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Novel Approaches to Fine-Tune Therapeutic Targeting of Platelets in Atherosclerosis: A Critical Appraisal

Thorsten Kessler[1,2] Heribert Schunkert[1,2] Philipp von Hundelshausen[2,3]

[1] Deutsches Herzzentrum München, Klinik für Herz- und Kreislauferkrankungen, Technische Universität München, Munich, Germany
[2] Deutsches Zentrum für Herz-Kreislauf-Forschung (DZHK) e.V., Partner Site Munich Heart Alliance, Munich, Germany
[3] Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Klinikum der Universität, Ludwig-Maximilians-Universität, Partner Site Munich Heart Alliance, Munich, Germany

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Address for correspondence Thorsten Kessler, MD, Deutsches Herzzentrum München, Klinik für Herz- und Kreislauferkrankungen, Technische Universität München, Lazarettstraße 36, 80636 Munich, Germany (e-mail: thorsten.kessler@tum.de).

Philipp von Hundelshausen, MD, Institute for Cardiovascular Prevention, Ludwig-Maximilians-University (LMU) Munich, Pettenkoferstraße 9, 80336 Munich, Germany (e-mail: philipp.von_hundelshausen@med.uni-muenchen.de).

Abstract

The pathogenesis of atherosclerotic vascular disease is driven by a multitude of risk factors intertwining metabolic and inflammatory pathways. Increasing knowledge about platelet biology sheds light on how platelets take part in these processes from early to later stages of plaque development. Recent insights from experimental studies and mouse models substantiate platelets as initiators and amplifiers in atherogenic leukocyte recruitment. These studies are complemented by results from genetics studies shedding light on novel molecular mechanisms which provide an interesting prospect as novel targets. For instance, experimental studies provide further details how platelet-decorated von Willebrand factor tethered to activated endothelial cells plays a role in atherogenic monocyte recruitment. Novel aspects of platelets as atherogenic inductors of neutrophil extracellular traps and particularities in signaling pathways such as cyclic guanosine monophosphate and the inhibitory adaptor molecule SHB23/LNK associating platelets with atherogenesis are shared. In summary, it was our intention to balance insights from recent experimental data that support a plausible role for platelets in atherogenesis against a paucity of clinical evidence needed to validate this concept in humans.

Keywords
- platelets
- acquired
- atherogenesis
- lipids
- adhesion molecules
- inflammation

Introduction

We have experienced during the last decades a continuous progress in treating the complications of atherosclerosis such as myocardial infarction (MI), stroke, and acute limb ischemia by diagnostic improvements and continued development of new medical devices and drugs. Still, a large part of patients with acute ischemia does not manage in time to profit from these medical advances.[1] Thus, the quest for novel targets aimed at a further individual reduction of the risk for a cardiovascular event by preventing atherosclerosis is justified. It has been argued that the sole causal risk factor for atherosclerosis is simply hypercholesterolemia and that other epidemiologically associated factors are either exacerbating or only bystander phenomena.[2] It may be that by abolishing circulating low-density lipoprotein (LDL)-cholesterol atherogenesis might be completely preventable; whether such an approach is realistic remains questionable. In the meantime, it will be worthwhile to understand the mechanisms of atherogenesis to identify novel targets. Beyond the metabolic, there is

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an intricate chronic inflammatory component to consider. With the CANTOS trial, the hypothesis that cardiovascular events can be prevented by blocking a potent inflammatory target such as interleukin-1β has been proven.[3] However, a clear cardiovascular benefit was outbalanced by the moderate effect size and fatal infections warranting further refinement through an individualized approach or testing new inflammatory targets.[4] Especially T cells or monocytes, giving rise to intimal macrophages, but also almost every circulating blood cell type, have been described in plaques or to contribute to the inflammatory infiltrate and stability of the plaque.[5] Irrespective of their pivotal role in arterial thrombosis, a causative role of platelets in atherogenesis has been suggested in the 1960s based on the concept that platelets represent a link between hemodynamic factors, lipids, and the characteristic localization of plaques.[6] The concept of platelets as important inflammatory agents has been refreshed again in 2005 by Gawaz and colleagues explaining the proinflammatory machinery of platelets that intimately links thrombosis and atherosclerosis.[7] In recent years, rare genetic mutations with atherosclerosis phenotypes have been discovered and genome-wide association studies as well as Mendelian randomization studies have emerged as powerful tools generating big data that shed light and spark discussions about how platelets and blood cells may contribute to human atherosclerosis, defining novel targets for primary prevention.

Platelets, Initiators, and Amplifiers of Atherogenic Leukocyte Recruitment

Nearly our entire knowledge about the mechanisms of how platelets affect inflammation and atherosclerosis originates from animal models; preclinical data, however, have only partially been translated to the human system. The current understanding is that platelet activation is a requirement for their atherogenic properties. Hyperreactive platelets are associated with greater atherosclerotic plaque burden and increased plaque vulnerability, especially in culprit lesions in patients undergoing percutaneous coronary intervention (PCI) as measured by intravascular ultrasound (IVUS), and patients with more extensive coronary atherosclerosis have a higher number of hyperreactive platelets.[8,9] It is thus possible that increased platelet reactivity may potentiate arterial thrombosis at the time of rupture, thereby driving inflammation and atherosclerotic lesion progression.

