Dissertation

The impact of advanced oxidized protein products on platelets and platelet-endothelial crosstalk

submitted by

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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 organizations 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, 15.8.2016

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig angefertigt und abgefasst, und jene Personen und Institutionen, die am Zustandekommen der Forschungsdaten beteiligt waren, namentlich genannt habe. Andere als die angegebenen Quellen habe ich nicht verwendet und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen habe ich als solche kenntlich gemacht. Die Arbeit an der Dissertation und daraus entstandener Publikationen wurde gemäß den Regeln der „Good Scientific Practice“ durchgeführt.

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Summary

Platelets patrol at high numbers in the blood stream to fulfil their essential function in primary hemostasis in times of vessel stress. While platelet function is beneficial on the one side, activated platelets also participate as key players in thrombosis, making inappropriate platelet function a major health issue. The multifaceted role of platelets was underestimated for a long time, but now their important role in inflammation as well as their contribution in all stages of atherosclerosis is more frequently perceived and investigated. Platelets are involved in inflammation by releasing their pro-coagulant and proinflammatory granule content at the site of inflammation and actively participate by directly interacting with endothelial cells and/or neutrophils. By doing so, platelets are able to trigger cell activation or amplify activation dependent cell functions. Individuals with increased platelet reactivity are at a higher potential risk for thrombosis which can lead to complete vessel occlusion and severe outcomes such as myocardial infarction and stroke. Several inflammatory diseases are associated with high levels of oxidative stress and increased platelet functionality. Moreover, there is a direct link between inflammatory diseases and high prevalence of thrombotic events.

During inflammation, activated neutrophils release myeloperoxidase (MPO), the only enzyme that is able to form the oxidant hypochlorous acid (HOCl). Of note, albumin which is the most abundant protein in the vasculature, is the primary target of HOCl, with limited damage to other materials. HOCl-modified albumin was shown to present the major fraction of advanced protein products (AOPPs), which accumulate in several inflammatory diseases, such as renal disease, and are markers for oxidative stress and inflammation. Whether AOPPs contribute to coagulation abnormalities which are frequently seen in uremic patients, has not been elucidated so far. In our study we could show a novel role for AOPPs as potent activators of platelet function. In the present study we revealed several AOPPs mediated changes on platelets, such as an increase in platelet adhesion molecules (P-selectin, phosphatidylserine and cluster of differentiation 40 ligand (CD40L) as a result of platelet granule release and protein integration in the outer platelet membrane. Since AOPPs induced platelet aggregation and P-selectin surface

expression could be considerably reduced when interfering with cluster of differentiation 36 (CD36) receptor binding, we can clearly argue that CD36 is the main platelet receptor facilitating the interactions between platelets and AOPPs. In this thesis project we elucidated that platelets upon AOPPs exposure were activated via a signalling cascade involving phospholipase C (PLC), phosphokinase C (PKC) and Calcium (Ca^{2+}) mobilisation as well as production of intracellular reactive oxygen species (ROS). By acting on platelets, AOPPs induce platelet-endothelial interactions as indicated by increased platelet adhesion to cultured human coronary artery endothelial cells (HCAECs) and, of note, endothelial tissue factor (TF) expression results from this cell-cell crosstalk.

Moreover, results obtained with *in vitro* modified AOPPs could be translated in a physiological relevant context, as AOPPs isolated from sera of uremic patients enhanced platelet aggregation dependent on their levels of oxidative modifications in a CD36 dependent manner.

In our study cohort of end stage renal disease patients on hemodialysis, AOPPs and serum TF levels were significantly increased. Notably, a significant correlation of AOPPs and serum TF was found, suggesting that platelets link oxidative stress and a prothrombotic phenotype.

Our results provide clear evidence that AOPPs formed in conditions underlying chronic oxidative stress can contribute to increased platelet reactivity. The resulting procoagulant phenotype on endothelial cells might contribute to the coagulation abnormalities in uremia and other inflammatory diseases. Our findings reveal previously unknown pro-thrombotic activities of oxidized albumin.

Zusammenfassung

Thrombozyten zirkulieren in hoher Anzahl im Blut, wo sie ihrer essentiellen Funktion in der primären Hämostase nachgehen. Während die Thrombozytenfunktion einerseits physiologisch relevant ist, sind aktivierte Thrombozyten auch in Thrombosen involviert. Eine erhöhte Thrombozytenaktivierung stellt somit auch ein großes gesundheitliches Problem dar. Personen mit erhöhter Thrombozytenaktivierung haben ein erhöhtes potenzielles Risiko für Thrombosen, die zu Gefäßverschluss und schweren Folgeerkrankungen, wie Herzinfarkt und Schlaganfall führen können. Verschiedene entzündliche Erkrankungen sind mit hohem oxidativen Stress und erhöhter Thrombozytenfunktionalität verbunden. Darüber hinaus gibt es eine direkte Verbindung zwischen entzündlichen Erkrankungen und einer hohen Prävalenz von thrombotischen Ereignissen. Die vielfältige Rolle der Thrombozyten wurde lange Zeit unterschätzt, aber ihre wichtige Rolle bei der Entzündungsreaktion, sowie ihr Beitrag in allen Stadien der Atherosklerose, wird nun häufig wahrgenommen und untersucht. Durch die Freisetzung ihrer prokoagulanten und proinflammatorischen Granula am Entzündungsherd, und auch ihre direkte Interaktion mit Endothelzellen und/oder neutrophilen Granulozyten, die zur Zellaktivierung oder Verstärkung von aktivierungsabhängigen Zellfunktionen führt, sind Thrombozyten ein wesentlicher Faktor bei Entzündungen.

Neutrophile Granulozyten sind die Hauptquelle des Enzyms Myeloperoxidase, das einzige Enzym, das das Oxidationsmittel Hypochlorit herstellen kann. Eine aktivierende Rolle von Hypochlorit modifiziertem Lipoproteinen niedriger Dichte (LDL) oder Lipoproteinen hoher Dichte (HDL) auf Thrombozyten, die über den Scavenger-Rezeptor CD36 vermittelt wird, ist bereits beschrieben worden. Im Gegensatz dazu ist der Einfluss modifizierter Proteine auf Thrombozyten bisher nicht geklärt. Albumin ist das häufigste Protein im Blutkreislauf, und auch das Angriffsziel von Hypochlorit, das kaum andere Substanzen angreift. In unserer Studie konnten wir zeigen, dass Hypochlorit oxidiertes Albumin, sogenannte “Advanced Oxidized Protein Products“, Thrombozyten aktivieren können.

AOPPs akkumulieren bei verschiedenen Entzündungserkrankungen, wie z.B. im Plasma von Patienten mit Nierenerkrankungen, und sind Marker für oxidativen Stress. Ob AOPPs zu den bei urämischen Patienten häufig auftretenden Gerinnungsstörungen beitragen, wurde bisher nicht untersucht.

In der vorliegenden Studie zeigen wir, dass AOPPs eine Erhöhung der Thrombozyten Adhäsionsmoleküle (P-Selektin, Phosphatidylserin und CD40L) induzieren. Dies resultiert aus der aktivierungsabhängigen Freisetzung von Thrombozytengranula und darauffolgender Proteinintegration in die äußere Thrombozytenmembran. Die durch AOPPs ausgelöste Thrombozytenaggregation und P-Selektin-Oberflächenexpression konnten erheblich vermindert werden, wenn der Scavenger-Rezeptor CD36auf Thrombozyten geblockt wurde. Daraus lässt sich klar schließen, dass CD36 ein entscheidender Rezeptor für die Interaktion zwischen Thrombozyten und AOPPs ist.

In der vorliegenden Arbeit konnten wir die Signalkaskade, über die AOPPs Thrombozyten aktivieren, aufklären. Sie beinhaltet die Aktivierung von Phospholipase C, Phosphokinase C und Ca^{2+} Mobilisierung, sowie die intrazelluläre Produktion reaktiver Sauerstoffspezies. Durch ihre Wirkung auf Thrombozyten induzierten AOPPs Wechselwirkungen zwischen Thrombozyten und Endothelzellen, die durch eine erhöhte Thrombozytenadhesion an kultivierten humanen Endothelzellen gezeigt werden konnte. Von großer Bedeutung ist auch die Expression von endothelialem Gewebsfaktor, der aus dieser Zell-Zell-Interaktion resultiert. AOPPs, die aus dem Serum von uremischen Patienten isoliert wurden, konnten die Thrombozytenaggregation abhängig von ihrer oxidativen Modifikation und dem CD36 Rezeptor verstärken. Die Resultate, die mit *in vitro* modifizierten AOPPs erzielt wurden, konnten somit in einen physiologisch relevanten Kontext gesetzt werden.

AOPPs und Gewebsfaktor waren im Serum von Patienten mit Nierenerkrankung signifikant erhöht. Interessant dabei ist die signifikante Korrelation von AOPPs und Gewebsfaktor, die eine starke Verbindung zwischen oxidativem Stress und einem prothrombotischen Phänotyp zeigt. Unsere Ergebnisse liefern klare Beweise, dass AOPPs, die unter Bedingungen mit chronischem oxidativen Stress gebildet werden, zu einer erhöhten Thrombozytenaktivität beitragen können. Diese führt zu einem

prokoagulanten Phänotyp bei Endothelzellen, was wiederum zu Gerinnungsstörungen in Urämie und anderen entzündlichen Erkrankungen beitragen könnte. Unsere Ergebnisse zeigen bisher unbekannte prothrombotische Aktivitäten von oxidiertem Albumin.

List of Abbreviations

ADP	Adenosine diphosphate
AOPPs	Advanced Oxidized Protein Products
Ca^{2+}	Calcium
CD	Cluster of Differentiation
Cys	Cysteine
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ESRD	End stage renal disease
FBS	Fetal Bovine Serum
GP	Glycoprotein
HCAECs	Human coronary artery endothelial cells
HD	Hemodialysis
HSA	Human serum albumin
ICAM	Intercellular adhesion molecule-1
DAG	Diacyl glycerol
IL	Interleukin
L-NAME	L-N6-Nitroargenine methyl ester
LPDS	Lipoprotein deficient serum
MCP-1	Monocyte chemotactic protein-1
Met	Methionine
MK	Megakaryocyte
MnTMPyP	Manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin
MPO	Myeloperoxidase
NCD	Noncommunicable disease
NET	Neutrophil extracellular trap
NO	Nitric Oxide
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PKC	Phosphokinase C
PLC	Phospholipase C
PRP	Platelet rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
ROS	Reactive oxygen species
SEM	Standard error of mean
SOD	Superoxide dismutase
TF	Tissue factor
vWF	Von Willebrand Factor
WHO	World health organisation

List of Figures

Index

I. Introduction

1. The anucleate platelets - are they really cells?

The anucleate platelets are known to be the smallest and one of the most abundant cells in the human blood stream. Within their short life time of approximately 8-11 days, the bone marrow derived platelets patrol in the circulation before they get depleted by spleen macrophages. Whether platelets fulfil their primary and essential role in blood coagulation or show their “dark face“ as key players in thrombosis strongly depends on their activation state. This dual and activation dependent function of platelets was identified already in the 19th century (1).

Platelets originate from Megakaryocytes (MK), but the exact mechanism by which they are formed and then released from their precursors is still not clearly elucidated. Three different models have been proposed in platelet development (2–4), however, recent studies support the model of proplatelet formation rather than the cytoplasmic fragmentation or the platelet budding theory (5,6). This model suggests platelets to be released in the final step of MK development from elongated strands of MK cytoplasm (=proplatelets), a process that is additional supported by shear from the blood flow. MK cytoskeletal reorganization occurs in the final step of proplatelet formation, and was described to be partially mediated by serotonin (5). This process results in high numbers of circulating platelets (usually between 150-400 * 10^9/L), making them - beside erythrocytes - the most abundant cells in the human blood stream (7,8). Of note, their numbers are approximately three times higher in rodents (9). In platelet development, one single MK gives rise to up to 1,000 non-nucleated cell segments (7,8) that express the platelet markers CD41 and CD42b, while the MK marker CD34 cannot be detected on their surface anymore (10).

1.1 Platelet structure – a brief overview

Still, it is a matter of debate whether platelets fulfil all the criteria to be classified as „cells“. Due to a lack of the nucleus, they have no DNA and therefore no genes they could reorganize, pointing out a strong argument for platelets to be cell fragments rather than fully accepted cells. Nevertheless, platelets contain cytoplasm from their progenitor cell including several specialized organelles such as granules (α- and dense granules), mitochondria for their energy supply, lysosomes and endosomes. Of particular importance, platelets contain mRNA and are therefore capable to newly synthesize proteins with their inherit translational machinery (11–14). Platelet associated soluble proteins like thrombospondin and vWF (von Willebrand factor) that are stored in the α-granules are exclusively synthesized in MKs, while others like fibrinogen and albumin are collected from plasma by MK facilitated endocytosis and then stored in granules (15). So, α- and dense granules contain important factors for platelet-platelet interactions and are needed to enable platelets to actively participate in the coagulation cascade. The platelet´s inner canalicular system includes a series of invaginated membranes that are folded in the quiescent state but allows the platelet to significantly increase their surface upon activation, when they undergo a “shape change” (9).

1.2 Platelets behave as cells

Although anucleate, platelets share some common features of all cells. They express a wide range of receptors for different purposes, such as glycoproteins (GPs) that sense exposed structures of the extracellular matrix (ECM) in times of vessel stress or bind the respective ligands expressed on other cell types to allow cell-cell interactions (16,17). Via their receptors they can respond to various molecules involved in the regulation of thrombosis and haemostasis or interact with cytokines and chemokines to get either activated or inhibited (18,19). Upon activation, intracellular signalling is induced via a protein phosphorylation cascade (20), and *de novo* protein synthesis is accomplished by a translation machinery and a spliceosome (21). Platelets can furthermore act in an „intelligent“ way to dangers of the surrounding. For instance, lipopolysaccharide (LPS) acting on platelet toll-like receptor 4 stimulate an intracellular signalling pathway that finally results in

secretion of distinct molecules. So, platelets can differ between signals, and effectively react to a given danger (22,23).

Recent research indicates an increasing role for platelets as modulators of lymphocyte functions. On the one hand, they control extravasation of lymphocytes, but on the other hand they were also reported to be able to trigger the release of neutrophil extracellular traps (NETs) that execute important functions in infection or cancer and are associated with thrombosis (24). Interestingly, although platelets lack the nucleus, platelets undergo activation-dependent apoptosis as shown by several apoptosis associated markers (25,26).

When addressing the question of platelet cellularity, the issue of platelets as „immune cells“ has gained huge interest since platelets are sensors of innate immunity, affect immune cells of the adaptive immunity and are capable to initiate inflammation. This issue was recently covered by several reviews (27–29).

2. Platelet function – a double edged sword

A simplified scheme about the dual function of platelets is given in **Figure 1.**

2.1 Platelet function in hemostasis

Under normal physiological conditions platelets circulate in the blood stream and endothelial cells prevent hemostasis by the release of nitric oxide (NO) and prostacyclin, both interfering with platelet activation (30,31). When the endothelial barrier is disrupted in the case of injury, several subendothelial matrix proteins such as von vWF, fibrillar collagens, fibronectin, and laminin become exposed to circulating blood platelets and support platelet binding by interacting with platelet receptors. Thereby, vWF binds the platelet GPIb-V-IX complex (32), collagen interacts with glycoprotein receptor GPVI (33,34) and integrin α2β1 (35), and fibronectin-platelet interactions are mediated by α5β1 and laminin by α6β1(32). vWF is crucial for initial platelet binding, especially in high shear vessels as arterioles (36), and the absence of GPIb-V-IX is associated with bleeding disorders (37). Ligation of GPVI with collagen activates platelets and as a consequence mediates

the shift of the main fibrinogen receptor GPIIbIIIa to an activated state. GPIIbIIIa is the most abundant glycoprotein and changes its conformation from a low to a high affinity state through inside-out signalling resulting in platelet aggregation as the final event of platelet activation (38). Additionally, collagen binding induces the generation and release of the soluble platelet agonist adenosine diphosphate (ADP) and thromboxane A_2 at sites of injury. ADP acts on the G-protein-coupled receptors P_2Y_1 (39) and P_2Y_{12} (40), while TXA_2 mediates its effects via and the thromboxane receptors TPα and TPβ (39). This amplifies platelet integrin adhesiveness in an autocrine and paracrine manner, thereby promoting clot formation. Therefore, interfering with these interactions by targeting TxA_2 formation (41) and/or the P_2Y_{12} receptor (42) was shown to be beneficial in counteracting thrombus formation (43). Activated platelets undergo a change in shape and provide a surface for the assembly of coagulation complexes. This is important for local thrombin formation, an enzyme that mediates conversion of fibrinogen to fibrin and further activates platelets via their protease-activated receptors as one of the most potent stimuli. Thus, thrombin elicited effects notably contribute to growth and stability of a blood clot (43,44).

The adhesion of platelets to ECM proteins is the initial step in primary haemostasis that forms a barrier against blood loss, results in platelet activation and as final steps in aggregation and plug formation. In secondary haemostasis, the coagulation cascade is triggered mainly by exposure of TF resulting in fibrin generation and polymerisation that is essential to trap red blood cells and stabilize the developing thrombus (45).

2.2. The dark site: platelets in thrombosis

Beside its beneficial physiological function in preventing blood loss, platelets are also key players in thrombosis where their functional properties are associated with vessel occlusion and the potential development of cardiovascular diseases with severe outcomes. Thrombosis is mediated to a great extent by platelets and fibrin, and basically occurs after rupture of an unstable atherosclerotic plaque exposing a prothrombotic surface to the blood stream and its cellular components. In contrast to a hemostatic plaque that extends in the extracellular space where it fixes the wound at the site of injury, a thrombus starts to form in the intima, extends into the

intraluminal space and leads to hemodynamic changes. When a clot is formed in the coronary or cerebral circulation, this can lead to severe pathophenotypes, like myocardial infarction and stroke (46).

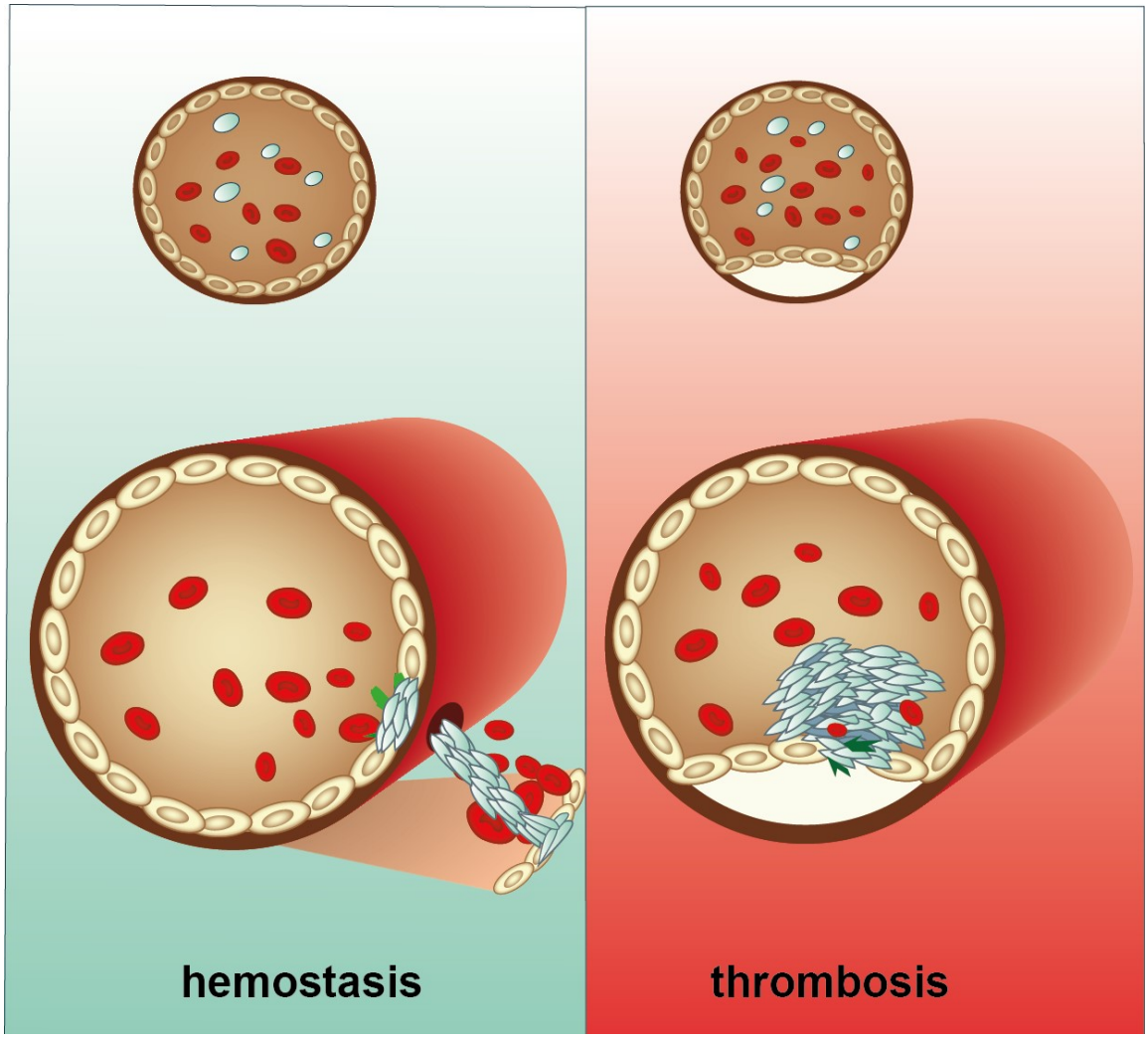

Figure 1: The dual function of platelets

Platelets and erythrocytes circulate in the blood stream as indicated in a vessel cross section (upper left panel: healthy vessel, upper right panel: atherosclerotic vessel). Upon vessel wall injury of a healthy artery (indicated in green), platelets get activated and seal the wound by forming a primary hemostatic plaque that extends into the extracellular space, with minimal clot extension into the lumen. In contrast, upon plaque rupture of an atherosclerotic vessel (lower right panel), the activated platelets form a thrombus that extends within the vessel. Complete vessel occlusion is a potential result and is furthermore associated with severe pathological outcomes.

