

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

Elisa d'Alessandro<sup>1</sup> Christian Becker<sup>2</sup> Wolfgang Bergmeier<sup>3</sup> Christoph Bode<sup>4</sup> Joshua H. Bourne<sup>5</sup>  
 Helena Brown<sup>6</sup> Harry R. Buller<sup>7</sup> Arina J. ten Cate-Hoek<sup>1</sup> Vincent ten Cate<sup>8</sup>  
 Yvonne J. M. van Cauteren<sup>9</sup> Yam F. H. Cheung<sup>10</sup> Audrey Cleuren<sup>11</sup> Danielle Coenen<sup>12</sup>  
 Harry J. G. M. Crijns<sup>9</sup> Ilaria de Simone<sup>12</sup> Sophie C. Dolleman<sup>13</sup> Christine Espinola Klein<sup>14</sup>  
 Delia I. Fernandez<sup>12</sup> Lianne Granneman<sup>15</sup> Arnoud van t Hof<sup>9</sup> Peter Henke<sup>16</sup>  
 Yvonne M. C. Henskens<sup>17</sup> Jingnan Huang<sup>12</sup> Lisa K. Jennings<sup>18</sup> Natalie Jooss<sup>12</sup> Mieke Karel<sup>12</sup>  
 Danique van den Kerkhof<sup>12</sup> Frederik A. Klok<sup>19</sup> Bram Kremers<sup>1</sup> Bernhard Lämmle<sup>20</sup> Avi Leader<sup>1,21</sup>  
 Annika Lundstrom<sup>22</sup> Nigel Mackman<sup>23</sup> Pier M. Mannucci<sup>24</sup> Zahra Maqsood<sup>6</sup>  
 Paola E. J. van der Meijden<sup>1,12</sup> Marc van Moorsel<sup>15</sup> Luis A. Moran<sup>25</sup> John Morser<sup>26</sup>  
 Manouk van Mourik<sup>9</sup> Stefano Navarro<sup>6</sup> Raluca A. I. Neagoe<sup>5</sup> Renske H. Olie<sup>1</sup> Pauline van Paridon<sup>1</sup>  
 Jens Posma<sup>1</sup> Isabella Provenzale<sup>12</sup> Pieter H. Reitsma<sup>13</sup> Billy Scaf<sup>1</sup> Leon Schurgers<sup>12</sup>  
 Jaap Seelig<sup>1,27</sup> Agneta Siegbahn<sup>28</sup> Bob Siegerink<sup>29</sup> Oliver Soehnlein<sup>30</sup> Eva Maria Soriano<sup>31</sup>  
 Marcin A. Sowa<sup>31</sup> Henri M. H. Spronk<sup>1</sup> Robert F. Storey<sup>32</sup> Chukiat Tantiwong<sup>12</sup> Alicia Veninga<sup>12</sup>  
 Xueqing Wang<sup>5</sup> Steve P. Watson<sup>5</sup> Jeff Weitz<sup>33</sup> Sacha S. Zeerleder<sup>34</sup> Hugo ten Cate<sup>1</sup> Scientific  
 Reviewer Committee\*

<sup>1</sup> Laboratory for Clinical Thrombosis and Hemostasis, Department of Biochemistry and Internal Medicine and Thrombosis Expert Center, Maastricht University Medical Center and CARIM School for Cardiovascular Diseases, Maastricht, The Netherlands

<sup>2</sup> Department of Dermatology, University Medical Center, Johannes Gutenberg-University Mainz, Mainz, Germany

<sup>3</sup> Department of Biochemistry and Biophysics, McAllister Heart Institute, University of North Carolina, Chapel Hill, United States

<sup>4</sup> Department of Cardiology and Angiology I, Medical Center - University of Freiburg, University Heart Center Freiburg, Bad Krozingen, Germany

<sup>5</sup> Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom

<sup>6</sup> Rudolf-Virchow-Zentrum, DFG Forschungszentrum für Experimentelle Biomedizin, Würzburg, Germany

<sup>7</sup> Department of Vascular Medicine, Amsterdam University Medical Center, Amsterdam, The Netherlands

<sup>8</sup> Clinical Epidemiology and Systems Medicine, Center for Thrombosis and Hemostasis, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany

<sup>9</sup> Department of Cardiology, Maastricht University Medical Center and CARIM School for Cardiovascular Diseases, Maastricht, The Netherlands

<sup>10</sup> Leibniz-Institut für Analytische Wissenschaften – ISAS, Dortmund, Germany

<sup>11</sup> Life Sciences Institute, University of Michigan, Ann Arbor, Michigan, United States

<sup>12</sup> Department of Biochemistry, Maastricht University and CARIM School for Cardiovascular Diseases, Maastricht, The Netherlands

\* Scientific Reviewer Committee Members: Lina Badimon, Christoph Binder, Marc Hoylaerts, Peter Karlheinz, Rory Koenen, Gregory Y. H. Lip, Steffen Massberg, Philipp von Hundelshausen, Christian Weber, Johann Wojta.

Address for correspondence Hugo ten Cate, MD, PhD, Department of Internal Medicine, Cardiovascular Research Institute, Maastricht University, P.O. Box 616, UNS50:BOX8, 6200 MD Maastricht, The Netherlands (e-mail: h.tencate@maastrichtuniversity.nl).