A crucial factor after platelet activation is the upregulation and activation of adhesion receptors that initiate and enhance the contact of platelets with (1) leukocytes to form aggregates and with (2) endothelial cells or their underlying matrix when exposed after injury. Key players are αIIbβ3, P-selectin, the von Willebrand factor (VWF) receptor complex (GPIbα/V/IX), and glycoprotein VI (GPVI).[7] These adhesion molecules and their binding partners lead to an increase in rolling and firm adhesion of platelets on endothelial cells, and tether circulating leukocytes to the artery as a requirement for subsequent migration into the intima.[10] Other adhesion molecules behave in a counterintuitive way: the selective genetic deficiency of JAM-A in platelets results in hyperreactive platelets and an increase in the formation of atherosclerotic lesions as JAM-A interacts with and inhibits αIIbβ3 activation, which also results in chemokine release (►**Fig. 1**).[11] However, a cell-type-dependent expression of JAM-A may be decisive whether JAM-A is atherogenic or atheroprotective. The expression of JAM-A on endothelial cells guides monocytes into flow-dependent predilection sites of atherosclerosis and JAM-A plasma levels are increased in coronary artery disease (CAD).[12,13] Therefore, generalized JAM-A inhibition could turn out to be a two-sided sword.

Several functionally relevant chemokines are expressed and released by platelets.[14] Chemokines tend to oligomerize which leads to the formation of mostly homodimers and heterodimers of either a CC-type (interaction of the N-terminal part) or a CXC-type (extension of the β-sheet). This is important because atherogenic monocyte recruitment by CCL5, CXCL4, and their heterodimers depends also on these features. It can be therapeutically addressed by peptide inhibitors that are derived from amino acid sequences of the interface and protect from atherosclerosis.[15,16]

GP1bα Interactions with von Willebrand Factor Mediate Platelet Adhesion and Promote Atherosclerosis

Platelets have been described as initiators of atherosclerosis because they adhere to the arterial endothelium of the carotid artery in *ApoE*$^{-/-}$ mice before atherosclerotic lesions become visible in a VWF- and GP1b-dependent process.[17] VWF is stored and released upon injury or under inflammatory conditions from endothelial Weibel–Palade bodies or platelet α-granules. It bridges collagen and activates a receptor complex upon multimerization (GPIbα/V/IX), which is exclusively expressed by megakaryocytes and platelets leading to platelet adhesion on endothelial cells and driving early as well as midstage atherosclerosis in mouse models.[17] The presence of activated VWF on atherosclerosis-prone endothelium has been confirmed by molecular imaging detecting GP1b-conjugated microbubbles by ultrasound in vivo.[18] Notably, the source of VWF affects its functionality, possibly by altered glycosylation such as reduced N-terminal sialylation and reduced affinity for GPIbα.[19] This might explain why in mouse models only endothelial cell-derived VWF but not platelet-derived VWF promotes atherosclerosis.[20]

After binding and activation of endothelial CD40 by CD40L, ultralarge VWF–platelet strings arise and facilitate monocyte diapedesis.[21] In conjunction with a reduced activity of ADAMTS13, which cleaves ultralarge VWF, and consequently higher amounts of ultralarge VWF in plasma of patients with CAD, this mechanism has been proposed to contribute to enhanced monocyte recruitment at atherosclerotic predilection sites.[21] Elevated VWF levels in humans are strongly associated with an increased risk of ischemic cardiovascular events. Whether this relation is causal or whether increased VWF levels just reflect disturbances of endothelial function remains to be elucidated.[22] It would be very interesting to translate these finding to human genetic disorders that are comparable to mouse models. Robust large-scale prospective

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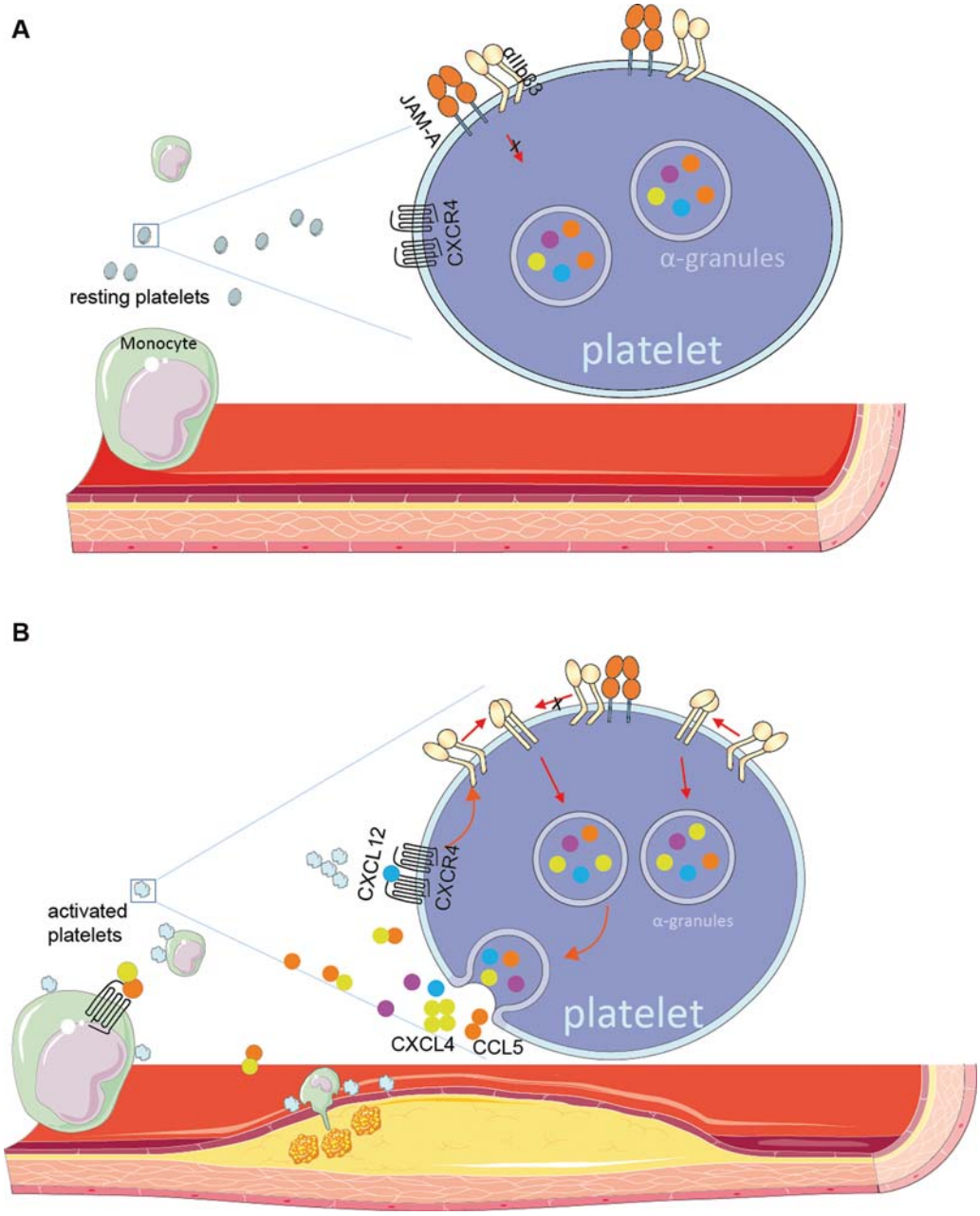