2.3 Thrombosis: a continuing global challenge

According to the world health organisation (WHO), cardiovascular diseases account for one-third of all annual deaths (47), whereas atherothrombotic diseases represent the major healthcare issue (48). What has changed within the last years is the long considered perception that it's primarily a disease of industrial counties. Although the prevalence of noncommunicable diseases (NCDs) increases all over the world (see **Figure 2** for a statistic of annual deaths resulting from chronic diseases) the highest rise is expected in low- and middle-income countries that already account for approximately 80 % of atherothrombotic deaths (47). So, it is obvious that novel and more effective antithrombotic drugs are needed. Although, research focuses on antithrombotic strategies already for decades, the outcome in regard to mortality is still quite small (41), thus, justifying the need of novel, more effective antithrombotic drugs. Seen in the context of continuously rising diseases like obesity, diabetes or other inflammatory disorders that are associated with a severe prothrombotic phenotype, a challenging future situation is definitively given (49). Recent reports indicate that patients suffering from diabetes are more resistant to conventional antithrombotic drugs, further aggravating the existing situation (50).

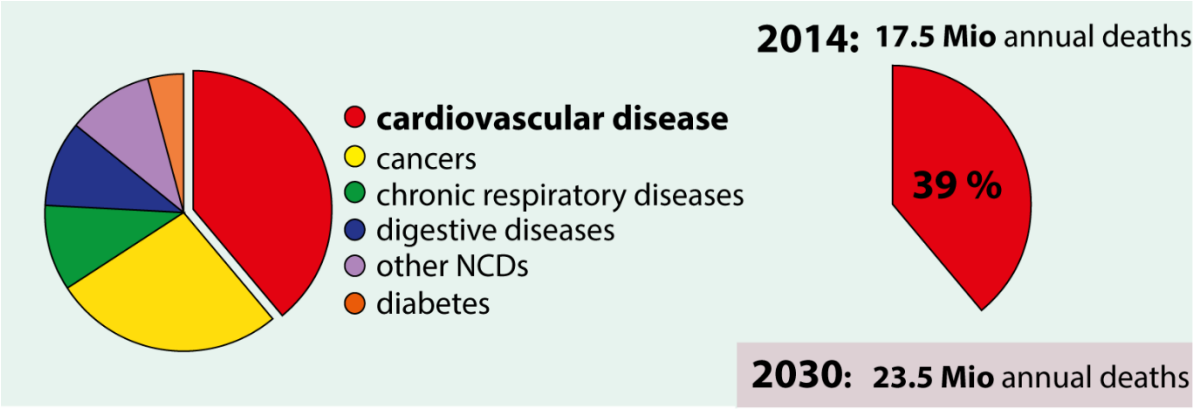

Figure 2: Proportion of noncommunicable disease deaths in 2008

Noncommunicable diseases (NCDs) also known as chronic diseases account for approximately 70 % of all deaths worldwide. Cardiovascular diseases were responsible for almost 40 % of annual NCD deaths in 2008. According to the WHO, deaths that underlie cardiovascular disease will rise from 17.5 Mio to 23.5 Mio annual deaths in 2030, whereas individuals of low- and middle-income countries are at higher potential risk.

3. Platelets in inflammation – the promiscuous platelet

It has been shown in several studies that platelets are not only central to thrombosis, but also play important roles in inflammation *per se*. By doing so, they participate either directly by releasing soluble ligands like interleukin 1 (IL-1β) (28) or favour inflammatory reactions indirectly due to activation of endothelial cells and neutrophils by complex cell-cell interactions. This chapter describes the potential of platelets as mediators of inflammation, and further focuses on atherosclerosis as example of an inflammatory disease strongly associated with thrombosis.

3.1. Platelet granules

There are several inflammatory molecules that are released from platelet granules without a clear described function in haemostasis, thus, strengthen the role of platelets as inflammatory cells (51). Platelets have three different type of granules: α-granules, lysosomes and dense granules, whereas α-granules are the most abundant, and their proteins involve factors that have described functional roles in haemostasis, thrombosis and inflammation. Upon activation, the content of α-granules is either released or expressed on the platelet surface, as it is known in the case of P-selectin that is upregulated in the membrane surface during platelet stimulation. Amongst others, α-granules contain vWF, coagulation factor V, Interleukin (IL)-8, RANTES, fibrinogen and large amounts of platelet factor 4, a CXC chemokine best known for affecting neutrophil and monocyte chemotaxis (52,53). With specific regard to inflammation, α-granules released cytokines affect leukocyte functions by regulating their movement and tissue migration as well as intracellular ROS production (53).

Factors released from dense granules mainly act on amplifying platelet activation responses and involve factors such as ADP and serotonin. An additional role for serotonin in supporting platelet adhesion to the endothelium was recently shown (54). The importance of lysosomes is mainly given by its bactericidal effects (55).

3.2 Platelet-endothelial crosstalk

Endothelial cells that line the vessels are not only a mechanical barrier between platelets and components of the extracellular matrix but also present an anti-thrombotic surface to the blood stream by antagonizing known platelet activation pathways (by release of NO and prostacyclin) and degradation of the important feedback agonist ADP (via ectonuclease activity of CD39). As a result, early studies thus assumed that endothelial injury is needed to allow platelet binding. However, recent *in vitro* studies show the potential of platelets to directly adhere to endothelial cells upon activation of either ECs and/or platelets (56–58). Platelet adhesion to endothelial cells was shown in several inflammatory states including ischemia and hypercholesterolemia (59).

Platelet-endothelium adhesion also occurs under high shear stress conditions as confirmed in several *in vivo* studies (60–63) indicating a multistep process that involves platelet tethering, rolling on the endothelial lining, and strong adhesion as final event. A simplified scheme of how platelets attach to the endothelium and inflame endothelial cells is depicted in **Figure 3**. The ongoing receptor interactions during this process are not elucidated in detail, but participation of both, selectins and integrins are proven in this crosstalk (64). Thereby, selectins mediate the initial loose contact of “platelet rolling”. Endothelial derived P-selectin is rapidly released from α-granules known as Weibel-Palade bodies upon activation, and is associated with several inflammatory conditions. In addition, E-selectin that is also expressed upon inflammation, enables loose platelet binding (61). Rolling along the endothelial vessel is independent of the activation state of platelets and is triggered solely by an inflamed endothelial layer, as shown in experiments using platelets from mice lacking P-and/or E-selectin (60).

Platelets interact with endothelial P-selectin mainly via binding with their GPIb complex that is also known as von Willebrand factor receptor, but do also express the ligand of P- selectin PSGL-1. It is important to mention that endothelial vWF is also released from Weibel bodies, and P-selectin supports anchoring of vWF to endothelial cells, providing an even more adhesive surface for platelets and their GPIb complex (65). Nevertheless, these initial interactions are reversible and unstable and depend on additional contacts mediated by integrins (66).

GPIIbIIIa is not only the major integrin involved in aggregation but due to its ability to bind factors deposited on endothelial cells, GPIIbIIIa enables platelet-endothelial interactions. Such factors involve fibrinogen, fibronectin or vWF.

GPIIbIIIa and αvβ3 have been shown to be key players in firm platelet-endothelium adhesion under static conditions (57,67). Furthermore, platelets with a functional inactive GPIIbIIIa fail to firmly adhere to activated ECs, as identified *in vivo* by specifically blocking GPIIbIIIa function (68). Although the molecular fundamentals are incompletely understood so far, there is evolving evidence that platelet-endothelial interactions allow cell-cell crosstalk and, thus, contribute in the initiation and also progression of vascular inflammation.

Platelets get stimulated as a result of platelets vessel binding, thus, leading to an activation dependent release of their proinflammatory granules content into their local environment. Doing so, platelets alter endothelial properties in diverse ways, including chemotactic, adhesive but also proteolytic properties (69). Of huge importance is platelet derived IL-1β that is not only a major stimulator of endothelial activation but also initiates the release of IL-6 and IL-8 from endothelial cells (70), and further increases adhesive molecules expression such as intercellular adhesion molecule-1 (ICAM-1) and αvβ3 (71). The expression of these early inflammatory genes involves activation of nuclear factor “kappa-light-chain-enhancer” of activated B-cells. (NF-κB), whereas platelet binding directly support NFκB activation by degradation of inhibitor of κB (IκB) (72,73).

Activated platelets can deliver chemokines and vice versa stimulate cells of the vessel wall to locally release their chemokines. In turn, endothelial cell derived chemokines promote platelet aggregation and adhesion, resulting in monocyte recruitment to the inflamed endothelium (74).

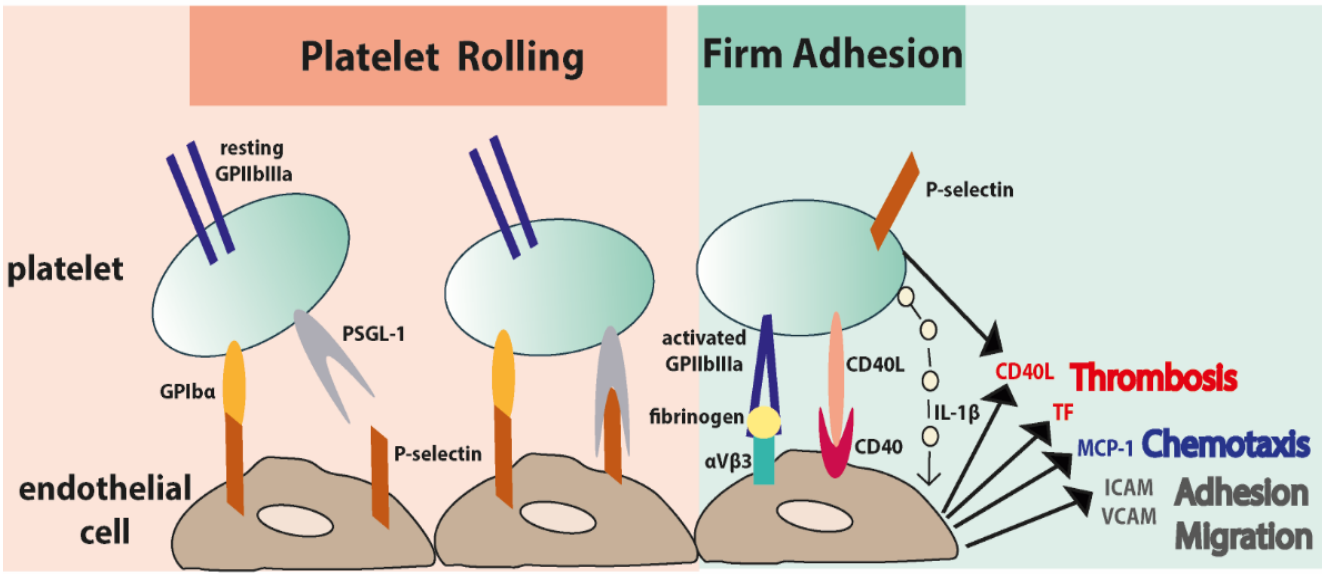

Figure 3: Endothelium attached platelets promote endothelial inflammation

An activated endothelium expresses P-selectin on its surface. Platelet rolling depends on the platelet surface proteins GPIbα and PSGL-1, both interacting with endothelial P-selectin. Selectins are needed for initial contact, but only provide reversible binding of platelets to endothelial cells. β3 integrins sufficiently mediate stable and firm adhesion, mainly via the interaction of the activated form of GPIIbIIIa (αIIbβ3) with endothelial αVβ3. Activated platelets express P-selectin (promoting platelet-neutrophil interactions) and CD40L that further stabilize platelet-endothelial interactions via binding to endothelial CD40L. Platelets inflame the endothelial lining by releasing IL-1β and CD40L, thus, allowing proatherogenic changes of the endothelium that might be important in the development of vascular inflammation. This involves MCP-1 mediated chemoattraction of leukocytes, increased VCAM-1 and ICAM-1 expression that promote the attachment of monocytes, neutrophils, and lymphocytes to the inflamed endothelium. CD40-CD40L interactions lead to endothelial TF expression.

3.3 The important role of CD40L

So far, the most important platelet derived inflammatory mediator is platelet-derived CD40 ligand (CD40L, CD154) that rapidly appears on the surface of activated platelets and, as functionally active molecule, triggers a proinflammatory phenotype on ECs (75,76). CD40L is a transmembrane protein of the tumor necrosis factor family that was primarily identified on immune cells. Although CD40L is expressed by several cells of the vasculature (including B-cells, T-cells, macrophages, basophiles and natural killer cells, endothelial cells and smooth muscle cells) (77), platelets are the major source of CD40L, accounting for almost 95 % of soluble CD40L (sCD40) (78), as it was further indicated by the correlation between platelet count and sCD40L (79). Elevated levels of CD40L are associated with hypercholesterolemia, diabetes, ischemic stroke, and acute coronary syndromes (80–83). Furthermore, it was investigated that high levels of sCD40L are associated with risk of future cardiovascular events in healthy women (84), indicating a role of CD40L as prognostic marker.

Depending on the strength of the respective stimulus, the cleavage from the platelet surface occurs within seconds to minutes in which the released CD40L fragment remains trimeric and functional. Ligation of CD40 on ECs by its ligand expressed on platelets promotes the release of IL-8 and monocyte chemotactic protein-1 (MCP-1), both important chemoattractants for neutrophils and monocytes. Moreover, platelet CD40L upregulates the expression of endothelial adhesion proteins (like E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1) that mediate binding of neutrophils, monocytes and lymphocytes to the inflamed vessel. Of particular importance, platelet CD40L was shown to induce TF on endothelial cells, that strongly links inflammation with thrombosis (85).

Its thrombotic activity is furthermore strongly linked to GPIIbIIIa. Due to a role of GPIIbIIIa antagonists in attenuating the release of sCD40L even in the absence of platelet aggregation clearly indicate a direct involvement of GPIIbIIIa in the shedding process of CD40L (86). Moreover, direct interaction of GPIIbIIIa with CD40L under high shear stress conditions promotes and stabilizes platelet thrombi, allowing localisation of platelet induced inflammation on the vessel wall (87).

3.4 Platelet-neutrophil interactions

When studying the platelet-neutrophil-axis, the ligation of platelet P-selectin with its high affinity ligand (PSGL-1) is definitely in the focus of ongoing research. PSGL-1 is continuously integrated in the membrane of neutrophils. As a result of the velocity gradient, platelets and neutrophils mainly circulate at the vessel periphery what increases the chances of collisions (88). When platelets got activated beforehand by inflammatory stimuli, they express P-selectin as a result of activation dependent granules release, thus, enabling the formation of platelet-neutrophil-aggregates (PNA (89). The relevance of these aggregates and the P-selectin dependent recruitment of neutrophils was revealed in atherosclerosis and also in studies that had the focus on damaged arterial surfaces. P-selectin enables tethering of platelets to neutrophils (90,91), thus, inducing platelet-neutrophil cross-talk that results in elevated levels of αMβ2 integrin CD11b/CD18 (Mac-1) on the surface of neutrophils. This upregulation subsequently supports firm adhesion that is mediated by neutrophil Mac-1 and the platelet glycoprotein GPIb (92). Further stabilisation is given by CD11a/CD18 binding to platelet adhesion molecule-2 (ICAM-2) (93) and GPIIbIIIa-bound fibrinogen (94,95).

Since the amount of P-selectin is significantly higher on platelets compared to endothelial cells, leukocytes can be easily recruited to the site of inflammation. Neutrophils activated via PNA formation have increased binding properties to the endothelium. Furthermore, platelets that are already attached to the endothelium form a bridge for platelet primed neutrophils and the endothelium, thereby recruiting neutrophils to a growing thrombus. Thrombus associated neutrophils facilitate pro-thrombotic actions by enhancing fibrin deposition (96).

Platelets might further contribute together with CD40L to neutrophil recruitment, by enhancing the proinflammatory phenotype of endothelial cells. Furthermore, CD40L can directly interact with neutrophil CD40, amplifying neutrophil activation.

Of particular interest, the role of PNAs is not limited solely to neutrophil recruitment to inflamed tissue, platelets also enhance the neutrophil defence mechanism.

Platelets are capable to enhance the production of ROS via their soluble mediators, thus, it is not surprising that neutrophils in PNA produce more ROS than free neutrophils (97). This particular process was shown to be P-selectin dependent (98).

Recent research stated that platelets can induce the formation of neutrophil extracellular traps, which are crucial for antimicrobial defence (99,100). Thereby, neutrophils release DNA and granular components such as MPO, histones and serine proteases (101,102). NETosis has been distinguished from other forms of cell death (103) and contributes to coagulation and platelet aggregation (104). NETs were shown to be able to colocalize with fibrin and von Willebrand factor within a thrombus (105) thus providing a scaffold that protects thrombi from fibrinolysis (106).

In thrombosis, neutrophil NET formation leads to a vicious circle where direct interactions with platelets and endothelial cells induce their activation, which in turn promotes further NETosis. Furthermore, NET derived histones enhance the activation of blood coagulation (104).

