Thrombo-Inflammation in Cardiovascular Disease: An Expert Consensus Document from the Third Maastricht Consensus Conference on Thrombosis

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Abstract

Thrombo-inflammation describes the complex interplay between blood coagulation and inflammation that plays a critical role in cardiovascular diseases. The third Maastricht Consensus Conference on Thrombosis assembled basic, translational, and clinical scientists to discuss the origin and potential consequences of thrombo-inflammation in the etiology, diagnostics, and management of patients with cardiovascular disease, including myocardial infarction, stroke, and peripheral artery disease. This article presents a state-of-the-art reflection of expert opinions and consensus recommendations regarding the following topics: (1) challenges of the endothelial cell barrier; (2) circulating cells and thrombo-inflammation, focused on platelets, neutrophils, and neutrophil extracellular traps; (3) procoagulant mechanisms; (4) arterial vascular changes in atherogenesis; attenuating atherosclerosis and ischemia/reperfusion injury; (5) management of patients with arterial vascular disease; and (6) pathogenesis of venous thrombosis and late consequences of venous thromboembolism.

Keywords
- thrombosis
- inflammation
- coagulation
- pulmonary embolism
- myocardial infarction
- stroke
- platelets

Introduction

Thrombo-inflammation is a commonly used term to describe the complex interplay between blood coagulation and inflammation,[1] in relation to the pathophysiology of cardiovascular diseases (CVD), including atherosclerosis and acute atherothrombotic complications like myocardial infarction and ischemic stroke, as well as venous thromboembolic disease.[2] The third Maastricht Consensus Conference on Thrombosis was held to bring together basic, translational, and clinical scientists to intensely discuss with the audience mechanisms and consequences of thrombo-inflammation in the context of CVD diagnostics and management. This article summarizes current evidence and research perspectives derived from presentations and discussions among faculty and audience. Speakers and students worked together on the elements that comprise this document, which is organized in sections representing the mechanistic elements covering the origin, mechanisms, and consequences of thrombo-inflammation in relation to CVD.

Theme 1: Challenges of the Endothelial Cell Barrier

The Role of Air Pollution

Ischemic CVDs downstream from atherosclerosis (myocardial infarction, nonembolic ischemic stroke, and peripheral artery disease) are the consequences of a complex interplay of multiple risk factors.[3] One of these is air pollution, a major environmental risk factor. Of 56 million deaths per year attributable to CVD (33% of total mortality), and 6.5 million of those are due to air pollution. Most recent estimates exceed previous numbers in showing an excess of 800,000 deaths/year in Europe due to air pollution: 40% related to ischemic heart disease and 20% due to ischemic stroke.[4,5] One of the key triggers is fine particulate matter with a diameter below 2.5 µm (PM2.5), which presents in natural and anthropogenic sources including fossil fuel and biomass combustion, industry, agriculture, wildfires, and wind-blown dust. PM2.5 behaves as a sponge that adsorbs an array of toxic substances of different compositions, thereby inducing inflammation and vascular (endothelial) dysfunction (►**Fig. 1**) The latter involves potential endothelin-1 related

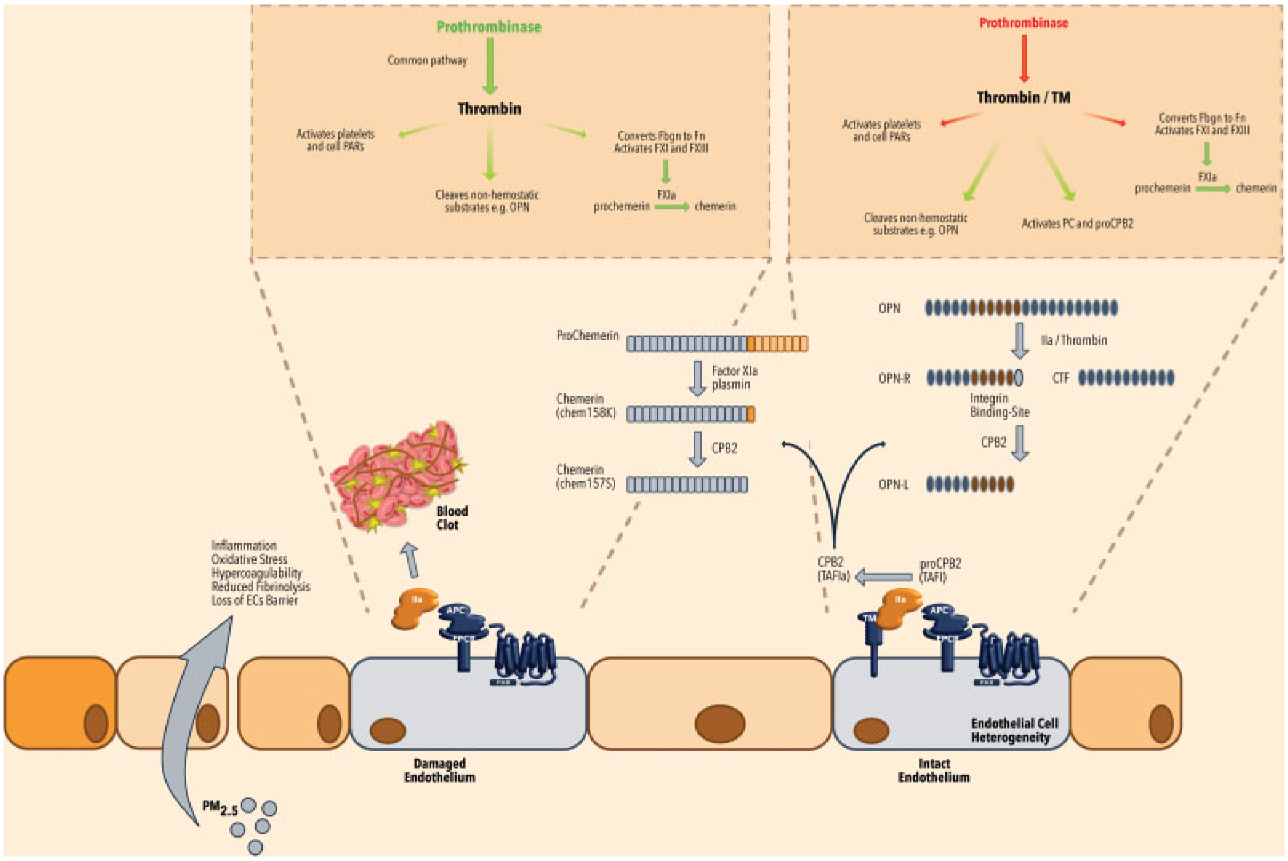

Fig. 1 Schematic representation of the potential areas for investigation for theme 1. Endothelial cell heterogeneity might contribute to initiation and progression of atherothrombotic disease providing potential new biomarkers and new targets for drug development. Particulate matter induces cell destructive effects such as inflammation, oxidative stress, and hypercoagulability, thereby linking endothelial cell heterogeneity to downstream thrombin effects. Inserts: schematic of the consequences of thrombin binding to TM. In the top panel, thrombin is generated from its inactive precursor, prothrombin, by the common pathway. Thrombin cleaves its canonical substrates such as protease-activated receptors, fibrinogen, and other proenzymes in the coagulation cascade, for example, FXI and Factor XIII, generating FXIa and FXIIIa, respectively. These reactions result in a clot containing both activated platelets and fibrin. Thrombin also cleaves proteins from outside the coagulation cascade such as osteopontin. Prochemerin is activated by FXIa. The bottom panel depicts the changes ensuing from thrombin binding to TM. Upon formation of the thrombin/TM complex the rates at which the canonical substrates are cleaved are greatly reduced while activation of protein C and procarboxypeptidase B are greatly enhanced. This leads to a change in substrate specificity of approximately 1 million fold. FXI, factor XI; TM, thrombomodulin.

mechanisms,[6] oxidative stress, aggravated by hypercoagulability, and reduced fibrinolytic activity in blood.[7–9] Air pollutants containing PM2.5 penetrate the lungs and translocate into the circulation, where they induce increased oxidative stress through mechanisms that are strikingly similar to those underlying vascular dysfunction in diabetes and hypertension. In addition, thrombogenic pathways are engaged through direct (contact activation) as well as tissue factor linked mechanisms.[10]

Endothelial Heterogeneity

In the circulation, vascular endothelial cells (ECs) contribute to the host defense against inflammatory and toxic substances like particulate matter. ECs are highly heterogeneous in structure and function, related to the specific vascular bed they reside in.[11] However, data on tissue-specific *in vivo* endothelial gene expression contributing to this heterogeneity, as well as their response to inflammatory triggers, has been relatively limited that is mainly due to the small fraction of ECs and interspersed distribution within tissues.[11] Combining translating ribosome affinity purification with high-throughput RNA sequencing analysis allows for the isolation and transcriptional profiling of ECs from multiple tissues captured in their *in vivo* microenvironment.[12,13] These data demonstrate remarkable EC heterogeneity under physiologic conditions; in addition to vascular bed-specific shifts in gene expression following lipopolysaccharide (LPS) exposure, this induces a general procoagulant, antifibrinolytic shift of the endothelium with reduced levels of thrombomodulin (TM) and tissue factor pathway inhibitor, and an upregulation in plasminogen activator inhibitor 1 (PAI-1) expression. However, protein C receptor (EPCR) mRNA levels show tissue-specific EC reactivity as levels in brain, heart, and kidney are lower in LPS-treated animals than in controls, while they are increased in liver and lung,[14] which could explain the tissue-specific susceptibility to increased vascular leakage in LPS-treated EPCR deficient mice.[15] This *in vivo* mouse model has the marked advantage of evaluating diversity in EC expression profiles *in situ* in a high-throughput fashion under different physiologic and pathologic conditions. Based on these data, it is possible to identify vascular bed-specific markers, as well as potentially identifying new biomarkers for disease progression and novel targets for therapeutic intervention.

Noncanonical Substrates Downstream from Thrombin

In addition to substrates directly leading to clot formation such as fibrinogen and the protease-activated receptors, thrombin's activity plays a central role in both short term as well as chronic outcomes following activation of the coagulation cascade. This is because other thrombin substrates such as the matricellular protein, osteopontin (OPN), and protein C (PC) modulate effectors for other indications such as inflammation and diabetes. One critical component in determining the outcome of the generation of thrombin is the presence of its cofactor, TM. When thrombin binds to TM, its activity is altered from pro-coagulant and pro-inflammatory to being anticoagulant and antiinflammatory.

TM is a constitutively expressed receptor on vascular ECs as well as some leukocytes that has a high affinity for thrombin.[16] Binding of thrombin to TM enhances activation of PC to activated protein C (aPC) which inactivates coagulation factors (F) Va and VIIIa. The thrombin-TM complex also activates the plasma basic carboxypeptidase, pro-carboxypeptidase B2 (proCPB2; thrombin-activatable fibrinolysis inhibitor [TAFI]). The activated enzyme, CPB2 (TAFIa), stabilizes fibrin clots by inhibiting plasmin generation and reducing fibrinolysis.[17–20] Apart from CPB2's role in inhibiting fibrinolysis, it is also antiinflammatory as it inactivates pro-inflammatory mediators such as bradykinin, anaphylatoxins C3a and C5a, and thrombin-cleaved OPN.[19,21–23] Thus, CPB2 and aPC have complementary roles in maintaining homeostasis as a result of their activation as both are antiinflammatory via different mechanisms while aPC inhibits further clot formation and CPB2 protects the clot from early dissolution, thereby preventing rebleeds.

