Blair, and E. J. Smart. 2000. High density lipoprotein prevents oxidized low density lipoprotein-induced inhibition of endothelial nitric-oxide synthase localization and activation in caveolae. *J. Biol. Chem.* 275: 11278-11283.

226. Fleisher, L. N., A. R. Tall, L. D. Witte, R. W. Miller, and P. J. Cannon. 1982. Stimulation of arterial endothelial cell prostacyclin synthesis by high density lipoproteins. *J. Biol. Chem.* 257: 6653-6655.

227. Brodde, M. F., S. J. Korporaal, G. Herminghaus, et al. 2011. Native high-density lipoproteins inhibit platelet activation via scavenger receptor BI: role of negatively charged phospholipids. *Atherosclerosis* 215: 374-382.

228. Assinger, A., W. Schmid, S. Eder, D. Schmid, E. Koller, and I. Volf. 2008. Oxidation by hypochlorite converts protective HDL into a potent platelet agonist. *FEBS Lett.* 582: 778-784.

229. Bodin, S., H. Tronchere, and B. Payrastre. 2003. Lipid rafts are critical membrane domains in blood platelet activation processes. *Biochim. Biophys. Acta* 1610: 247-257.

230. Bodin, S., S. Giuriato, J. Ragab, et al. 2001. Production of phosphatidylinositol 3,4,5-trisphosphate and phosphatidic acid in platelet rafts: evidence for a critical role of cholesterol-enriched domains in human platelet activation. *Biochemistry* 40: 15290-15299.

231. Locke, D., H. Chen, Y. Liu, C. Liu, and M. L. Kahn. 2002. Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI. *J. Biol. Chem.* 277: 18801-18809.

232. Boesze-Battaglia, K., S. T. Clayton, and R. J. Schimmel. 1996. Cholesterol redistribution within human platelet plasma membrane: evidence for a stimulus-dependent event. *Biochemistry* 35: 6664-6673.

233. von Hundelshausen, P. and C. Weber. 2007. Platelets as immune cells: bridging inflammation and cardiovascular disease. *Circ. Res.* 100: 27-40.

234. Russwurm, S., J. Vickers, A. Meier-Hellmann, et al. 2002. Platelet and leukocyte activation correlate with the severity of septic organ dysfunction. *Shock* 17: 263-268.

235. Vincent, J. L., A. Yagushi, and O. Pradier. 2002. Platelet function in sepsis. *Crit. Care Med.* 30: S313-7.

236. Zarbock, A., R. K. Polanowska-Grabowska, and K. Ley. 2007. Platelet-neutrophil-interactions: linking hemostasis and inflammation. *Blood Rev.* 21: 99-111.

237. Schuligoi, R., R. Schmidt, G. Geisslinger, M. Kollroser, B. A. Peskar, and A. Heinemann. 2007. PGD2 metabolism in plasma: kinetics and relationship with bioactivity on DP1 and CRTH2 receptors. *Biochem. Pharmacol.* 74: 107-117.

238. Philipose, S., V. Konya, I. Sreckovic, et al. 2010. The prostaglandin E2 receptor EP4 is expressed by human platelets and potently inhibits platelet aggregation and thrombus formation. *Arterioscler. Thromb. Vasc. Biol.* 30: 2416-2423.

239. Curvers, J., J. de Wildt-Eggen, J. Heeremans, J. Scharenberg, D. de Korte, and P. F. van der Meer. 2008. Flow cytometric measurement of CD62P (P-selectin) expression on platelets: a multicenter optimization and standardization effort. *Transfusion* 48: 1439-1446.

240. Rossi, F., E. Rossi, F. I. Pareti, S. Colli, E. Tremoli, and L. Gallo. 2001. In vitro measurement of platelet glycoprotein IIb/IIIa receptor blockade by abciximab: interindividual variation and increased platelet secretion. *Haematologica* 86: 192-198.

241. Bakdash, N. and M. S. Williams. 2008. Spatially distinct production of reactive oxygen species regulates platelet activation. *Free Radic. Biol. Med.* 45: 158-166.

242. Krotz, F., H. Y. Sohn, T. Gloe, et al. 2002. NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood* 100: 917-924.

243. Bergmeier, W. and L. Stefanini. 2009. Novel molecules in calcium signaling in platelets. *J. Thromb. Haemost.* 7 Suppl 1: 187-190.

244. DiMinno, G., M. J. Silver, A. M. Cerbone, A. Rainone, A. Postiglione, and M. Mancini. 1986. Increased fibrinogen binding to platelets from patients with familial hypercholesterolemia. *Arteriosclerosis* 6: 203-211.

245. Drobnik, W., G. Liebisch, F. X. Audebert, et al. 2003. Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. *J. Lipid Res.* 44: 754-761.

246. Yan, J. J., J. S. Jung, J. E. Lee, et al. 2004. Therapeutic effects of lysophosphatidylcholine in experimental sepsis. *Nat. Med.* 10: 161-167.