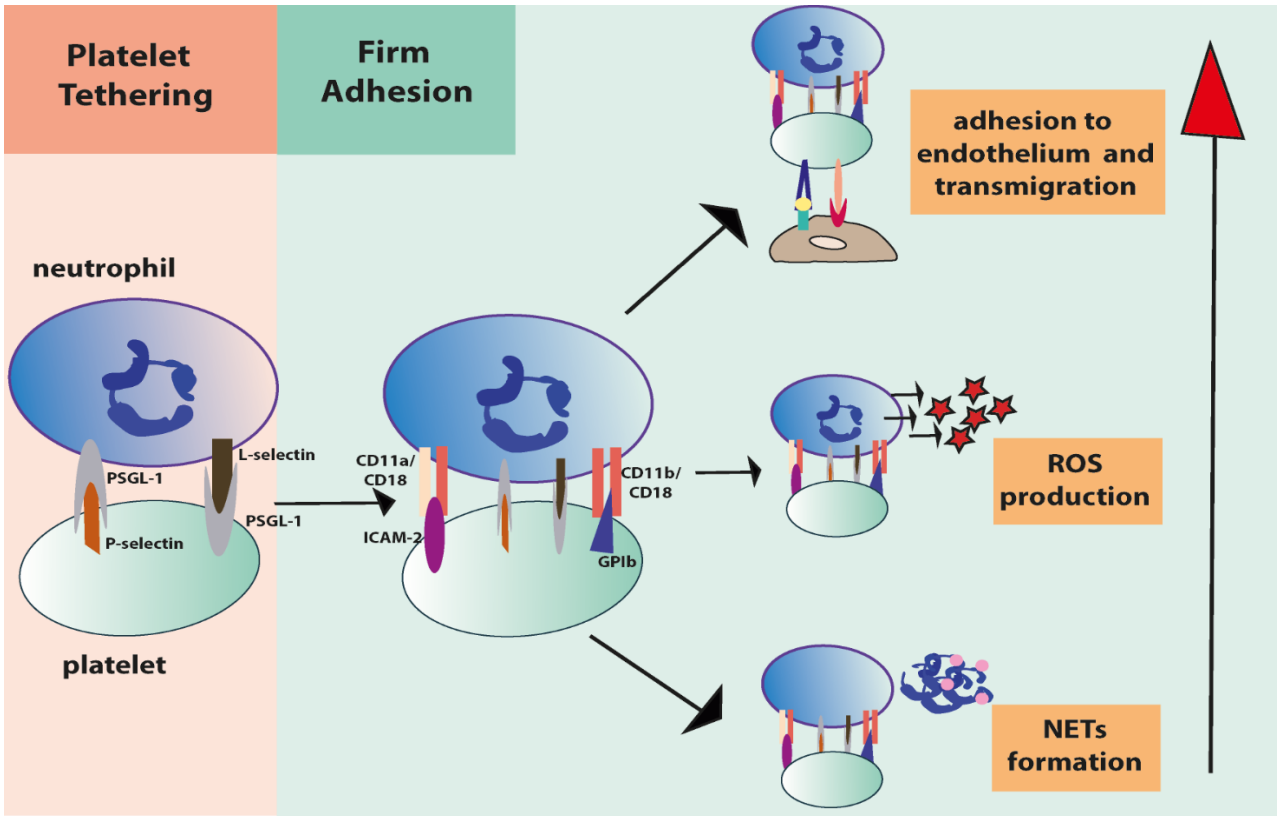

Figure 4: Impact of PNAs on neutrophil functions

Tethering of activated platelets to neutrophils depends on the interaction between platelet P-selectin and its high affinity counter ligand neutrophilic PSGL-1. Furthermore, L-selectin interacting with platelet PSGL-1 contributes to PNA formation. PNA formation during inflammation further induces neutrophil activation as indicated by neutrophilic integrin expression (CD11b/CD18). Firm adhesion is provided by CD11b/CD18 that interacts with GPIb on platelets and CD11a/CD18 that interacts with platelet ICAM-2. The contact dependent activation of neutrophils by circulating platelets results in increased adhesion to the endothelium and consequent transmigration. During inflammation, endothelium attached platelets build a bridge to enable binding of platelet primed neutrophils to endothelial cells. Neutrophils in PNAs show increased ROS production and NET formation.

3.5 Platelets in atherosclerosis

Atherosclerosis is the process that underlies most cardiovascular diseases and is regulated by genetic factors and aging but is also strongly associated with dyslipidemia, hypertension, smoking, diabetes and obesity (107). Nowadays, it is well established that atherosclerosis is a progressive chronic inflammatory disease characterized by lipid infiltration into the subendothelial space of large arteries (so called plaques) and complex inflammatory interactions, based on cell-cell-crosstalk leading to the release of various inflammatory stimuli (108). As indicated above, platelets function as key players in vascular inflammation through the release of pro-inflammatory granule content and their direct interactions with endothelial cells and leukocytes. Their role in atherosclerosis has been extended within the last years: platelets are known to contribute in all steps of atherosclerosis. Reduced inflammatory gene expression as a result of the blockade of platelet-endothelial interactions profoundly decreased the infiltration of leukocytes in the arterial wall. This result establish a clear role of platelets as contributors of endothelial dysfunction during the early steps of atherosclerosis (109). Platelet make a major contribution to monocyte arrest and monocyte recruitment by secreting MCP-1 and support macrophage differentiation by releasing RANTES and platelet factor-4(110). Despite their roles in the onset of atherosclerosis, platelets are capable to intensify inflammation in all stages of atherosclerosis by expressing membrane molecules (such as ICAM-2, P-selectin, and CD40L) that regulate adhesion and aggregation, chemotaxis, survival, differentiation and angiogenesis (46). Of particular importance are the roles of P-selectin and CD40L whose inflammatory roles have already been described in previous chapters. When plaque rupture occurs as the final event of this chronic inflammatory condition (111), platelets contribute as key players in thrombosis that might lead to cardiovascular complications such as myocardial infarction and stroke (112).

So, platelets are involved in all inflammatory processes of the disease from the initial chronic phase of atheroma initiation and its extension to the phase of acute disease, where they initiate thrombosis upon plaque rupture. Atherosclerotic plaques are highly procoagulant due to the presence of TF as well as various platelet activators, such as collagen.

3.6 TF bridges coagulation and inflammation

TF is a type I integral membrane glycoprotein and member of the cytokine receptor superfamily (113), and certainly the key trigger for coagulation. By doing so, TF binds to its ligand factor VII/VIIa (FVII/VIIa), forming the TF/FVIIa complex leading to thrombin generation, fibrin deposition, and platelet activation (114,115).

The presence of TF in atherosclerotic lesions largely contributes to its thrombogenic potential, thereby linking inflammation with coagulation. There is striking evidence for an important role of TF in inflammation as several experiments have demonstrated. TF could enhance the proinflammatory functions of macrophages (116), and upregulation of IL-8 was shown in both, keratinocytes and macrophages (117,118). By selectively inhibiting TF, a growing thrombus can be tremendously reduced on the site of a ruptured plaque (119).

Several cells are sources of TF, but from main importance are cells of the vasculature (endothelial cells and smooth muscle cells) (120) as well as monocytes (121). There is still a debate whether platelets are an additional source of TF. Due to its location, TF is exposed to the circulation in response to inflammatory cytokines and/or physical or chemical damage. When TF is exposed to the blood stream, most of its functions are rapidly induced (120). The function of TF is balanced by its counter player TF pathway inhibitor, a major anticoagulant protein that inhibits the FVIIa-TF-complex. Disruption of the tisse factor inhibitor gene leads to embryonic lethality, indicating the importance of TF regulation by this inhibitor.

In several pathological states, including hyperlipidemia, TF that circulate in the form of microvesicles show elevated levels in plasma compared to healthy subjects (122).

4. Platelet interactions with lipoproteins

Despite their carrier functions, lipoproteins are able to elicit intracellular signalling in various target cells. Interestingly, several studies revealed a correlation between hyperlipidemia and platelet hyperreactivity, whereas platelets of hypercholesteremic patients tended to have higher *in vivo* platelet activation, indicating a role for LDL in this context (123). It was revealed that the presence of LDL alone leads to elevated binding of fibrinogen by platelets (124), and is proposed to stimulate TXA_2 formation and PKC activation, thereby inducing platelet intracellular signalling cascades (125). Furthermore, LDL mediated effects on platelets were linked to activation of phospholipase C and Ca^{2+} mobilisation (126).

In later studies, the proaggregatory impact of the oxidized form of LDL (oxLDL) on platelets was clearly shown (127–129), and due to the prothrombotic phenotype in atherosclerosis oxLDL-platelet interactions are still in the focus of intense research.

It was assumed that the protein moiety of oxidized LDL is responsible for its stimulatory effects on platelets, and hypochlorite oxidized LDL was shown to be a stronger platelet agonist, compared to copper oxidized LDL (130). Additional, a recently published study revealed a novel role for hypochlorite oxLDL in promoting prothrombotic platelet signalling via inhibition of the NO/guanosine 3′,5′-cyclic monophosphate/protein kinase G pathway (129).

In contrast, HDL decreases agonist induced platelet aggregation in a dose dependent manner (131) and inversely correlates with P-selectin expression on human platelets (132). It was reported that HDL facilitates its atheroprotective and antithrombotic effects mainly via upregulation of endothelial NO synthase, increased production of prostacyclin and elevated selectin and TF expression on endothelial cells, that subsequently decrease the formation of thrombin (132). The major HDL apolipoprotein A1 was related to the protective features facilitated by HDL (133). The results obtained in studies were oxidized HDL was used are controversial, and mainly depend on the oxidant used for HDL modification (134–136).

4.1 Lipoprotein-receptor-interactions

Scavenger receptors are a group of heterologous surface receptors that share the ability to bind modified/oxidized forms of LDL. Within the last 2 decades, the list of scavenger receptors has been enormously increased. Among them, class B scavenger receptor CD36 and SR-BI, class E scavenger receptor LOX-1, class D scavenger receptor CD68 were discovered on platelets, but some of these findings could not be repeated and are therefore still a matter of debate. Especially, reports regarding the presence of scavenger receptors type A on platelets are controversial. Whether scavenger receptors on platelets strongly contribute to cardiovascular pathology as well as their general role in platelet physiology has to be revealed adequately in future studies(137,138).

CD36 (fatty acid transporter), mainly known for its role in lipid uptake and atherosclerosis, is expressed on various cell types including endothelial cells, macrophages and platelets. CD36 interacts with native HDL, LDL, VLDL, thrombospondin-1, collagen but also fatty acids and pathogen derived particles (139,140). It has been suggested that CD36 has proactivatory potential on platelets by interacting with arachidonic acid and, moreover, decreased binding efficacy to collagen was reported for human platelets lacking CD36 in the early stages of adhesion (141).

Recently, a novel family of oxidized choline phospholipids ($oxPC_{CD36}$) that accumulate in dyslipidemia and are formed via different pathways during oxLDL formation were elucidated as CD36 ligands (142). High plasma levels of $oxPC_{CD36}$ were linked to increased CD36 mediated platelet reactivity, but CD36-/- mice could respond in the same way to platelet agonists as the respective wild type mice, strengthening the hypothesis that the prothrombotic role of CD36 might be restricted to a dyslipidemic phenotype. These results clearly show a direct association of dyslipedemia with oxidative stress and increased platelet reactivity. Exposure of $oxPC_{CD36}$ to platelets furthermore sensitized platelets to other platelet stimuli at *in vivo* relevant concentrations. In addition, plasma levels of $oxPC_{CD36}$ are associated with high levels of human platelet activation (138). Another recent finding by Magkenzi *et.al* (129) extends the oxLDL-platelet CD36 knowledge, by presenting that oxLDL promote thrombosis via binding to CD36 by interfering with platelet

regulatory pathways. In short, oxLDL induced platelet signalling resulted in ROS/superoxide formation via PLC activation that directly lead to platelet activation. Of interest, ROS further contributed to platelet activation by interfering with the inhibitory NO/cGMP/PKG pathway. This finding might explain the decreased response of ADP and collagen that occur upon CD36 deficiency.

Distinct evidence is given that oxidized LDL binds with high affinity to platelet CD36, whereas some groups postulate a unique role of CD36 in this interaction (128,143,144) while others discuss a combined action of scavenger receptor-A (SR-A and CD36 in oxLDL binding on platelets (145). Nevertheless, there are findings ruling out a contribution of SR-A in inducing oxLDL triggered signalling in platelets (138).

Acetylated LDL, representing a classical ligand for SR-A receptors, failed to influence platelet activation state, raising the question whether SR-A is expressed on platelets surface at all (146). In contrast to this speculation, p38MAPK phosphorylation could be induced in CD36-deficient platelets, but phosphorylation did not occur in mice lacking both CD36 and SR-A (145). In addition, experiments using blocking antibodies for platelet CD36 indicate that CD36 is solely responsible for a part, but not the overall binding of oxLDL (147–149).

In addition, oxLDL induced effects are also elicited by the lectin like oxidized low density lipoprotein receptor 1 (LOX-1), which is expressed on endothelial cells, macrophages and smooth muscle cells. In a recent study, LOX-1 mRNA and protein were identified in human platelets and the presence of the receptor was further confirmed in megakaryocytic cell lines. In contrast to CD36, LOX-1 expression occurs in an activation dependent manner and is stored in α-granules in resting platelets. Of note, LOX-1 recognizes and binds activated platelets (150,151), and an important role for LOX-1 in thrombosis is supported by its ability to crosslink activated, LOX-1 expressing platelets, thereby LOX-1 might contribute to thrombus growth and stabilisation.

Class B scavenger receptor B1 (SR-BI,), a close relative of CD36, binds native as well as oxidized lipoproteins and is expressed on the surface of various cell types including hepatocytes and endothelial cells (152). While one group postulates SR-

BI to be the major receptor for oxHDL on platelets (153), others could not confirm the presence of SR-BI on platelets (154).

Despite these discrepancies, the described role of SR-BI on platelets involves oxHDL mediated antiaggregatory and antithrombotic effects, whereas oxidized phospholipid components are suspected to facilitate these beneficial effects

5. Oxidative stress in inflammation

Oxidative stress is a state of imbalance between oxidants and antioxidants, resulting from an improperly working antioxidant defence system. The insufficient inactivation of oxidants leads to tissue damage by accumulating reactive oxygen species (ROS) (155).

ROS are formed by the reduction of oxygen and include free radicals such as $O_2^{\cdot -}$ (superoxide), $ONOO^{\cdot -}$ (peroxynitrite) and OH^- (hydroxyl) but also non-radicals such as H_2O_2 (hydrogen peroxide) (156,157).

In general, there are many enzymatic sources of ROS including xanthine oxidase, cyclooxygenases, lipooxygenases, myeloperoxidases, cytochrome P450 monooxygenase, uncoupled nitric oxide synthase peroxidases, and NADPH oxidase. Of note, ROS production not necessarily occurs in intracellular compartments, but can be generated extracellularly (158). Oxidative stress and a chronic inflammatory phenotype are associated with the pathophysiology of atherosclerosis (158,159). Blood neutrophils constitute a major source of ROS in the circulation, and closely link oxidants to proinflammatory conditions (160). The heme enzyme MPO is abundantly expressed in neutrophils, accounting for 5% of total protein (161). MPO is released from neutrophil granules upon activation. Recently, MPO was found to be co-localized with macrophages in atherosclerotic plaques (162). MPO is the only enzyme that produces HOCl *in vivo* by conversion of H_20_2 in the presence of chloride ions (Cl^-) (163). Within the phagolysosome, HOCl contributes to host defence (161), but when excessively released, its strong oxidation properties harm other components such as proteins, lipids or DNA, thereby contributing to tissue injury (163).

In the context of atherosclerosis, the major role of HOCl is given by its strong ability to oxidize lipoproteins. Thereby formed oxHDL and oxLDL trigger the formation of macrophages to foam cells, inhibits endothelial nitric oxide synthase (164) and in a feed-back loop further enhance neutrophil degranulation and oxidants production (165). Furthermore, the oxidized form of LDL is involved in platelet activation (166). Beside lipoproteins, HOCl attacks plasma protein, mainly albumin and forms so called advanced oxidized protein products (AOPPs) (167).

5.1 Platelets, oxidative stress and vascular inflammation

Beside neutrophils, also platelets and endothelial cells in their activated states are capable to synthesize ROS (168,169), indicating a strong contribution of oxidative stress to vascular inflammation. Furthermore, it was shown that oxygen radicals can be generated within a growing thrombus (170), making a point for the relevance of the already intensively ongoing discussion regarding the interplay of oxidative stress, inflammation and risk of cardiovascular events.

Platelets were described to be capable to produce superoxide anion (171) and the more stable H_2O_2 that can be released or is directly involved in intraplatelet signalling cascades (172). In general, ROS were shown to have proactivatory potential on platelets in several on inflammation or thrombosis based pathophenotypes. Despite the direct impact on platelets, ROS furthermore contribute to enhanced thrombosis due to the conversion of NO (173). In mice, the link between oxidative stress and thrombosis has already been shown in a model of hyperhomocysteinemia (174).

5.2 Modified proteins as markers of oxidative stress

Proteins have gained full attention as markers of oxidative stress only within the last two decades. This is surprising, since their high vulnerability to oxidants is a well-documented finding (175), and the fact that extracellular fluids usually lack high levels of antioxidants, makes plasma proteins a primary target for oxidation (176).

Accordingly, albumin the most abundant protein in the circulation, is prone to oxidative attack (177). Oxidation may induce structural and functional alterations interfering with metabolic or enzymatic properties of the protein. Oxidation can range from modification of a single amino acid residue to complete protein denaturation or fragmentation. In contrast to mild oxidized proteins, severe damaged proteins are resistant to proteolysis, and modifications are often irreversible (178,179). The important role of modified albumin as marker of oxidative stress in several inflammatory conditions is introduced in the next chapter.

6. AOPPs are markers for oxidative stress and inflammation

The term “Advanced oxidized protein products” (AOPPs) was first used by Witko-Sarsat *et. al,* to describe a modified protein fraction that was found to accumulate in the plasma of chronic renal disease patients. Interestingly, the highest levels of AOPPs, mainly consisting of oxidized albumin, were measured in end stage renal disease patietns on hemodialysis. In line with this finding, AOPP levels increased in all stages of renal disease, thus, directly reflecting the levels of oxidative stress that appear to increase with the severity of the disease. It was shown that subjects in an advanced state of renal disease, but not yet on hemodialysis, had AOPP levels that were approximately three times higher than those measured in the respective control group (167). In this chapter, main characteristics of AOPPs, their prevalence in inflammatory diseases and their potential interaction partners will be discussed.

6.1 Characterisation of AOPPs

AOPPs are characterized as dityrosine-containing and crosslinked proteins that are formed by the reaction of plasma proteins with the myeloperoxidase product HOCl. Reactive chlorinating species are able to modify proteins in various ways. This includes the conversion of cysteine residues to disulfides or higher oxidation products, the conversion of cysteine and methionine residues to sulfoxides, the oxidation of tryptophan, but also the chlorination of amino groups and tyrosine (180).

As mentioned above the formation of AOPPs depends on the phagocytic enzyme MPO, indicating an important role for activated neutrophils in the context of plasma AOPP formation (167). Moreover, a correlation between the amount of circulating neutrophils and plasma concentration of AOPPs has been shown in patiens with uremia (181). *In vitro* AOPPs are formed by addition of reagent HOCl to plasma albumin, the *in vivo* major source of AOPPs in human plasma (177). Thereby, the concentration of the chlorinated oxidant added directly correlates with the measured AOPP levels, providing evidence that AOPP formation directly results from oxidation of plasma proteins with HOCl (167). Moreover, a kinetic model could predict plasma proteins as the major target of HOCl, that elicits almost no damage to other materials (180). AOPPs consist of two main fractions: the high molecular-weight AOPPs (600 kDa) which are albumin aggregates formed by disulfide bridges and/or dityrosine crosslinking, while the low molecular weight AOPPs (80 kDa) contain albumin in its monomeric form (167). When size exclusion chromatography was performed, low and high molecular AOPPs were almost not detectable in plasma of healthy (167,182). AOPP concentrations are measured by a spectrophotometric method at 340 nm and expressed in chloramine-T equivalents (183) (see method section for further details). AOPP levels correlate with plasma levels of dityrosine, a hallmark of protein oxidation, and the advanced glycation end-products (AGE)- pentosidine, both established markers of post translational mediated protein damage (167).

6.2 Albumin – the major AOPP in blood

Human serum albumin (HSA) represents the most abundant protein in plasma and accounts for more than 50% of total proteins. Once secreted from the liver to the blood stream, the 66kDa protein has a half-life of approximately 20 days. Within this lifespan, albumin can pass through the circulation around 15,000 times and can be recruited by neutrophils to the site of inflammation where it triggers antioxidative defence activities (184,185). So, beside its function as transporter protein and negative acute phase protein, albumin is the major extracellular antioxidant (186,187) accounting for around 70 % of free radical trapping activity in plasma (184).

Of note, the antioxidative properties of albumin are accomplished to a high extend by methionine (Met) and cysteine (Cys) (188). Only one of the 35 cysteine residues is in the reduced state (Cys 34), and majorly accounts for its antioxidative properties (185).

6.3 AOPPs in renal disease and other inflammatory conditions

As already mentioned above, AOPPs were first described in chronic kidney disease, where the highest levels were found in patients on hemodialysis followed by patients on peritoneal dialysis. Of note, traditional risk factors failed to explain the highly prevalent cardiovascular phenotype in patients with chronic renal disease. AOPPs were proposed as non-traditional risk factors for predialytic cardiovascular events that result from atherosclerosis (189). AOPPs as markers for oxidative stress and mediators of inflammation were found to be elevated in patients with coronary artery disease, diabetes mellitus, systemic sclerosis, and colorectal cancer among other chronic diseases (176).

The chronic inflammatory stress prevalent in renal disease is mainly a feature of the high oxidative stress levels caused due to neutrophil and monocyte activation and ROS production, respectively. While neutrophils are the main source of ROS that result from the NADPH complex, monocytes to a large extent, are provided for the production of proinflammatory cytokines such as IL-1, TNF-α, and IL-6. Importantly, both cells are capable to produce MPO, an enzyme strongly associated with

atherosclerosis (190,191). This link was shown by studies that discovered MPO in aortic atheromatous plaques, while the enzyme could not be detected in aortae of healthy subjects (192).