$CPB2^{-/-}$ mice have been used to study inflammatory diseases such as lung diseases including allergic bronchial asthma, chronic thromboembolic pulmonary hypertension (CTEPH) and alveolitis, but also autoimmune arthritis, sepsis, etc.[17,22,24–26] Outcomes in $CPB2^{-/-}$ mice can improve or worsen the disease depending on the particular model being studied; $CPB2^{-/-}$ mice had worse C5a-induced alveolitis than wild type, but in a polymicrobial sepsis model, $CPB2^{-/-}$ mice had improved survival, less lung edema, and less liver and kidney damage compared with wild type.[20] In the alveolitis model lack of CPB2 allowed unregulated C5a activity,[22] whereas in the polymicrobial sepsis model the key substrate leading to the phenotype of protection in the $CPB2^{-/-}$ mice was C3a despite the presence of C5a in exacerbating the disease.[20]

OPN has pleiotropic functions involved in both cell–cell and cell–matrix interactions while it can also circulate as a pro-inflammatory cytokine.[27] OPN is expressed by many inflammatory cells (e.g., T-cells and macrophages), and its expression is enhanced during inflammation or stress. OPN can interact with many different cells via integrin receptors resulting in, among others, leukocyte cell survival, differentiation, and mobilization but also changes in adhesion, migration, trafficking, etc.[28]

OPN contains a conserved thrombin cleavage site that generates OPN-R (the N-terminal fragment) and CTF (the C-terminal fragment) which have new activities not present in full-length OPN. OPN-R reveals a cryptic integrin binding site, enhancing cellular adhesion and survival.[19,28] Following its formation, the C-terminal of OPN-R is a substrate for CPB2 that removes the novel integrin binding site. Jurkat cells, an immortalized human T cell line have enhanced binding to OPN-R which was abolished by CPB2 treatment,[19] showing that cleavage of the newly exposed integrin binding site at the C-terminal of OPN-R is abolished by CPB2 treatment, removing its pro-inflammatory function.

Chemerin is an adipokine and chemoattractant that circulates in the blood in its inactive prochemerin form.[29] Its activation proceeds via proteolytic cleavages by enzymes from the coagulation and fibrinolytic cascades of the C-terminus, resulting in various chemerin forms with distinct C-terminal sequences which possess different levels of activity. The relatively low bioactivity of the chemerin form, chem158K, generated by FXIa- or plasmin- cleavage of prochemerin is enhanced by subsequent CPB2 proteolysis to the fully active form, chem157S.[29,30] This is the only known substrate of CPB2 that is activated by CPB2 cleavage rather than inactivated.[29,31] Activation of both of the proteases responsible for generation of chem158K (FXIa and plasmin) is downstream from generation of thrombin linking the coagulation and fibrinolytic systems to modulation of endocrine disorders.

All of the thrombin substrates considered here can affect both the immediate outcome of a thrombotic event but also the long-term vascular consequences. Activity of CPB2 and aPC directly affect the size and length of time that a clot will be present. Their anti-inflammatory effects will modulate the local and systemic inflammatory environment. The activity of thrombin-cleaved OPN controls infiltration by circulating leukocytes into the vessel wall while active chemerin may exacerbate obesity and diabetes.

Extreme Endothelial Cell Challenge: The Case of Thrombotic Thrombocytopenic Purpura

Auto-immune mediated thrombotic thrombocytopenic purpura (iTTP) and congenital thrombotic thrombocytopenic purpura (cTTP) are rare diseases with a historical mortality of >90%. Over the past two decades much knowledge has been gained regarding the pathophysiology of TTP,[32,33] but already in the 1980s the empirical introduction of plasma exchange (PEX) and fresh frozen plasma (FFP) replacement had resulted in a spectacular improvement in survival of 80%.[34] Predisposing factors for iTTP include female sex, African-American race, and certain HLA-DR types.[32] Both iTTP and cTTP may require a "second hit" besides acquired or congenital severe deficiency of ADAMTS13 activity, for example, an (often mild) prodromal infection or, especially for cTTP, pregnancy.[35] ADAMTS13 is a protease that cleaves ultralarge forms of von Willebrand factor, thereby controlling its prohemostatic activity; its deficiency triggers extensive and poorly controlled VWF-platelet vessel wall interactions. TTP is associated with significant comorbidity, caused by microthrombi leading to ischemic organ damage, mainly of the brain and heart. Long-term sequelae include neurocognitive disturbances, depression, arterial hypertension, and a significantly increased mortality in survivors of iTTP attacks.[36,37]

Differential diagnosis of acute TTP must exclude other forms of thrombotic microangiopathies (TMA), especially atypical hemolytic uremic syndrome (HUS)[33] which is essential for appropriate management. Prompt diagnosis of acute iTTP or cTTP based on severely deficient ADAMTS13 activity (<10%, and most often <3%), with (iTTP) or without (cTTP) ADAMTS13 inhibitor, is important considering new treatment strategies becoming available, such as caplacizumab or rhADAMTS13. The recent demonstration of an open conformation of ADAMTS13 specifically in all patients with acute iTTP and approximately 25% of those in remission having survived acute iTTP, but in none with a diagnosis of sepsis or HUS suggests that such open conformation, demonstrable using a monoclonal antibody against a cryptic epitope in the spacer domain of ADAMTS13, may become a specific biomarker for iTTP in the future.[38]

The cornerstone of treatment of acute iTTP still consists of therapeutic PEX with FFP replacement and corticosteroids, whereas an acute episode of cTTP may be treated by FFP.[39] The nanobody caplacizumab, blocking the VWF A1 domain—platelet glycoprotein (Gp) Ib-IX-V interaction, given upfront in acute iTTP by i.v. and then daily s.c. injection in addition to daily PEX, FFP replacement and corticosteroids, leads to faster resolution of thrombocytopenia, less exacerbation, and hopefully less organ damage by preventing the formation of microthrombi.[40] It is of importance to ascertain recovery of ADAMTS13 activity before stopping caplacizumab to avoid TTP exacerbation or recurrence. Bleeding risk is increased under caplacizumab, the VWF-platelet interaction being completely inhibited, but so far, no serious bleeding complications have been observed.[40] Besides upfront treatment with caplacizumab, aiming at immediately blocking the pathologically enhanced VWF-platelet interaction, immunosuppression is mandatory to eliminate the pathogenic anti-ADAMTS13 autoantibodies. In addition to corticosteroids, the anti-CD20 antibody rituximab is increasingly used for this purpose even though there is still discussion whether it should be reserved for therapy-resistant patients, given upfront to any acute iTTP patient, and/or preemptively in clinically asymptomatic survivors of acute iTTP with recurring severe acquired ADAMTS13 deficiency.[41]

The availability of rhADAMTS13, which has been successfully tested in a phase 1 pharmacokinetics and safety study in 15 cTTP patients, will probably facilitate standard prophylaxis and/or treatment for the rare patients with cTTP.[42]

Theme 1: Potential Areas for Investigation

- EC heterogeneity linked to (hallmarks of) disease initiation and progression to identify biomarkers and new targets for drug therapy; technical standardization of gene expression profiling, including defining "healthy" and "diseased" cells; collaborative approaches to process, analyze, interpret, and follow-up high-throughput datasets.
- Exploration of downstream products of the coagulation cascade including, for example, the thrombin-cleavage fragments of OPN or the different chemerin forms, as potential biomarkers.
- Evaluate the potential of therapeutic strategies including PC or aPC mutants with altered anticoagulant over anti-inflammatory activities, as well as soluble TM and CPB2 inhibitors for treatment of acute stroke, myocardial infarction and venous thromboembolism.
- The value of chemerin receptor antagonists to modify the course of diabetes and obesity in patients following a thrombotic event.
- The role of ADAMTS13 and von Willebrand factor as cardiovascular risk factors in epidemiologic studies.

Theme 2: Circulating Cells and Thrombo-Inflammation: Platelets, Neutrophils, and Neutrophil Extracellular Traps

New Mechanisms in Platelet-Mediated Thrombosis

The role of platelets in hemostasis and thrombosis is well established, but the mechanisms through which platelet surface GP's interact with surrounding cells and proteins in the vasculature require further elucidation (►**Fig. 2**). The immunoreceptor-tyrosine-based-activation (ITAM)-containing receptor receptor glycoprotein VI (GPVI) has been shown to directly interact with collagen to activate downstream SH2 domain-containing tyrosine kinase, Syk, thereby initiating platelet activation. However, more recently, $GPVI^{-/-}$ mice demonstrated a delay in vascular occlusion in response to ferric chloride ($FeCl_3$), but not in initiation of thrombus formation, with no fibrillar collagen found in the formed thrombus.[43] These unexpected results lead to speculation on a second ligand for GPVI in the growing thrombus with the proposal that this was fibrin. The interaction of GPVI with polymerized fibrin amplifies thrombin generation and platelet recruitment.[44] Further, platelet spreading on fibrin is abolished in human platelets deficient in GPVI due to a homozygous insertion in the extracellular domains which prevents membrane expression.[45] This confirms that fibrin activates platelets through the GPVI-Fc receptor-γ-chain complex. Further research is required to establish whether the binding sites for collagen and fibrin on GPVI are shared or distinct.

Fibrin binds selectively to monomeric GPVI, determined by surface plasmon resonance spectroscopy, in contrast to collagen which binds to dimeric GPVI.[45] On the other hand, fibrin can bind to dimeric GPVI.[46] The explanation for these opposing results may be related to sequence differences in recombinant GPVI. The availability of a large number of antibodies and related reagents (e.g., nanobodies) to GPVI is utilized to test if they block the interaction with collagen and fibrin. The use of aspirin and $P2Y_{12}$ inhibitors in thrombosis are limited because a proportion of patients have

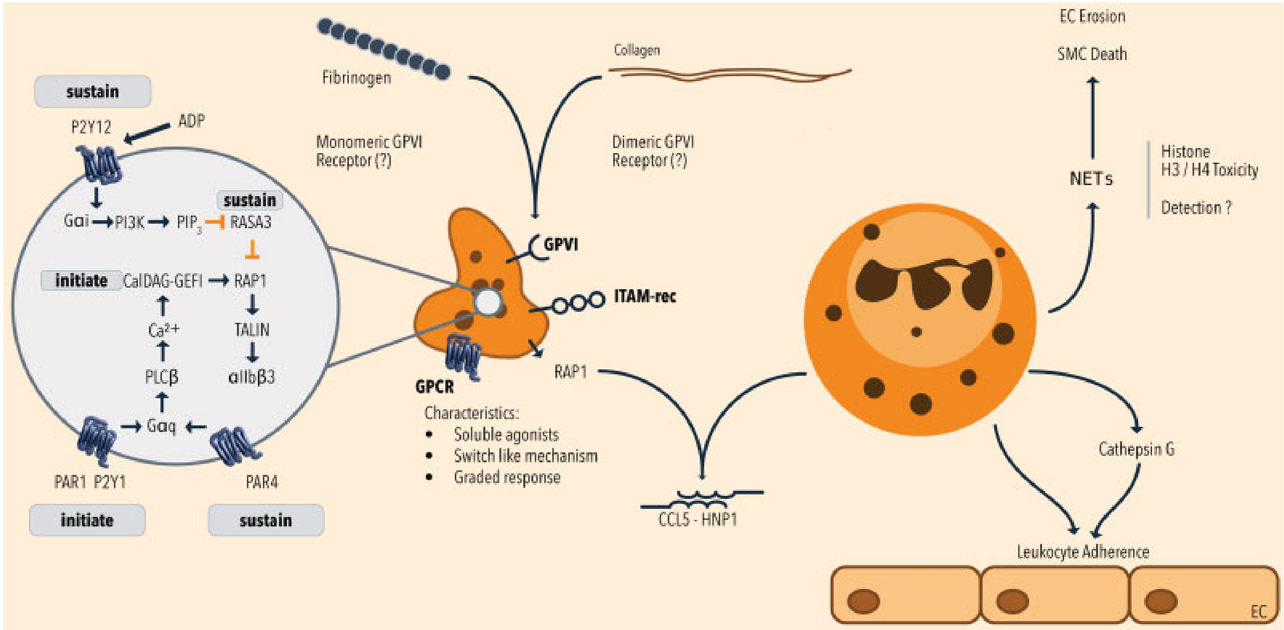

Fig. 2 Schematic representation of the potential areas for investigation for theme 2. Platelet signaling differs between individuals due to variability in GPCR signaling. One of the unexplored areas is the monomeric versus dimeric signaling of fibrinogen and collagen on glycoprotein VI Platelet integrin activation from low to high affinity depends on agonist receptor activation of mainly two classes: GPCRs (including PAR1, PAR4, P2Y1, and P2Y12) and immunoreceptor tyrosine-based activation motif-containing receptors. GPCR signaling has three main advantages: soluble agonists, switch-like mechanisms, and graded receptors. Crucial on the GPCR-signaling is the downstream activation of the GTPase RAP1, triggering immediate activation of integrin receptors through activation of talin. Platelets and neutrophils interact with each other, thereby enhancing thrombotic mechanisms. Upon activation, neutrophils release human neutrophil peptide 1, interacting with platelet-derived CCL5 into a heteromer, which enhances monocyte recruitment. In a second mechanism, neutrophils recognize CCL5 resulting in the release of cathepsin G, an interesting target to diminish adherence of leukocytes to large arteries. Neutrophils release extracellular traps consisting of genomic DNA and nucleosome proteins of which histones H3 and H4 are considered most toxic. GPCR, G-protein coupled receptor; PAR, protease-activated receptor.