<sup>13</sup> Department of Internal Medicine (Nephrology) and the Einthoven Laboratory for Experimental Vascular and Regenerative Medicine, Leiden University Medical Center, Leiden, The Netherlands

<sup>14</sup> Center of Cardiology/Cardiology I, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany

<sup>15</sup> Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands

<sup>16</sup> Michigan Medicine Vascular Surgery Clinic, Cardiovascular Center, Ann Arbor, Michigan, United States

<sup>17</sup> Central Diagnostic Laboratory, Maastricht University Medical Center and CARIM School for Cardiovascular Diseases, Maastricht, The Netherlands

<sup>18</sup> CirQuest Labs, LLC and the University of Tennessee Health Science Center, Memphis, Tennessee, United States

<sup>19</sup> Department of Thrombosis and Hemostasis, Leiden University Medical Center, Leiden, The Netherlands

<sup>20</sup> Department of Hematology and Central Hematology Laboratory, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland; Center for Thrombosis and Hemostasis, University Medical Center of the Johannes Gutenberg University, Mainz,

received

September 25, 2019

accepted after revision

January 22, 2020

© 2020 Georg Thieme Verlag KG  
 Stuttgart · New York

DOI <https://doi.org/10.1055/s-0040-1708035>.  
 ISSN 0340-6245.

Germany; Haemostasis Research Unit, University College London, London, United Kingdom

<sup>21</sup>Department of Hematology, Rabin Medical Center, Petah Tikva, Israel

<sup>22</sup>Division of Internal Medicine, Department of Clinical Sciences, Karolinska Institute, Danderyd Hospital, Stockholm, Sweden

<sup>23</sup>Department of Medicine, UNC McAllister Heart Institute, University of North Carolina, Chapel Hill, North Carolina, United States

<sup>24</sup>Scientific Direction, IRCCS Ca’ Granda Maggiore Policlinico Hospital Foundation, Milano, Italy

<sup>25</sup>CiMUS, University of Santiago de Compostela, Santiago de Compostela, Spain

<sup>26</sup>Division of Hematology, Stanford University School of Medicine and Palo Alto Veterans Administration Health Care System, California, United States

<sup>27</sup>Department of Cardiology, Rijnstate ziekenhuis, Arnhem, The Netherlands

<sup>28</sup>Department of Medical Sciences, Clinical Chemistry and Science for Life Laboratory, Uppsala University, Uppsala, Sweden

<sup>29</sup>Center for Stroke research Berlin, Charité Universitätsmedizin, Berlin, Germany

<sup>30</sup>Institute for Cardiovascular Prevention, Ludwig Maximilian University Munich, Munich, Germany

<sup>31</sup>Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, United Kingdom

<sup>32</sup>Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, Sheffield, United Kingdom

<sup>33</sup>Division of Hematology and Thromboembolism, Department of Medicine and Thrombosis and Atherosclerosis Research Institute, McMaster University, Hamilton, Ontario, Canada

<sup>34</sup>Department of Haematology and Central Haematology Laboratory, Inselspital, Bern University Hospital, University of Bern, and Department for BioMedical Research, University of Bern, Bern, Switzerland