End stage renal disease patients have a dramatically increased mortality rate when compared to the age matched general population (193). Cardiovascular complications account for almost half of these deaths, whereas atherosclerotic occlusive accidents are involved at least in every second cardiovascular case of death (194).

The high prevalence of cardiovascular disease complications upon dialysis therapy lead to the assumption that atherosclerosis is initiated already before starting dialysis in the earlier phases of renal disease (193). There is compelling evidence that in renal disease inflammation, oxidative stress markers and cardiovascular disease are strongly associated (167,195–197).

Beside the crucial role of uremic toxins in inflammation, AOPPs might be important mediators of cardiovascular disease. AOPPs were shown to competitively inhibit the HDL receptor SR-BI, thus, preventing its full potential for cholesterol ester uptake and respective plasma HDL-cholesterol clearance (198). Interestingly in a subsequent study it was shown that, AOPPs are irreversibly SR-BI antagonists(199). AOPPs were also shown to promote the formation of foam cells, by interfering with the cholesterol efflux capacity of macrophages (198) and to activate monocytes and neutrophils (200)

6.4 Receptors for AOPPs

As shown in several diseases, AOPPs accumulate in the circulation as well as in several tissues, and their functions were associated with severe conditions. As a consequence, research that focuses on the elucidation of potential interaction partners for AOPPs was intensified. The aim was to extend the current knowledge on how AOPPs facilitate their effects, and to find potential therapeutically targets.

The first receptors found in this regard were the receptor of AGE products (RAGE) on the surface of endothelial cells (201,202) and the scavenger receptor CD36 was identified to interact with AOPP-albumin (203). In the kidney, RAGE expression is upregulated upon chronic AOPP loading (204), and RAGE-AOPP interactions were shown to induce ROS production via activation of NAD(P)H oxidase. Interfering with the binding of AOPPs to CD36 decreased the activation of the AOPP induced renin–angiotensin system in tubular cells (176). Similar to RAGE, CD36 triggers a coagulation cascade that involves activation of PKC and NAD(P)H, superoxide formation and NF-κB activation, finally resulting in inflammation of the vascular endothelium and renin-angiotensin system activation (203).

With specific regard to inflammation and lipid disorders, SR-BI was identified to irreversible bind AOPP-albumin (199), thus, interfering with the beneficial effects of its ligand HDL. Several studies could clearly demonstrate a protective role of SR-BI in cardiovascular disease as reviewed by Rigotti *et al.* (205). In *in vivo* experiments, intravenous injections of AOPPs resulted in higher levels of total plasma cholesterol (206).

7. Aim of the study

Thrombosis is associated with high oxidative stress levels as found in several inflammatory conditions. As a result, oxidative modified proteins accumulate in the plasma of patients suffering from inflammatory diseases, such as renal disease. Previous studies could show that plasma albumin is the major target of chlorinated oxidants that are formed by the neutrophilic enzyme MPO. Neutrophil activation is yet another typical characteristic of inflammation.

While the impact of oxidative modified lipoproteins like oxLDL on platelets was already revealed in detail in the context of atherosclerosis and thrombosis, studies that elucidate a potential role of oxidized proteins on platelets have not been performed so far.

Given that (i) the half-life of albumin in plasma is significantly higher compared to the half-life of lipoproteins, and (ii) levels of oxidized albumin are associated with cardiovascular disease, we hypothesized a potential role of AOPPs on platelet function.

With regard to functional platelet properties, we wanted to determine platelet activation markers, platelet aggregation, and platelet adhesion upon treatment with AOPPs. Moreover, we were interested whether AOPPs via binding to platelet scavenger receptors induce signalling cascades necessary for platelet activation and a prothrombotic phenotype. In the broader context of thrombosis, we wanted to reveal whether AOPPs stimulated platelets promote a procoagulant phenotype in endothelial cells.

In short, the main focus of this work was to investigate whether oxidative modified albumin (AOPPs) alter platelet reactivity, thereby linking inflammation and a pro-thrombotic phenotype.

II. Material and Methods

1. Material

1.1 Reagents

3,3′-dihexyloxacarbocyanine iodide	Sigma
ADP	Probe & Go, Sigma
Antibody diluent	DAKO
BD Cell Fix	BD Biosciences
BD FACS Flow	BD Biosciences
BSA, Fraction V	PAA
$C_6H_5Na_3O_7 * 2H_2O$	Merck
$CaCl_2$	Merck
Collagen	Probe & Go
Cytochalasin B	Sigma
D(+)-Glucose monohydrate	Merck
NaN_3	Sigma
DMSO	Carl Roth GMBH
Dulbecco´s PBS 1x liquid - $CaCl_2$, $MgCl_2$	Invitrogen
Dulbecco´s PBS 1x liquid +$CaCl_2$, $MgCl_2$	Invitrogen
EDTA	Carl Roth GMBH
Fibrinogen	Sigma

Annexin V Binding Buffer	BD Biosciences
HEPES	PAA
KCl	Merck
$MgCl_2$	Carl Roth GMBH
Na_2HPO_4 * 2 H_2O	Merck
NaCl	Carl Roth GMBH
$NaHCO_3$	Merck
NaOH	Carl Roth GMBH
Sodium citrate	Sigma
FBS “Gold“ (EU approved)	PAA
Plastic labware for cell culture	PAA
Aqua Bidest	Fresenius Kabi
HiTrap Blue HP	GE Healthcare
JBS Methylation Kit	Jena Biosciences
PD-10 Desalting Columns	GE Healthcare
PD MiniTrap G-25	GE Healthcare
NaOH 1mM	Merck
KH_2PO_4	Merck
Superoxide detection kit	Enzo Life Sciences
NaOCl	Sigma
Potassium iodide (KI)	Sigma
Chloramine T-hydrate 98 %	Sigma
$C_6H_5Na_3O_7$	Merck
Fibronectin	Sigma

Gelatin powder	Merck
Trypsin	PAA
Citric acid monohydrate	Merck
Polyethylene glycol (PEG) solution	Sigma
Ethanol (EtOH) 99 %	AustrAlco
Vena8Flouro+ Biochips	THP
Vena8 Enothelial+ Biochips	THP
Thrombin	Probe&Go
U-73122	Sigma
Chelerythrine	Sigma
BAPTA-AM	Sigma
Annexin V	BD
L-NAME	Sigma
TF ELISA	American Diagnostica
Fucoidan	Sigma
HCl	Sigma
100 Sterican	Brown

1.2 Antibodies

anti-CD36 antibody [clone FA6-152]	Abcam
polyclonal anti-SR-A antibody	Abcam
anti-SRBI antibody (NB400-101)	Novus Biological
anti-CD32 antibody	Abcam
CD62P antibody-FITC	BD Biosciences
IgG Isotype control antibody-FITC	BD Biosciences
IgG Isotype control antibody-PE	BD Biosciences
anti-CD154 (CD40L) antibody-FITC	BD Biosciences
Anti-CD142 antibody-PE	BD Biosciences

1.3 Buffers and Solutions

Fix solution
30 ml FACS flow
10 ml distilled water
1 ml CellFix

Wash buffer for preparation of washed platelets
140 mM NaCl
10 mM $NaHCO_3$
2.5 mM KCl
0.9 mM Na_2HPO_4 * 2 H_20
2.1 mM $MgCl_2$
22 mM $C_6H_5Na_3O_7$
0.055 mM D(+)-Glucose monohydrate
0.35% BSA
pH 6.5

Detachment buffer for TF expression
PBS
25 mM HEPES
10 mM EDTA

Tyrode Buffer for preparation of washed platelets
10 mM HEPES
134 mM NaCl
1 mM $CaCl_2$
12 mM $NaHCO_3$
2.9 mM KCl
0.34 mM Na_2HPO_4 * 2 H_2O
1mM $MgCl_2$
0.055 mM D(+)-Glucose monohydrate
pH 7.4

Buffer for albumin isolation
Binding buffer: 50mM KH_2PO_4, pH=7
Elution buffer: 50mM KH_2PO_4, 1.5M KCl, pH=7

1.4 Equipment and Software

ABX Micros 60	Horiba Medical (Tulln, Austria)
Apact 4004	LABiTec (Ahrensburg, Germany)
CellixVenaFlux software	Cellix Ltd (Dublin, Ireland)
DucoCell software	Cellix Ltd
FACS Calibur flow cytometer	Becton-Dickinson (Mountain View, USA)
Hamamatsu ORCA-03G digital camera	Hamamatsu (Herrsching, Germany)
Mirus nanopump	Cellix Ltd
Olympus IX70 fluorescence microscope	Olympus (Vienna, Austria)
Zeiss Axiovert 40 CFL microscope	Zeiss (Vienna, Austria)
Nano Drop 1000	Thermo Scientific
xMark Microplate Spectophotometer	BioRad
Mini Spin	Eppendorf
Centrifuge 5810 R	Eppendorf
Megafuge 1.0 R	Kendro Labratory Products

2. Methods

2.1 Blood collection

The study was approved by the Institutional Review Board (Ethics committee of the Medical University Graz). Blood was taken from end stage renal disease patients undergoing dialysis treatment prior to dialysis sessions and from patients after kidney transplantation during a scheduled outpatient clinical visit. Patients with malignancy, pregnancy, chronic inflammatory bowel disease, active alcohol abuse, severe organ dysfunction unrelated to renal dysfunction or patients with clinical evidence of active infection were excluded from the study. Age and sex matched control subjects that did not take any medication and had no evidence of renal disease were included in the study. All blood volunteers signed an informed consent form in agreement with the Institutional Review Board of the Medical University of Graz. All methods were carried out in accordance with the approved guidelines. Serum samples were stored at -70 °C until further use.

2.2 Preparation of washed platelets

Blood was drawn from healthy volunteers after they signed an informed consent form. Whole blood was collected using sodium citrate (3.8 %) as anticoagulant and immediately centrifuged at 400 x g for 20 minutes to obtain platelet rich plasma (PRP). To prepare human washed platelets as used for this study, fresh PRP was mixed with 2 % ethylenediaminetetraacetic acid (EDTA) in a 1:20 ratio to the PRP, whereas usually 10 ml of PRP were taken. This was then followed by centrifugation at 1000 x g for 15 minutes. Supernatant was discarded and the pellet re-suspended in 1 ml of a low pH wash buffer. Additional 9 ml of wash buffer were added and platelets were washed twice by centrifugation at 1000 x g for 15 minutes. The pellet finally re-suspended in tyrode buffer at a physiological and the suspension was filled up to the initial volume of PRP used. Washed platelets were immediately used for functional platelet assays.

2.3 Isolation of albumin from serum

Albumin from end stage renal disease patients and controls was separated from other serum proteins by affinity chromatography using HiTrap Blue HP 1 ml columns (GE Healthcare) according to the instructions of the manufacturer. In brief, after equilibration of 5 column volumes of binding buffer lipoprotein deficient serum (LPDS) was applied at a flow rate of 0.5 mL/min using an ÄKTA FPLC system (GE Healthcare). Afterwards, the column was washed again with binding buffer (5 column volumes) before albumin was eluted with 5 column volumes of elution buffer. The purified samples were then passed over PD-10 desalting columns to exchange the high salt elution buffer for phosphate buffered saline (PBS).

2.4 AOPPs assay

The AOPPs - assay was performed as previously described (207). In brief, serum was depleted of apoB-containing lipoproteins with polyethylenglycol (PEG). 400 µL PEG-solution (20% PEG in 200 mmol/L glycine buffer, pH = 7.4) was added to 1 mL serum, thoroughly mixed and incubated for 20 minutes at room temperature. Precipitate was pelleted by centrifugation (10,000 × g, 30 min) and the supernatant, referred to as LPDS was used for AOPP detection. Subsequently, 10 µL LPDS was mixed with 40 µl of 0.2 M citrate buffer and incubated for 2 minutes on a shaker. Afterwards, absorbance at 340 nm and optical density at 595 nm were measured within one hour on a Nano Drop 1000 (Peqlab, Erlangen, Germany) spectrophotometer. Absorbance was converted into the respective AOPP concentrations by means of a standard curve ranging from 1 to 100 µ mol/L chloramine-T as described (199). AOPP concentrations were expressed as µ mol/L of chloramine-T equivalents.

2.5 In vitro AOPPs preparation

AOPPs were prepared by incubation of albumin with hypochlorous acid in the absence of free amino acid/carbohydrates/lipids to exclude the formation of AGEs-like structures as previously described (199). For albumin oxidation a 100-fold molar excess of hypochlorous acid over albumin was used. After gently mixing, the AOPPs preparation was shortly centrifuged and incubated for 30 minutes on ice. Afterwards, the modified albumin preparations were passed over PD MiniTrap G-25 columns (GE-healthcare) to remove excess reactants and immediately stored at − 70 °C until further use.

Reduction of N-Chloramines – preparation of "aged" AOPPs

To investigate whether N-chloramines are involved in AOPPs elicited effects on platelets, hypochlorous acid modified albumin was incubated for 5 days at 37 °C to allow total decomposition of N-chloramines. AOPPs that were formed that way are referred to as "aged" AOPPs and were directly used in platelet aggregation experiments and compared to "aged" albumin treated platelets.

Reductive Methylation – preparation of methylated AOPPs

Reductive Methylation was performed using the JBS methylation kit, according to the protocol of the manufacturer (JBS methylation kit, Jena Biosciences). Thereby, formaldehyde is used as alkylating reagent and dimethylamine borane complex as reducing reagent, a combination that was described to have the mildest effect on the biochemical properties of the target proteins. All reactions were carried out on ice and all reagents provided by the kit were freshly prepared before use. Methylation instructions recommended the use of 1 mL protein (dissolved in PBS) within the concentration range between 1 and 10 mg/ml. Respective concentrations of albumin and AOPPs were used as target proteins for methylation. Solution A was prepared by dissolving 6 mg of dimethylamine borane complex in 100 µl reagent grade water. Then 20 µl of the prepared solution A was given to the target protein solution before solution B (containing 1 M formaldehyde) was added. The reaction mixture was gently mixed and then incubated at 4 °C for 2 hours. Afterwards, this step was repeated by adding the same amount of reagents A and B to the protein solution. After another 2 hours of incubation, 10 µl of solution A were added and the final preparation was stored at 4 °C overnight. The next morning, the reaction was

stopped by adding solution C, containing 1 M Tris (pH= 7.5). If precipitates were observed after overnight incubation, they were removed in a centrifugation step before addition of solution C. To separate the methylated protein from the reaction mixture, size exclusion chromatography using PD-10 columns (GE healthcare) was performed. The methylated protein was stabilized by solution D, containing 50 mM dithiothreitol. The final concentration of dithiothreitol in the sample ranges between 1-5 mM. Methylated albumin and methylated AOPPs were directly used for platelet aggregation experiments.

2.6 Platelet aggregation

Platelet aggregation was recorded at 37 °C with constant stirring using the 4-channel platelet aggregometer APACT4004 (LABiTec, Ahrensburg, Germany) that works on the principle of light transmission aggregometry, as previously described (208). Platelet aggregation of freshly prepared washed platelets was induced with AOPPs (25–100 µ g/mL), methylated AOPPs (100 µg/ml) or “aged” AOPPs (100µg/ml) and respective albumin controls. If not stated otherwise, platelets were incubated with AOPPs for 2 minutes. In experiments using isolated albumin of end stage renal disease patients (patients AOPPs), platelets were pre-incubated with patient AOPPs for 10 minutes, before platelet aggregation was started by addition of ADP (5–20 µM) in the presence of fibrinogen (1 µg/mL). The concentration of ADP was adapted to yield 30–50% platelet aggregation in the presence of pooled albumin preparations that were isolated before from healthy control subjects. Platelet aggregation was measured for 4 min with constant stirring (1000 rpm) at 37° C. The blocking antibodies/inhibitors were added 10 min before *in vitro* prepared AOPPs or patient AOPPs were added. Data are expressed as percent of maximum light transmission, with non-stimulated washed platelets being 0% and tyrode buffer being 100%. In some cases data are expressed normalized to the aggregation response achieved by albumin or AOPPs isolated from control subjects. In general, washed platelets were used for aggregation experiments within 3.5 hours after blood take to assure proper platelet functionality.

2.7 P-selectin and CD40L surface expression

Activated platelets express certain receptors specific for their activation state, such as P-selectin and CD40L. Activation dependent surface expression experiments were carried out according to previous established protocols (208). In brief, washed platelets were incubated with albumin or AOPPs for 5 min. To compare levels of activation, P-selectin expression was induced with ADP (3 µM) in the presence of cytochalasin B in a final concentration of (5 µg/ml). Cytochalasin B facilitates the translocation of P-selectin from platelet granules to the surface. CD40L was stimulated with thrombin (200 U/mL), known as one of the strongest platelet agonists, shown several times to significantly induce CD40L expression. Inhibitors/blocking antibodies were added 20 min before platelet stimulation. For P-selectin and CD40L staining, platelets were incubated at room temperature for 30 min in the presence of an anti-CD62P-FITC labelled antibody (15 µg/mL) against P-selectin or anti-CD40L-FITC labelled antibody (15 µg/mL). Afterwards, the reaction was stopped with ice cold PBS without $CaCl_2$ and $MgCl_2$ and samples were centrifuged at 400 x g for 7 min. The supernatant was discarded and cells fixed with fix solution (1 ml Cellfix, 10 ml distilled water and 30 ml cold FACS flow). Fixed platelets were measured with a FACS Calibur flow cytometer (Becton-Dickinson). FITC labelled antibodies were detected in FL-1 channel and an increase in P-selectin and CD40L expression was given when an increase in FL-1 channel could be measured. Fluorescence values were normalized to vehicle treated samples.

2.8 Phosphatidylserine surface exposure

To measure the exposure of the late activation marker phosphatidylserine on the surface of platelets, 200 µl of washed platelets (3×10^7/mL) were incubated for 10 minutes with albumin, AOPPs or the positive control collagen (10 µg/ml). To each sample 2.5 µl FITC labelled Annexin V was added and platelet samples were incubated at room temperature for further 15 minutes. Platelets were washed by centrifugation and re-suspended in binding buffer according to the manufacturer's protocol (BD Biosciences, Germany), and phosphatidylserine exposure on platelets was measured as Annexin V staining within one hour using flow cytometry. Data are expressed as % Annexin positive cells, whereas gating occurred with untreated and unlabelled platelets.

2. 9 Intracellular ROS production

Washed platelets (1 × 10^8/mL) were mixed with superoxide detection probe (Enzo Life Sciences, Lausen, Switzerland) and incubated according to the manufacturer's protocol for 30 min at 37 °C. Subsequently, platelets were pre-incubated with vehicle, the superoxide dismutase (SOD) mimetic Manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP, 50 µg/mL) or blocking antibodies at indicated concentrations for another 30 minutes, respectively. This was followed by incubation with AOPPs or collagen (10 µg/mL) for additional 15 minutes at room temperature. Superoxide/ROS production was immediately assessed by a FACS Calibur flow cytometer (Becton-Dickinson) as an increase in fluorescence intensity (FL-2 channel). Superoxide production was normalized to vehicle treatment.

2.10 Platelet adhesion under flow conditions

Platelet adhesion experiments under flow were performed to address two different issues. First, biochips were coated either with collagen or fibrinogen to determine whether AOPPs stimulated platelets adhere to ECM proteins. Second, washed platelets were perfused over a biochip pre-coated with HCAECs to reveal whether platelet adhesion to an intact endothelial monolayer is altered upon AOPPs treatment.

Platelet adhesion to collagen/fibrinogen

Vena 8 biochips (Cellix Ltd, Dublin, Ireland) were coated with fibrinogen (200 µg/mL) or collagen (200 µg/mL) at 4°C overnight as described (208). On the next day, the chips were blocked with BSA (10 µg/mL) for 30 minutes at room temperature and subsequently rinsed with PBS containing 0.9 mmol/L Ca^{2+} and 0.5 mmol/L Mg^{2+}. Washed platelets were incubated with AOPP-albumin (100 µg/mL) and immediately perfused over the fibrinogen/ collagen coated channels at constant shear stress of 0.5 dyne/cm^2 for 6 minutes using the Mirus nanopump (Cellix). Platelet adhesion was recorded on an Olympus IX70 fluorescence microscope and an Olympus UPlanFI-20/0.40 lens, using a Hamamatsu ORCA-ER digital camera and the Olympus CellP software. Cell images of 3 microscopic fields from each channel were captured, and images were analysed using DucoCell software (Cellix).