Fig. 1 Atheroprotective role of platelet JAM-A. The integrin αIIbβ3 and JAM-A are coexpressed on platelets. Either by direct interaction or possibly via contacts through intermediary proteins lead the presence of JAM-A to reduced outside-in signaling of αIIbβ3 (**A**). Genetic depletion of JAM-A from platelets results in increased outside-in signaling and consequently hyperreactive platelets (**B**). These hyperreactive platelets tend to form aggregates and complexes with monocytes and interact with dysfunctional endothelium to release chemokines such as CXCL4, CCL5, and CXCL12 which drives the generation of early atherosclerotic lesions.

clinical data that confirm a protection from atherosclerosis are, however, missing so far for both the rare Bernard–Soulier syndrome (GP1bα deficiency) and the common von Willebrand disease (VWD) where VWF is dysfunctional and levels are decreased. Lately, a Swedish registry of 2,790 individuals found that cardiovascular disease (CVD) mortality was more than halved in patients with VWD compared with controls while hospitalization due to a cardiovascular event was increased by 30%.[23] This might indicate an increase of rupture-prone lesions or lower stability of atherosclerotic lesions, whereas the lower mortality might be associated with a protection from arterial thrombosis. In aggregate, VWF appears to be a therapeutic target but experimental and clinical data are not consistent and require further investigation.

P-selectin

P-selectin is expressed on both endothelial cells and platelets. It is known for quite some time that the presence of P-selectin on both cellular entities can exacerbate atherosclerosis as shown by bone marrow transplantation models and adoptive transfer of P-selectin-deficient platelets.[24,25] More recently, based on these findings and aiming to prevent the anti-

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inflammatory and antithrombotic platelet-dependent processes that are instrumental in atherogenesis, a P-selectin blocking monoclonal antibody (inclacumab) has been developed and tested in humans with NSTEMI, which resulted in decreased peri-interventional myocardial damage.[26] As a side note, P-selectin expression is enhanced in platelets and endothelial cells in patients with sickle cell disease contributing to the risk of vaso-occlusive crises. A humanized monoclonal antibody, crizanlizumab, has been shown in the SUSTAIN trial to protect patients with sickle cell disease from vaso-occlusive crises and has recently been approved by the Food and Drug Administration for this indication.[27]

Although promising, randomized controlled trials over a longer period in a preventive setting and monitoring plaque development would be warranted to draw conclusions on beneficial effects of blocking P-selectin on atherogenesis in humans.

Glycoprotein VI

GPVI is one more target in atherogenesis. GPVI is a platelet-specific membrane protein that is primarily known for its interaction with fibrillar collagen but other ligands such as fibronectin or vitronectin are also known.[28] These ligands are thought to be the binding partners for GPVI in atherogenesis since the subendothelial localization of collagen precludes their encounter at intact endothelium.[29] Platelets adhere to the endothelium of early atherosclerotic arteries which can be diminished by antagonists like GPVI-Fc that can be coupled to microbubbles or by monoclonal antibodies, which goes hand in hand with a lesser extent of atherosclerosis.[29,30] The inhibition of GPVI appears to be especially attractive as side effects are expected to be low since humans with a genetic deficiency of GPVI and respective knockout mice have only a mild bleeding diathesis.[31] GPVI-Fc is envisioned to be powerful in preventing atherothrombosis without causing bleeding because collagen is an important component of atherosclerotic plaque activating platelets via GPVI and GPVI-Fc is most effective under high shear stress, but not low shear rates.[32,33] GPVI-Fc (revacept) has entered clinical trials. A phase I trial showed that the drug was well tolerated and currently phase II trials are being performed to test its effectiveness in preventing periprocedural PCI-associated events.[34,35] The parenteral application of GPVI-Fc however hampers its use in primary prevention. An alternative option is orally available inhibitors of the Bruton's tyrosine kinase that interfere with the downstream signaling of GPVI and GPIb.[36–38]