further thrombotic episodes and both drugs increase the risk of bleeding. GPVI is an attractive target for a new class of antiplatelet agents as it appears to play a minor role in hemostasis. The challenge, however, is the high cost of clinical trials to test this general hypothesis. The trial design could be problematic as the patients may have to be taking a second antiplatelet agent, leading to an increase in bleeding. A smaller trial in patient subgroups with a high risk of thrombosis would have a reduced cost and also demonstrate efficacy in blocking GPVI in human, thereby translating results from both *in vitro* models and mouse models that mimic the microfluidics and nanofabrication in the vasculature. Revacept is a recombinant dimeric GPVI which competes with platelet GPVI for binding to collagen. Revacept is a weaker inhibitor of collagen signaling than the humanized blocking Fab to GPVI, ACT017, which binds directly to the Ig receptor. In addition, ACT017 blocks activation of platelets by fibrin. Revacept has completed two phase II clinical trials and ACT017 is undergoing phase 2.[47]

Regulators of Platelet Adhesion and Inflammation

Platelet adhesion to areas of vascular injury depends on the cell's ability to rapidly convert its integrin receptors from a low to a high affinity state. Platelets express two main classes of agonist receptors, which sense changes in the environment and thus initiate intracellular signaling required for integrin activation[48]: G protein-coupled receptors (GPCRs) and immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors. GPCRs have three important advantages over ITAM receptors as initiators of platelet activation during hemostatic plug formation (1): they are activated by soluble agonists, that is, they can mediate cellular activation in the core of the hemostatic plug where there is no direct contact with the extracellular matrix (2); their switch-like activation mechanism allows for a near-immediate generation of intracellular second messengers; and (3) they facilitate a graded response as key agonists like thrombin and ADP activate cells via two distinct receptors, one that initiates signaling and another that is required for the signal to be sustained. Most GPCRs directly activate phospholipase C, a key enzyme in the formation of the second messengers, diacylglycerol and calcium (Ca^{2+}). Downstream of second messengers, the small GTPase RAP1 has a crucial role in the near-immediate activation of integrin receptors. Similar to the GPCR system, RAP1 activity is controlled by GDP for GTP exchange, mediated in a switch-like fashion by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs).[49] Only small changes in the cytosolic Ca^{2+} concentration are required to trigger the activation of CalDAG-GEFI,[50] a major GEF for RAP1 in platelets. However, this signal will not be sustained unless inhibitory signaling by the GAP, RASA3, is inhibited the latter regulated downstream of the platelet ADP receptor, $P2Y_{12}$ and consequent PI3 kinase-mediated generation of phosphatidylinositol[3–5]-trisphosphate (PIP_3).[51] Once activated, RAP1 communicates with TALIN, a direct interactor and activator of integrin receptors. In contrast to cells of the innate and adaptive immune system, RAP1 in platelets does not rely on an adapter protein like RIAM1 (RAP1-GTP interacting molecule-1) for recruiting TALIN.[52,53] A functionally relevant direct interaction between RAP1 and TALIN has recently been demonstrated,[54–56] likely another adaptation required for platelets to be able to rapidly adhere and aggregate under high shear stress conditions. In summary, the "G protein highway to integrin activation" is crucial for classical hemostasis. Genetic disruption or pharmacological intervention with individual components of this pathway often cause severe bleeding. Other signaling systems (ITAM receptors, kinases, etc) play a less important role during hemostatic plug formation. However, these proteins may be more important for other forms of hemostasis, such as inflammatory hemostasis and vascular development, where platelet aggregation under flow is not required. The relative contribution of individual signaling pathways to pathological thrombus formation in arterial and venous thrombosis needs further investigation. Additional experimental studies should also include more mechanistic studies on the RAP1-TALIN interaction, other downstream effectors of RAP1 in platelets, the contribution of RAP2 to platelet function, and whether and how RAP1 regulators with low expression levels affect platelet function.

Neutrophils and Atherosclerosis

Neutrophils in Early Stages of Atherosclerosis

In recent years, neutrophils have received recognition for their role in chronic inflammation including atherosclerosis.[57] Depletion of neutrophils during early stages of atherosclerosis reduced lesion sizes as well as the accumulation of monocyte and macrophages, an effect partially driven by neutrophil-derived chemotactic granule proteins.[58,59] Neutrophils themselves are in part recruited to large arteries through action of platelet-borne CCL5 which is deposited on atherosclerotic endothelium. However, centered on CCL5, neutrophils have been shown to engage in mechanisms that form a detrimental alliance between neutrophils and platelets and stimulate monocyte recruitment.[60,61] In acute and chronic inflammation, neutrophils and platelets, both of which promote monocyte recruitment, are often activated simultaneously. HNP1 (human neutrophil peptide 1) from neutrophils forms heteromers with CCL5 derived from platelets enhancing the recruitment of monocytes at the site of inflammation. The recruitment of classical monocytes can be inhibited by disturbing heteromers of neutrophil HNP1 and platelet CCL5. These heteromers stimulate monocyte adhesion through CCR5 ligation. Based on understanding the structural features of HNP1-CCL5 heteromers, stable peptides that disturbed pro-inflammatory HNP1-CCL5 interactions were generated and successfully used to limit monocyte recruitment.[62] As a second mechanism, neutrophils recognizing CCL5 were shown to deposit cathepsin G on inflamed large arteries. Importantly, this mechanism was restricted to large arteries and does not occur in postcapillary venules. Mechanistically, cathepsin G is immobilized on arterial endothelium where it activates leukocytes to firmly adhere by engaging integrin clustering, a process of crucial importance to achieve effective adherence under high-shear flow. Therapeutic neutralization of cathepsin G specifically abrogated arterial leukocyte adhesion without affecting myeloid cell adhesion in the microcirculation of mice.[63]

Circadian Control of Arterial Myeloid Cell Adhesion

The clinical manifestation of CVD exhibits daily variation, with an increased incidence in the early morning hours. This coincides with circadian oscillations of glucocorticoids, blood pressure, leukocyte counts, and other parameters regulating inflammatory processes.[64] Myeloid cell adhesion in atherosclerotic regions is controlled in a circadian fashion. During the day, a threefold amplitude in adherent myeloid cells was seen.[65] In the morning hours in mice, there was a higher influx and adhesion of myeloid cells into the site of injury. CCL2 concentration also varied throughout the day, being higher during the morning and decreasing in the evening. The circadian recruitment pattern differed between macro- and microcirculation.[65–67] In the microcirculation, there was a lower adhesion in the morning than the evening, while the opposite happened in the macrocirculation, where the higher adhesion level was seen in the morning. Time optimized inhibition of the CCL2-CCR2 axis reduced atherosclerosis with limited side effects. The advantage of this method is the reduction of lesion size, with no impact on microvascular recruitment or circulating myeloid cell counts.

Role of Neutrophils in Advanced Stages of Atherosclerosis

Clinical studies show a striking association between circulating neutrophil counts (in particular neutrophil:lymphocyte ratio) and the incidence of acute coronary syndromes (ACS).[68,69] There were, however, few mechanistic studies on the role of neutrophils in plaque destabilization or plaque erosion. Neutrophils were found to release NETs at arterial sites of disturbed flow.[70,71] NETs in this location promote erosion of ECs and subsequent cardiovascular complications.[72,73] In the context of plaque destabilization, the number of intimal neutrophils correlates with plaque instability.[73] Mechanistically, activated smooth muscle cells (SMCs) attracted neutrophils and induced release of NETs through CCL7. NETs in close proximity to SMCs induce their death and consequently accelerate plaque destabilization. The cytotoxicity evoked by NETs is centered on histone H4, a highly cationic nuclear protein found abundantly in NETs.[74] The N-terminus of histone H4, especially exhibits membrane activity, causing membrane bending and ultimately leading to pore formation and subsequent cell lysis. Antibody-assisted histone H4 neutralization or charge inhibition by tailored cyclical peptides could successfully lower plaque destabilization in mice.[74]

Nucleosomes, Neutrophil Extracellular Traps, DNAses, and Vascular Injury

The evolutionary role of neutrophil activation in the form of NETs is to scavenge and finally prevent spread of bacteria and fungi. Beyond infectious diseases, there is interest in NETs in a variety of pathologies including thrombo-inflammation or so called "immune-thrombosis"[75].

Importantly, although NETosis implies that only neutrophils release extracellular traps, many other cells (e.g., monocytes and eosinophils) can undergo NETosis. Hence, the name "extracellular traps" without specifying the cellular origin should be considered since it is difficult to identify the cellular source.

Upon NETosis neutrophils expel a meshwork of DNA which is decorated with histones and neutrophilic proteases.[76] Each component of this meshwork may play an individual role in pathophysiology, or they may act in concert. Nucleosomes, consisting of DNA and an octamer of histone H2A, H2B, H3, and H4, respectively, form the backbone of the meshwork released by the neutrophil.[77] Nucleosomes as such are not cytotoxic, but cell-free histones are highly cytotoxic most probably due to their strong positive charge defined by their high levels of lysine and arginine residues.[78] Arginine-rich H3/H4 are the most toxic since neutralization of these two histones dampens inflammation.[79,80] Interestingly, distortion of nucleosome structure, for example, by using benzonase nuclease results in cytotoxicity, most probably due to exposure of the toxic parts of histones.[78]

Although the detection of NETs (components) is an appealing approach, there are many caveats that obscure interpretation of results.[81,82] First, in detecting NETs, histone preparations always contain other proteins, making it difficult to conclude that observed effects are due to histones. Specificity problems also occur with immunohistochemistry, as nucleic acid solutions may be contaminated with polyphosphates that trigger coagulation. In addition, cross reactivity of antibodies to nuclear proteins and DNA also affects interpretation of results. NETosis induction *in vitro* in the absence of protease inhibitors may result in the degradation of proteins and antibodies used for analysis.

In developing tools for diagnosis or experimental research related to extracellular traps, the focus should be on: quantification, determination of the cellular origin, translation of *in vitro* data to *in vivo* distinguishing between DNA from extracellular traps and DNA from dying cells. Isolation of neutrophils is very challenging and the different purification protocols result in different levels of neutrophil priming, impacting the interpretation of the results. In addition, a standardization of stimuli used to induce NETosis is urgently needed since the results obtained using different protocols are difficult to compare and make a translation into the *in vivo* situation very difficult. An example of this is NETosis induced by phorbol 12-myristate 13-acetate, which takes at least 3 hours, whereas using LPS, NETosis is achieved in 60 minutes *in vivo*.[83] Documented assays to measure NETs in plasma targeting components released upon NETosis, for example, citrullinated histones, complexes between DNA and neutrophilic proteins, often lack specificity. Finally, it remains open whether the data on NETs acquired in mice reflect the biology of NETs in humans, since mice have unique neutrophil subpopulations that may differ in NET formation as compared with human neutrophils.

To study the impact of NETs *in vivo* in disease requires models to follow NET formation *in vivo,* allowing research into NETs as therapeutic targets for treating inflammatory and thrombotic diseases. Timing of DNAse treatment to disintegrate NETs seems crucial. Too early administration of DNAse may result in harmful effects due to, for example, inefficient "wall off" of bacteria.[84,85] In addition, DNase cleavage may result in the liberation of unwanted cell-free DNA and DNA-binding proteins, which in turn may propagate inflammation.

Inhibition of histone modification by PAD4 in specific inflammation models is beneficial in mice for survival and thrombosis.[86–90] However, the translation of PAD4 inhibition into a therapeutic intervention in systemic inflammation and thrombosis is not yet "established." Currently, there is still considerable uncertainty on whether NETs are important in pathology and healing, or merely innocent bystanders.

Theme 2: Potential Areas for Investigation

- Interindividual variability in G protein signaling and its impact on efficacy and safety of antiplatelet agents.
- The efficacy and safety of novel interventions aimed at GpVI, up to and including focused clinical studies in high risk for thrombosis patients.
- Usefulness of new engineered systems, like organ-on-a-chip as well as *in silico* modeling approaches, in combination with traditional biochemical, cell biological, and *in vivo* approaches to study how genetic or pharmacological alterations of platelet function affect the hemostatic (and thrombotic) role of cells.
- The role of neutrophils in different stages of atherosclerosis and determine whether there is a specific subset of neutrophils that is prone to undergo NETosis; implications for clinical outcomes.
- The impact of NETs in disease needs to be studied in models that track/follow NETs *in vivo,* which would also enable research of NETS as therapeutic targets.
- Interventions aimed at cleaving and inactivating NETs, for example with DNAse, although potential side effects may emerge due to release of more toxic histones.