Thromb Haemost 2020;120:538–564

## 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

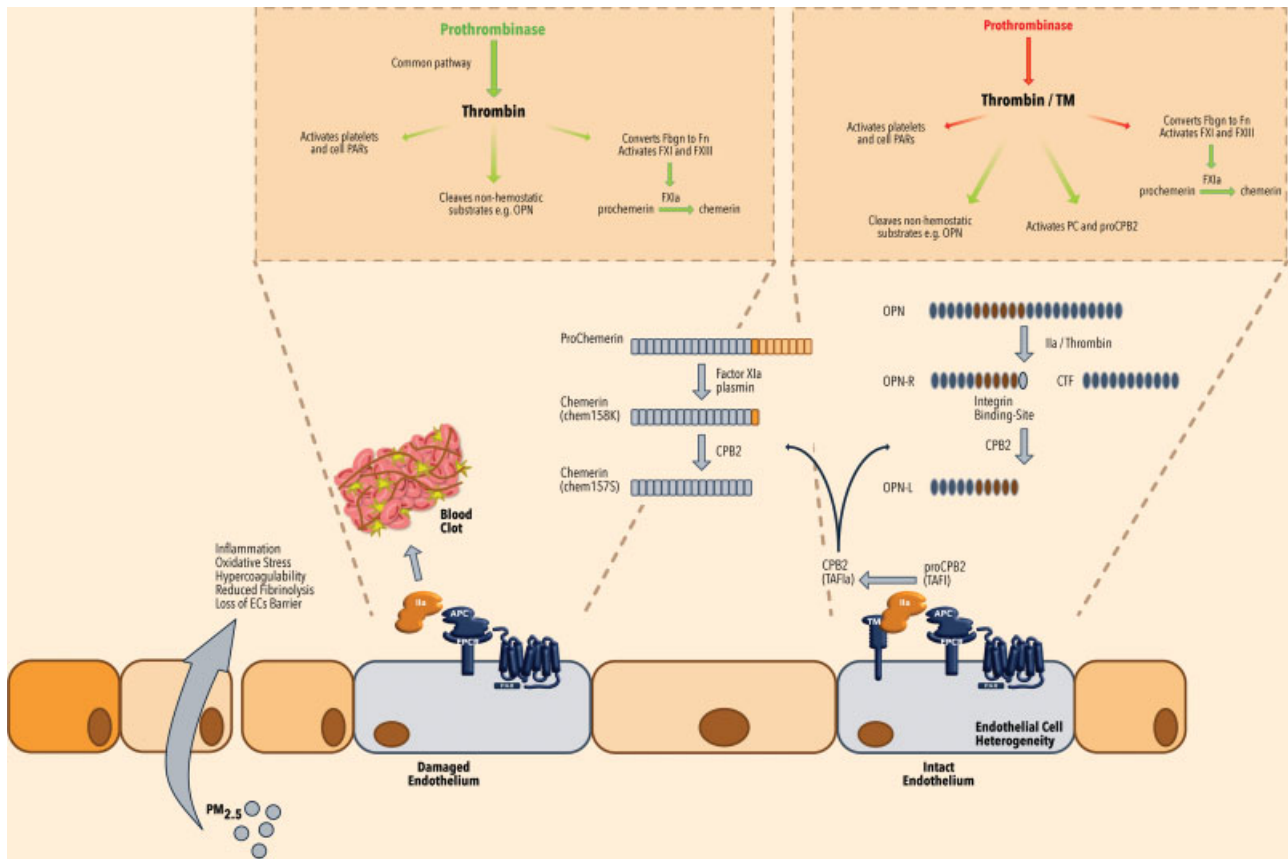
## Introduction

Thrombo-inflammation is a commonly used term to describe the complex interplay between blood coagulation and inflammation,<sup>1</sup> 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.<sup>2</sup> 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.<sup>3</sup> 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.<sup>4,5</sup> One of the key triggers is fine particulate matter with a diameter below 2.5 μm (PM<sub>2.5</sub>), which presents in natural and anthropogenic sources including fossil fuel and biomass combustion, industry, agriculture, wildfires, and wind-blown dust. PM<sub>2.5</sub> 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. Prochimerin 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,<sup>6</sup> oxidative stress, aggravated by hypercoagulability, and reduced fibrinolytic activity in blood.<sup>7-9</sup> Air pollutants containing PM<sub>2.5</sub> 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.<sup>10</sup>

**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.<sup>11</sup> 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 tis-

sues.<sup>11</sup> 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.<sup>12,13</sup> 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,<sup>14</sup> which could explain the tissue-specific susceptibility to increased vascular leakage in LPS-treated EPCR deficient mice.<sup>15</sup> 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 anti-inflammatory.

TM is a constitutively expressed receptor on vascular ECs as well as some leukocytes that has a high affinity for thrombin.<sup>16</sup> 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.<sup>17–20</sup> Apart from CPB2's role in inhibiting fibrinolysis, it is also anti-inflammatory as it inactivates pro-inflammatory mediators such as bradykinin, anaphylatoxins C3a and C5a, and thrombin-cleaved OPN.<sup>19,21–23</sup> Thus, CPB2 and aPC have complementary roles in maintaining homeostasis as a result of their activation as both are anti-inflammatory via different mechanisms while aPC inhibits further clot formation and CPB2 protects the clot from early dissolution, thereby preventing rebleeds.

CPB2<sup>-/-</sup> 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.<sup>17,22,24–26</sup> Outcomes in CPB2<sup>-/-</sup> mice can improve or worsen the disease depending on the particular model being studied; CPB2<sup>-/-</sup> mice had worse C5a-induced alveolitis than wild type, but in a polymicrobial sepsis model, CPB2<sup>-/-</sup> mice had improved survival, less lung edema, and less liver and kidney damage compared with wild type.<sup>20</sup> In the alveolitis model lack of CPB2 allowed unregulated C5a activity,<sup>22</sup> whereas in the polymicrobial sepsis model the key substrate leading to the phenotype of protection in the CPB2<sup>-/-</sup> mice was C3a despite the presence of C5a in exacerbating the disease.<sup>20</sup>

OPN has pleiotropic functions involved in both cell–cell and cell–matrix interactions while it can also circulate as a pro-inflammatory cytokine.<sup>27</sup> 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.<sup>28</sup>

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.<sup>19,28</sup> 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,<sup>19</sup> 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.<sup>29</sup> 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.<sup>29,30</sup> This is the only known substrate of CPB2 that is activated by CPB2 cleavage rather than inactivated.<sup>29,31</sup> 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,<sup>32,33</sup> 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%.<sup>34</sup> Predisposing factors for iTTP include female sex, African-American race, and certain HLA-DR types.<sup>32</sup> 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.<sup>35</sup> 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.<sup>36,37</sup>

Differential diagnosis of acute TTP must exclude other forms of thrombotic microangiopathies (TMA), especially atypical hemolytic uremic syndrome (HUS)<sup>33</sup> 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.<sup>38</sup>

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.<sup>39</sup> 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.<sup>40</sup> 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.<sup>40</sup> 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.<sup>41</sup>

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.<sup>42</sup>

### 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.

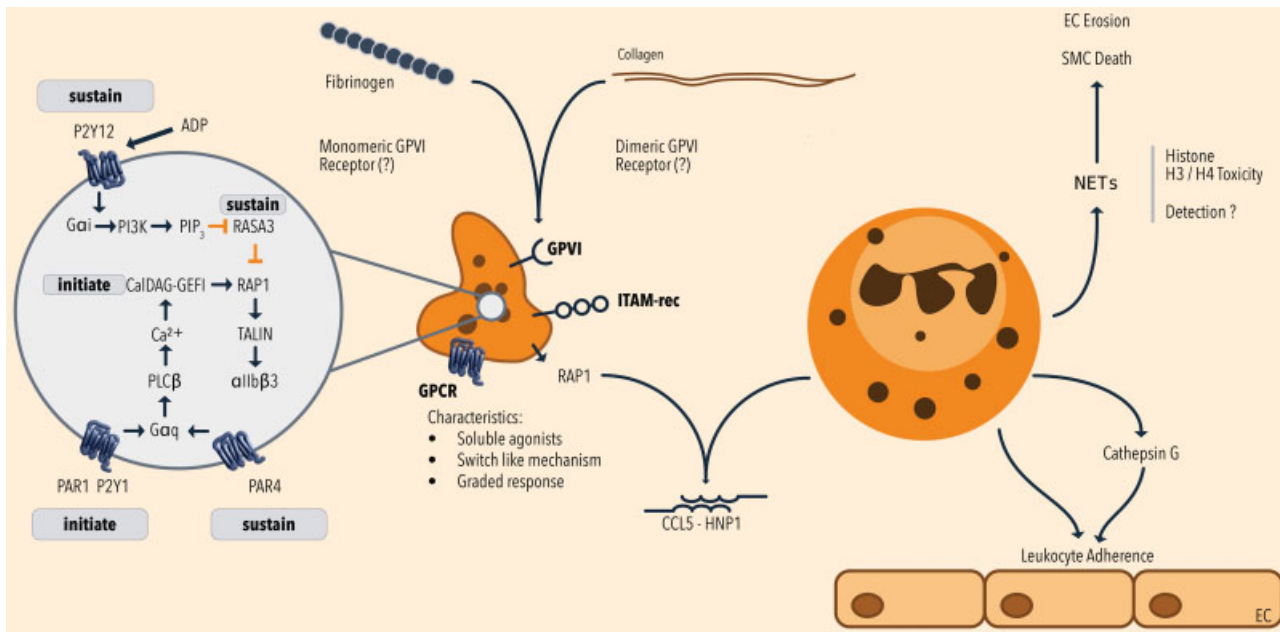
- Exploration of downstream products of the coagulation cascade including, for example, the thrombin-cleavage fragments of OPN or the different chemerin forms, as potential biomarkers.
- Evaluate the potential of therapeutic strategies including PC or aPC mutants with altered anticoagulant over anti-inflammatory 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 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<sup>-/-</sup> mice demonstrated a delay in vascular occlusion in response to ferric chloride (FeCl<sub>3</sub>), but not in initiation of thrombus formation, with no fibrillar collagen found in the formed thrombus.<sup>43</sup> 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.<sup>44</sup> 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.<sup>45</sup> 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.<sup>45</sup> On the other hand, fibrin can bind to dimeric GPVI.<sup>46</sup> 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<sub>12</sub> 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. GPIIb/IIIa 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 GPIIb/IIIa 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 GPIIb/IIIa which competes with platelet GPIIb/IIIa for binding to collagen. Revacept is a weaker inhibitor of collagen signaling than the humanized blocking Fab to GPIIb/IIIa, 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.<sup>47</sup>