Platelet adhesion to endothelial cells

To investigate platelet adhesion to human coronary artery endothelial cells (HCAECs) under flow conditions, Vena 8 Endothelial + biochips (Cellix Ltd, Dublin, Ireland) were coated with fibronectin (20 µg/mL) at 4 °C overnight. On the next day, the chip was first rinsed with Dulbecco's modified PBS containing 0.9 mmol/L Ca^{2+} and 0.5 mmol/L Mg^{2+} and HCAECs (75.000 cells in 14 µL medium) were seeded in each channel. Cells were incubated for 20 minutes at 37 °C to allow cells to attach to the channels. Subsequently, medium was added and cells were then incubated for 2 hours at 37 °C. Afterwards, washed platelets were incubated with albumin (100 µg/ml) or AOPPs (100 µ g/mL) and immediately perfused over the HCAEC monolayer at constant shear stress of 0.5 dyne cm^2 for 6 minutes using the Mirus nanopump (Cellix). Platelet adhesion was recorded on an Olympus IX70 fluorescence microscope and an Olympus UPlanFI-20/0.40 lens, using a Hamamatsu ORCA-ER digital camera and the Olympus CellP software. Cell images of 3 microscopic fields from each channel were captured.

2.11 Cell culture of human coronary artery endothelial cells (HCAECs)

HCAECs were cultured as previously described (209). Cells were purchased from Lonza (Verviers, Belgium) and cultured in EGM-2 MV Bullet medium (Lonza) containing 5% fetal bovine serum (FBS) at 37 °C in humidified 5% CO_2. Endothelial cells from two different HCAEC donors were passaged at 80–90% confluence and were used from passage 6–9. 50,000 cells were plated on 48-well plates (Greiner, Germany) and used for experimental procedures one day after reaching confluence.

2.12 Determination of TF

TF surface expression on HCAECs

Washed platelets were treated with albumin (100µg/ml), AOPPs (100 µg/mL) or thrombin (200 U/mL) for 10 minutes before platelet suspensions were diluted 1:1 with pre-warmed endothelial growth media (EGM-2 MV Bullet medium + 5 % FBS). Subsequently, platelets were added to confluent HCAECs that were plated on 48-well culture plates. Cell culture plates were centrifuged for 2 minutes at 750 × g to allow cell-cell interaction during the following incubation period (3 hours, 37 °C). For control experiments, albumin (100 µg/ml) or AOPPs (100 µg/ml) in the absence of platelets (control experiment 1) or the supernatants of albumin (100 µg/ml) or AOPPs (100µg/ml) treated platelets (control experiment 2) were added to HCAECs. After co-cultivation with HCAECs, supernatants were removed and detachment buffer was added to all wells and cells were incubated for 30 minutes at 37 °C. Afterwards, the well content was collected and transferred to FACS tubes. After a washing step (5 min, 400 × g), the supernatant was discarded and cell pellets were incubated with anti-TF antibody (0,5 µg/mL) at 4 °C for 30 minutes. Cells were washed again (5 min, 400 × g) and the pellet re-suspended in fixative solution before samples were measured by flow cytometry.

TF surface expression on platelets

For experiments where TF expression on platelets was determined, albumin (100 µg/ml) and AOPPs (100 µg/mL) were added to freshly prepared washed platelets. Platelets were then incubated for 30 min in the presence of an anti-TF antibody (0.5 µg/mL). Further steps were performed as described for P-selectin surface expression.

Determination of serum TF levels

Serum TF levels of uremic patients and controls were determined using the IMUBIND ELISA kit (American Diagnostica Inc, Stamford, CT) according to the manufacturer's protocol. In brief, 100 µl of TF standard or diluted serum sample (1:4 with sample buffer containing 1 % BSA) was added in microwells pre-coated with capture antibody. Wells were covered and incubated overnight at 4 ° C.The next day, wells were washed four times with wash buffer (provided by the company) to

minimise unspecific binding to the capture antibody. This was followed by addition of 100 µl of a biotinylated detection antibody that specifically recognizes bound TF. After incubation of 1 hour at room temperature, wells were again washed four times with wash buffer. Afterwards, 100 µL of diluted enzyme conjugate (12 µL of Enzyme Conjugate to 12 mL of Enzyme Conjugate Diluent) were added to each well and incubated for one hour. The enzyme conjugate contains a streptavidin conjugated horseradish peroxidase (HRP) that finally allows the formation of the antibody - enzyme detection complex. Wells were washed four times before 100 µl of TMB substrate solution were added. Within the incubation time of 20 minutes the substrate reacts with HRP, creating a blue coloured solution. Afterwards, the reaction was stopped by adding 50 µl of stop solution (0.5M H_2SO_4) to increase the sensitivity. TF levels of the yellow coloured samples were determined by reading the absorbance on a microwell platereader at a wavelength of 450 nm within 30 minutes. Measured sample values were compared with those of the standard curve. TF levels of patient and control serum samples were expressed as pg/mL.

2.13 Statistical analysis

Data are shown as mean + SEM for n observations using platelets from different donors. Washed platelets that could not be induced by AOPPs or ADP were assumed to be functional ineffective and were excluded from the study. Each n in each experiment represents an individual donor. Comparisons of groups were performed using one-way ANOVA with Bonferroni's post-hoc test. Results obtained with patient samples were analysed with non-parametric tests due to the small sample size. Differences in between the three groups were analysed with the Kruskal–Wallis with Dunn's post-hoc test. Probability values of $P < 0.05$ were considered as statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Correlation between patient AOPPs and serum TF levels was determined with linear regression analysis, P and r^2 value are shown in the respective figure. All graphs of the calculated data were drown with Graph Pad Prism 5. Adobe Illustrator was used for figures in the introduction and discussion section.

III. Results

AOPPs used for the following experiments were formed *in vitro* by exposure of hypochlorous acid to human serum albumin as described previously (199). The AOPP content of *in vitro* modified AOPPs was comparable to AOPP levels that were measured in albumin isolated from end stage renal disease patients, referred to as "patient AOPPs". This chapter outlines our findings regarding the impact of *in vitro* generated or isolated patient AOPPs on platelet function and the resulting prothrombotic endothelial phenotype.

1. AOPPs increase ADP and collagen induced platelet aggregation of washed platelets

First, potential effects of AOPPs on platelets were tested using washed platelets isolated from the plasma of healthy donors. When *in vitro* formed AOPPs (100 µg/ml) were exposed to freshly prepared washed platelets, both, ADP and collagen induced platelet aggregation was increased compared to albumin treated platelets 4-fold **(Figure 5)**, indicating a gain of proaggregatory properties of oxidative modifications.

Of note, albumin and vehicle (PBS without Ca^{2+} and Mg^{2+}) treated platelets did not differ in their aggregation response **(Figure 5)**. If not stated otherwise, no difference between vehicle and albumin treated platelets was achieved in all following platelet assays performed.

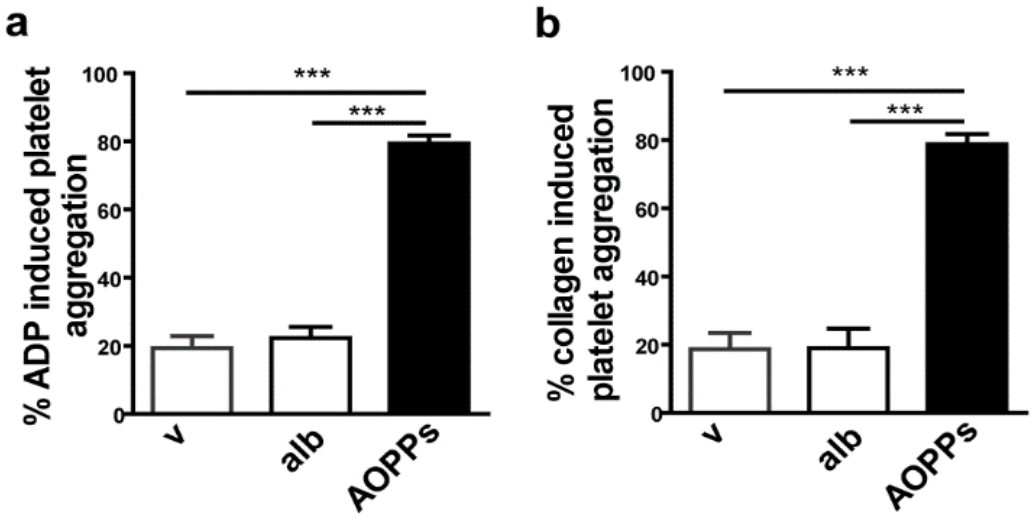

Figure 5: AOPPs increase ADP and collagen stimulated platelet aggregation of washed human platelets

*(**a,b**) Platelets were pre-incubated with vehicle (v), albumin (alb, 100 µg/ml) or AOPPs (100µg/ml) for 2 minutes, before stimulation occurred with either (**a**) ADP (5 -10µM) in the presence of fibrinogen (5 µg/ml) or (**b**) collagen (5 -10 µg/ml) to achieve approximately 20 % aggregation. Recording of light transmission aggregometry was started directly after stimulation of platelets and platelet aggregation was measured for 5 minutes (n=5). All values are shown as mean + SEM. ***P < 0.001 as indicated.*

2. AOPPs induce aggregation of washed platelets

Based on the strong and rapid increase in platelet aggregation, AOPPs were tested for their ability to trigger aggregation independent of platelet pre-stimulation with known platelet agonists. AOPPs (25-100 µg/ml) induced platelet aggregation in a concentration dependent manner, whereas albumin failed to transmit proaggregatory effects **(Figure 6a, b).**

Moreover, platelet aggregation assays were performed in the presence of EGTA to exclude platelet agglutination. In the case of platelet agglutination, platelet-platelet interaction results from crosslinking of nearby platelets, independent of platelet activation and participation of the major platelet integrin GPIIbIIIa that is

continuously expressed on platelets but only able to bind fibrinogen in its activated state.

A functional active GPIIbIIIa depends on Ca^{2+} mobilisation. As the calcium chelator EGTA (1 and 5 mmol/l) concentration dependently decreased, AOPPs induced platelet aggregation **(Figure 6c)**, activation of GPIIbIIIa inside-out signalling i.e. platelet activation is definitely involved in AOPP mediated effects on platelets.

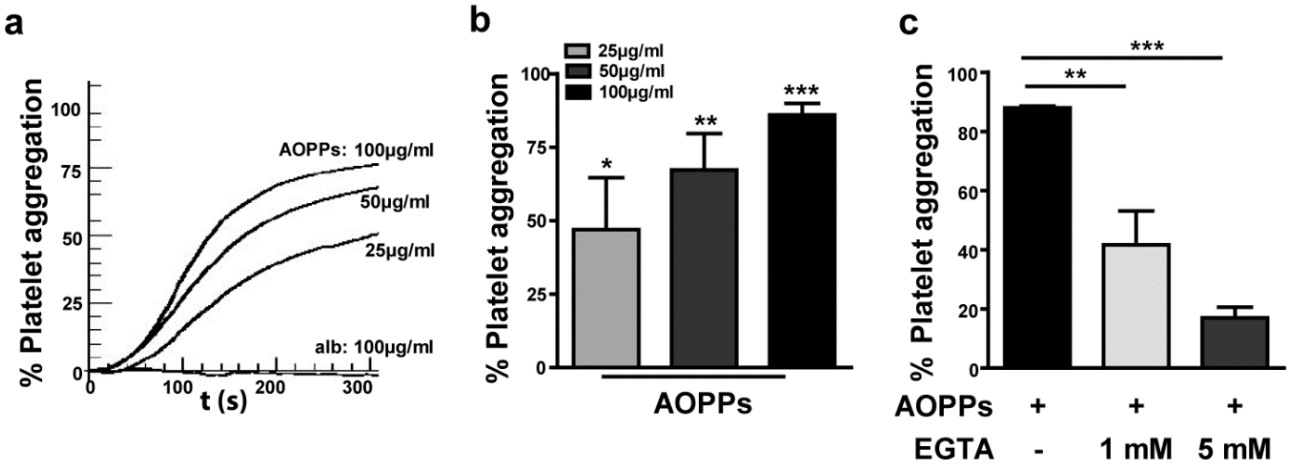

Figure 6: AOPPs trigger platelet aggregation, not agglutination, in a concentration dependent manner

(a,b) *Albumin (alb) or AOPPs at indicated concentrations were added to washed platelets two minutes before aggregation was recorded using light transmission aggregometry. One representative tracing out of 5 is shown in* ***(a)****, whereas quantification of the concentration dependent increase in platelet aggregation is shown in* ***(b)****.* ***(c)*** *EGTA (1 mM and 5mM) was added to platelets 10 minutes prior to stimulation with AOPPs (100 µg/ml) and reduced the proaggregatory effect of AOPPs in a concentration dependent manner, thereby excluding platelet agglutination (n=3). All values are shown as mean + SEM. *P< 0.05, **P< 0.01 and ***P< 0.001 as indicated.*

3. AOPPs induce upregulation of platelet activation markers

The activation of platelets by proaggregatory stimuli leads to degranulation of platelet α-granules, allowing the procoagulant glycoprotein P-selectin to integrate in the outer platelet membrane. The crucial role of P-selectin is given by its contribution to inflammatory and prothrombotic cell-cell interactions, as it occurs between platelet P-selectin and its ligand P-selectin glycoprotein ligand-1 (PSGL-1) present on human neutrophils.

The activation dependent surface expression of P-selectin was determined by flow cytometry using a labelled anti-P-selectin antibody. AOPPs promoted the upregulation of platelet P-selectin comparable to stimulation with ADP (3 µM) as shown in **Figure 7a**. Addition of cytochalasin B (5µg/ml) is necessary to induce degranulation of ADP stimulated platelets. In contrast, AOPPs facilitated α-granule secretion, and consequent P-selectin upregulation was not altered in the presence of cytochalasin B (data not shown), suggesting a different mechanism of activation. While P-selectin is a marker for early platelet activation, the exposure of phosphatidylserine on the platelet outer membrane is a marker of late and irreversible platelet activation. However, only strong agonists (like thrombin or collagen) are capable of triggering the conversion to procoagulant phosphatidylserine exposing platelets (210).

Phosphatidylserine exposure on platelets can be determined with labelled Annexin V, which binds specifically to surface expressed PS. AOPPs lead to a 5-fold increase in Annexin V positive platelets compared to albumin as presented in **Figure 7b**. Thus, the AOPPs induced expression of the procoagulant marker phosphatidylserine was similar potent when compared to collagen stimulation (10 µg/mL) which was performed as positive control **(Figure 7b)**.

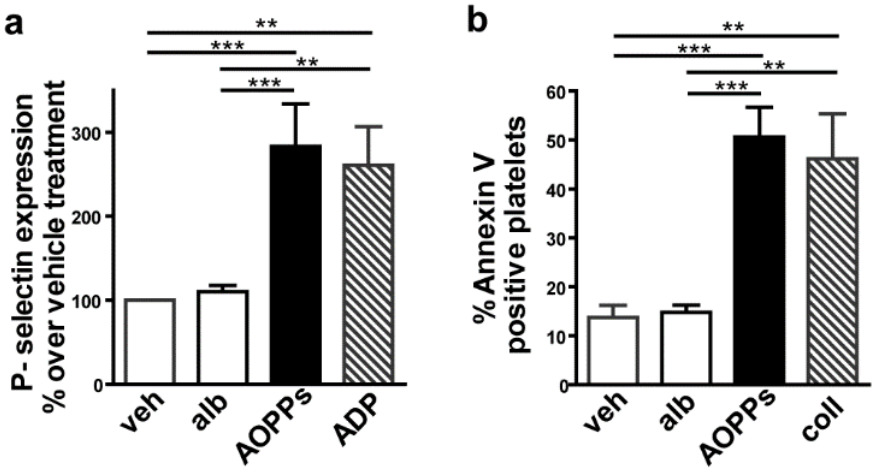

Figure 7: AOPPs induce upregulation of the platelet activation markers P-selectin and phosphatidylserine

(a) *Upregulation of P-selectin on the surface of platelets was measured by flow cytometry using an FITC-labelled anti-CD62P antibody against P-selectin. Platelets were incubated with albumin (100 µg/mL), AOPPs (100 µg/ml) or the positive control ADP (3 µM) in the presence of cytochalasin B (5 µg/mL) for 10 minutes. Obtained FL1 fluorescence levels were normalized to vehicle (veh) treated platelets and shown as percentage over vehicle (n= 7).* ***(b)*** *Platelets were exposed to vehicle (veh), albumin (100µg/mL), AOPPs (100µg/mL) or the positive control collagen (coll, 10µg/mL) for 10 minutes and FITC-labelled Annexin V was used to determine surface expression of PS. Results are shown as percentage Annexin V-positive platelets (n= 7). All values are shown as mean+ SEM. **P< 0.01, ***P< 0.001 as indicated.*

4. AOPPs promote platelet adhesion to fibrinogen under flow conditions

As shown in platelet aggregation experiments, addition of AOPPs to washed platelets induced activation of the platelet glycoprotein GPIIbIIIa, which *in vivo* facilitates adhesion to the ECM protein fibrinogen that is exposed to the blood stream in times of vessel stress. Consequently, platelet adhesion to fibrinogen was evaluated in the presence of AOPPs using a system that mimics vessel flow conditions. Thereby, washed platelets treated with AOPPs or albumin were perfused

over fibrinogen (100 µg/ml) coated biochip channels, while respective adhesion was recorded after three minutes of platelet perfusion.

AOPPs treated platelets showed significantly higher platelet adhesion to fibrinogen when compared to albumin, where only small aggregates could be detected **(Figure 8a)**. Platelet adhesion images of repeated experiments were analysed with a standardized image quantification program that counts the area covered by platelets. Image quantification clearly confirmed the significant increase in platelet adhesion **(Figure 8b)**.

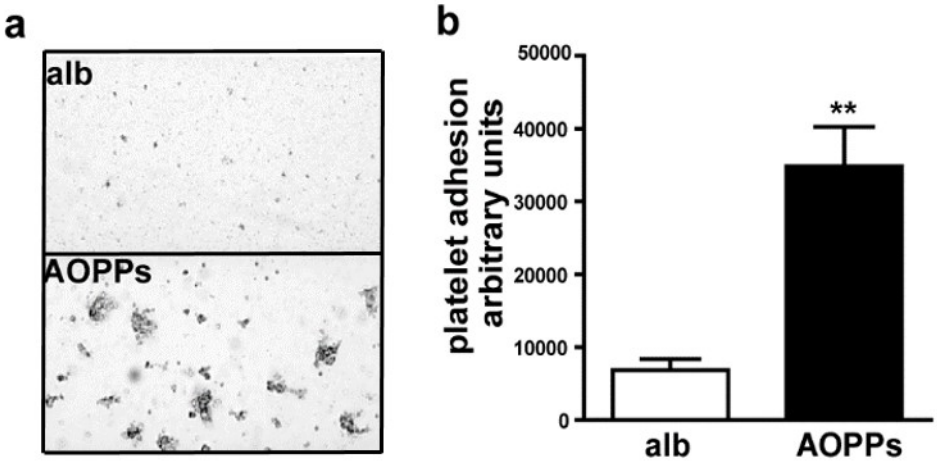

Figure 8: Platelet adhesion to fibrinogen is elevated upon AOPPs exposure

(a, b) *Platelets were treated with albumin (alb, 100 µg/ml) or AOPPs (100 µg/ml) and then perfused over fibrinogen (100µg/ml) coated biochip channels at constant shear stress. Platelet adhesion was video monitored and one representative picture is shown in* ***(a)****, whereas the area covered by platelets was calculated from 3 different pictures of each platelet donor (n=4) after 3 minutes of platelet perfusion* ***(b).*** *AOPPs induced increase in platelet adhesion is indicated in arbitrary units given by the calculation with DucoCell Software. Values are shown as mean+ SEM.*
***P< 0.01 as indicated.*

5. N-Chloramine formation is responsible for the proaggregatory effects of AOPPs

It is known that hypochlorous acid, the oxidant formed *in vivo* by neutrophilic MPO and used for *in vitro* preparation of AOPPs, primary targets ε-amino groups of lysine residues. As a result, formation of lysine chloramines occurs along with the loss of the positive charge. It was previously reported in our group that the amino acids tyrosine, lysine, histidine and arginine are modified upon exposure of HOCl to albumin, whereas lysine residues showed the highest extend of oxidative modifications.

To test whether lysine residues specifically play a role in AOPP triggered platelet activation, the chlorination of lysines was prevented by reductive methylation, or freshly prepared *in vitro* AOPPs were incubated for 5 days at 37 °C to allow total decomposition of N-chloramines. Thereby formed AOPPs are referred to as methylated and “aged” AOPPs. After respective albumin modifications, methylated and aged AOPPs were tested on its effects on platelet aggregation of washed platelets as shown in **Figure 9**.