Theme 3: Procoagulant Mechanisms

Tissue Factor Expressing Extracellular Vesicles in Cancer

Tissue factor is a transmembrane glycoprotein and receptor for FVII/VIIa.[91] TF in the intravascular compartment is mostly confined to leukocytes, existing in a hidden or encrypted form that can be decrypted to allow complex formation and factor X activation.[92] The decryption process depends on several factors including externalization of phosphatidylserine to the outer membrane leaflet, thiol-disulfide exchange pathways, and sphingomyelin in the outer membrane.[93] The TF:FVIIa complex is the primary physiologic trigger of coagulation and plays an essential role in hemostasis. However, aberrant TF expression can promote thrombosis in several pathological settings.[94] The focus here is on circulating TF+ extracellular vesicles (EVs) as a biomarker for thrombotic risk as well as the role of TF in arterial and venous thrombosis (►**Fig. 3**). EVs, formerly known as microvesicles or microparticles, refer to submicron vesicles that are released from apoptotic cells, activated cells and tumor cells.[95] Plasma levels of TF + EVs can be measured by activity- and antigen-based assays, with the former being more sensitive and reliable.[96] In healthy adults, levels of TF+ EVs are undetectable.[97] However, increased levels of TF + EVs in different diseases may trigger thrombosis.[96] This has led to the idea of using TF + EVs as a potential biomarker to identify patients at risk of developing thrombosis.

Arterial thrombosis is triggered by erosion or rupture of atherosclerotic plaques resulting in the exposure of thrombogenic components, including TF. The majority of TF in human atherosclerotic lesions is present on macrophages and macrophage-derived EVs. Importantly, inhibition of TF significantly reduced thrombosis after ultrasonic disruption of carotid plaques in $ApoE^{-/-}$ mice, indicating that it plays a critical role in thrombus formation after plaque rupture.[98]

Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE),[99] is also triggered by TF. DVT initiation occurs in valve pockets of larger veins due to low oxygen levels and turbulent blood flow.[99] This leads to activation of the endothelium, changing it from an anticoagulant to a procoagulant surface. There are many risk factors for VTE including cancer.[99] Interestingly, the rate of VTE varies in patients with different types of cancer with pancreatic cancer, having one of the highest rates.[100] Circulating TF + EV activity correlates with VTE in pancreatic cancer patients.[101] Tumor cells might release TF+ EVs into the circulation and initiate VTE. Using a mouse model bearing human pancreatic BxPc-3 tumors to evaluate the role of tumor-derived TF + EVs in thrombosis,[102] tumor-bearing mice had significantly larger thrombi in an inferior vena cava stasis model compared with mice without tumors. Furthermore, an antihuman TF antibody significantly reduced thrombus size in tumor bearing but not control mice. The results suggest that inhibition of tumor TF expression and/or reducing the generation of tumor-derived TF + EVs may represent a new approach to prevent cancer-associated thrombosis.[102]

Extracellular Vesicles and Thrombin Generation in Acute Ischemic Stroke

Although the management of acute ischemic stroke (AIS) has much improved with the introduction of catheter guided thrombectomy on top of antithrombotic and thrombolytic agents, particularly in the early phase of stroke development (<4.5 hours), many challenges remain. First, bleeding risk is a feared complication of these treatments. Second, rates of recurrent stroke remain high despite secondary prevention, in particular in the first months after AIS.[103,104] Third, stroke is a heterogeneous condition with different causes.[105] In contrast to acute myocardial infarction (AMI), intensified antiplatelet treatment for noncardioembolic AIS has not provided net benefit but instead increased the rates of major bleeding,[106–108] except for short-term treatments in the first few weeks after AIS.[109,110] Biomarkers to help assess risk or outcome are scarce.[111]

We investigated EVs and thrombin generation variables as candidate biomarkers for risk stratification after AIS or TIA.[112] Platelet-derived EVs (PEV) can reflect platelet

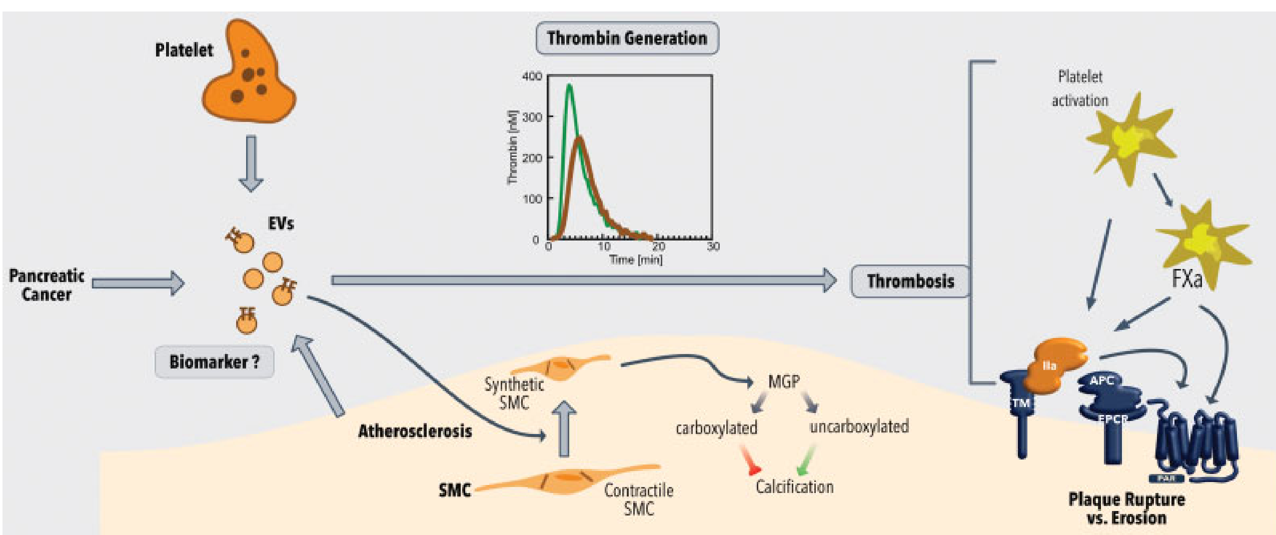

Fig. 3 Schematic representation of the potential areas for investigation for themes 3 and 4. EVs from platelets or, for example from pancreatic tumors, can trigger coagulation through activation of the TF-mediated extrinsic pathway or the intrinsic pathway, thereby contributing to both venous and arterial thrombosis. TF-bearing EVs are potential biomarkers and are mechanistically of interest for atherogenic effects such as enhancing atherosclerosis through the phenotypical switch of vascular smooth muscle cells from contractile to synthetic. This process stimulates vascular calcification in which the vitamin K dependent matrix Gla proteinplays an important role. Overall, hypercoagulability and thrombosis enhance pro-atherogenic processes through activated platelets and PAR-dependent thrombin or factor Xa signaling. Although atherothrombosis is a net result of the effect of hypercoagulability on and in the plaque, the underlying mechanisms can be distinctly different, depending on whether plaque rupture or erosion occurs. EV, extracellular vesicle; PAR, protease-activated receptor; TF, tissue factor.

activation, in particular if they are expressing P-selectin. Tissue factor positive EVs (TF + EV) may reflect monocyte activation; thrombin generation *ex vivo* and *in vivo* likely reflects thrombogenicity. Baseline EV concentrations of all types were higher for patients than matched healthy controls, but only a few specific subpopulations were associated with risk of new ischemic events. Notably neither phosphatidylserine positive P-selectin + PEV nor PS +/TF + PEV, the dominant TF + EV population showed any association with outcome. Instead only PS-negative TF + PEV resulted in increased risk of recurrent AIS or AMI. Surprisingly PS +/PEV tended to be associated with reduced risk, suggesting that certain EV subpopulations may have protective effects after AIS/TIA. Similarly, high levels of endogenous thrombin potential and peak thrombin in the acute phase were associated with an unexpected reduced risk; in contrast high EV-induced peak thrombin was associated with increased risk. Overall results suggest that the hemostatic balance is disturbed in the acute phase of AIS/TIA, with unexpected consequences for long-term risk. Specific EV subpopulations appear to play a role in this imbalance such as those lacking PS. The ischemic/postischemic brain may be involved as it is rich in TF and expresses coagulation proteins and their inhibitors.[113]

Theme 3: Potential Areas for Investigation

- The role of the intrinsic pathway of coagulation in the enhanced venous thrombotic phenotype observed in mice bearing pancreatic tumors.
- The addition of biomarkers, such as PEV + TF, to improve the ability of risk assessment scores to identify cancer patients that are at risk of VTE.
- Mechanisms regulating the blood-brain barrier (in health and during ischemia/reperfusion (I/R) injury) and the transmission of proteins and cells/EVs; the impact on interpretation of soluble biomarkers (including TF positive EVs) originating from the brain.
- Mechanisms of production and clearance of EVs from different platelet populations with different ratios of PS/PC exposure, preferably assessed by different analytic methods; this can improve the interpretation of the pathophysiological significance of EVs in thrombosis.

Theme 4: Arterial Vascular Changes in Atherogenesis; Attenuating Atherosclerosis and Ischemia/Reperfusion Injury

Vascular Inflammation and Calcification

Vascular calcification is considered a late stage event in atherosclerosis but already appears at early stages of the disease (►**Fig. 3**). Microcalcification, at a scale undetectable by conventional computed tomography scanning, causes inflammation and plaque instability.[114] Vitamin K-dependent proteins require carboxylation for their biological activity and play a crucial role in vascular calcification with a key role for matrix Gla protein (MGP).[115] The calcification inhibitory function of MGP became clear from studies in $MGP^{-/-}$ mice that were born to term, but all developed vascular calcification early on and died within a few weeks after birth. MGP is produced by vascular smooth muscle cell (VSMC), which play a central role in vascular calcification. Platelet EVs induce changes in VSMC, directing them toward a pro-inflammatory and pro-calcifying phenotype.[116] This process is associated with a prothrombotic phenotype in mice.[117] Specific coagulation proteases like thrombin promote calcification[118] and this effect can be counteracted with dabigatran (Kapustin et al, unpublished). Interestingly, the GLA domain of prothrombin as well as of protein S inhibits calcification. Synthetic VSMCs start shedding EVs in contrast to contractile VSMC, correlating with increased calcification, which seems TF dependent.[118] Drugs that interfere with vitamin K dependent carboxylation, that is vitamin K antagonists (VKA), induce calcification in VSMCs, in mice as well as in humans. Vitamin K treatment reduces VKA induced calcification, in part by carboxylation of MGP and by reducing reactive oxygen species.[119] Warfarin increases EV release from SMC and these vesicles are loaded with inactive uncarboxylated MGP.