### 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<sup>48</sup>: 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 ( $\text{Ca}^{2+}$ ). 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).<sup>49</sup> Only small changes in the cytosolic  $\text{Ca}^{2+}$  concentration are required to trigger the activation of CalDAG-GEFI,<sup>50</sup> 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<sub>12</sub> and consequent PI3 kinase-mediated generation of

phosphatidylinositol<sup>3-5</sup>-trisphosphate (PIP<sub>3</sub>).<sup>51</sup> 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.<sup>52,53</sup> A functionally relevant direct interaction between RAP1 and TALIN has recently been demonstrated,<sup>54-56</sup> 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.<sup>57</sup> 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.<sup>58,59</sup> 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.<sup>60,61</sup> 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.<sup>62</sup> 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.<sup>63</sup>

### *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.<sup>64</sup> Myeloid cell adhesion in atherosclerotic regions is controlled in a circadian fashion. During the day, a threefold amplitude in adherent myeloid cells was seen.<sup>65</sup> 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.<sup>65-67</sup> 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).<sup>68,69</sup> 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.<sup>70,71</sup> NETs in this location promote erosion of ECs and subsequent cardiovascular complications.<sup>72,73</sup> In the context of plaque destabilization, the number of intimal neutrophils correlates with plaque instability.<sup>73</sup> 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.<sup>74</sup> 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.<sup>74</sup>

### **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”<sup>75</sup>.

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.<sup>76</sup> 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.<sup>77</sup> 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.<sup>78</sup> Arginine-rich H3/H4 are the most toxic since neutralization of these two histones dampens inflammation.<sup>79,80</sup> 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.<sup>78</sup>

Although the detection of NETs (components) is an appealing approach, there are many caveats that obscure interpretation of results.<sup>81,82</sup> 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 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*.<sup>83</sup> 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.<sup>84,85</sup> 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.<sup>86–90</sup> 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.<sup>91</sup> 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.<sup>92</sup> 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.<sup>93</sup> 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.<sup>94</sup> 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.<sup>95</sup> Plasma levels of TF+ EVs can be measured by activity- and antigen-based assays, with the former being more sensitive and reliable.<sup>96</sup> In healthy adults, levels of TF+ EVs are undetectable.<sup>97</sup> However, increased levels of TF+ EVs in different diseases may trigger thrombosis.<sup>96</sup> 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<sup>-/-</sup> mice, indicating that it plays a critical role in thrombus formation after plaque rupture.<sup>98</sup>

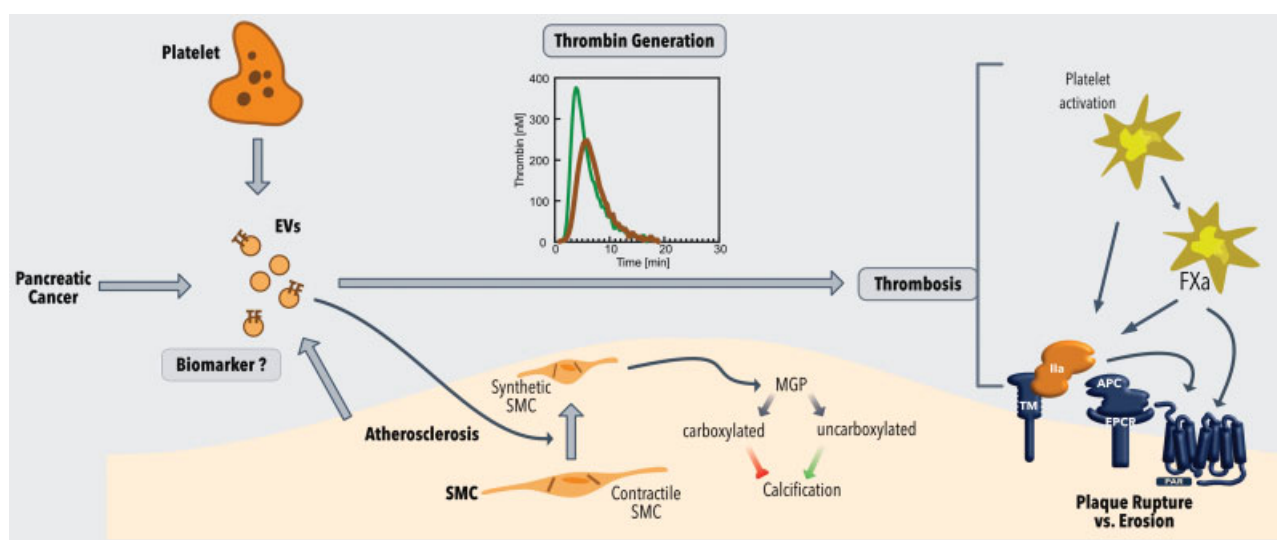
Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE),<sup>99</sup> is also triggered by TF. DVT initiation occurs in valve pockets of larger veins due to low oxygen levels and turbulent blood flow.<sup>99</sup> This leads to activation of the endothelium, changing it from an anticoagulant to a procoagulant surface. There are many risk factors for VTE including cancer.<sup>99</sup> Interestingly, the rate of VTE varies in patients with different types of cancer with pancreatic cancer, having one of the highest rates.<sup>100</sup> Circulating TF+ EV activity correlates with VTE in pancreatic cancer patients.<sup>101</sup> 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,<sup>102</sup> 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.<sup>102</sup>