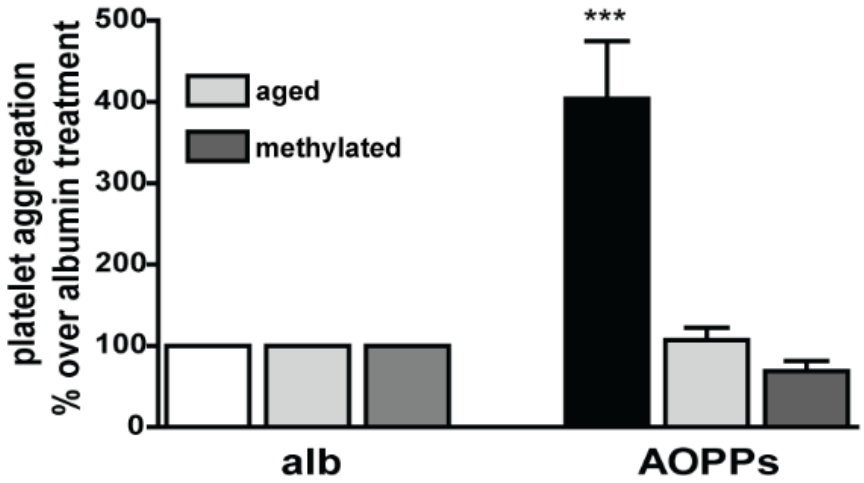

Figure 9: N-Chloramines are responsible for AOPPs triggered aggregation

*Platelets were pre-incubated with albumin (alb, 100 µg/ml), aged albumin (100 µg/ml), methylated albumin, AOPPs (100µg/ml), aged AOPPs (100µg/ml) or methylated AOPPs for 2 minutes before stimulation with ADP (5 -10µM) in the presence of fibrinogen (5 µg/ml) to achieve approximately 20 % aggregation. Recording of light transmission aggregometry was started directly after stimulation of platelets, and platelet aggregation was measured for 5 minutes (n=5). All values are shown as mean + SEM. ***P < 0.001 as indicated.*

6. AOPPs activate platelets via CD36

The induction of a rapid and strong platelet response let us assume that AOPPs elicit their effects on platelets via directly binding to platelet surface receptors. In this context, we assessed a potential role of scavenger receptors that were reported to interact with oxidized lipoproteins. Although literature has not yet been able to clearly identify which scavenger receptors are present on platelets, there are studies that report the expression of class A scavenger receptor LOX-1 (211), class B scavenger receptors SR-BI (153), and CD36 (146) on the surface of platelets. Of additional importance, AOPPs were already described to be SR-BI (199) and CD36 (203) ligands on other cell types.

To test interactions between AOPPs and receptors on platelets, washed platelets were pre-incubated with several blocking antibodies prior to addition of AOPPs. Of note, only blocking of scavenger receptor CD36 could significantly reduce AOPP-induced platelet aggregation, whereas blocking antibodies against SR-BI and SR-A failed to show any effects **(Figure 10a)**. The anti-CD36 antibody used in this experimental setting is known to specifically block an epitope responsible for oxLDL-binding and since it was reported that the Fc portion of this antibody is capable of inducing platelet aggregation independently (212), platelets were pre-incubated with a specific antibody (anti-CD32, 1µg/ml) against Fcγ receptors. Blocking the oxidized LDL binding site of CD36 was concentration dependent and blocking of the Fcγ receptor was necessary to counteract Fcγ induced platelet aggregation **(Figure 10b)**. In addition, no intrinsical effect of CD32 was found **(Figure 10b)**. However, blocking of CD36 did not result in total inhibition of platelet aggregation like described for oxLDL (213), suggesting a role for another, yet unknown platelet receptor that might be involved in AOPPs induced effects.

In the context of SR-A receptors, we also used fucoidan, since its role as SR-A blocking agent in platelet assays was described (145). Although preincubation with fucoidan (50 µg/ml) did not affect AOPPs mediated platelet aggregation (data not shown), its recently demonstrated proaggregatory role via platelet CLEC-2 receptor (214) exclude fucoidan as a suitable substance in this context. Therefore, a specific SR-A blocking antibody was used to explicitly rule out a contribution of SR-A receptors **(Figure 10a)**.

To provide further evidence for the crucial role of CD36 in AOPP mediated effects on platelets, P-selectin upregulation was detected in the presence of an anti-CD36 antibody. P-selectin expression was diminished approximately 60 % when anti-CD36 (4 µg/ml) was exposed to platelets prior to AOPPs stimulation **(Figure 10c)**.

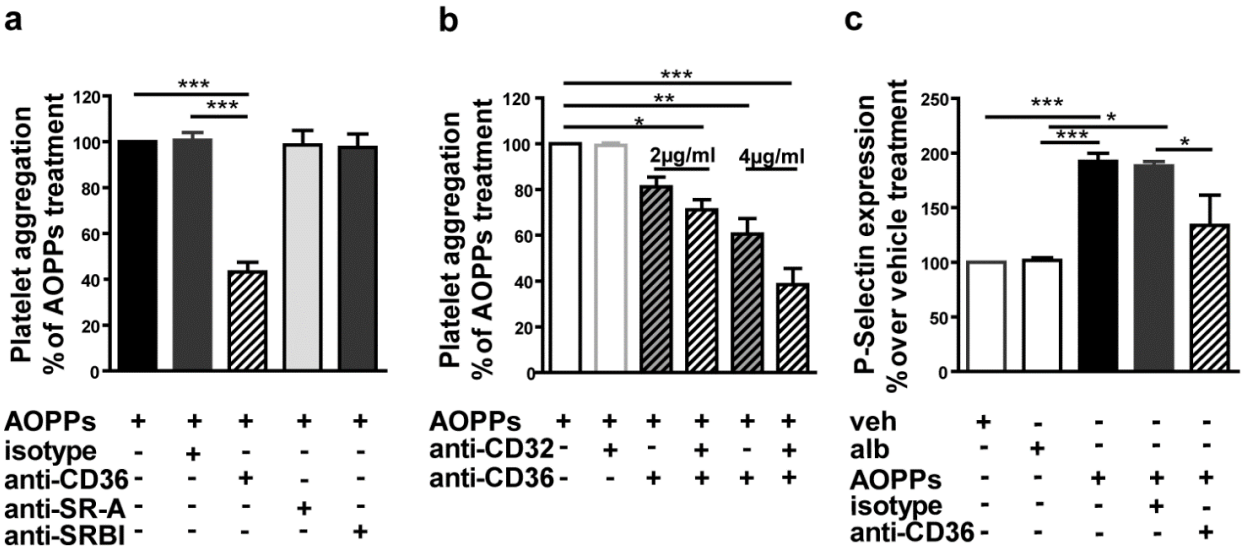

Figure 10: Scavenger receptor CD36 is involved in AOPPs triggered platelet aggregation and P-selectin upregulation

(a) *Platelets were incubated with an anti-CD36 blocking antibody (4 µg/mL), an anti-SR-A blocking antibody (4 µg/mL), an anti-SRBI blocking antibody (4 µg/mL) and a respective isotype control for 10 minutes before AOPPs (100µg/mL) were added. In all cases, platelets were preincubated with a specific antibody against the Fcγ receptor (anti-CD32, 1 µg/ml). Data are presented normalized to AOPPs induced platelet aggregation (n= 3–7).* ***(b)*** *Platelets were preincubated with indicated concentrations of anti-CD36 antibodies in the presence or absence of anti-CD32 antibodies before platelets were stimulated with AOPPs (n=4).* ***(c)*** *Expression of the activation dependent marker P-selectin upon exposure of albumin (alb, 100µg/ml) or AOPPs (100 µg/ml) was detected in the presence of an anti-CD36 blocking antibody (4µg/mL) or isotype control. Results were normalized to vehicle treated platelets (n= 3–6). All values are shown as mean + SEM. *P< 0.05, **P< 0.01, ***P< 0.001 as indicated*

7. AOPP-induced platelet aggregation depends on intracellular ROS/superoxide production

CD36 was recently associated with platelet hyperactivity and platelet intracellular ROS production (138). Superoxide was shown to be produced by activated platelets to increase aggregation, and to be furthermore involved in integrin activation (129). Therefore, a superoxide detection probe was used to clarify whether AOPPs promote ROS production in platelets.

As depicted in **Figure 11**, treatment of platelets with AOPPs resulted in ROS formation that was comparable to platelet ROS formation upon collagen (10 µg/ml) exposure. Induction of this response was almost completely reversed by the SOD mimetic MnTMPyP, (50 µg/ml), whereas the eNOS inhibitor L-N6-Nitroargenine methyl ester (L-NAME) did not affect superoxide production in platelets **(Figure 11a)**. In line with these results, MnTMPyP pentachloride diminished AOPPs triggered platelet aggregation **(Figure 11b)**, while different concentrations of L-NAME did not result in any aggregation changes **(Figure 11c)**. Therefore we concluded that interfering with platelet NO production has no impact on platelet aggregation mediated by AOPPs.

Taken together, these results clearly indicate a role of ROS in AOPP triggered platelet activation and aggregation. Of note, L-NAME and MnTMPyP showed no intrinsic effects neither when measuring platelet superoxide production nor in aggregation assays (data not shown).

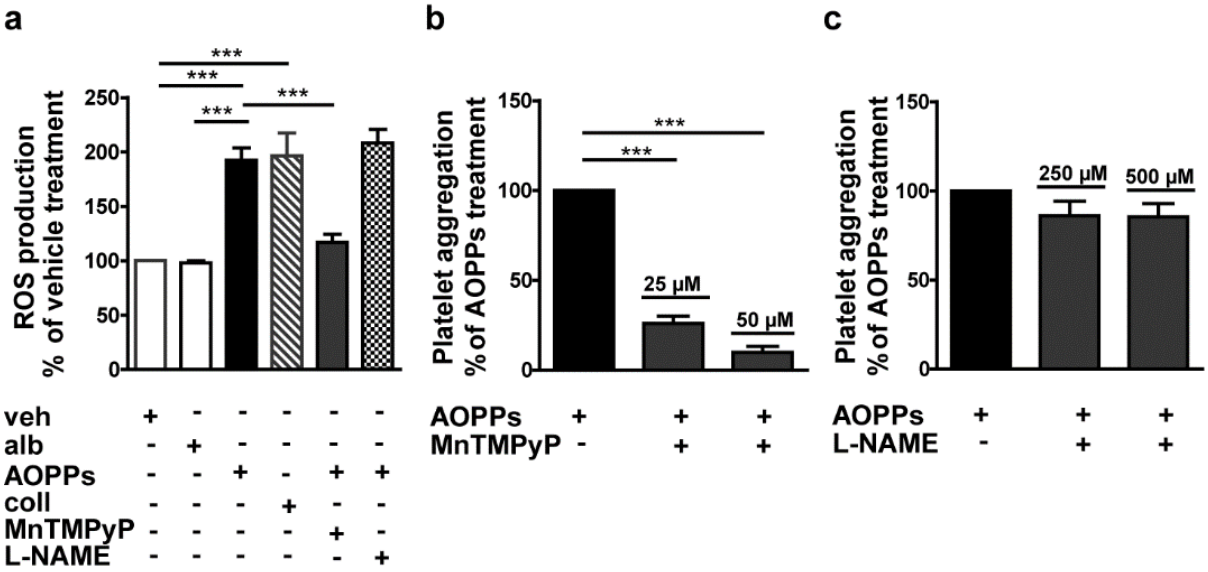

Figure 11: AOPPs triggered platelet aggregation depends on ROS production

(a) ROS/superoxide production of platelets treated with vehicle (veh), albumin (alb, 100 µg/mL), AOPPs (100 µg/mL) or the positive control collagen (coll, 10 µg/mL) were performed with or without pretreatment of the SOD mimetic MnTMPyP (50µmol/L) or the eNOS inhibitor L-NAME. Levels of intracellular ROS are shown relative to vehicle treatment. ROS were assessed by flow cytometry (n= 3). **(b, c)** Platelets were incubated with different concentrations of MnTMPyP **(b)** or L-Name **(c)** respectively, to test whether superoxide or eNOS play a role in AOPPs induced platelet aggregation. All values are shown as mean + SEM. ***P< 0.001 as indicated

8. Platelet evoked intracellular ROS production is not related to CD36 activation

Since recent literature pointed out ROS production as a downstream event of CD36 activation (213), we hypothesized that ROS production in platelets occur via this signalling pathway. Furthermore, based on our findings that incubation of platelets with the superoxide dismutase mimetic almost completely abolished AOPPs induced proaggregatory effects, a contribution of CD36 in this context seemed to be most likely. Nevertheless, blocking of CD36 did not change intracellular ROS levels (**Figure 12**), suggesting that additional receptors might play a role in AOPPs triggered ROS production.

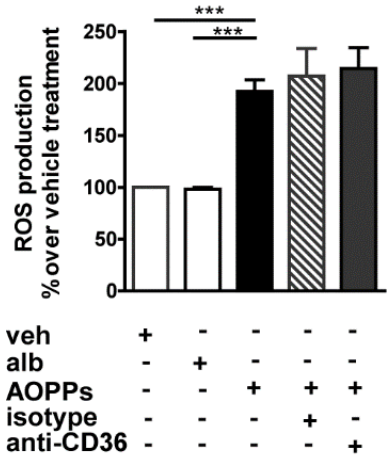

Figure 12: Scavenger receptor CD36 is not involved in AOPPs evoked ROS production

*Platelets were incubated with vehicle (veh), albumin (alb, 100µg/ mL) or AOPPs (100 µg/ml), in the presence of anti-CD36 (4 µg/ml) or isotype control. Antibodies were added 10 minutes in advance. All values are shown as mean + SEM. ***P< 0.001 as indicated*

9. AOPPs mediate the activation of PLC, PKC and mobilisation of Ca^{2+}

Next, we wanted to gain insights in AOPPs triggered intraplatelet signalling. A common signalling pathway that was elucidated in several cell types links PLC and PKC activation to intracellular ROS production. We assumed that PLC and PKC activation might also play a role in AOPP mediated platelet effects, as it was also recently reported that ligation of CD36 by oxLDL caused activation of PLC and this further extends to activation of PKC (143). Moreover, ligation of CD36 by oxLDL was shown to stimulate PKC-dependent generation of ROS (129). In general, PKC was shown to play an important role in granule secretion and subsequent platelet aggregation (215). Ca^{2+} mobilisation occurs downstream of PLC activation, is activated by diacyl glycerol (DAG), and is essential for platelet activation, e.g. platelet aggregation.

To deepen our understanding of the processes whereby AOPPs induced platelet aggregation, we investigated whether the presence of several inhibitors could interfere with AOPPs mediated proaggregatory effects. We observed that AOPPs triggered platelet aggregation was inhibited by the PLC inhibitor U-73122 and the

PKC inhibitor chelerythrine in a concentration dependent manner as depicted in **Figure 13a** and Figure **13b**, respectively.

For agonist induced elevation of free intracellular Ca^{2+} levels, Ca^{2+} is released from intracellular stores, but also enters the cell from the outside. Extracellular Ca^{2+} is required for AOPPs induced platelet aggregation as shown in **Figure 13c.** The chelation of intracellular Ca^{2+} with BAPTA-AM (20 µM) resulted in a significant reduction in AOPP-induced platelet aggregation (**Figure 13c**).

Since BAPTA-AM is solved in DMSO, and an antiaggregatory function of DMSO is described (216), the impact of BAPTA-AM on AOPPs treated platelets was compared to a DMSO vehicle. DMSO reduced platelet aggregation by approximately 20 % **(Figure 13c)**.

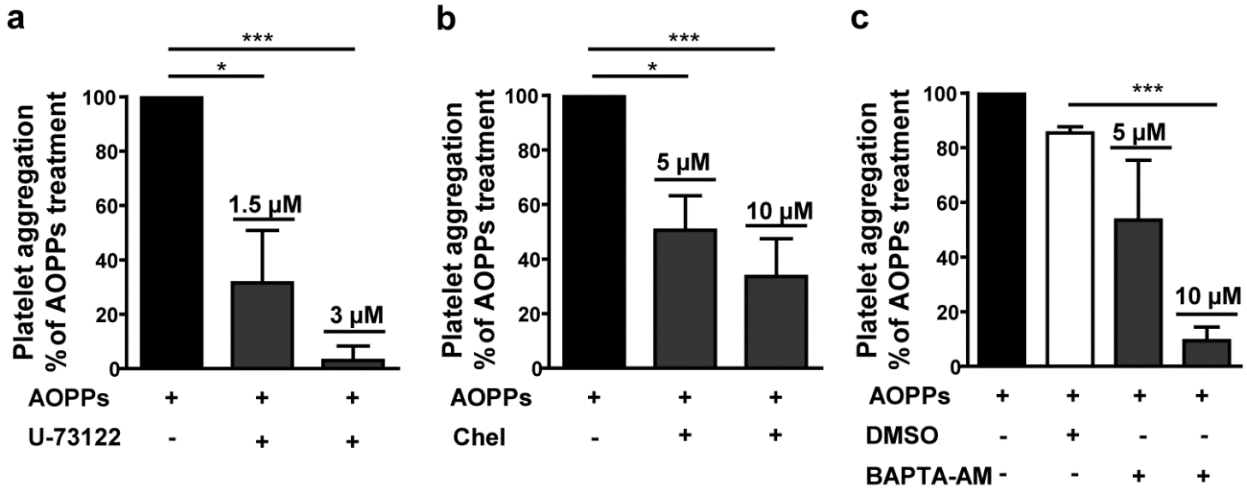

Figure 13: AOPP-induced platelet activation depends on activation of PLC and PKC and Ca2+ mobilisation

(a, b, c) *Washed platelets were preincubated with different concentrations of the PLC inhibitor U-73122 (1.5 and 3 µmol/L)* ***(a)****, the PKC inhibitor chelerythrine (Chel, 5 and 10 µmol/L)* ***(b)*** *as well as the Ca^{2+} scavenger BAPTA-AM (BAPTA, 20µmol/L)* ***(c)****, before AOPPs (100 µg/mL) were added. The decrease of platelet aggregation is shown as percentage of normalized AOPPs response. BAPTA-AM effects were compared to DMSO treated platelets (n= 3–5). All values are shown as mean + SEM. *P< 0.05, ***P< 0.001 as indicated*

10. AOPPs upregulate CD40L on platelets and increase platelet adherence to endothelial cells

Activated platelets intensify inflammatory processes by expressing membrane molecules on their surface that influence several biological functions in the vessel wall. Platelets were shown to express the ligand of CD40 (CD154) within seconds of exposure to distinct agonists (85).The CD40–CD40L interaction represents an important link between platelet activation and inflammation.

For that purpose we assessed whether AOPPs induce expression of CD40L. As indicated in **Figure 14a**, CD40L was upregulated upon exposure of AOPPs.

This finding raises the question whether adhesion properties of AOPP-activated platelets are changed under flow conditions. Therefore, human coronary artery endothelial cells were cultivated in a Vena Biochip **(Figure 14, left panel)** and platelets were perfused over the biochip at constant shear stress. Photographs were taken after 3 minutes of perfusion to visualize platelet binding properties. Notably, AOPPs (100 µg/ml) caused increased adherence to HCAECs as indicated by arrows in **Figure 14 (right panel)**.