Coagulation Proteases and Their Impact on Atherosclerosis

Hypercoagulability is a driver of atherosclerosis.[120] In concert with cells and EVs, coagulation proteases are generated that, when not inhibited by natural anticoagulants, interact with PARs at the cell surfaces, including those of EC. Physiologically, thrombin binds to TM to generate aPC in complex with EPCR, thereby providing EC protective effects through noncanonical activation of PAR-1. Inflamed ECs show changes in receptor presence and configuration, which may make them more susceptible to effects of coagulation proteases including thrombin and FXa in activating PARs.[121] One of the consequences is a shift in aPC- toward thrombin-mediated PAR-1 activation, and this biased signaling directs protective signaling toward inflammatory signaling effects.[122,123] This shift toward pro-inflammatory actions may also involve PAR-mediated contributions of FVIIa and FXa that drive and/or aggravate atherogenesis and convert atherosclerosis into a more unstable phenotype. Overall, coagulation proteases are intimately associated with all stages of atherosclerosis and contribute to plaque instability in preclinical studies.[124] Vice versa, plaque instability triggers coagulation. Thrombotic coagulation mechanisms may be different depending on rupture or erosion of the atherosclerotic plaque.[125,126] Due to changes in atherosclerotic phenotype (e.g., influence of statins and antismoking campaigns), erosion is becoming more prevalent than rupture. Whether indeed erosion and rupture trigger fundamentally different thrombogenic mechanisms in which either platelets (collagen) or clotting factors (TF exposure, NETs and contact activation) are mobilized with a dominance favoring one over the other, has been poorly explored. The contribution of cells like VSMCs in driving prothrombotic mechanisms deserves further attention, as well as the potential differences between vascular beds and the impact of vascular calcification. Clinically, there is a need for diagnostic imaging techniques to distinguish eroded from ruptured plaques, which is presently being addressed by optical coherence tomography.[127–129]

Although preclinical studies clearly demonstrate these links between coagulation activity and atherosclerosis, clinical evidence is still scarce and mostly circumstantial.[130,131] The clinical application of direct oral anticoagulants against thrombin or FXa could affect cardiovascular changes driven by coagulation proteases. Observations from preclinical studies demonstrate that inhibiting thrombin or FXa attenuates atherogenesis in atherosclerosis prone mice.[132] Moreover, regression of atherosclerosis during prolonged rivaroxaban treatment occurred, suggesting cardiovascular protection through anticoagulant therapy.[133] The majority of preclinical models show a phenotypical switch toward enhanced plaque stability upon attenuation of coagulation activity. Limited clinical data support a possible advantage of the direct anticoagulants over either no anticoagulation or VKA. Whether combination therapy of anticoagulant and platelet inhibition such as applied in the COMPASS trial (see "PAD, where do the guidelines lead us?" and further) offers additional vascular protection due to their synergistic actions is still unknown.[134] Vascular protection could also be achieved with aPC variants that lack the anticoagulant activity but can still induce cellular protective effects through EPCR-dependent PAR-1 activation. This strategy is currently being employed to provide protection for the endothelial blood brain barrier in patients with ischemic stroke.[135,136] Whether molecules such as recombinant aPC may also offer systemic vascular protection remains unanswered.

Pleiotropy of Antiplatelet Agents: Impact on Ischemia/Reperfusion Injury?

The pathogenesis of cell damage following reperfusion of ischemic tissue (I/R injury) is due to enhanced production of inflammatory mediators, recruitment of polymorphonuclear leukocytes (PMNs), and blockage of blood flow.[137–139] Leukocyte-platelet-EC interactions are important for microvascular dysfunction and release of cytotoxic mediators such as reactive oxygen intermediates and proteases, and imply a role for the bridging molecule, P-selectin. Myocardial I/R injury can potentially be limited by conditioning of the heart. In spite of abundant *in vitro* and preclinical data supporting benefits of ischemic preconditioning, such strategies are not yet implemented in clinical practice guidelines.

Antiplatelet agents may potentially contribute to limitation of I/R injury, depending on the type of agent (class), dose (low vs. high), and timing of conditioning (pre- and post-conditioning PCI).

Aspirin benefits exceed TXA_2 inhibition as it may increase platelet nitric oxide (NO) synthesis, protect NO from inactivation, improve endothelial dysfunction and exert antiinflammatory effects.[140] Combining aspirin with a $P2Y_{12}$ antagonist (dual antiplatelet therapy, DAPT) is recommended by the clinical guidelines for the management of ACS. Either for secondary prevention, or for patients undergoing a revascularization procedure, oral antiplatelets agents are utilized: clopidogrel, prasugrel, and ticagrelor. These agents (either thienopyridines or nonthienopyridines) indirectly or directly inhibit the $P2Y_{12}$ ADP receptor. Although DAPT is better than single APT in reducing the risk of stent thrombosis, it has been suggested that when combined with a high level of $P2Y_{12}$ blockade, the net effect of higher dose aspirin could be removal of antithrombotic and vasodilating prostanoids that lessen the antithrombotic effectiveness of the combined treatment.[141] Lower dose aspirin with the adenosine reuptake inhibitor, dipyridamole, started during ischemia augmented the effects of simvastatin in limiting infarct size.[142] In contrast, high-dose aspirin blocked the protective effect of simvastatin. Combination of low-dose atorvastatin with either the phosphodiesterase-III inhibitor cilostazol or dipyridamole synergistically limited infarct size. The combination of dipyridamole with low-dose aspirin and simvastatin resulted in the smallest infarct size, suggesting that antiplatelet regimens may require modification for patients who are receiving statins.[142,143] Patients receiving $P2Y_{12}$ receptor antagonists may already be cardioprotected through the conditioning pathways. If it is confirmed that patients receiving $P2Y_{12}$ receptor antagonists are already benefiting from conditioning cardioprotection, other mechanisms should be targeted for further protection. Clopidogrel and cangrelor protect the monkey heart against infarction via a mechanism involving inhibition of platelet signaling pathways activated during reperfusion to prevent reperfusion injury.[144] Ticagrelor affects the adenosine compartment as it inhibits the equilibrative-nucleoside-transporter 1 and thereby adenosine cell reuptake.[145] The PLATO trial comparing ticagrelor and clopidogrel in ACS patients demonstrated an all-cause mortality benefit for ticagrelor prompting a hypothesis of pleiotropic effects beyond its antiplatelet properties.[145]

In a rat model, ticagrelor and rosuvastatin when given in combination have an additive effect on local myocardial adenosine levels in the setting of I/R. Increased adenosine concentrations translate to further platelet inhibition, regulation of inflammatory mediators, and arterial vasodilation that may reduce I/R injury.[146]

In a canine model, tirofiban, a GPIIb-IIIa antagonist, administered at the time of myocardial reperfusion, which produced a modest reduction of tissue necrosis during reocclusion and prolonged occlusion times. In conclusion, limiting platelet aggregation during reperfusion impacted infarct development.[147] Thus, short-acting GPIIb-IIIa antagonists such as tirofiban and epitifabtide may not only reduce thrombus burden and microembolization but also limit consequences of I/R injury.

Other targets to enable conditioning in conjunction with antiplatelet agents include DNA glycosylase/AP lyase repair enzyme activity that confers cytoprotection in several injury models. Endonuclease III (Endo III), a mitochondrial DNA glycosylase/APlyase, was studied in terms of infarct size reduction in a myocardial I/R injury model.[148] In this study, an i.v. bolus of 8 mg/kg EndoIII, just prior to reperfusion, reduced infarct size from approximately 44 to 25%. This effect was amplified and the infarct size was reduced to 15% when EndoIII was combined with cangrelor. EndoIII protects the heart from necrosis by avoiding the release of pro-inflammatory fragments of mitochondrial DNA (mtDNA) into the myocardium. EndoIII and DNase have been proposed as agents that can be administered at reperfusion to add their protective effect to those of a P2Y12 antagonist.[148,149]

Clinically, there is still no therapy aimed at reducing I/R injury (MI size) that is clearly associated with improved clinical outcomes.

Theme 4: Potential Areas for Investigation

- Determine triggers that drive VSMCs to calcification and/or fibrosis; contribution of microcalcification to plaque instability; role of EVs as mechanistic link between activated platelets, hypercoagulability, and VSMC's vascular calcification.
- Contribution of specific PARs in mediating the atherogenic effects of coagulation proteases, as well as the required anticoagulant level to maintain sufficient APC generation and its cytoprotective effects.
- Impact of sex, age, and menopause on mechanisms that relate to eroded versus ruptured plaque triggered atherothrombosis.
- Improvement of imaging techniques to differentiate thrombosis caused by plaque rupture from erosion.
- Mechanism(s) by which platelet $P2Y_{12}$ inhibitors, aspirin, or agents such as dipyridamole induce protection against I/R injury in man.
- Whether there is a beneficial role of "healthy" platelets in the context of myocardial I/R injury and how this potentially protective role is changed in, for example, type 2 diabetes.
- Effects of combination therapies to target multifactorial mechanisms of I/R injury.

Theme 5: Management of Patients with Arterial Vascular Disease

Acute Coronary Syndrome: Management before Admission

Management of patients with acute coronary syndrome (ACS) has dramatically changed over the past decades. Patients with chest pain undergo triage already in the ambulance, differentiating cardiac from noncardiac. In cases of suspected cardiac ischemia, treatment with vasodilators (nitroglycerin) and aspirin is started. Based on the electrocardiogram additional management can be initiated during transport and this information is forwarded to the acute coronary care department. Part of this risk and management stratification could be started even earlier by the attending physician (general practitioner [GP] in most cases). Risk scores like HEART,[150] could be used in the general practitioner setting, but several issues need to be explored, including pretest probability in the target populations. Education and training would be needed, in particular in handling point-of-care (POC) devices correctly. Implementation, maybe involving integrating eHealth solutions, needs to be addressed systematically, starting with central GPs as referral centers.

Theoretically, POC biomarkers, including troponin, could be routinely used by GPs when evaluating a patient with chest pain. Important issues include demonstrating that such a test has a high negative predictive value; rather troponin testing has been implemented for positive predictive value. Results of early triage would include timely decisions on antithrombotic medication, such as early $P2Y_{12}$ inhibition, to be administered by ambulance personnel in the future. For example, patients with chest pain and a HEART score ≥ 3 could be treated in the ambulance. A pitfall is that only 25 to 30% of patients would benefit from antithrombotic treatment, but the remainder is exposed to their potential bleeding risk that increases along with thrombotic risk, notably age. Therefore, short-acting (or reversible) antithrombotic drugs may be helpful in the early phase of the triage process.

Ischemic Stroke; Risk Estimation and Prognosis

Ischemic stroke is an acute heterogeneous thrombo-inflammatory disorder requiring diagnosis, triage, and therapy as soon as possible. The old adage of "time is brain" remains relevant, even with the field looking to expand the current time window for thrombolysis and thrombectomy.[151,152] Because of this there is worldwide interest in so-called mobile stroke treatment units, dedicated ambulances with computed tomography capability that allow early diagnosis and subsequent treatment of ischemic stroke, reducing time-to-treatment by almost 30 minutes.[153,154] Evidence for effectiveness on clinical outcomes is expected soon.[155,156]

As discussed in "EVs and thrombin generation in AIS," stroke is a highly heterogeneous disease when considering clinical presentation, severity, imaging, location of the lesion, and functional outcome which are captured by numerous scores, scales, and classification systems. Probably, the most widely known is the modified Rankin Scale, a seven-category ordinal outcome focused on the functional outcome after stroke. Using the modified Rankin Scale measured at 90 days after stroke as the primary endpoint in clinical trials has given the stroke field a powerful, simple, and standardized way to evaluate whether a new intervention indeed delivers benefit for the patient. Researchers in the field of thrombosis should consider using a similar approach in studies evaluating functional outcome after VTE.[157]

Coagulation is a sine qua non for ischemic stroke, but the role of hypercoagulability, an increased clotting propensity within the limits of normal hemostasis, is not so clear. Hypercoagulability may increase the risk of ischemic stroke in the young, suggesting its role in cryptogenic stroke.[158,159] Although interesting from a causal point of view, this finding has yet to lead to actionable clinical insights to prevent strokes. However, even though there is substantial data on hemostasis biomarkers to predict outcome after stroke, their added predictive value is limited—partly due to varying methodologies in the different studies, especially in acute phase blood sampling.[111] Still, some emerging treatment targets can be identified such as coagulation FXI for which now small molecule and antisense oligonucleotide treatments are being developed and patented at an unprecedented rate.[160] FXI's relatively minor role in hemostasis coupled to its possible critical role in thrombus formation suggest that thrombosis risk might be reduced without an increase in bleeding. The role of NETs with their negatively charged long DNA molecules that act as a scaffold in clot formation, is also not completely understood. New treatments that limit NET formation or target NETs directly (e.g., DNAse) could be tested as an adjunct to thrombolysis.[161]

Peripheral Arterial Diseases, Where do the Guidelines Lead Us?