### 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.<sup>103,104</sup> Third, stroke is a heterogeneous condition with different causes.<sup>105</sup> 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,<sup>106-108</sup> except for short-term treatments in the first few weeks after AIS.<sup>109,110</sup> Biomarkers to help assess risk or outcome are scarce.<sup>111</sup>

We investigated EVs and thrombin generation variables as candidate biomarkers for risk stratification after AIS or TIA.<sup>112</sup> 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 protein plays 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.<sup>113</sup>

### 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.<sup>114</sup> 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).<sup>115</sup> The calcification inhibitory function of MGP became clear from studies in MGP<sup>-/-</sup> 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.<sup>116</sup> This process is associated with a prothrombotic phenotype in mice.<sup>117</sup> Specific coagulation proteases like thrombin promote calcification<sup>118</sup> 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.<sup>118</sup> 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.<sup>119</sup> 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.<sup>120</sup> 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.<sup>121</sup> 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.<sup>122,123</sup> 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.<sup>124</sup> Vice versa, plaque instability triggers coagulation. Thrombotic coagulation mechanisms may be different depending on rupture or erosion of the atherosclerotic plaque.<sup>125,126</sup> 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.<sup>127–129</sup>

Although preclinical studies clearly demonstrate these links between coagulation activity and atherosclerosis, clinical evidence is still scarce and mostly circumstantial.<sup>130,131</sup> 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.<sup>132</sup> Moreover, regression of atherosclerosis during prolonged rivaroxaban treatment occurred, suggesting cardiovascular protection through anticoagulant therapy.<sup>133</sup> 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.<sup>134</sup> 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.<sup>135,136</sup> 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.<sup>137–139</sup> 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<sub>2</sub> inhibition as it may increase platelet nitric oxide (NO) synthesis, protect NO from inactivation, improve endothelial dysfunction and exert anti-inflammatory effects.<sup>140</sup> Combining aspirin with a P2Y<sub>12</sub> 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 antiplatelet agents are utilized: clopidogrel, prasugrel, and ticagrelor. These

agents (either thienopyridines or nonthienopyridines) indirectly or directly inhibit the P2Y<sub>12</sub> 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<sub>12</sub> 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.<sup>141</sup> Lower dose aspirin with the adenosine reuptake inhibitor, dipyridamole, started during ischemia augmented the effects of simvastatin in limiting infarct size.<sup>142</sup> 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.<sup>142,143</sup> Patients receiving P2Y<sub>12</sub> receptor antagonists may already be cardioprotected through the conditioning pathways. If it is confirmed that patients receiving P2Y<sub>12</sub> 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.<sup>144</sup> Ticagrelor affects the adenosine compartment as it inhibits the equilibrative-nucleoside-transporter 1 and thereby adenosine cell reuptake.<sup>145</sup> 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.<sup>145</sup>

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.<sup>146</sup>

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.<sup>147</sup> Thus, short-acting GPIIb-IIIa antagonists such as tirofiban and eptifibatid 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/AP lyase, was studied in terms of infarct size reduction in a myocardial I/R injury model.<sup>148</sup> 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 P2Y<sub>12</sub> antagonist.<sup>148,149</sup>

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<sub>12</sub> 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,<sup>150</sup> 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<sub>12</sub> inhibition, to be administered by ambulance personnel in the future. For example, patients with chest pain and a HEART score  $\geq 3$  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.<sup>151,152</sup> 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.<sup>153,154</sup> Evidence for effectiveness on clinical outcomes is expected soon.<sup>155,156</sup>

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.<sup>157</sup>

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.<sup>158,159</sup> 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.<sup>111</sup> 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.<sup>160</sup> 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.<sup>161</sup>

### 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, mesenteric arteries, renal arteries, and lower extremity arteries.<sup>162</sup> 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<sup>163</sup> 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.<sup>164</sup> In the Euclid trial ticagrelor was noninferior to clopidogrel in patients with LEAD.<sup>165</sup> Surprisingly, in patients with asymptomatic LEAD, aspirin was not better than placebo in spite of the similarly elevated mortality in such patients.<sup>166</sup> 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.<sup>167</sup> The use of oral anti-coagulants (mostly VKA) does not add any benefit in patients with LEAD except for those that underwent venous bypass grafting.<sup>168</sup> 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).<sup>169</sup> 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.<sup>170</sup> 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.<sup>171</sup> 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.<sup>172</sup>

In the Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy Study (STABILITY) which tested the effect of the selective Lp-PLA<sub>2</sub> 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.<sup>173,174</sup>

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.<sup>175</sup> 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.<sup>176,177</sup> 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.<sup>178</sup> 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).<sup>179</sup>

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)<sup>180,181</sup> in which the effect of Canakinumab, a monoclonal antibody targeting IL-1 $\beta$ , 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 anti-inflammatory therapy—neither reduced IL-1 $\beta$ , IL-6, or hsCRP nor lowered cardiovascular event rates.<sup>182</sup> 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.<sup>182</sup> Most recently, low dose colchicine reduced recurrent ischemic events in patients after a recent AML.<sup>183</sup>