Platelet and NETs in Atherosclerosis: Guilty by Association

Activities of platelets and neutrophils are closely intertwined and join forces in inflammation and atherosclerosis. Formation of neutrophil extracellular traps (NETs; NETosis) emerges as a potential important link between these cellular entities.[39] Upon activation, neutrophils release decondensed chromatin decorated with granule proteins forming extracellular fibers that bind and kill bacteria.[40] Critical for the unfolding of the chromatin structure is the enzyme peptidylarginine deiminase (PAD4) that catalyzes the citrullination of histones thereby uncoiling chromatin.[41]

The presence of NETs has been shown in sections of human atherosclerotic lesions, both at the luminal aspect and within murine atherosclerotic lesions.[42,43] A role of NETs in propagating atherosclerosis is further supported by the finding that pharmacological interventions blocking NET formation via PAD4 inhibition can reduce atherosclerosis and arterial thrombosis in mice.[44] Several mechanisms have been proposed to explain the proatherosclerotic role of NETs: e.g., neutrophil-derived granule proteins (e.g., cathelicidin) stimulate a type I interferon response and cause endothelial dysfunction[45] and cholesterol crystals induce NETs which prime macrophages for atherogenic IL-1β release.[43] Furthermore, smooth muscle cells (SMCs) from atherosclerotic lesions attract neutrophils and trigger NETosis which in turn causes arterial tissue damage and inflammation.[46]

A role of platelets in NETosis was noted by observing that plasma from humans suffering from severe sepsis induced TLR4-dependent platelet–neutrophil interactions, leading to the production of NETs and clearing of bacteria.[47] Under various conditions of activation, platelets have been demonstrated to trigger neutrophils to expel their NETs. In addition, platelet inhibitors proved to be protective by preventing NET formation in neutrophil–platelet-dependent diseases such as acute lung injury and atherosclerosis.[39,48] Furthermore, in chronic inflammation platelet microparticles contribute to vasculopathy. This is fostered by their interaction with neutrophils and depends on the nuclear danger molecule HMGB1, which triggers neutrophils to cast their NETs.[49,50]

Platelets store and release chemokines that can form heteromers such as the chemokine CXCL4 that binds to CCL5 leading to synergistic effects on leukocyte recruitment and which can elicit NETosis in combination but not alone.[51] Blocking this heterodimerization reduces atherosclerosis, lung injury, and the formation of NETs.[51,52] Reciprocally, cell-free NETs induce platelet aggregation which depends on cathepsin G.[53]

In summary, both activated platelets and NETs alone have been shown to play a role in experimental atherosclerosis. However, there is interdependency as activated platelets bind to and activate neutrophils which eventually leads to NETosis.[39,45,54] What is unknown so far is to what extent each component drives atherosclerosis independently. Although it is known that platelets induce NETs which then play a role in atherosclerosis, the concept that NETs represent a causative link between activated platelets and atherosclerosis has yet to be proven.

Platelets and Lipids in Atherosclerosis: A Complex Relationship

Based on the LIPID MAPS classification system, lipids can be classified into eight categories (fatty acids [FAs], glycerolipids, [glycerol-]phospholipids, sphingolipids, sterols, prenols, saccharolipids, and polyketines).[55] While all these are detectable in platelets, only few examples of prenols, saccharolipids, and

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polyketines have been detected and are therefore not further discussed in this section.[56] Nevertheless, several prenols, saccharolipids, and polyketines have important functions in platelet biology and have been reviewed elsewhere.[57–59]

Polyunsaturated Fatty Acids Are Regulators of Platelet Activity and Affect Atherosclerosis

FAs can harbor multiple double bonds (PUFAs, polyunsaturated FAs). The position of the first double bond at the methyl end (omega, opposite the carboxyl group) explains the terminology so that omega-3 (n-3) FAs such as eicosapentaenoic acid (EPA) are differentiated from omega-6 FAs like arachidonic acid (AA). Integrated into phospholipids of the plasma membrane, FAs influence the fluidity and stability of cell membranes. Bulkier molecules such as the n-3 FA docosaheptaenoic acid result in greater membrane fluidity than FAs that fit better into the membrane geometry such as EPA.[60] This may have important implications for the activity of platelets and other cell types and be one of various reasons why clinical trials in cardiovascular prevention using PUFA (consisting of mixtures) reported ambiguous results. Nevertheless, the REDUCE-IT trial, which corroborates the significant protective effects of pure EPA on cardiovascular events in the JELIS study, reignited the interest in using omega-3 acids in preventing atherosclerosis.[61,62] Various preclinical data shed some light on the manifold, incompletely understood mechanisms, including reduced inflammation by effects on T cells and enhancement of resolution by lipid mediators, enhanced cholesterol efflux, antioxidant properties, and last but not least inhibitory effects on platelets.[60] A hint that these effects on platelet activation might be clinically relevant comes from the tendency toward more bleeding events under EPA in REDUCE-IT.[61] Feeding EPA to rabbits increases the incorporation of EPA into platelets and reduces collagen-induced platelet aggregation.[63] FAs are part of phospholipids in the platelet membrane and get released by cytoplasmic phospholipase A2 to serve as a substrate for platelet cyclooxygenase (COX-1), lipoxygenase (12-LOX), and CYP50 epoxygenases yielding various platelet inhibitors and activators. While AA serves as a precursor for the potent platelet activator thromboxane A2 and an opponent of the potent platelet inhibitor PGI2 (prostacyclin), EPA can be metabolized in platelets to various inhibitory lipids like thromboxane A3.[59] These lipid mediators have a short half-life so that they are generated on demand. Adding AA to whole blood rapidly induces platelet activation in an autocrine manner via thromboxane binding to its G_i-coupled receptor TP on platelets, whereas PGI2 is produced by endothelial cells and activates in a paracrine fashion its G_s-coupled receptor IP. Deletion of TP results in diminished platelet reactivity and reduced atherosclerosis, whereas the knockout of IP accelerated atherosclerosis and decreased the stability of the lesions. Hence it appears that platelet activation through lipid mediators can be an important regulator of atherosclerosis.[64–66] The platelet-specific effects of low-dose aspirin result from irreversible inhibition of COX-1 in platelets. Effects on PGI2 and thromboxane A2 are only the tip of the iceberg as low-dose aspirin in humans leads to drastic changes of the platelet FA profile and of other lipids that on top seem to vary considerably between donors.[56] Aspirin is established in secondary but not in primary cardiovascular prevention. In humans low-dose aspirin completely abrogates platelet-derived thromboxane generation without reducing C-reactive protein (CRP) levels.[67] Whether aspirin can reduce atherosclerosis in humans remains unclear; in light of many disappointing results in the setting of primary prevention, the latest being the ASPREE trial, large effects on atherogenesis seem unlikely.[68] A failure of low-dose aspirin to reduce residual inflammation, as assessed with the most established prognostic risk marker in CVD, CRP, could be a possible explanation.