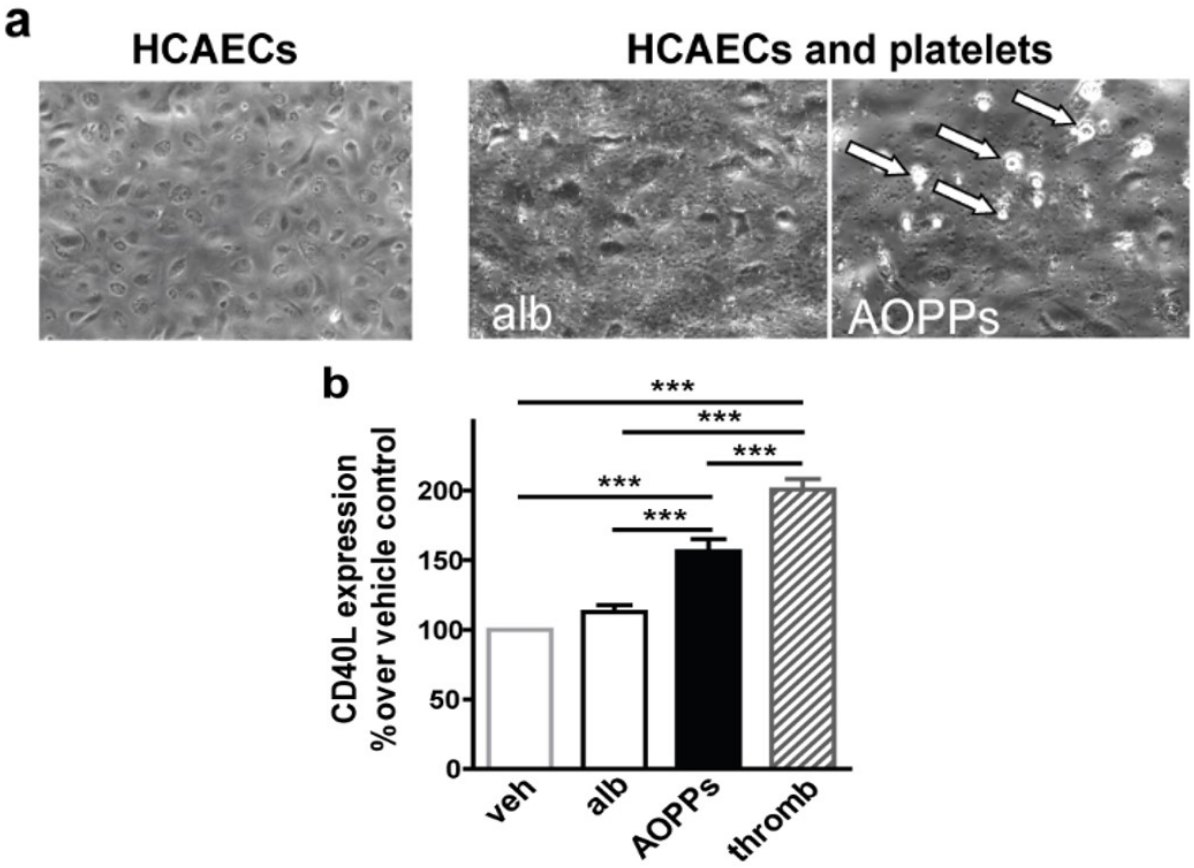

Figure 14: AOPPs cause increased platelet adhesion to HCAECs and trigger upregulation of CD40L on platelets

(a) Platelets were treated with AOPPs (100 µg/mL) or albumin (alb, 100 µg/mL) for 2 minutes and then perfused over a HCAEC monolayer (left panel) with constant shear stress. Adhesion of platelets to HCAECs as a result of the respective treatment is shown in the right panel. Platelet aggregates are indicated by arrows. Images were taken 6 minutes after the start of perfusion (n = 3) **(b)** Platelets were preincubated with vehicle (veh), albumin (alb, 100 µg/mL), AOPPs (100 µg/mL) or thrombin (thromb, 200 U/mL), and CD40L expression was assessed by flow cytometry. Data indicate the percentage of CD40L expression over vehicle treatment (n = 5). Results are shown as mean + SEM of percentage TF expression compared to vehicle treatment. ***P < 0.001 as indicated.

11. AOPPs induce TF expression on HCAECs via platelet-endothelial interactions

Ligation of CD40-CD40L upon exposure of several platelet agonists is associated with TF expression on endothelial cells, thereby promoting a prothrombotic phenotype (75). When AOPPs stimulated platelets were exposed to HCAECs, they caused a considerable induction of endothelial TF as measured by flow cytometry **(Figure 15a)**. Thrombin (200 U/ml) was used as positive control, since it was described to be a strong inducer of platelet mediated TF expression on endothelial cells (75).

To make sure that soluble factors released by platelets are not sufficient to induce TF on endothelial cells, we incubated HCAECs with supernatants of platelets that were prior incubated with AOPPs. In addition, albumin or AOPPs were added to cultured HCAECs in the absence of platelets, to exclude direct effects of AOPPs on endothelial TF expression. This control experiment was from particular importance, since it was described that AOPPs induced the expression of several endothelial surface proteins. Of note, the levels of TF were not altered when endothelial cells were treated with AOPPs alone **(Figure 15b)** or with supernatants of platelets incubated with AOPPs **(Figure 15c)**.

Moreover, to rule out platelet derived TF in this context, we measured TF expression on platelets stimulated with AOPPs. Surface expression pattern of TF was also unchanged on platelets upon AOPPs exposure **(Figure 15d)**.Taken together, these results provide strong evidence for direct platelet-endothelial interaction via CD40L-CD40.

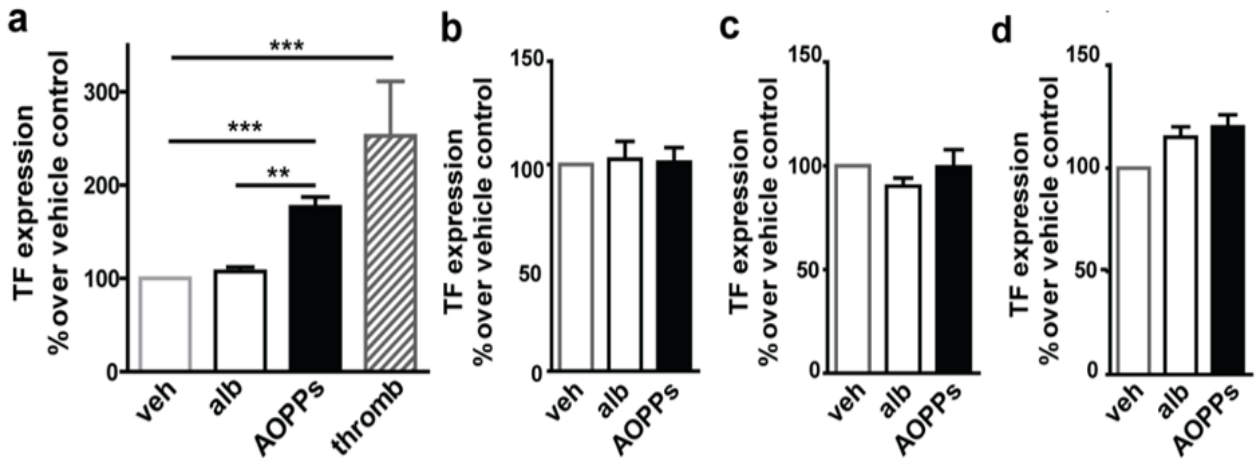

Figure 15: AOPPs mediate endothelial TF expression via a direct platelet-endothelium interaction

(a) Platelets were pre-exposed to vehicle (veh), albumin (alb, 100 µg/mL), AOPPs (100 µg/mL) or the positive control thrombin (thromb, 200 U/ml) before they were added to a HCAECs monolayer to allow cell-cell interaction (n = 3). **(b)** Supernatants that were obtained by centrifugation of vehicle (veh), albumin (alb, 100 µg/mL) or AOPPs (100 µg/mL) treated platelets were added to a HCAECs monolayer (n = 3–4) **(c)** Vehicle (veh), albumin (100 µg/mL) or AOPPs (100 µg/mL) were directly added on a HAECs monolayer in the absence of platelets (n = 3–4). **(d)** Platelets were exposed to albumin (100 µg/mL) or AOPPs (100 µg/mL). **(a–d)** TF surface expression on HCAECs **(a-c)** or platelets **(d)** was determined with a PE-labelled anti-TF-antibody by flow cytometry. All results are shown as mean + SEM of percentage TF expression compared to vehicle treatment. **P < 0.01, ***P < 0.001 as indicated.

12. Patient AOPPs enhance platelet aggregation dependent on their oxidative modifications

Elevated levels of AOPPs were shown to accumulate in plasma of end stage renal disease patients and were strongly associated with disease severity. Furthermore, they and are independent cardiovascular risk factors (176,206).

All patient samples we used for this study were collected at the Department of Internal Medicine, Clinical Division of Nephrology at the Medical University of Graz. To demonstrate that our *in vitro* experiments can be translated into the *in vivo*

situation we isolated albumin from patient and control sera. End stage renal disease patients are known to have the highest AOPPs levels (167), whereas AOPP levels have not been determined in the sera of kidney transplant recipients (KTx) so far. Further information about the respective patient group is given in the method section and the clinical characteristics of all study subjects are shown in **Table 1**.

	Control (ctrl)	End stage renal disease patients	
		Hemodialysis (HD)	Kidney transplant (KTx)
n	14	28	29
Age (y)	40 (25–73)	54 (18–81)	53 (24–75)
Male/female	7/7	14/13	11/19
Dialysis (months)	–	111 ± 22	–
Cardiovascular disease	–	17/27	15/30
Statins	–	4/27	6/30
Antihypertensive agents	–	12/27	21/30
Antiaggregant agents	–	8/27	7/30
EPO	–	2/27	2/30
Anticoagulants	–	4/27	4/30
Albumin (g/dL)	4.7 ± 0.9	3.9 ± 0.6[a]	4.0 ± 0.8
Hemoglobin (g/dL)	14.3 ± 0.2	11.1 ± 2.1[a]	12.4 ± 2.2[a]
Phosphate (mmol/L)	1.2 ± 0.1	1.6 ± 0.3[b]	1 ± 0.2
Creatinine (mg/dL)	0.9 ± 0.0	8.1 ± 1.6[a]	1.6 ± 0.3[a]
Ca^{2+} (mmol/L)	2.4 ± 0.0	2.2 ± 0.4[b]	2.5 ± 0.5

Table 1: Clinical characteristics of study subjects

Results are given as medians with the interquartile range or as mean ± SD. Significance from the Kruskal-Wallis with Dunn post hoc test was accepted at the level of 0.001 versus control ([a]) and at the level of 0.05 versus control ([b])

To determine the levels of protein modifications, AOPPs were detected in ApoB depleted serum of hemodialysis patients, kidney transplant recipients (KTx) and controls. Thereby, relative absorbance of samples and a chloramine-T standard was measured on a photospectrometer at a certain wavelength. Afterwards, AOPP concentration was expressed as µmol/L of chloramine-T equivalents.

In good agreement with previously published papers (180) (199) HD-patients showed significantly increased AOPP levels compared to controls **(Figure 16a)**. Interestingly, AOPPs measured in KTx patients were significantly decreased compared to HD-patients, almost reaching levels of healthy subjects **(Figure 16a).** This clearly indicates that oxidative stress is reduced after kidney transplantation, resulting in decreased protein oxidation.

To elucidate whether the levels of *in vivo* protein modifications are sufficient to induce platelet aggregation we isolated albumin from serum of uremic patients (patient AOPPs) and respective controls. Of note, patient AOPPs amplified ADP stimulated platelet aggregation dependent on the levels of their oxidative modifications **(Figure 16b)**. In these experiments, the aggregation response of patient AOPPs treated platelets was shown relatively to control albumin treated platelets.

In line with our *in vitro* data, elevated levels of platelet aggregation as a result of patient AOPPs treatment could be reduced by blocking CD36 on platelets **(Figure 16c)**.

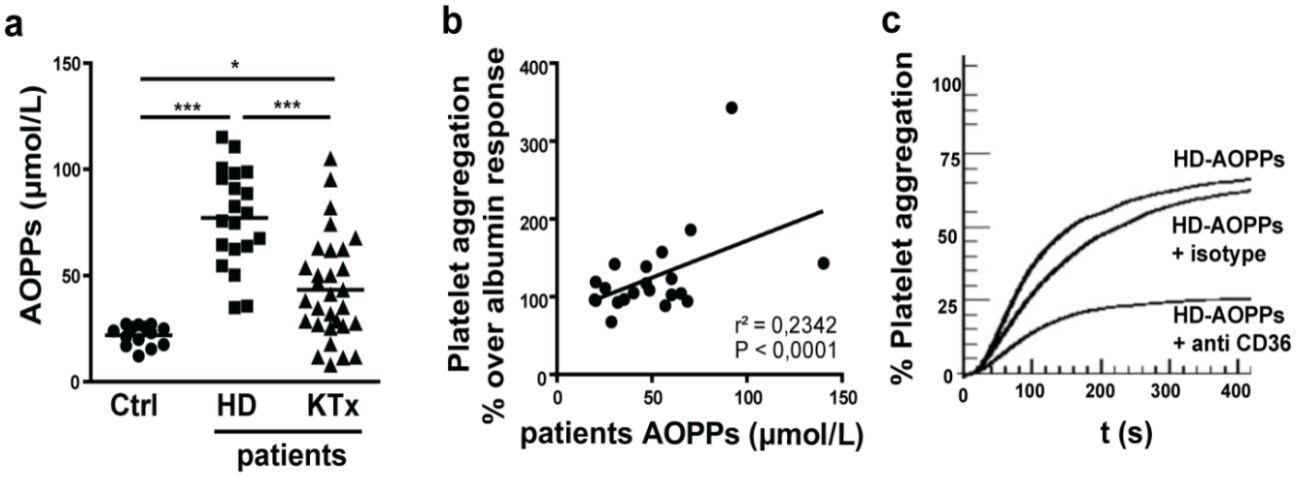

Figure 16: The proaggregatory potential of AOPPs isolated from ESRD patients depends on oxidative modifications and is mediated via CD36

(a) Levels of AOPPs (µmol/l) from ApoB depleted serum samples were determined. Study cohort consisted of end stage renal disease patients on hemodialysis (n = 28, HD), subjects after kidney transplantation (n = 29, KTx) and control subjects (n = 14, Ctrl) **(b)** Patient AOPPs (1 mg/mL) isolated from end stage renal disease patients on HD (n = 21) were added 10 minutes before aggregation was stimulated with ADP (5–20 µM) in the presence of fibrinogen (5 µg/mL). The ADP concentration used lead to approximately 30-40 % platelet aggregation in the presence of albumin (1 mg/mL) isolated from control subjects. A significant correlation between serum levels of AOPPs and platelet aggregation in the presence of AOPPs isolated from HD-patients is depicted. Platelet aggregation is normalized to albumin isolated from control subjects. **(c)** AOPPs (1mg/mL) isolated from a HD-patient was added to platelets in the presence of an anti-CD36 antibody (4 µg/mL) or isotype control. Platelets were incubated 10 minutes with the respective antibodies in the presence of an anti-CD32 antibody (1 µg/ml). One representative tracing is shown. *P< 0.05 ***P < 0.001 as indicated.

13. Serum levels of AOPPs and TF significantly correlate in renal disease patients and controls

Elevated serum TF levels are a marker of inflammation, and TF expression was shown to be induced by oxLDL (217). Furthermore, blood TF is likely to contribute to a thrombotic phenotype after rupture of a plaque. Since our results were in line with other groups that could show a role for activated platelets to potently trigger TF expression on endothelial cells (75), we were interested in serum TF levels of our study cohort. Levels of soluble TF in serum was determined with a commercially available ELISA using the same patient samples as for AOPP measurements.

TF levels were profoundly increased in HD patients, whereas TF levels in kidney transplant patients were not significantly higher compared to controls **(Figure 17a)**. This suggests that the high oxidative stress and prothrombotic phenotype is markedly reduced after kidney transplantation. Interestingly, measured TF concentration and AOPP levels in this study cohort showed significant correlation **(Figure 17b).**

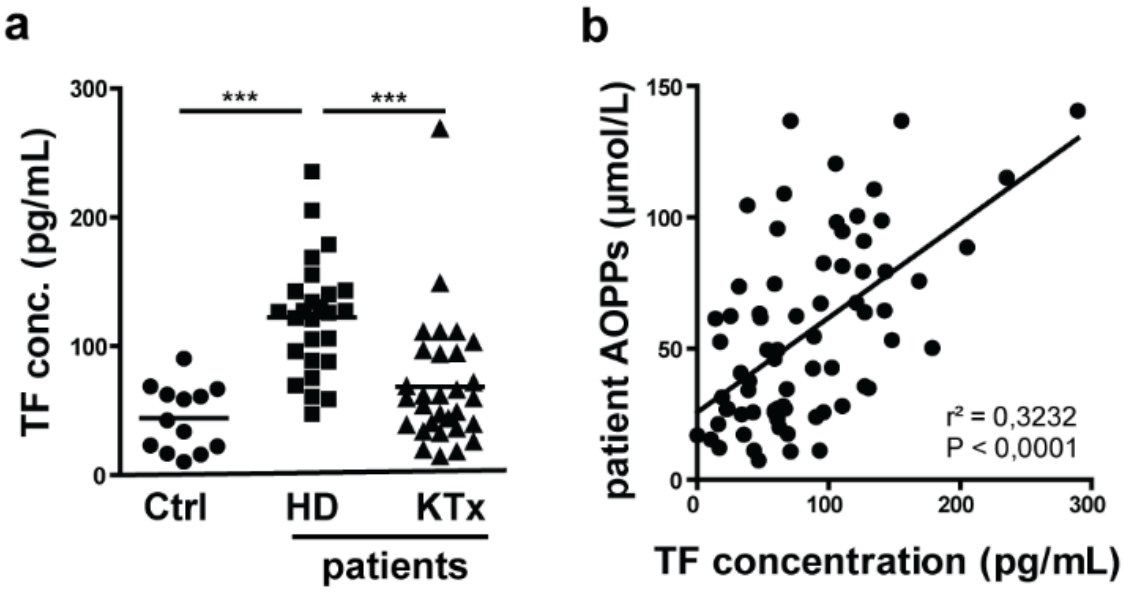

Figure 17: Serum TF levels are upregulated in ESRD disease patients on HD, and TF levels correlate significantly with AOPPs levels

(a) *TF concentration was determined from serum samples of end stage renal disease patients on HD (n = 28), subjects after kidney transplantation (KTx, n = 29) and controls (n = 14) with ELISA.* ***(b)*** *Correlation between serum levels of AOPPs and concentration of TF measured with ELISA. ***P < 0.001 as indicated.*

IV. Discussion

In the present work, we provide compelling evidence that albumin upon oxidative modification acts as a potent activator of platelet functions. AOPPs thereby trigger the expression of early and late platelet activation markers (P-selectin, CD40L and phosphatidylserine), and promote platelet adhesion to fibrinogen and collagen (the major protein present in the extracellular matrix exposed upon vascular injury). Furthermore, treatment with AOPPs allows platelets to strongly attach to cultivated endothelial cells (HCAECs), whereas a contribution of the CD40-CD40L-axis is most likely in enabling platelet-endothelial interactions. Moreover, AOPP activated platelets promote TF expression on endothelial cells via direct cell-cell interactions. Of note, the platelet scavenger receptor CD36 was identified as the main transmitter of AOPP triggered functions. A scheme of the proposed procoagulant activity of AOPPs on platelets is depicted in **Figure 18** and results are discussed in detail in the following chapters.

It is a general accepted finding that increased platelet reactivity is associated with enhanced prospective risk for coronary events with a deadly outcome (218). Since blood platelets are the key players in the process of hemostasis and thrombosis, it is hardly surprising that changes in their reactivity can lead to severe outcomes. Many of these lethal phenotypes result from thrombosis that can occur as a direct consequence from abnormalities in platelet function, i.e. platelet hyperreactivity (219). Inappropriate platelet activation combined with a high prothrombotic potential can be found in several pathophysiological states like atherosclerosis (220), diabetes (221) or the metabolic syndrome (222).

Several lines of evidence were given that atherosclerosis, the major source of morbidity and mortality in the Western world (47), is an inflammatory disease.

Inflammation indeed drives all phases of atherosclerosis, from inception, through progression, and ultimately acute thrombotic complications (plaque rupture and probably plaque erosion) (223).
Among others, C-reactive protein and MPO, as a marker of leukocyte activation, have been clearly linked with atherosclerosis and leukocytes per se have been

detected in atherosclerotic lesions (224). In the case of atherosclerosis, increased platelet reactivity coincide with inflammation and systemic high oxidative stress levels (225).

Accelerated atherosclerosis has been observed in chronic renal disease, an inflammatory condition associated with accumulating AOPPs resulting from chronic oxidative stress levels (181). Patients that receive hemodialysis live in a state of chronic platelet activation that is related on the one hand to uremia and on the other hand directly to the dialysis procedure (226,227). In addition, renal disease is linked to a prothrombotic phenotype (228).

(Lipo)protein modification occurs as a result of high oxidant levels in the blood stream. There are several oxidants involved in the oxidation of lipoproteins such as lipoxygenase, peroxynitrite and hypochlorous acid (229–231) and among these, MPO derived hypochlorous acid is of special interest since it was reported that the functional properties of hypoxLDL differ from all other forms of oxLDL. During hypoxLDL formation, hypochlorous acid almost exclusively attacks the protein part of the lipoprotein, without inducing the production of lipid peroxides (232). *In vivo* relevance was illustrated for hypochlorous acid modified lipoproteins (192) and MPO *per se* (233) in human atherosclerotic lesions. Of note, hypoxLDL was shown by several groups to directly activate platelets via platelet expressed surface receptors (126-128).