The recent 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases (PAD), in collaboration with the European Society for Vascular Surgery, cover all arterial beds outside the heart, including carotid and vertebral arteries, upper extremities, mesenterial arteries, renal arteries, and lower extremity arteries.[162] Patients with particular lower extremity arterial disease (LEAD) present with stable symptoms of intermittent claudication or with critical limb ischemia. LEAD is associated with an increased cardiovascular event rate[163] and therefore secondary prevention is very important to improve prognosis. In addition to lifestyle improvement (smoking cessation, walking, and healthy diet), specific medical interventions include statins and more recently, PCSK9 inhibitors (aiming for low density lipoprotein (LDL) cholesterol <1.8 mmol/L), strict diabetes and blood pressure control and antithrombotic medication. The latter should minimally be an antiplatelet agent, with a preference for clopidogrel over aspirin.[164] In the Euclid trial ticagrelor was noninferior to clopidogrel in patients with LEAD.[165] Surprisingly, in patients with asymptomatic LEAD, aspirin was not better than placebo in spite of the similarly elevated mortality in such patients.[166] In general, combined APT does not add benefit to the patient and increases bleeding risk; it is confined to short-term use, for example, after endovascular interventions.[167] The use of oral anticoagulants (mostly VKA) does not add any benefit in patients with LEAD except for those that underwent venous bypass grafting.[168] The most recent addition to the antithrombotic arsenal is the so-called COMPASS regimen, comprising rivaroxaban 2.5 mg bd plus low dose aspirin, a combination that reduced cardiovascular mortality as well as major acute limb events in patients with PAD (LEAD or carotid artery disease).[169] The next guidelines will probably be modified based on COMPASS and the results of the ongoing Voyager trial. A very useful intervention for patients with intermittent claudication is exercise training that may, in various ways, reduce the burden of the vicious cycle of thrombo-inflammation associated with this vascular disease.[170] Exercise has documented beneficial effects on endothelium, reduces inflammation, stimulates vascular angiogenesis, and improves muscle metabolism and blood flow. This includes changes in monocytic function toward a less inflammatory phenotype.[171] With currently available interventions including a plethora of medication, developing individually tailored management of patients with LEAD is imperative.

Risk Stratification with Biomarkers: Promises and Deliverables

The availability of high specificity, high sensitivity, and high throughput methods to measure circulating biomarkers of cellular stress, organ dysfunction, and inflammation have led to testing and validation of their diagnostic and prognostic utility in patients with acute and chronic coronary heart disease (CHD), and atrial fibrillation (AF) as well as in apparently healthy individuals.

Utility of Circulating Protein Biomarkers in Coronary Heart Disease

Inflammatory biomarkers have attracted considerable interest and in a recent meta-analysis of 29 population-based prospective cohort studies, the importance of inflammatory cytokines and the risk of nonfatal AMI and CHD was analyzed. Some of cytokines showed an increased risk of between 10 and 25%, including interleukin (IL)-6 when adjusted for clinical risk factors. This indicates that circulating levels of pro-inflammatory cytokines in initially healthy persons are associated with CHD outcomes independent of traditional clinical risk factors.[172]

In the Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy Study (STABILITY) which tested the effect of the selective Lp-PLA_2 inhibitor Darapladib, in patients with chronic CHD, five different biomarkers, N-terminal portion of the prohormone of B-type natriuretic peptide (NT-proBNP), troponin T, LDL-C, IL-6, and growth differentiation factor 15 (GDF-15), showed strong prognostic capabilities for prediction of cardiovascular (CV) events and death.[173,174]

Multivariable Cox regression analysis was used to develop a clinical prediction model based on the most important biomarkers for CV death. Among clinical variables and biomarkers NT-proBNP had the strongest prognostic value, with a Chi-square value over 170. Clinical variables that contributed to discrimination concerning CV death were age, diabetes, smoking and prior PAD, and the biomarkers GDF-15, LDL-C, and IL-6. Based on these data, a biomarker-based model for prediction of CV death was developed and validated. The final prediction model entailed (1) age, (2) biomarkers NT-proBNP, troponin T, and LDL-cholesterol, and (3) clinical variables; smoking, diabetes, and PAD. This ABC-model was well calibrated and had high discriminatory ability for CV death (C-index 0.81) in both the derivation STABILITY study and the validation Luric cohort.[175] Thus, this ABC-score provides a robust tool for the prediction of CV death in patients with stable CHD. It is based on a small number of readily available factors and can be widely used for clinical assessment and guide management-based CV risk.

New analytical high-throughput technologies, such as modified aptamers (Somalogic) and Proximity Extension Assay (Olink Proteomics), allow simultaneous measurements of hundreds of biomarkers in a small volume of plasma for screening multiple protein biomarkers for associations with CVD and use of combination of biomarkers to predict adverse events.[176,177] In the Heart and Soul study, a prospective cohort of patients with CHD from 12 clinics in the San Francisco Bay Area with enrollment from September 2000 to December 2002, follow-up to 2011 (derivation cohort) and HUNT3, a Norwegian population based study (the validation cohort), enrollment 2006 to 2008 and follow-up 2012 (5.6 years), the Somalogic aptamer technology was used. Out of 1,054 proteins, nine biomarkers were associated with CHD and some of which are novel.[178] A 9-Protein Model was developed for the combined endpoint of MI, stroke, HF, and death. The participants had 4-year cumulative event rates of less than 10% in the first deciles (lowest score) and between 60 and 80% event rates in the 10th deciles (highest score). The combination of the 9-Protein Model with the standard Refit Framingham Model also outperformed Refit Framingham Model alone (c-indices, 0.71 vs. 0.64 in the validation cohort) in predicting patient's risk.

Another multimarker tool to identify incident major adverse coronary events (MACE), a composite of CV death, MI and stroke, in patients referred for coronary angiography, was recently developed in 649 patients (derivation cohort) and 278 patients (validation cohort) (the Casablanca study). This score includes four biomarkers; NT-proBNP, KIM-1, osteopontin, and tissue inhibitor of metalloproteinase (TIMP-1) and has a promising performance with an area under the curve (AUC) of 0.79 better than clinical variables alone (AUC = 0.75).[179]

Thus, protein biomarker profiles reflecting different pathophysiologic mechanisms of MACE in several populations with stable CHD might be useful for prognostication and decision support. Further external validation studies are needed to elucidate the importance of these novel biomarker tools.

Biomarkers and Antiinflammatory Therapy

Statins have a beneficial effect in reducing inflammation with a decrease in high sensitivity (hs) C-reactive protein (CRP) as a biomarker. In the Jupiter study, rosuvastatin treatment decreased LDL-C and hsCRP but did not address whether reduction of inflammation in the absence of cholesterol lowering might reduce CV events. This question was addressed in the Cardiovascular Risk Reduction Study (CANTOS)[180,181] in which the effect of Canakinumab, a monoclonal antibody targeting IL-1β, in stable post-MI patients with elevated hsCRP, level was studied. Increased doses of Canakinumab reduced the hsCRP and IL-6 levels without affecting LDL-C level. Canakinumab also reduced the cumulative incidence of CV events over a 4-year period further discussed in "promising strategies for prevention and treatment of arterial thrombo-inflammation," below.

In CIRT (Cardiovascular Inflammation Reduction Trial), however, low-dose methotrexate—a broad-spectrum antiinflammatory therapy—neither reduced IL-1β, IL-6, or hsCRP nor lowered cardiovascular event rates.[182] The different outcomes might result from the different levels of hsCRP, at the time of inclusion in these two studies, signifying different levels of ongoing inflammation.[182] Most recently, low dose colchicine reduced recurrent ischemic events in patients after a recent AMI.[183]

Biomarkers for Determining Thromboembolic Risk and Bleeding during Antithrombotic Therapy in Atrial Fibrillation

AF is the most common sustained arrhythmia and confers an independent increased risk of stroke, heart failure, and death. Total 20 to 30% of all strokes are due to AF. Biomarkers that include cardiovascular stress, myocardial injury, cardiac and renal dysfunction, coagulation activity, and inflammation are associated with underlying pathophysiology and clinical events and may help refine risk assessment in patients with AF.[184] Circulating EVs and microRNAs are involved in the pathophysiological process of AF and may contribute to inflammation, activation of coagulation, and angiogenesis in AF.

Inflammation may be associated with AF as well as the pathogenesis of the arrhythmia. The utility of inflammatory biomarkers as indicators of stroke or other cardiovascular events was therefore investigated in the Apixaban for the Prevention of Stroke in Subjects with AF (ARISTOTLE) study. Two biomarkers of inflammation, IL-6 and CRP, were significantly related to CV death as well as all-cause mortality but were not associated with stroke or systemic embolic events after adjustment for clinical risk factors and other biomarkers.[185]

The ARISTOTLE study demonstrated that Troponin I/T and NT-proBNP contained more prognostic information than most clinical parameters in AF. Based on the stroke and bleeding cases in the ARISTOTLE study, the ABC-stroke score and the ABC-bleeding score were established for the prediction of risk for these events. Three factors—age (A), NT-proBNP, and troponin I/T (biomarkers = B) and prior stroke (clinical event = C)—were shown to have a high correlation with stroke occurrence ($\chi^2 > 20$), while five other factors including age, GDF-15, troponin T, hemoglobin level, and previous bleeding were shown to be highly correlated with bleeding events ($\chi^2 > 10$).[186,187] The ABC-stroke and bleeding scores were further validated in the ENGAGE AF-TIMI 48-trial with samples from over 8,700 patients and outperformed the clinically used CHA_2DS_2-VASc score for predicting stroke in both the ARISTOTLE study (c-indices, 0.68 vs. 0.62) and the ENGAGE study (c-indices, 0.67 vs. 0.59) and the HAS-Bled score for bleeding, ARISTOTLE (c-indices, 0.68 vs. 0.61), ENGAGE (0.69 vs. 0.62).[188]

In conclusion the biomarkers, NT-proBNP and troponin I/T, were very valuable in evaluation of patients with CHD and AF. Inflammatory biomarkers, IL-6 and hsCRP, were also effective in monitoring a patient's inflammatory activity and effectiveness of antiinflammatory treatment in patients with CHD. The 9-Protein Model, ABC-stroke score, and ABC-bleeding score developed using multibiomarker approaches were also shown to provide better risk prediction than Refit Framingham Model, CHA_2DS_2-VASc, and the HAS-BLED score, respectively.

Current Limitations in Biomarker Implementation

In many studies, biomarkers have been determined at onset while clinical outcomes occur throughout follow-up at different time intervals. Hence, this dynamic aspect is missing in most biomarker assessment studies and biomarkers are often nonspecific in relation to complex diseases. This may limit their clinical relevance.[189]

The New Era of Antithrombotic Management

Personalized Antithrombotic Management

In patients with chronic coronary artery disease, vascular protection strategies beyond current guideline-based interventions (e.g., aspirin, statin, and ACE-I/antihypertensive agents) have become available including four new options: PCSK9i, SGLT2 inhibitors and GLP-1 RA, dual pathway inhibition (DPI), and antiinflammation (canakinumab). These interventions make use of available biomarkers including LDL, Hba1c, and hsCRP; for DPI no current biomarker is available.

The new interventions add substantially to risk reduction, showing mortality reductions of 15% in Odyssey,[190] 32% in EMPA-REG,[191] 18% in Compass,[169] and 14% in Cantos.[180]

In addition to biomarkers, is there a role for genotyping for a more personalized approach? Although genotyping for the CYP2C19 gene in clopidogrel resistance did not predict clinical events,[192] managing patients using a genotype-based strategy for clopidogrel provided noninferiority in efficacy when compared with standard use of ticagrelor or prasugrel in patients with a PCI indication.[193] Other current options to individualize treatment include stroke risk estimation based on CHA_2DS_2-VASc score, although refinement might allow dissection of risk subclasses even further.[2]

Implant Antithrombotic Management

What is the optimal antithrombotic strategy post-TAVI (transcatheter aortic valve implantation)? The prevalence of subclinical leaflet thrombosis after intervention might have been underestimated and may range from approximately 15 to 40%.[194–197] Currently, DAPT is prescribed but would oral anticoagulation be better? Whether a direct oral anticoagulant (DOAC) could be applied instead of VKA remains questionable. The GALLILEO trial was stopped because in the rivaroxaban arm more thromboembolisms and more bleeding with higher mortality were seen compared with the aspirin arm.[198] Further research on the most effective way of preventing thrombosis at these artificial surfaces is warranted. In addition, biomarkers like thromboelastography post-TAVI may be helpful in documenting clotting tendency (RISTRATAVI study NCT0364 9594). Other improvements may come from platelet profiling with whole blood tests as surrogate parameter for leaflet thrombosis, or eventually the use of other devices, including left atrial appendix closure devices, left ventricular assist devices (LVAD), or extracorporeal membrane oxygenation circulation, which may require less intense antithrombotic therapy.