Sphingolipids

Sphingolipids are components of the cell membrane and regulate signaling. They comprise a group of molecules that are derivatives of ceramide which is mainly generated at the cytosolic side of the endoplasmic reticulum by assembling the amino acid serine and palmitoyl-CoA. Enzymatic reactions with ceramide as a substrate result in sphingosine and further steps produce sphingosine-1-phosphate (S1P), glucosylceramide, lactosylceramide, and sphingomyelin, which are important signaling molecules in inflammation and atherogenesis.[69–71] Analysis of atherosclerotic plaques revealed that sphingolipids are important plaque components and contribute to plaque inflammation and stability.[72]

Different to the on-demand metabolism of FA, platelets store S1P in granules and in nongranular compartments.[73] Platelet activation leads to a release of S1P upon activation. As platelets express S1P receptors such as S1PR1, this results in a positive feedback mechanism.[74] Moreover, expression of S1PR1 on megakaryocytes is required for normal thrombopoiesis as S1P drives cytoplasmic extensions of megakaryocytes into bone marrow sinusoids to shed proplatelets into the circulation.[75] Although S1P is considered a proinflammatory mediator, platelet-derived S1P may have atheroprotective properties: it accelerates endothelial cell proliferation and drives endothelial cell migration.[76] Sphingomyelin affects platelet reactivity through its incorporation into and formation of specialized regions within platelet membranes, lipid rafts, where important platelet adhesion receptors including GPVI and GPI-V-IX or scavenger receptors such as CD36 are embedded and that are required for a proper function.[57] Another lipid species, phospholipids, is essential in these rafts fostering signaling through G-protein-coupled receptors by providing important second messengers.[77]

(Glycero-)Phospholipids

Phospholipids are the main component of cell membranes composed of glycerol derivatized by two hydrophobic acyl groups and a polar phosphate group. Depending on the molecules attached to the phosphate, phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and the phosphatidylinositol-related species (PI-PIP3) are classified. Cleavage of phospholipids by phospholipase C generates phosphoinositide second messengers in signal transduction (e.g., PIP, IP_3) and diacylglycerol, whereas lysophosphatidylcholine (LPC) arises after removal of an alkyl group of PC and a subsequent

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step by the phospholipase D autotaxin forms lysophosphatidic acid (LPA).

Under resting conditions the aminophospholipids PE and PS remain located at the inner leaflet of the platelet membrane. Platelet activation and apoptosis lead to Ca^{2+}-dependent activation of a phospholipid transporter, the scramblase TMEM16F, that leads to the translocation of PS and PE to the outer leaflet thereby exposing binding sites for Annexin V and coagulation factors.[78]

Furthermore, the comparison of the platelet lipid profiles from CAD patients with matched controls reveals that LPC is weakly detectable in platelets of healthy persons, but increased by several orders of magnitude in CAD patients and also found in vulnerable atherosclerotic plaques.[79,80] LPC has been reported to be concentrated in microvesicles from activated platelets which are important markers and factors in vascular inflammation and atherosclerosis.[81] Lipid profiling of platelet MV identified differences in the composition with higher amounts of PC and LPC compared with the activated parent platelets.[80] Platelets express the lysophospholipid receptor G2A/GPR132 that is responsible for platelet activation through LPC.[80]

LPA, a derivative of LPC, is implicated in platelet activation and atherosclerosis signaling through G-protein-coupled receptors of the LPAR (EDG) family. LPA is produced by platelets through phospholipase A1 and autotaxin and is one active component of mildly oxidized LDL and atherosclerotic plaques in platelet activation.[82,83]