Interestingly, there was no study that revealed potential effects of hypochlorous acid modified albumin in the context of platelet functions. We hypothesized that this might be of particular interest, since albumin is the major target for hypochlorous acid in the circulation, and AOPPs were shown to accumulate in several inflammatory diseases, with unchallenged high levels in chronic renal disease (183). Given that inflammatory diseases are strongly associated with increased platelet reactivity and thrombotic complications, it was the aim of this study to reveal potential effects of AOPPs on platelet reactivity.

1. Platelet aggregation and receptor signalling

Our first results indicated that freshly modified AOPPs not only enhance the aggregatory response of ADP or collagen pre-treated platelets, but are furthermore able to trigger platelet aggregation independent of pre-stimulation. Induction of platelet aggregation occurred in a concentration dependent manner and AOPPs were identified as strong platelet stimuli. Of note, the AOPP-mediated effects on platelets strongly depend on N-chloramines since “aged” AOPPs, consisting of albumin fractions in which chloramines are almost completely eliminated, lose their ability to affect platelet functions. To strengthen this result, reductive methylation was performed to interfere with protein associated chloramine formation. In line with the results obtained with “aged” AOPPs, no difference could be observed between hypochlorous acid treated and native albumin, thus providing evidence for the relevance of unstable, protein-associated N-chloramines in AOPP-induced platelet activation.

As already shown for LDL, the underlying oxidation procedure seems to strongly influence its functional properties, even though when oxidation resulted in comparable modifications. In contrast to hypoxLDL, platelet aggregation properties were not altered when LDL was oxidised by trace metal (130). Although there is still a lack of knowledge regarding *in vivo* occurring oxidation mechanism, a hallmark of oxLDL oxidation is the increased binding affinity to scavenger receptors (234), key players in the development of atherosclerosis (235).

Of note, several scavenger receptors have been reported to be expressed on platelets. Based on the rapid and strong aggregation response that occurred upon AOPPs exposure, an involvement of platelet receptors was considered most likely.

A large body of evidence supports the role of CD36 as a major glycoprotein expressed on platelets, but its distinct function remained obscure for a long time. Among others, CD36 was identified to directly interact with thrombospondin-1, oxLDL, fatty acids and microbial DAGs (236). Recently, platelet CD36 has moved back into the focus of research when Podrez *et. al* could described a role for a novel family of oxidized choline glycerophospholipids ($oxPC_{CD36}$) that accumulate in dyslipidemia and are formed during LDL oxidation. $oxPC_{CD36}$ were shown to interact

with platelet CD36, thereby linking this scavenger receptor with oxidative stress, dyslipidemia and a prothrombotic phenotype (142).

This was in line with previous results that dyslipidemia associated pathophysiological states show higher platelet reactivity and enhanced thrombogenic potential (138). Especially for hypoxLDL, a role as potent trigger of platelet aggregation and granule dependent P-selectin expression as well as GPIIbIIIa activation was clearly established by several independent groups (145,166,237).

In our study, the scavenger receptor CD36 was identified as a main transmitter of AOPP triggered functions, since platelet aggregation, as well as P-selectin expression could be significantly reduced in the presence of a CD36 antibody. Since AOPP-induced platelet function could not be reversed completely by blocking CD36, so we checked for the contribution of other scavenger receptors in this context.

The scavenger receptor SR-BI was recently described in our group to interact with AOPPs due to its modified lysine residues. Furthermore, it was observed that N-chloramines transform plasma albumin into irreversible inhibitors of SR-BI (199). Based on this findings, we also investigated a potential role for platelet SR-BI in interacting with AOPPs. Interestingly, blocking SR-BI using an anti-SR-BI blocking antibody failed to influence platelet aggregation triggered by AOPPs. Beside the possibility that platelet CD36 is the predominant receptor on platelets for oxidized lipoproteins, with only minimal contribution of SR-BI, there is also a huge discrepancy in literature regarding the presence of SR-BI on platelets (153,154).

A distinct role for scavenger receptors type A (SR-A) in mediating oxidized lipoprotein signals on platelets is also a matter of debate. While some groups propose a mechanism in which platelet activation pathways are initiated by a combined action of CD36 and SR-A (145), others provide evidence for a unique role for CD36 (128,143,144). (. Groups that propose fucoidan as appropriate inhibitor of SR-A receptors in platelet aggregation might not be familiar with its additional role as platelet agonist via CLEC-2 (238). Nevertheless, we could rule out a contribution of SR-A receptors in AOPPs mediated platelet aggregation since the presence of a specific antibody against SR-A receptors did not change the aggregation response upon AOPPs exposure. With specific regard to scavenger receptor LOX-1, we

considered a contribution of this receptor in AOPP-mediated platelet activation unlikely, since LOX-1 has been described to be expressed on platelets only in an activation-dependent manner (211).Taken together, CD36 was the only scavenger receptor we could clearly define a role in platelet activation and aggregation.

Platelet aggregation triggered by the activated form of GPIIbIIIa is the final result of agonist dependent platelet activation and strongly depends on intraplatelet signalling pathways which are receptor specific, but usually converge into common signalling events (239). Our findings indicate that AOPPs, in line with almost all platelet agonists, activate PLC, which is known to catalyse the hydrolysis of phosphatidyl inositol 4, 5 bisphosphate to the second messengers inositol triphosphate and DAG. DAG further activate Ca^{2+} mobilisation from internal stores and promote activation of PKC, key events in the intraplatelet activation cascade that finally result in platelet aggregation. Although the intracellular release of Ca^{2+} in response to stimuli is assumed to be smaller compared to the extracellular Ca^{2+} influx (240), it seems to be crucial in AOPP-induced platelet activation, as chelation of intracellular Ca^{2+} by BAPTA-AM almost completely abolished platelet aggregation. The chelation of extracellular Ca^{2+} also interferes with platelet activation and furthermore allows the exclusion of platelet agglutination in this context. Classical PKCs, particularly PKC α were described to play a critical role in the release of platelet granule and subsequent platelet aggregation (215).

AOPPs were previously demonstrated to activate NAD(P)H oxidase through a PKC dependent pathway, thereby promoting ROS production in several cell types (202,241). In platelets, hypoxLDL was identified to stimulate ROS through a pathway involving CD36 and PKC activation (129). Platelet intracellular ROS/superoxide was upregulated in the presence of AOPPs, and the SOD mimetic MnTMPyP potently reduced AOPP triggered platelet aggregation. This let us assume that CD36 might be relevant in this context. Surprisingly, blocking of CD36 did not interfere with ROS production, and the mechanism how AOPPs stimulate ROS production remains obscure and needs to be further investigated.

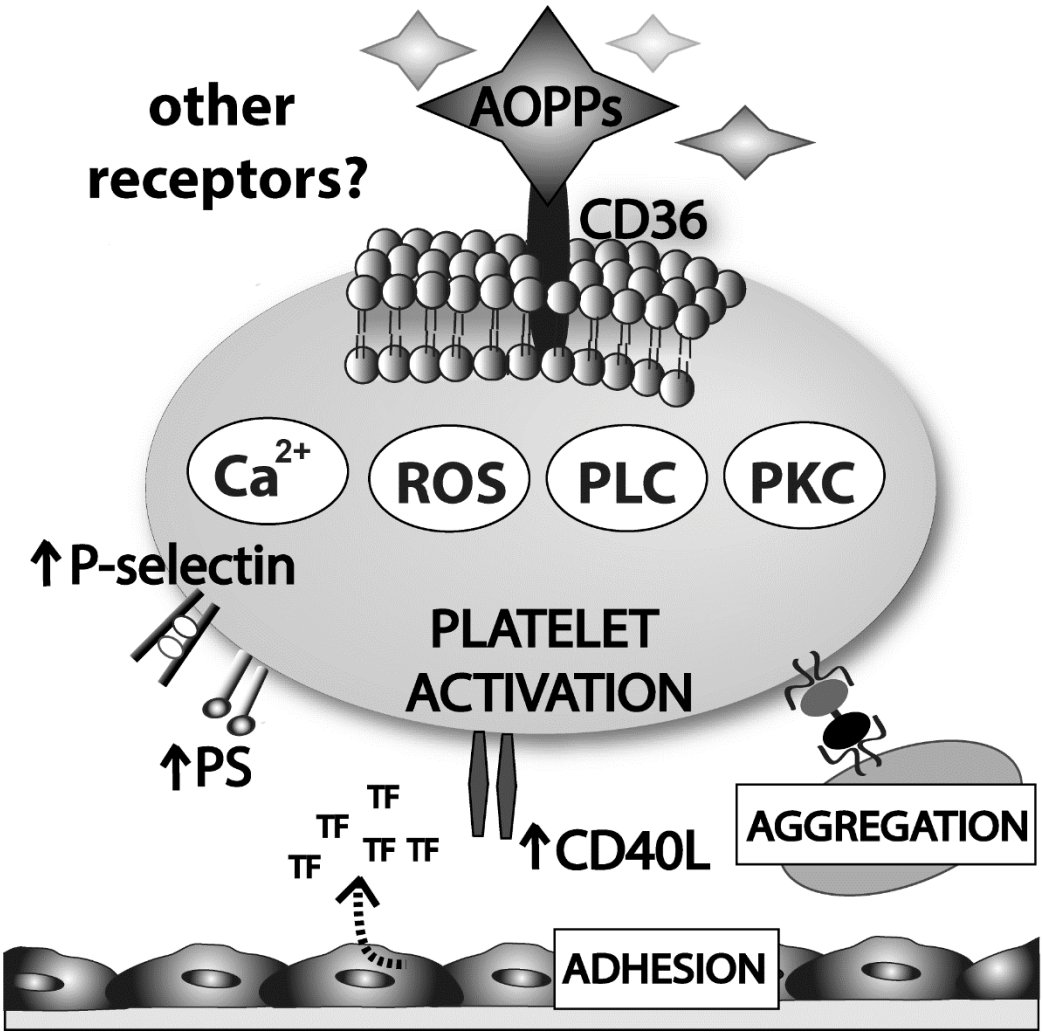

Figure 18:

AOPPs triggered platelet activation and its prothrombotic effects

AOPPs promote platelet activation as indicated by upregulation of P-selectin, phosphatidylserine (PS) and CD40L. These effects are mediated via interaction of AOPPs with the platelet receptor CD36, although additional AOPP-receptor-interactions cannot be ruled out in this context. AOPPs trigger intracellular signalling involving ROS formation, activation of PLC and PKC and calcium mobilisation. Platelet aggregation, as the final event of platelet activation, is induced by AOPPs. AOPP activated platelets adhere to the fibrinogen and endothelial cells, whereas they induce the expression of endothelial TF as a result of direct cell-cell interactions.

2. Platelet activation markers and platelet-endothelial interactions

P-selectin is released from α-granules and integrated in the platelet membrane in an activation dependent manner. A significant upregulation of P-selectin could be determined in the presence of AOPPs. Of note, the role of P-selectin is not limited as indicator of early platelet activation, it is furthermore a critical mediator of platelet-neutrophil interaction (242).

Platelet-neutrophil aggregates are known to circulate in several inflammatory diseases. They are required to recruit neutrophils to inflamed tissue by inducing the upregulation of adhesive molecules, thereby “priming” neutrophils to efficiently adhere to the endothelium. The surface interactions between platelets and neutrophils contribute to several neutrophil elicited functions such as chemotaxis (required for neutrophil tissue migration), phagocytosis and ROS production. Of note, P-selectin has a unique role in these activities (242). Higher levels of platelet neutrophil aggregates were shown to be prevalent in several conditions associated with cardiovascular disease and they might contribute equally to the thrombotic and inflammatory processes (243,244). Evidence is given for the absolute requirement of the platelet P-selectin/PSGL-1 axis for neutrophil recruitment in atherosclerosis and injured arterial surfaces (245,246), and platelet P-selectin is directly involved in intracellular ROS production of neutrophils as shown with platelets from patients with inflammatory bowel disease (17). ROS production is potentiated in neutrophils complexed to platelets and furthermore enhanced when platelet neutrophil aggregates are activated by ADP, released from activated platelets (244).

Via platelet P-selectin, AOPPs are expected to contribute to the inflammatory and thrombotic potential of neutrophils. Neutrophil activation, i.e. ROS production, leads to increased production of MPO derived hypochlorous acid, thus, potentially creating a positive feedback loop. In general, targeting P-selectin/PSGL-1 and thereby interfering with the formation of complexes between platelets and neutrophils, might be an effective novel antiinflammatory strategy. The performance of several antibodies in clinical trials is currently under investigation (247).

Beside P-selectin, AOPPs also significantly increased the surface expression of phosphatidylserine, a marker for strong and late platelet activation that is associated

with platelet apoptosis. Negatively charged phospholipids, such as phosphatidylserine are usually located in the inner leaflet, thus explaining the procoagulant activity of the inner membrane. Surface expression of phosphatidylserine is necessary to provide a suitable surface for the pro-thrombinase complex. Thereby, surface-exposed phosphatidylserine allows binding of activated factor V, in turn recruiting Ca^{2+} dependent factor X and prothrombin attach to the platelets surface and lead to increased thrombin generation (248). Of interest in this regard, thrombin generation as indicator of pro-coagulant activity was found to be more than 2-fold higher in uremic patients compared to healthy controls. This was assumed to result from increased phosphatidylserine externalisation of platelets from uremic patients that was determined by flow cytometry. These results contribute to the view that the thrombophilic potential in patients with renal disease, might be, at least partially, ascribed to increased levels of platelet phosphatidylserine exposure (249).

While P-selectin upregulation is crucial for neutrophil binding, and phosphatidylserine might mainly contribute to the procoagulant functions of activated platelets, CD40L expression allows binding of platelets to endothelial cells. In addition, a recent finding is that CD40 is expressed on neutrophils (250). Although there is little knowledge given so far, a study that investigated the distinct role of CD40-CD40L interactions on platelets and neutrophils found increased neutrophilic ROS levels, further promoting platelet activation and sCD40L release, thus, contributing to a redox controlled positive feedback reaction (251).

It was shown that intraplatelet ROS formation is associated with CD40L integration in the outer platelet membrane (252). We observed that platelets treated with AOPPs showed elevated levels of CD40L surface expression as well as enhanced binding properties to cultured endothelial cells under flow conditions. This is in line with the recent assumption that activated platelets are capable to bind to an intact endothelial layer, regardless of a pre-activated or injured i.e. collagen exposing vessel wall (77). Although CD40L is expressed on several cells of the vasculature, about 95 % of the soluble CD40L is platelet derived (253). Platelet bound as well as soluble CD40L show proinflammatory properties(75), further establishing the role of activated platelets as important modulators of inflammation. Moreover, several lines of evidence suggest that CD40L might be at the heart of the atherosclerotic process,

since it contributes to the progression of atherosclerotic lesions, but also in the inflammatory aspects of thrombosis *per se* (78). Amongst others, one study clearly showed the prothrombotic activity of CD40L, as infused recombinant sCD40L was effective in normalising the thrombosis defect prevalent in CD40L-/- mice (87).

Of interest, the prothrombotic potential of platelet sCD40L seems to be ultimately linked to the major platelet glycoprotein GPIIbIIIa. Antagonizing GPIIbIIIa interferes with the shedding of CD40L from activated platelets (86), and direct interactions of these proteins stabilize thrombi under conditions of high shear stress (87).

Pioneering work of Henn *et. al* describe that a direct interaction between endothelial CD40 and its ligand lead to a procoagulant endothelial phenotype by directly inducing surface expression of TF (75). TF is the most important player in the initiation of the extrinsic way of blood coagulation. TF, together with factor VII forms the extrinsic pathway of coagulation. Thereby, the complex of TF and factor VIIa activates factor IX and factor X, leading to fibrin formation as a final result of this cascade activation (254). TF can be formed and released by several different cell types of the vasculature such as monocytes, endothelial cells and smooth muscle cells (120,121). High amounts of TF can be found in atherosclerotic plaques (255), and this might be the major source that drives thrombosis. Statins interfere with monocyte/macrophage TF production and are expected to reduce the prothrombotic potential of a plaque after rupture (217). Of note, sCD40L is associated with increased risk of cardiovascular events in patients with coronary artery disease (256), indicating an important role for the CD40L-TF-axis in promoting a prothrombotic phenotype.

In addition, when we incubated AOPPs activated platelets with endothelial cells, elevated levels of TF were observed on HCAECs. AOPPs were previously shown to directly act on endothelial cells and promote the expression of adhesive molecules like ICAM-1 or VCAM, both shown on mRNA and protein levels (202). In addition, an active compound of oxidized LDL was shown to directly induce TF expression on endothelial cells (257).Therefore, we had to rule out the possibility that TF upregulation did occur independent of platelets as a consequence of AOPPs directly acting on endothelial cells. Our findings strongly suggest a contribution of the platelet CD40L-CD40 axis, since AOPPs alone or the supernatant of AOPPs

treated platelets failed to influence TF expression on endothelial cells. Furthermore, to rule out a contribution of platelet TF in this setting, we revealed TF expression on AOPPs activated platelets. In line with several other groups we failed to identify TF on platelets. While early publications suggested blood platelets as major sources of TF, being stored in their α-granules and released upon activation (258), a recent report failed to detect TF or its activity after platelet stimulation with collagen (259). Interestingly, it was shown that upon LPS stimulation of whole blood, TF is directly transferred from monocytes to platelets (260), what might explain the discrepancies regarding platelet derived TF. Nevertheless, it was also reported that platelets in a quiescent state express TF pre-mRNA that is spliced during platelet activation, finally leading to an increase in procoagulant activity (14).

3. AOPPs in chronic kidney disease patients

In our study cohort, AOPPs were found to be significantly higher in chronic kidney disease patients compared to healthy controls, a result that was in line with previous investigations (166) (199). Levels of AOPPs determined in serum of patients after kidney transplantation were still higher than levels of AOPPs observed in controls, but significantly reduced compared to hemodialysis patients. Kidney transplantation is the preferred therapy for the majority of patients with end stage renal disease because overall survival and quality of life are better than with hemodialysis (261), hence reduced AOPPs might be one of the factors contributing to the decreased cardiovascular mortality of renal transplant patients.

We further investigated the impact of AOPPs isolated from patient and control samples on platelet functions. Patient AOPPs enhanced platelet aggregation, and this effect was directly dependent on their levels of oxidative modifications. This is an important finding that might contribute to the understanding of high platelet reactivity in oxidative stress associated inflammatory conditions (262,263). Given that patient AOPPs had the functional ability to increase platelet aggregation via CD36, effects seen before with *in vitro* modified albumin can be considered in a physiological relevant context. The AOPP content of 1 mg/ml of our *in vitro* generated albumin was 7.0 µmol/L and therefore comparable to the content of isolated AOPPs (2-10 µmol/L). Nevertheless, one has to keep in mind that *in vivo*

generated AOPPs are not identical to the *in vitro* modified proteins, given that endogenous modification pathways of proteins are by far more complex.

Another major finding of our study was that levels of AOPPs and TF concentrations exhibited a significant correlation, thereby linking high oxidative stress levels directly with a prothrombotic phenotype. Interestingly, TF levels of kidney transplant patients were in a similar range when compared to controls that might, at least in part reflect decreased oxidative stress in this patient group, thereby interfering with the platelet CD40L-CD40 axis that favours TF factor expression.

4. Summary and conclusion

To sum up, our study presents a novel role for AOPPs as potent platelet activators. AOPPs potential to activate platelets strongly depends on N-chloramines, formed upon oxidative modification of albumin with hypochlorous acid. AOPP-induced platelet activation involves platelet scavenger receptor CD36, common intraplatelet signalling events involving ROS production, and expression of surface proteins such as P-selectin, phosphatidylserine and CD40L. These activation markers are integrated in the platelet membrane upon granule release, thereby allowing cell-cell interactions with other cell types and the formation of procoagulant platelets. Via direct interactions with endothelial cells, AOPP-stimulated platelets induce a prothrombotic and proinflammatory phenotype on endothelial cells by inducing endothelial TF expression. Interestingly, AOPPs isolated from renal disease patients and controls significantly correlated with TF levels measured from the respective serum sample, indicating a strong link between oxidative stress in inflammation, platelet reactivity and a prothrombotic phenotype. Our data suggest that oxidative modified proteins, as occurring in several inflammatory diseases, can directly affect platelet functionality. AOPPs might therefore play a considerable role in the onset and progression of cardio-thrombotic diseases and might therefore present novel pharmacological targets.

V. References

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