CURRICULUM VITAE

Sanja Curcic

Personal Information

Date of birth	16 July 1986, Novi Sad, Serbia
Affiliation	Institute for Experimental and Clinical Pharmacology Medical University of Graz Universitätsplatz 4 A-8010 Graz, Austria Tel: 43 (316) 380 4307

Education

2005-2010	Pharmacy studies, University of Novi Sad, Faculty of Medicine, Department of pharmacy
2010-2011	Internship in a pharmacy, Novi Sad, Serbia
2011-2015	Ph.D. program in Molecular Medicine Institute for Experimental and Clinical Pharmacology, Medical University of Graz, Austria

Awards

2013	Poster Prize at the Doctoral Day Medical University of Graz, Austria
2005-2010	Awarded by the University of Novi Sad, Serbia, for the success achieved in 2005/6, 2006/7, 2007/8, 2008/9, 2009/10 academic years

Publications

- Curcic S, Holzer M, Frei R, Pasterk L, Schicho R, Heinemann A, Marsche G. Neutrophil effector responses are suppressed by secretory phospholipase A_2 modified HDL. Biochim Biophys Acta. 2014;
- Holzer M, PhD, Schilcher G, Curcic S, Trieb M, Ljubojevic S, Stojakovic T, Scharnagl H, Kopecky C, Rosenkranz AR, Heinemann A, Marsche G. Dialysis modalities and HDL composition and function. JASN.2014;

- Holzer M, Wolf P, Inzinger M, Trieb M, Curcic S, Pasterk L, Weger W, Heinemann A, Marsche G. Anti-psoriatic therapy recovers high-density lipoprotein composition and function. J Invest Dermatol. 2014; 134(3):635-642
- Holzer M, Wolf P, Curcic S, Birner-Gruenberger R, Weger W, Inzinger M, El-Gamal D, Wadsack C, Heinemann A, Marsche G. Psoriasis alters HDL composition and cholesterol efflux capacity. J Lipid Res. 2012; 53(8):1618-1624
- Holzer M, Zangger K, El-Gamal D, Binder V, Curcic S, Konya V, Schuligoi R, Heinemann A, Marsche G. Myeloperoxidase-derived chlorinating species induce protein carbamylation through decomposition of thiocyanate and urea: novel pathways generating dysfunctional high-density lipoprotein. Antioxid Redox Signal. 2012; 17(8):1043-1052

Presentations

- Sanja Curcic, Michael Holzer, Robert Frei, Lisa Pasterk, Akos Heinemann and Gunther Marsche. Secretory phospholipase A_2 modified HDL fully suppresses neutrophil effector responses: Central role of palmitoyl-lysophosphatydilcholine Doctoral Day 2014; DEZ 10, 2014; Graz, AUSTRIA. [Poster]
- Sanja Curcic, Michael Holzer, Robert Frei, Lisa Pasterk, Akos Heinemann and Gunther Marsche. Secretory phospholipase A_2 modified HDL fully suppresses neutrophil effector responses: Central role of palmitoyl-lysophosphatydilcholine 20th Scientific Symposium of the Austrian Pharmacological Society (APHAR); SEP 26-27, 2014; Innsbruck, Austria. [Poster]
- Sanja Curcic, Michael Holzer, Robert Frei, Lisa Pasterk, Akos Heinemann and Gunther Marsche. Secretory phospholipase A_2 modified HDL shows potent anti-inflammatory effects on neutrophils ZMF (Center for Medical Research) 10th Anniversary; JUL 17-18, 2014; Graz, AUSTRIA. [Poster]
- Sanja Curcic, Michael Holzer, Robert Frei, Lisa Pasterk, Akos Heinemann and Gunther Marsche . Secretory phospholipase A_2 modified HDL fully suppresses neutrophil effector responses: Central role of palmitoyl-lysophosphatydilcholine 4th European Lipidomic Meeting; SEP 22-24, 2014; Graz, AUSTRIA. [Poster]

- Curcic, S; Holzer, M; Frank, S; Frei, R; Heinemann, A; Marsche, G; . Secretory phospholipase A_2 drives reprogramming of neutrophils Doctoral Day 2013; DEZ 9, 2013; Graz, AUSTRIA. [Poster]
- Curcic, S; Holzer, M; Heinemann, A; Marsche, G. Acute-phase secretory phospholipases have a potent antiinflammatory activity. Joint Meeting of the Austrian Neuroscience Association (13th ANA Meeting) and the Austrian Pharmacological Society (19th Scientific Symposium of APHAR); SEP 16-19, 2013; Vienna, AUSTRIA. [Oral Communication]
- Curcic, S;Holzer, M; Schilcher, G; Rosenkranz, A; Stojakovic,T;Heinemann, A; Marsche, G; . Chronic kidney disease alters anti-inflammatory properties of HDL. Doctoral Day 2012; Dec 7, 2012; Graz, AUSTRIA. [Oral Communication]