Sterols/Cholesterol

Hypercholesterolemia in humans correlates with platelet count.[84] As specified above, the relevance of the platelet count and platelet indices in humans for atherosclerosis remains vague/obscure. However, antibody-induced selective depletion of platelets inhibits atherogenesis significantly.[85] Inversely, it is conceivable that an increased generation of platelets by hypercholesterolemia is atherogenic although the mechanisms how cholesterol levels modulate thrombopoiesis are unresolved.[86] Reducing cholesterol and circulating LDL levels affects platelet reactivity by cholesterol-dependent and pleiotropic, cholesterol-independent effects.[87] Clinically, pleiotropic, antithrombotic actions of statins have been concluded from the JUPITER trial that showed a significant reduction of deep vein thrombosis in the cohort treated with rosuvastatin.[88] Which molecular mechanisms lie behind these assumed antiplatelet effects have been investigated in preclinical studies. Statins inhibit the synthesis of cholesterol by blocking HMG-CoA-reductase and as a side effect other lipids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate are missing for posttranslational prenylation of the small GTP-binding proteins Rho and Rac so that their activity is reduced.[89] Rho and Rac are ubiquitously expressed and are indispensable regulators of the platelet cytoskeleton with various effects on downstream pathways including nitric oxide signaling.[90] However, the size of the attributed pleiotropic effects on platelet reactivity in vivo is difficult to separate from the effects that occur as a result of the reduction of cholesterol: hypercholesterolemia alone increases platelet activation via binding of LDL and oxLDL to platelet CD36.[91,92] Novel treatment options for hypercholesterolemia as antibodies against PCSK9 also display antiplatelet characteristics: in a small clinical study the treatment with PCSK9 inhibitors resulted in a decrease of platelet P-selectin, platelet aggregation, and released proteins from α-granules like CXCL4.[93] Levels of circulating PCSK9 correlate with platelet activity in acute coronary syndrome patients.[94]

Genetic Evidence for a Role of Platelets in Coronary Artery Disease

Platelet Parameters

Hematologic parameters including platelet count or mean platelet volume (MPV) have been associated with atherosclerosis and cardiovascular risk.[95,96] Larger platelets have been described to be more reactive and to have a greater prothrombotic potential so that MPV has been found to be a useful prognostic parameter in MI.[95] Although MPV is a readily available parameter with implications to platelet function, it is not standardized. Multiple factors including preanalytical issues such as time-dependent swelling of platelets in EDTA affect MPV so that using MPV is not a standard in clinical practice.[97] As hematologic parameters show high interindividual variability, large genome-wide analyses of hematologic parameters have been undertaken to identify genetic variants that influence traits of red and white blood cell counts, but also platelet indices. ►**Supplementary Table S1** (available in the online version) gives an overview of studies reporting genetic associations with platelet phenotypes. In 2009 a first large systematic genome-wide meta-analysis[98] identified 15 loci determining the MPV that jointly explained 8.6% of the total genetic variance in MPV, but only to 0.5% of the platelet count.[98] The most interesting region associated with platelet count and MPV was a haplotype restricted to Europeans located on chromosome 12q24 comprising 10 common single nucleotide polymorphisms (SNPs) including a nonsynonymous SNP Arg262Trp (rs3184504) in the gene *SH2B3* associated with atherosclerosis and MI (see below). This haplotype is of importance because it significantly associates with premature CAD.[99] In more recent and even larger studies searching for cardiometabolic risk factors in Europeans, more loci and variants have been discovered and refined that are more strongly associated with platelet count and explain the variance of platelet count to more than 8%.[100] Signals for platelet count were mostly found within genes for congenital (*GFI1B*, *THPO*) or acquired (*APOH*) platelet disorders, underscoring that more subtle genetic variation within genes known to contain loss-of-function variants may reflect interindividual differences in these complex traits.[100] In the last and so far most powerful study, more than 1,000 variants were identified to define platelet indices. In a Mendelian randomization study these variants displayed a weak, unexpectedly inverse relationship of coronary heart disease (CHD) and MPV suggesting shared causal pathways for CHD and MPV, although the mechanisms behind remain to be clarified.[101]

Taken together the results of these studies suggest that functional properties are more important for the role of platelets in atherosclerosis than the number of platelets

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(►**Supplementary Table S1**, available in the online version).[101–108]

As a prominent example, the cytochrome P450 2C19 genotype has been associated with a response to clopidogrel therapy.[109,110] Genome-wide analyses also identified several genetic variants identified with platelet aggregation after stimulation with different agonists.[111] Additionally, genes or pathways involved in platelet biology or function have also been identified to be associated with coronary atherosclerosis (for a review, see Erdmann et al[112]), e.g., the *SH2B3* gene and nitric oxide signaling.

SH2B3/LNK—A Coronary Artery Disease Risk Gene with a Role in Platelet Function

As stated above, the *SH2B3* gene is located on 12q24 at a very complex genomic locus which shows associations with a variety of traits, e.g., type 1 diabetes,[113] blood pressure,[114,115] celiac disease,[116] but also CAD[99] and platelet count.[98,101] The gene encodes LNK, an inhibitory adaptor protein regulating cytokine signaling and cell cycle in endothelial and hematopoietic cells.[117,118] LNK prevents the signal transduction from a receptor tyrosine kinase to downstream JAK2, such as the signaling from TPO via MPL that triggers thrombopoiesis. Therefore, loss of LNK signaling results in thrombocytosis and increased platelet activation by αIIbβ3 outside-in signaling.[117,119,120] In a study designed to explain the mechanism of the common CAD risk variant which results in a loss of function of LNK, Wang et al demonstrated that this variant causes an increase in platelet count especially under proatherogenic conditions, i.e., high-cholesterol levels. *Lnk*$^{-/-}$ mice displayed enhanced platelet activation, more leukocyte–platelet complexes, and accelerated arterial thrombosis and atherosclerosis. Specifically, hypercholesterolemia in *Lnk*$^{-/-}$ mice led to enhanced interleukin-3/granulocyte–macrophage colony-stimulating factor receptor signaling but also increased platelet activation.[121] In summary, platelet LNK is now an experimental and genetic link between, on the one side, high cholesterol levels, high platelet counts, high platelet reactivity and on the other side increased atherosclerotic plaque formation and MI. Enhancing LNK to inhibit platelet generation and activation might be an innovative strategy to reduce cardiovascular risk