*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.<sup>184</sup> 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.<sup>185</sup>

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 ( $\chi^2 > 10$ ).<sup>186,187</sup> 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<sub>2</sub>DS<sub>2</sub>-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).<sup>188</sup>

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<sub>2</sub>DS<sub>2</sub>-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.<sup>189</sup>

### 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,<sup>190</sup> 32% in EMPA-REG,<sup>191</sup> 18% in Compass,<sup>169</sup> and 14% in Cantos.<sup>180</sup>

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,<sup>192</sup> 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.<sup>193</sup> Other current options to individualize treatment include stroke risk estimation based on CHA<sub>2</sub>DS<sub>2</sub>-VASc score, although refinement might allow dissection of risk subclasses even further.<sup>2</sup>

#### *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%.<sup>194-197</sup> 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.<sup>198</sup> 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.<sup>169</sup> Here, the DPI combination was superior

in efficacy compared with either agent alone. Interestingly, the curve for rivaroxaban only starts to deviate after approximately 1.5 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,<sup>199</sup> 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.<sup>200</sup> Platelet-related targets include Gp-VI, CLEC-2, P-selectin, vWF/ADAMTS13, CD39, platelet  $\alpha$ 2-adrenoreceptor, and platelet kinases/phosphatases, as discussed in part in previous sections. Other targets include the contact pathway (FXII) and related inhibitors of neutrophil activation (NET formation), polyphosphates, and targeted thrombolytic strategies.<sup>2</sup>

### 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.<sup>201</sup> Often multiple active plaques are present in patients with ACS, which explains the propensity to further atherothrombotic events.<sup>202</sup> 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.<sup>180</sup>

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<sub>12</sub> 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<sub>12</sub> 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”<sup>203</sup>). Comparison of the PEGASUS-TIMI 54 platelet function sub-study and the STEEL PCI study demonstrates an extensive overlap between ADP-induced platelet aggregation with placebo and with clopidogrel, respectively.<sup>204,205</sup> The more effective and reliable P2Y<sub>12</sub> inhibition observed with ticagrelor explains its markedly greater efficacy in preventing stent thrombosis compared with clopidogrel.<sup>206,207</sup>

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.<sup>208</sup> 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.<sup>209</sup> 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.<sup>210</sup> 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.<sup>209,211</sup> 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.<sup>169,209</sup>

Impaired fibrinolysis is an independent predictor of poor outcome after ACS and represents a potential target for new therapies.<sup>212</sup> 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<sub>2A</sub> receptor antagonists. To tackle the inflammatory components, there is evidence from the PLATO study that clopidogrel attenuates systemic inflammation via an off-target effect<sup>213</sup> 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.<sup>214</sup> Low-dose aspirin does not have any detectable antiinflammatory effects and may even promote inflammation under some circumstances.<sup>215</sup>

### 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.<sup>216</sup>

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.<sup>217,218</sup> 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.<sup>219</sup> 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.<sup>220</sup> Therefore, for successful DPI, selection of the appropriate dose regimen of DOAC is essential.

The benefits of DPI were revealed in the COMPASS trial.<sup>169</sup> In that study, 27,395 patients with stable CAD or PAD were randomized to one of three treatment 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-1 $\beta$  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.<sup>180</sup> 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.<sup>221</sup> 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.<sup>222,223</sup> 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)<sup>224</sup>—and possibly mast cells,<sup>225</sup> other immune cell types that regulate clot inflammation and degradation have been identified (NK cells and T cells).<sup>226,227</sup>

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- $\gamma$ .<sup>228</sup> 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.<sup>229</sup> 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).<sup>230</sup>

While the frequency of thromboses increases with age,<sup>231</sup> 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.<sup>232</sup> However, a more recent large trial<sup>233</sup> 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.<sup>234</sup> A third trial in patients selected for ileo-femoral vein thrombosis only also failed to show a clear benefit of catheter-guided thrombolysis.<sup>235</sup> 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.<sup>236–238</sup> 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,<sup>239–241</sup> LMWH may however still have the advantage of potentially inhibiting P-selectin mediated inflammation, and has some clinical precedent in humans with PTS.<sup>242,243</sup> One inference is that the more consistent anticoagulation achieved with a DOAC may, in general, be slightly more effective than VKA, with its inher-

ent variability, in general.<sup>244,245</sup> Other potential targets for therapeutic interventions aimed at reducing PTS are II-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.<sup>246</sup> 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.<sup>247</sup> 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.<sup>248,249</sup> A proposed mechanism is that increased PAI-1 ultimately leads to reduced 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 II-6.<sup>250</sup> 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.<sup>251</sup>

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.<sup>252</sup> Ly6C<sup>Lo</sup> monocyte/macrophages may be important in pro-resolution activities and drive vein wall healing.<sup>253</sup> 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.<sup>254</sup>

#### **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.<sup>255</sup> One at least equally important outcome has been mostly overlooked: the post-PE syndrome.<sup>256</sup> 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.<sup>257,258</sup> 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.<sup>259</sup> In contrast, CTEPH may be cured after surgical removal of the chronic clots.<sup>259</sup> Notably, due to the nonspecific clinical presentation, the delay in diagnosis of CTEPH after PE is more than 1 year,<sup>260</sup> resulting in more advanced disease stage at diagnosis and higher mortality.<sup>261</sup> 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.<sup>262-264</sup>

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.<sup>157</sup>

#### **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 ligation 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,  $\gamma\delta$  T-cells and B-cells, have not yet been considered and should be investigated.<sup>219</sup>
- 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.

### Funding

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement number 766118." E.D., S.C.D., and B.S. are supported by a grant from the Netherlands Heart Foundation (CVON2014-09, RACE V, reappraisal of AF: interaction between hypercoagulability, electrical remodeling, and vascular destabilization in the progression of AF).

### Conflict of Interest

None declared.