Dual Pathway Antithrombotic Therapy

Given the importance of TF in initiating thrombosis, as well as in the context of a ruptured plaque, the use of combined anticoagulant and antiplatelet therapy makes sense. This was the basis for the regimen tested in the COMPASS trial discussed above.[169] Here, the DPI combination was superior in efficacy compared with either agent alone. Interestingly, the curve for rivaroxaban only starts to deviate after approximately1.5 year, which is comparable to the previous observation for statins. It remains unknown whether the rivaroxaban 5 mg bd survival line would eventually have merged with the rivaroxaban 2.5 mg plus aspirin arm since the trial was prematurely stopped.

Novel Antithrombotic Targets

Targeting factor XI is promising in VTE prevention,[199] see previous section "ischemic stroke; risk estimation and prognosis" and further in "promising strategies for prevention and treatment for arterial thrombo-inflammation." Factor IX targeting by aptamer was stopped at phase 3 stage for futility.[200] Platelet-related targets include Gp-VI, CLEC-2, P-selectin, vWF/ADAMTS13, CD39, platelet a2-adrenoreceptor, and platelet kinases/phosphatases, as discussed in part in previous sections. Other targets include the contact pathway (FXII) andrelated inhibitors of neutrophil activation (NET formation), polyphosphates, and targeted thrombolytic strategies.[2]

How to Improve Secondary Prevention after Coronary Thrombosis?

Patients that suffered from AMI had approximately 10 years lower life expectancy compared with those without an AMI in the Framingham study.[201] Often multiple active plaques are present in patients with ACS, which explains the propensity to further atherothrombotic events.[202] Preventive measures after ACS include, in addition to revascularization procedures, improved lifestyle and modification of active risk factors that includes treatment of dyslipidemia, blood pressure, diabetes, and thrombotic risk. Antiinflammatory drugs may show some benefit in selected patients as suggested by the CANTOS trial, as discussed in the next section.[180]

Antiplatelet therapy is a cornerstone in the management of all patients with CAD. Multiple platelet activation pathways can be targeted among which aspirin and $P2Y_{12}$ receptor inhibitors have become standard agents for a prolonged duration after ACS and/or PCI. Clopidogrel is a second-generation thienopyridine that inhibits the $P2Y_{12}$ receptor via an active metabolite generated in the liver. However, the pharmacodynamic response to clopidogrel is highly variable among subjects, such that in approximately 30% the antiplatelet effect is insufficient (clopidogrel "resistance"[203]). Comparison of the PEGASUS-TIMI 54 platelet function substudy and the STEEL PCI study demonstrates an extensive overlap between ADP-induced platelet aggregation with placebo and with clopidogrel, respectively.[204,205] The more effective and reliable $P2Y_{12}$ inhibition observed with ticagrelor explains its markedly greater efficacy in preventing stent thrombosis compared with clopidogrel.[206,207]

In the PEGASUS-TIMI 54 trial, ticagrelor was superior to placebo in combination with aspirin in reducing CV events beyond 1 year after MI, while fatal bleeding was not increased.[208] Long-term DAPT reduces ischemic events following MI in patients at high CV risk at the cost of more nonfatal bleeding. The most pronounced benefit of long-term DAPT is seen in AMI patients with unmodifiable risk factors: multivessel CAD, diabetes, CKD (epidermal growth factor receptor <60 mL/min), coexistent PAD, and greater age.[209] Excluding patients with risk factors for bleeding, such as anemia and prior hospitalization for bleeding, may further enhance the benefit of long-term DAPT. The biomarker GDF-15 may play a role in assessing bleeding risk although prospective studies are warranted to determine its utility.[210] Selecting patients with multivessel CAD, either very severe and/or associated with diabetes, CKD, PAD or recurrent AMIs, is likely to reduce CV death among other ischemic outcomes.[209,211] Rivaroxaban 2.5 mg bd in combination with aspirin offers an alternative therapy in high-risk patients with stable multivessel CAD or prior MI, including patients with prior nonlacunar ischemic stroke.[169,209]

Impaired fibrinolysis is an independent predictor of poor outcome after ACS and represents a potential target for new therapies.[212] Moreover, there is a need to further investigate how safety of combined antithrombotic treatments can be improved, in particular regarding bleeding complications. Potentially safer strategies include drugs like Revacept (GPVI antagonist) and $5HT_{2A}$ receptor antagonists. To tackle the inflammatory components, there is evidence from the PLATO study that clopidogrel attenuates systemic inflammation via an off-target effect[213] although the mechanism is not clear yet. This explains why clopidogrel has more antiinflammatory effects than ticagrelor despite the greater ability of ticagrelor to inhibit the promotion of inflammation by activated platelets.[214] Low-dose aspirin does not have any detectable antiinflammatory effects and may even promote inflammation under some circumstances.[215]

Promising Strategies for Prevention and Treatment of Arterial Thrombo-Inflammation

Atherosclerotic plaque disruption triggers platelet activation and initiation of coagulation and subsequent thrombin generation. Thrombin not only converts fibrinogen to fibrin but also serves as a potent platelet agonist and driver of inflammation. Therefore, thrombin links thrombosis with platelet activation and inflammation.[216]

Antiplatelet therapy is a cornerstone for prevention and treatment of atherothrombosis because platelets predominate in arterial thrombi. The principles of (D) APT have been discussed in the previous sections.

Despite single APT or DAPT, up to 5% of patients with chronic atherothrombosis and up to 11% of patients with ACS have recurrent ischemic events each year. The limited utility of APT suggests that these events are triggered by a stimulus that is unresponsive to suppression of platelet activation. This stimulus is TF that is exposed at sites of atherosclerotic plaque disruption and initiates coagulation and triggers thrombin generation.[217,218] Therefore, concomitant suppression of thrombin generation and platelet activation may be better than antiplatelet therapy alone for prevention of atherothrombosis.

The dose of rivaroxaban for stroke prevention in patients with AF is 20 mg once daily; the dose is reduced to 15 mg once daily in patients with a creatinine clearance between 15 and 50 mL/min. When administered in combination with DAPT in ACS patients, low-dose rivaroxaban (2.5 mg twice daily) had a better benefit-risk profile than a higher dose regimen (5 mg twice daily) for the prevention of recurrent ischemic events.[219] The importance of using the lowest effective dose is highlighted by the results of the APPRAISE trial. In that study, administration of the treatment dose of apixaban (5 mg twice daily) on top of DAPT increased the risk of bleeding in ACS patients without reducing the risk of recurrent ischemic events.[220] Therefore, for successful DPI, selection of the appropriate dose regimen of DOAC is essential.

The benefits of DPI were revealed in the COMPASS trial.[169] In that study, 27,395 patients with stable CAD or PAD were randomized to one of three treatments arms after a run-in phase: rivaroxaban 2.5 mg twice daily with aspirin 100 mg once daily; rivaroxaban 5 mg twice daily alone, or aspirin 100 mg once daily alone. The primary outcome was a composite of cardiovascular death, stroke, or nonfatal MI. About 90% of participants had CAD and 27% had PAD. The primary outcome was significantly lower in the rivaroxaban plus aspirin group than in the aspirin alone group (4.1 and 5.4%, respectively; hazard ratio [HR]: 0.76, 95% confidence interval [CI]: 0.66–0.86; $p < 0.001$). This translates to an absolute risk reduction of 1.3%, a relative risk reduction of 24%, and a number needed to treat of 76. The primary outcome was not significantly lower with rivaroxaban alone compared with aspirin (4.9 and 5.4%, respectively; HR: 0.90, 95% CI: 0.79–1.03; $p = 0.12$). All-cause mortality was reduced by 0.7% with the rivaroxaban and aspirin combination compared with aspirin alone (HR: 0.82, 95% CI: 0.71–0.66; $p = 0.01$). The rate of major bleeding was significantly higher in the rivaroxaban plus aspirin group than in the aspirin alone group (3.1 and 1.9%; respectively; HR: 1.70, 95% CI: 1.40–2.05; $p < 0.001$). Most of the excess bleeds were in the gastrointestinal tract, and there was no significant increase in the rates of intracranial or fatal bleeds. The rate of the net clinical benefit, the composite of cardiovascular death, stroke, MI, fatal, or symptomatic bleeding into a critical organ, was lower in the rivaroxaban plus aspirin group than in the aspirin alone group (4.7 and 5.9%, respectively; HR: 0.80, 95% CI: 0.70–0.91; $p < 0.001$). Therefore, the combination of low-dose rivaroxaban and aspirin has a clear net benefit for the prevention of recurrent ischemic events compared with aspirin alone.

Thrombosis and inflammation are intimately connected, and inflammation contributes to atherothrombosis. Modified lipoproteins, such as oxidized LDL, promote the inflammatory reactions that characterize and drive atherosclerosis. Leukocyte recruitment to the arterial wall is an important step in this process. Inflammatory cells elaborate cytokines such as IL-1, IL-6, and tumor necrosis factor and cytokine levels are elevated in most, if not all, inflammatory states. IL-1β is central to the inflammatory response and drives the so-called IL-6 signaling pathway.

In the CANTOS trial, which enrolled 10,061 patients with prior MI and high-sensitivity CRP levels $\geq$2 mg/dL, patients were randomized to treatment with canakinumab (at doses of 50, 150, or 300 mg every 3 months) or to placebo.[180] Compared with placebo, the primary efficacy endpoint, a composite of nonfatal MI, nonfatal stroke, or cardiovascular death, was reduced by approximately 15% in the 150-mg canakinumab group (HR: 0.85, 95% CI: 0.74–0.98; $p = 0.021$) and the 300-mg group (HR: 0.86, 95% CI: 0.75–0.99; $p = 0.031$) but not in the 50-mg canakinumab group (HR: 0.93, 95% CI: 0.80–1.07; $p = 0.30$). Canakinumab did not reduce all-cause mortality compared with placebo (HR: 0.94, 95% CI: 0.83–1.06; $p = 0.31$), and it was associated with a higher incidence of fatal infections. Therefore, although the results of the CANTOS trial advances the hypothesis that inflammation contributes to coronary artery disease, routine use of canakinumab is not warranted because of its modest net clinical benefit and high cost.

A second trial aimed at testing the inflammatory hypothesis of CAD compared methotrexate, which inhibits IL-6, with placebo. The study was stopped early because there was no evidence of a reduction in cardiovascular events with methotrexate.[221] Therefore, the data available to date suggest that the inflammatory process driving atherosclerosis is mediated by the IL-1 signaling pathways and not by IL-6 signaling.

Conclusion and Future Directions

The results of the COMPASS trial provide new insights into the pathogenesis of atherothrombosis by highlighting the importance of thrombin as a driver of recurrent ischemic events. To best translate the findings into practice, patients at highest risk for recurrent ischemic events need to be identified. Patients with PAD, those with polyvascular disease and high-risk CAD patients such as those with diabetes mellitus, hypertension, or heart failure are likely to derive the greatest benefit from the combination of low-dose rivaroxaban and aspirin. Still to be determined is when and which ACS patients to transition from DAPT to the combination of aspirin plus rivaroxaban. Nonetheless, the COMPASS trial will change treatment paradigms for atherothrombosis prevention in CAD and PAD patients.

The major side effect of DPI is bleeding. As briefly discussed, current research is focused on development of safer anticoagulants such as factor XI inhibitors.[222,223] Additional studies are needed to determine whether these next generation anticoagulants will provide a safer platform than rivaroxaban for the addition of single or dual antiplatelet therapies.

Finally, despite the promising results of the CANTOS trial, the role of antiinflammatory agents for prevention of cardiovascular events remains uncertain. More studies are needed to confirm the importance of the IL-1 signaling pathways in this process. To move forward, agents that are more effective, safer, and less expensive than canakinumab are needed. Until such agents are available, and more studies are performed, low-dose rivaroxaban plus aspirin will be the mainstay for the secondary prevention of atherothrombosis.

Theme 5: Potential Areas for Investigation

- Early risk stratification including biomarkers and noninvasive imaging (coronary CT/ MRI) before antithrombotic Rx; availability of quicker acting simple-to-administer drugs.
- Following ACS diagnosis, careful work-up is essential and this should be organized in the most patient friendly manner in close collaboration between GP and cardiologists. This may involve specialized GP's and requires triage of low versus high complexity patients.
- New clinical trials on the use of multibiomarker analysis to improve early diagnostics of CVD, and to explore the associated mechanisms and kinetics of biomarkers; independent validation trials to evaluate the usefulness of those biomarkers.
- Strategies for personalization of the duration of DAPT need to be refined and potentially informed by biomarkers such as GDF-15.
- Improved strategies for preventing progression of atherosclerosis with due consideration of vascular inflammation, lipids, and thrombotic pathways and the effects that different drugs have on these parameters.
- More effective strategies for reducing bleeding risk during dual antithrombotic therapy are required, informed by greater understanding of the mechanisms behind life-threatening bleeding events.
- Determine whether the next generation anticoagulants, including inhibitors of the FXII/FXI pathways, will provide a safer platform than current DOACs for the addition of single or dual antiplatelet therapies.
- Safer and less expensive agents than canakinumab are needed to provide clinically meaningful antiinflammatory therapy for preventing atherothrombosis. Similarly, safer and ultimately less expensive antiplatelet and anticoagulant agents are needed.