Multiple Genes Involved in Nitric Oxide Signaling

Several genes which encode proteins that play a prominent role in nitric oxide signaling have been associated with CAD in genome-wide association studies (for a review see Wobst et al[122]): *NOS3*, which encodes the endothelial nitric oxide synthase (eNOS),[123] *GUCY1A1* (formerly named *GUCY1A3*), which encodes the α1-subunit of the soluble guanylyl cyclase (sGC),[124] *MRVI1*, which encodes inositol 1,4,5-trisphosphate receptor-associated cyclic guanosine monophosphate (cGMP) kinase substrate (IRAG),[125] and *PDE5A*, which encodes phosphodiesterase 5A (PDE5A), are the most prominent examples. However, several genes encoding proteins in related pathways, e.g., *PDE3A*, encoding phosphodiesterase 3A,[126] or the genes *EDN-1*[127,128] and *EDNRA*,[124] encoding endothelin 1 and its receptor, respectively, have also been associated with CAD.

In the vasculature, nitric oxide is produced by eNOS mainly in endothelial cells leading to production of the second messenger cGMP in, e.g., vascular SMCs (VSMCs) and platelets by sGC. Accumulation of cGMP leads to relaxation of VSMC[129] and inhibition of platelet aggregation, respectively.[130,131] One mechanism is that the elevation of endogenous NO levels leads to reducing the thiol reductase activity of protein disulfide isomerase by S-nitrosylation which prevents platelet aggregation, α-granule release, and thrombin generation on platelets.[132] These NO effects, which also have been shown to influence, e.g., vascular remodeling[133] or vascular inflammation,[134,135] are limited by the breakdown of cGMP into GMP by PDE5A. Pharmacological modulation of these processes is used in a variety of diseases: supplementation of nitric oxide donors to relief angina pectoris, stimulators of sGC in pulmonary hypertension and heart failure, and PDE5A inhibitors in pulmonary hypertension and erectile dysfunction. *MRVI1*, the gene encoding IRAG, which represents a target of cGMP-dependent intracellular signaling, has also been associated with platelet aggregation.[111] The variants at all of these loci, i.e., *NOS3*, *GUCY1A1*, *PDE5A*, and *MRVI1*, are located in noncoding regions. However, at least for *NOS3*, *GUCY1A1*, and *PDE5A*, an association between genotype and gene expression has been reported, i.e., the risk alleles of *NOS3* and *GUCY1A1* lead to reduced gene expression.[136–138] As a consequence cGMP availability is reduced whereas the *PDE5A* risk allele is associated with increased gene expression.[139,140] While the effect at *NOS3* and *GUCY1A1* loci has been reported to be mediated via altered promoter activity,[138,141] the link between genotype and gene expression at the *PDE5A* locus is not yet understood.

While the connection between variants involved in nitric oxide signaling and platelet aggregation is obvious, it is still not known how exactly this pathway is involved in plaque formation and atherosclerosis in general. Hints that the pathway might not be only important in atherothrombotic but also preceding processes come from both experimental studies rendering an involvement in the recruitment of inflammatory cells to the vessel wall likely.[134,135] Furthermore, there is genetic evidence that at least impaired sGC activity might primarily affect atherosclerosis: in a family with high prevalence of premature manifestation of CAD due to a digenic mutation in *GUCY1A1* and the gene encoding the chaperone protein CCTeta, four of 11 family members carrying at least one mutation underwent PCI or coronary artery bypass surgery at a young age but did not suffer from MI.[142] It will thus be a challenge to identify the underlying cellular and molecular mechanisms to evaluate the potential of modifying this pathway in atherosclerosis. In this respect, cumulative effects of multiple risk alleles which share effects in the NO–cGMP-signaling pathway or respective coexpression networks may be informative.[123–125,139,143]

Despite these data suggesting a role for platelet phenotypes and/or function in atherosclerosis, it has to be mentioned that monogenic diseases influencing platelet phenotypes have not

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been associated with reduced incidence of CVDs. In Glanzmann's thrombasthenia in particular, a disease which is a consequence of deficient αIIbβ3 integrin function in platelets, cardiovascular events as arterial thrombosis or deep vein thrombosis have been reported (for a review, see Nurden[144]). Another example is the rare Bernard–Soulier syndrome that is characterized by defects in the VWF–receptor complex (GPIb-V-IX), where also MI has been reported.[145] However, these reports have to be taken with caution as it is obvious that CVDs and in particular CAD are influenced by several further risk factors that cause the disease despite altered platelet function.

Pharmacological Approaches Targeting Platelets in Atherosclerosis

Given the findings from basic research, inhibiting platelet function seems like a plausible strategy to prevent atherosclerotic plaque formation and progression. Current platelet treatment targets include COX-1, which mediates AA metabolism, $P2Y_{12}$ adenosine diphosphate receptors, and the αIIbβ3 glycoprotein receptor. Whereas the latter two are rather targeted/utilized in specific situations, e.g., after coronary stenting or in acute coronary syndromes, the role of aspirin in both primary and secondary prevention of atherosclerotic disease as well as in animal models has been extensively studied.