### Acknowledgments

We are grateful to Mrs Lidewij Bos for substantial support throughout the conference and for Susanne Pauwels and her team for logistic support.

### References

- 1 Jackson SP, Darbousset R, Schoenwaelder SM. Thromboinflammation: challenges of therapeutically targeting coagulation and other host defense mechanisms. *Blood* 2019;133(09):906–918
- 2 Spronk HMH, Padro T, Siland JE, et al. Atherothrombosis and thromboembolism: position paper from the Second Maastricht Consensus Conference on Thrombosis. *Thromb Haemost* 2018; 118(02):229–250
- 3 Mozaffarian D, Wilson PW, Kannel WB. Beyond established and novel risk factors: lifestyle risk factors for cardiovascular disease. *Circulation* 2008;117(23):3031–3038
- 4 Lelieveld J, Münzel T. Air pollution, chronic smoking, and mortality. *Eur Heart J* 2019;40(38):3204
- 5 Mannucci PM, Harari S, Franchini M. Novel evidence for a greater burden of ambient air pollution on cardiovascular disease. *Haematologica* 2019;104(12):2349–2357
- 6 Finch J, Conklin DJ. Air pollution-induced vascular dysfunction: potential role of endothelin-1 (ET-1) system. *Cardiovasc Toxicol* 2016;16(03):260–275
- 7 Rudez G, Janssen NA, Kilinc E, et al. Effects of ambient air pollution on hemostasis and inflammation. *Environ Health Perspect* 2009;117(06):995–1001
- 8 Varela-Carver A, Parker H, Kleinert C, Rimoldi O. Adverse effects of cigarette smoke and induction of oxidative stress in cardiomyocytes and vascular endothelium. *Curr Pharm Des* 2010;16(23):2551–2558
- 9 Miller MR, Shaw CA, Langrish JP. From particles to patients: oxidative stress and the cardiovascular effects of air pollution. *Future Cardiol* 2012;8(04):577–602
- 10 Kilinç E, Van Oerle R, Borissoff JJ, et al. Factor XII activation is essential to sustain the procoagulant effects of particulate matter. *J Thromb Haemost* 2011;9(07):1359–1367
- 11 Shapiro NI, Aird WC. Sepsis and the broken endothelium. *Crit Care* 2011;15(02):135
- 12 Sanz E, Yang L, Su T, Morris DR, McKnight GS, Amieux PS. Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. *Proc Natl Acad Sci U S A* 2009;106(33):13939–13944
- 13 Everett LA, Cleuren AC, Khoriaty RN, Ginsburg D. Murine coagulation factor VIII is synthesized in endothelial cells. *Blood* 2014; 123(24):3697–3705
- 14 Cleuren ACA, van der Ent MA, Jiang H, et al. The in vivo endothelial cell transcriptome is highly heterogeneous across vascular beds. *Proc Natl Acad Sci U S A* 2019;116(47): 23618–23624
- 15 von Drygalski A, Furlan-Freguia C, Ruf W, Griffin JH, Mosnier LO. Organ-specific protection against lipopolysaccharide-induced vascular leak is dependent on the endothelial protein C receptor. *Arterioscler Thromb Vasc Biol* 2013;33(04):769–776
- 16 Ziedins K, Mann K. Molecular Basis of Blood Coagulation. 2018: 1885–1905.e8. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/3286010>. Accessed February 14, 2020
- 17 Fujiwara A, Taguchi O, Takagi T, et al. Role of thrombin-activatable fibrinolysis inhibitor in allergic bronchial asthma. *Lung* 2012;190(02):189–198
- 18 Morser J, Gabazza EC, Myles T, Leung LL. What has been learnt from the thrombin-activatable fibrinolysis inhibitor-deficient mouse? *J Thromb Haemost* 2010;8(05):868–876
- 19 Myles T, Nishimura T, Yun TH, et al. Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation. *J Biol Chem* 2003;278(51):51059–51067
- 20 Shao Z, Nishimura T, Leung LL, Morser J. Carboxypeptidase B2 deficiency reveals opposite effects of complement C3a and C5a in a murine polymicrobial sepsis model. *J Thromb Haemost* 2015; 13(06):1090–1102
- 21 Naito M, Taguchi O, Kobayashi T, et al. Thrombin-activatable fibrinolysis inhibitor protects against acute lung injury by inhibiting the complement system. *Am J Respir Cell Mol Biol* 2013;49(04):646–653
- 22 Nishimura T, Myles T, Piliponsky AM, Kao PN, Berry GJ, Leung LL. Thrombin-activatable procarboxypeptidase B regulates activated complement C5a in vivo. *Blood* 2007;109(05):1992–1997
- 23 Relja B, Lustenberger T, Puttkammer B, et al. Thrombin-activatable fibrinolysis inhibitor (TAFI) is enhanced in major trauma patients without infectious complications. *Immunobiology* 2013;218(04):470–476
- 24 Renckens R, Roelofs JJ, ter Horst SA, et al. Absence of thrombin-activatable fibrinolysis inhibitor protects against sepsis-induced liver injury in mice. *J Immunol* 2005;175(10): 6764–6771
- 25 Satoh T, Satoh K, Yaoita N, et al. Activated TAFI promotes the development of chronic thromboembolic pulmonary hypertension: a possible novel therapeutic target. *Circ Res* 2017;120(08): 1246–1262