Theme 6: Pathogenesis of Venous Thrombosis and Late Consequences of Venous Thromboembolism

The Role of Leukocyte Populations in Venous Thrombosis

Immune cells perform key functions in venous thrombosis including (1) initiation of blood clotting, (2) local inflammation, (3) tissue remodeling, and eventually (4) controlled clot resolution. In addition to the cell types that trigger coagulation (thrombocytes, monocytes, and neutrophils)[224]—and possibly mast cells,[225] other immune cell types that regulate clot inflammation and degradation have been identified (NK cells and T cells).[226,227]

T cells regulate the function of numerous cells inside and outside the immune system; the nature and extent of their recruitment and activation are crucial for the resolution or persistence of inflammatory immune responses. A specific subgroup of T cells, effector memory T cells, is recruited into the thrombus and vascular wall of thrombotic veins, where they are antigen-independently activated and delay the resolution of the thrombus by the formation of interferon-γ.[228] Migrated T cells become tissue resident, but the significance and possible role of these and possibly-other tissue-resident immune cells in venous thrombosis are unknown.

To study the cellular and molecular basis and mechanisms of venous thrombosis, different models have been used, which differ widely in their triggering mechanisms (tissue damage, stasis and stenosis, and hypercoagulable state.[229] Consequently, the role of individual immune cell populations in these models may differ (e.g., the type and extent of neutrophil recruitment certainly varies with the amount of tissue damage). Equally important are genetic differences that affect individual cell populations but are often ignored (e.g., the prominent difference in neutrophil activity between mouse BL/6 substrains).[230]

While the frequency of thromboses increases with age,[231] age-related changes in the immune system are not well covered in thrombosis research.

Experimental Insight into Postthrombotic Syndrome

Postthrombotic syndrome (PTS) is a syndrome occurring in almost half of patients with DVT, characterized by long-term morbidity and loss in quality of life. There is no specific treatment available to prevent PTS or to diminish its burden. The mainstay of medical therapy of DVT relies on rapid and therapeutic anticoagulation, leg elevation in the acute phase, and compression therapy. The high prevalence of postthrombotic morbidity, its societal burden and the associated reduction in health-related quality of life renders this syndrome an important clinical conundrum to be solved.

Elimination of the acute venous thrombotic occlusion can be achieved with a combination of thrombolysis and catheter guided clot removal, potentially reducing the burden of PTS.[232] However, a more recent large trial[233] did not show any significant impact of catheter-guided thrombolysis, as compared with standard treatment, on PTS incidence although a reduction in PTS severity was observed.[234] A third trial in patients selected for ileofemoral vein thrombosis only also failed to show a clear benefit of catheter-guided thrombolysis.[235] Thus, the jury is still out on thrombus removal to reduce PTS. Thrombolysis and adjunctive stenting induces endothelial damage and therefore enhancing peri-procedural anticoagulant therapy might be necessary. Theoretically, the addition of a platelet inhibitor like aspirin to anticoagulation, may offer benefit as platelets also contribute to venous thrombogenesis.[236–238] The increased bleeding risk of combined full dose anticoagulation and aspirin is a potential downside that needs to be addressed.

DOAC-treated patients may have a reduced incidence of PTS as compared with low-molecular-weight heparin (LMWH)/VKA treatment,[239–241] LMWH may however still have the advantage of potentially inhibiting P-selectin mediated inflammation, and has some clinical precedent in humans with PTS.[242,243] One inference is that the more consistent anticoagulation achieved with a DOAC may, in general, be slightly more effective than VKA, with its inherent variability, in general.[244,245] Other potential targets for therapeutic interventions aimed at reducing PTS are Il-6, P-selectin and TLR-9. Administration of the P-selectin inhibitor aptamer promoted iliac vein recanalization, preserved venous valve competence and reduced vessel wall fibrosis in a baboon model of venous thrombosis.[246] The use of statins might provide benefit in patients with venous thrombosis but so far, no prospective controlled trials aimed at PTS patients alone have been undertaken (see ClinicalTrials.gov Identifier: NCT02679664).

Alterations in interindividual fibrinolytic activity may also impact thrombus resolution. Fibrinolytic enzymes also have other effects, including macrophage infiltration in thrombi (uPA dependent) and vessel wall changes including collagen content and fibrosis, with PAI-1 and vitronectin as cofactors in some of these actions.[247] The effects of PAI-1 and vitronectin may be mediated by effector molecules such as metalloproteinase (MMP)-2, MMP-9, and TIMP-1, via molecular cascades modifying matrix destruction, inactivation of cytokines and shedding of cell surface molecules.[248,249] A proposed mechanism is that increased PAI-1 ultimately leads to reduced reduces plasmin activity, thereby lowering MMP-2/-9 activity, resulting in increased thrombus volume and reduced vessel fibrosis. These divergent outcomes illustrate the complexity of the system; hence upregulation of fibrinolytic stimuli like uPA may result in untoward consequences and could explain why active fibrinolysis did not achieve better results clinically. It is important not to develop drugs that destabilize clots that otherwise would not (or not as quickly) embolize. An ideal agent could be one that increases the natural uPA expression in ECs, preferably utilizing specific receptors on local ECs in the microenvironment of the clot.

Experimental evidence suggests that other ways of stimulating thrombus resolution that may have therapeutic potential include inhibition of FXI, P- and E-selectin, NETs, TLR9, MMP-2 and -9, PAI-1, and Il-6.[250] In addition, knowledge of the biology of DVT resolution and the impact of recurrent thrombosis on the vessel wall is still scarce. There are clear differences in vessel wall inflammatory responses to a first as compared with repeated thrombotic occlusion, with more fibrotic changes in the latter.[251]

In the vena cava ligation model in mice, monocytes are not essential in thrombogenesis, however they are necessary for thrombus resolution. Differentiated macrophages infiltrate the thrombus and their secreted mediators augment plasminogen release.[252] Ly6C^{Lo} monocyte/macrophages may be important in pro-resolution activities and drive vein wall healing.[253] Monocyte phenotype is highly plastic and dictated by the local environment but it is uncertain if they can be modified to polarize the monocytes/macrophages to a healing phenotype. Modulating the immune system generally may entail unexpected, and potential harmful consequences and should therefore be approached with a high degree of caution, but local modulation of monocyte activity could decrease off target effects. Moreover, phenotypic differences between murine and human monocytes should be taken into account.

In addition, lifestyle interventions such as weight reduction and physical exercise might reduce symptoms. Supportive therapy such as compression therapy is recommended as it also reduces associated symptoms such as pain and edema. Moreover, compression therapy may reduce PTS incidence if there is adequate patient compliance.[254]

Pulmonary Embolism and Chronic Thrombo-Embolic Hypertension: How to Improve Outcomes?

Published literature on the outcome after acute PE mostly focuses on recurrent VTE, anticoagulation-associated bleeding, occult cancer, arterial cardiovascular events, and overall mortality.[255] One at least equally important outcome has been mostly overlooked: the post-PE syndrome.[256] This syndrome involves long-term functional limitations as a direct consequence of the PE, including CTEPH, chronic thromboembolic vascular disease, and any other PE-induced changes of cardiac and/or pulmonary function as well as deconditioning.[257,258] The post-PE syndrome is associated with a decreased quality of life, higher risk of depressive disorders, unemployment, and increased utilization of healthcare resources.

CTEPH is the most severe presentation of the post-PE syndrome with poor outcome if not diagnosed in time.[259] In contrast, CTEPH may be cured after surgical removal of the chronic clots.[259] Notably, due to the nonspecific clinical presentation, the delay in diagnosis of CTEPH after PE is more than 1 year,[260] resulting in more advanced disease stage at diagnosis and higher mortality.[261] Earlier CTEPH diagnosis and improved patient outcomes can likely be realized by interventions aimed at improving healthcare utilization during follow-up of acute PE, closer attention to signs of CTEPH on standard CT scans performed to diagnose PE and routine evaluation of the presence of CTEPH in the course of PE in all patients.[262–264]

For the less severe presentations of the post-PE syndrome, application of cardiopulmonary rehabilitation programs may be of great benefit, achieving full recovery in most patients. Newly developed (patient or physician reported) outcome measures should allow comparison of the effects of different treatments, for example reperfusion therapies, on long-term functional outcome.[157]

Theme 6: Potential Areas for Investigation

- Most current venous thrombosis models are best suited to study initial clotting. This focus, however, ignores important aspects of the disease, in particular chronic syndromes and side effects. There is thus a need to develop experimental methods for repetitive thrombogenesis and models for chronic venous insufficiency (CVI), PTS, and CTEPH.
- The Vena Cava ligature or damage model is very invasive. Further standardization of the procedure and its effects on the immune system should be examined (e.g., by sham operation). There is a need for a less invasive model that is easy to monitor and does not require the administration of painkillers or narcotics.
- There is a knowledge gap on the role of immune cells and their interaction with tissue cells during thrombosis and subsequent tissue remodeling which should be remedied. Numerous immune cell types whose participation and significance for inflammatory reactions are known, but others such as dendritic cells, ILCs, γδ T-cells and B-cells, have not yet been considered and should be investigated.[219]
- Optimization of anticoagulant treatment, especially in the acute phase of venous thrombosis, concerning the intensity, type, or combination of different anticoagulants and pleiotropy of LMWH and/or DOACs. Investigate the addition of antiplatelet therapy (mainly in the acute phase, such as P2Y12 inhibitors) and addition of direct p-selectin inhibition (mainly in the acute phase) to determine effect on PTS.
- Clot structure may give insights into interindividual heterogeneity toward etiology and thrombus resolution. Consider a thrombus biopsy study to focus and direct this work. Study the contribution of valvular function to the phenotype and PTS severity. Improve thrombolytic and stenting strategies to reduce endothelial damage, via timing and dosage assessment.
- The role of the fibrinolytic system (upregulation of uPA, in particular in ECs) in thrombus resolution and vessel wall remodeling deserves further study; including on vehicles (nanoparticles) that carry plasminogen for activation at thrombus site. Identify mechanisms to enhance the local endogenous fibrinolytic system.
- Immune modulation: monocyte manipulation toward a "pro-healing" monocyte phenotype, to accelerate thrombus resolution and vessel wall healing from inflammation, in a time specific manner. Use of matrix targeted nanoparticles to direct certain inhibitors to problematic (fibrotic) regions. Target the thrombus specifically to induce thrombus resolution (without having to use systemic anticoagulation). Selective inhibition of pro inflammatory cytokines (e.g., IL-6 and IL-1) with time dependent assessment.
- Patient and/or physician reported functional outcome measures that also allow comparison of the effects of different treatments in patients with VTE, for example, reperfusion therapies, on long-term functional outcome are needed.

Conclusion

This third consensus conference assembled an interactive group of young and seasoned investigators in the broad area of "thrombo-inflammation" related cardiovascular disorders. While this document summarizes the presentations and discussions, it is not comprehensive in the sense that certain elements that could have been discussed, like the potential value of genetic multimarker testing for risk stratification, were not included simply because relevant experts in such areas were not present at this meeting. At the same time, the sum of the state-of-the-art presentations provides a foundation for further research in the mechanisms of thrombo-inflammation and all potential clinical consequences.

What is known about this topic?

- Thrombo-inflammation is a driver of CVD
- Key players have been identified: endothelium, blood coagulation, and inflammation
- Details about molecular and cellular mechanisms comprising thrombo-inflammation emerge

What does this paper add?

- This symposium paper summarizes new insights into details of several mechanisms that comprise "thrombo-inflammation."
- Inflammatory challenges of the endothelium (cells, EVs, and inflammatory cells) alter the barrier function and allow procoagulant reactions to start.
- Thromboinflammation is a driver of atherogenesis and atherothrombosis, but similarly has impact on venous thromboembolism and its late complications.

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Conflict of Interest
None declared.

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