In secondary prevention, aspirin has been shown to significantly reduce the incidence of vascular events in patients with acute stroke (absolute reduction: 0.9%) or MI (absolute reduction: 3.8%), previous stroke/transient ischemic attack (absolute reduction: 3.6%) or MI (absolute reduction: 3.5%), but also other high-risk situations (absolute reduction: 2.1%).[146] These benefits clearly outweigh the risk of bleeding, i.e., the number needed to treat (NNT) to prevent a serious vascular event ranges between 50 and 100, whereas the number needed to harm ranges between 500 and 1,000 and 5,000 and 10,000 for gastrointestinal bleeding events and hemorrhagic strokes, respectively.[147] The effect in secondary prevention is thought to be a consequence of preventing atherothrombosis.[148]

In primary prevention of atherosclerotic plaques using aspirin, the situation seems more complex. In animal models, several studies have suggested that AA-related pathways and their inhibition are associated with atherosclerotic plaque formation and/or progression. In *Ldlr*$^{-/-}$ mice fed a Western-type diet, indomethacin, a nonselective COX inhibitor reduced atherosclerotic plaque formation and reduced expression of, e.g., soluble intercellular adhesion molecule and monocyte chemotactic protein-1.[149] That this effect is mediated via COX-1 is suggested by the fact that selective inhibition of COX-1 but not COX-2 led to reduced atherosclerotic plaque formation in *ApoE*$^{-/-}$ mice fed a high-cholesterol diet.[150] An impact of reduced thromboxane A_2, the product of COX-1-mediated AA metabolism, on atherosclerotic plaque formation has also been shown by specifically inhibiting its receptor; in this study, however, the unselective inhibition of COX-1 and -2 by indomethacin did not lead to reduced atherosclerotic plaque formation.[151] Data from human studies do not ultimately clarify the role of aspirin in primary prevention. Although there are early data which suggested a benefit from high-dose aspirin in atherosclerotic plaque progression,[152] randomized clinical trials in healthy subjects[153] and meta-analyses[154] have not been able to show a benefit from aspirin intake that outweighs the increased risk in bleeding. Two recent studies in diabetic or high-risk patients also failed to prove a benefit from aspirin in primary prevention.[155,156] There may be a subgroup of patients without previous cardiovascular events, but at a risk comparable to that of patients in secondary prevention (predicted 10% mortality in 10 years) or individuals with a particular genetic background. A very interesting example is the reduction of LDL-cholesterol and cardiovascular events using statins. Here, it has been shown that a high genetic risk score (including 27 variants associated with incident CHD) is associated with a stronger reduction of cardiovascular risk by statin therapy compared with individuals with a low genetic risk score. In an analysis of the JUPITER trial, the NNTs to prevent a cardiovascular event within 10 years were 66, 42, and 25 in the low, intermediate, and high genetic risk groups, respectively.[99] It is possible that in the sense of precision medicine, individuals could also be identified to specifically benefit from antiplatelet treatment with an unspecific drug such as aspirin if this is identified as the disease-driving pathway.[157] Also here, knowledge from genome-wide association studies might be useful. Whereas the Womens' and Physician's Health Study did not show a clear benefit from aspirin in primary prevention in the overall study population,[153] individuals carrying the homozygous *GUCY1A3* risk genotype had a benefit from aspirin treatment with a 17% risk reduction in women and a 51% reduction in men. In women the NNT treated to avoid one major CVD event was 121.[158] Considering higher risk and stronger effects in men, the NNT for *GUCY1A3*-guided prescription of aspirin could enter the range of clear benefit unless otherwise contraindicated (NNT $\leq$ 100).[159] Surprisingly, carriers of the nonrisk allele, either heterozygous or homozygous, did not only lack benefit from aspirin but rather experienced an increased risk compared with placebo.[158] This is a peculiar observation that remains to be validated and explained. However, it has been shown that the *GUCY1A3* genotype is also associated with a response to aspirin therapy with nonrisk allele carriers showing lower on-aspirin platelet reactivity.[160] One could speculate that in nonrisk allele carriers aspirin shifts platelets toward an increased risk of bleeding which is itself—directly and indirectly via anemia—associated with cardiovascular events.[161–163] Of note, it also needs to be taken into account that the effect of particular SNPs on CAD risk—and thereby also the presumed effect on the responsiveness to antiplatelet therapies— is rather mediated by altered gene expression than protein function. In contrast to *CYP2C19* alleles leading to slower metabolism of clopidogrel to active metabolites and —as a consequence—to increased risk of ischemic events in patients with acute coronary syndromes[109,164,165], the *GUCY1A3* risk allele is associated with reduced expression of the gene.[138] The influence of reduced sGC protein levels on response to aspirin treatment is, however, thought to be a result of changes in intracellular equilibria: as such, AA influences platelet nitric oxide levels[166] and *GUCY1A3* risk allele carriers presenting reduced sGC protein levels and activity

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might benefit from aspirin to outweigh this effect.[160] While this remains speculative, such complex interactions need to be taken into account.

Conclusion and Outlook

In this brief review we have pointed out some recent advances in the understanding how platelets influence atherogenesis, but a comprehensive reporting on all concepts was not within the scope. There is still a large gap to be closed between the clear notion of platelets as inflammatory and atherogenic cellular particles derived from experimental data and prove for this concept in humans. This is based primarily on the fact that platelets have a dual role as drivers of atherosclerosis and executers of arterial thrombosis after plaque rupture. Human data evaluating prognosis originate to a large part from registries, trials, and observational studies that mainly include symptomatic patients after hospitalization and therefore the thrombotic role of platelets in plaque erosion and rupture masks their impact on earlier stages in plaque development. Still, some studies were able to relate platelet phenotypes to CAD by recording PCI or coronary artery bypass grafting in a nonacute setting. An obstacle that needs to be overcome generally in evaluating atherosclerosis, which is a slowly progressive disease, is the possibility to assess plaque phenotypes in asymptomatic humans over long time ranges. High-resolution imaging such as optical coherence tomography and IVUS are able to characterize coronary plaques, but are invasive techniques precluding a screening of asymptomatic patients. Therefore, a translational realization of all these interesting concepts remains challenging.

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Conflict of Interest
None declared.

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