- 26 Song JJ, Hwang I, Cho KH, et al; Consortium for the Longitudinal Evaluation of African Americans with Early Rheumatoid Arthritis (CLEAR) Registry. Plasma carboxypeptidase B downregulates inflammatory responses in autoimmune arthritis. *J Clin Invest* 2011;121(09):3517–3527
- 27 Ono N, Nakashima K, Rittling SR, et al. Osteopontin negatively regulates parathyroid hormone receptor signaling in osteoblasts. *J Biol Chem* 2008;283(28):19400–19409
- 28 Shao Z, Morser J, Leung LL. Thrombin cleavage of osteopontin disrupts a pro-chemotactic sequence for dendritic cells, which is compensated by the release of its pro-chemotactic C-terminal fragment. *J Biol Chem* 2014;289(39):27146–27158
- 29 Ge X, Yamaguchi Y, Zhao L, et al. Prochemerin cleavage by factor XIa links coagulation and inflammation. *Blood* 2018;131(03):353–364
- 30 Du XY, Zabel BA, Myles T, et al. Regulation of chemerin bioactivity by plasma carboxypeptidase N, carboxypeptidase B (activated thrombin-activable fibrinolysis inhibitor), and platelets. *J Biol Chem* 2009;284(02):751–758
- 31 Leung LLK, Morser J. Carboxypeptidase B2 and carboxypeptidase N in the crosstalk between coagulation, thrombosis, inflammation, and innate immunity. *J Thromb Haemost* 2018. Doi: 10.1111/jth.14199
- 32 Kremer Hovinga JA, Coppo P, Lämmle B, Moake JL, Miyata T, Vanhoorelbeke K. Thrombotic thrombocytopenic purpura. *Nat Rev Dis Primers* 2017;3:17020
- 33 Saha M, McDaniel JK, Zheng XL. Thrombotic thrombocytopenic purpura: pathogenesis, diagnosis and potential novel therapeutics. *J Thromb Haemost* 2017;15(10):1889–1900
- 34 Rock GA, Shumak KH, Buskard NA, et al; Canadian Apheresis Study Group. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. *N Engl J Med* 1991;325(06):393–397
- 35 Fuchs TA, Kremer Hovinga JA, Schatzberg D, Wagner DD, Lämmle B. Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. *Blood* 2012;120(06):1157–1164
- 36 Deford CC, Reese JA, Schwartz LH, et al. Multiple major morbidities and increased mortality during long-term follow-up after recovery from thrombotic thrombocytopenic purpura. *Blood* 2013;122(12):2023–2029
- 37 Falter T, Schmitt V, Herold S, et al. Depression and cognitive deficits as long-term consequences of thrombotic thrombocytopenic purpura. *Transfusion* 2017;57(05):1152–1162
- 38 Roose E, Schelpe AS, Joly BS, et al. An open conformation of ADAMTS-13 is a hallmark of acute acquired thrombotic thrombocytopenic purpura. *J Thromb Haemost* 2018;16(02):378–388
- 39 Masias C, Cataland SR. Novel therapies in thrombotic thrombocytopenic purpura. *Res Pract Thromb Haemost* 2017;2(01):19–26
- 40 Scully M, Cataland SR, Peyvandi F, et al. Caplacizumab treatment for acquired thrombotic thrombocytopenic purpura. *New Engl J Med* 2019;380(04):335–346
- 41 Coppo P, Froissart A; French Reference Center for Thrombotic Microangiopathies. Treatment of thrombotic thrombocytopenic purpura beyond therapeutic plasma exchange. *Hematology (Am Soc Hematol Educ Program)* 2015;2015:637–643
- 42 Scully M, Knöbl P, Kentouche K, et al. Recombinant ADAMTS-13: first-in-human pharmacokinetics and safety in congenital thrombotic thrombocytopenic purpura. *Blood* 2017;130(19):2055–2063
- 43 Alshehri OM, Hughes CE, Montague S, et al. Fibrin activates GPVI in human and mouse platelets. *Blood* 2015;126(13):1601–1608
- 44 Mammadova-Bach E, Ollivier V, Loyau S, et al. Platelet glycoprotein VI binds to polymerized fibrin and promotes thrombin generation. *Blood* 2015;126(05):683–691
- 45 Onselae MB, Hardy AT, Wilson C, et al. Fibrin and D-dimer bind to monomeric GPVI. *Blood Adv* 2017;1(19):1495–1504
- 46 Slater A, Perrella G, Onselae MB, et al. Does fibrin(ogen) bind to monomeric or dimeric GPVI, or not at all? *Platelets* 2019;30(03):281–289
- 47 Voors-Pette C, Lebozec K, Dogterom P, et al. Safety and tolerability, pharmacokinetics, and pharmacodynamics of ACT017, an antiplatelet GPVI (glycoprotein VI) Fab. *Arterioscler Thromb Vasc Biol* 2019;39(05):956–964
- 48 Bergmeier W, Stefanini L. Platelets at the vascular interface. *Res Pract Thromb Haemost* 2018;2(01):27–33
- 49 Stefanini L, Bergmeier W. RAP GTPases and platelet integrin signaling. *Platelets* 2019;30(01):41–47
- 50 Cook AA, Deng W, Ren J, Li R, Sondek J, Bergmeier W. Calcium-induced structural rearrangements release autoinhibition in the Rap-GEF CalDAG-GEFI. *J Biol Chem* 2018;293(22):8521–8529
- 51 Stefanini L, Paul DS, Robledo RF, et al. RASA3 is a critical inhibitor of RAP1-dependent platelet activation. *J Clin Invest* 2015;125(04):1419–1432
- 52 Su W, Wynne J, Pinheiro EM, et al. Rap1 and its effector RIAM are required for lymphocyte trafficking. *Blood* 2015;126(25):2695–2703
- 53 Stritt S, Wolf K, Lorenz V, et al. Rap1-GTP-interacting adaptor molecule (RIAM) is dispensable for platelet integrin activation and function in mice. *Blood* 2015;125(02):219–222
- 54 Lagarrigue F, Gingras AR, Paul DS, et al. Rap1 binding to the talin 1 F0 domain makes a minimal contribution to murine platelet GPIIb-IIIa activation. *Blood Adv* 2018;2(18):2358–2368
- 55 Gingras AR, Lagarrigue F, Cuevas MN, et al. Rap1 binding and a lipid-dependent helix in talin F1 domain promote integrin activation in tandem. *J Cell Biol* 2019;218(06):1799–1809
- 56 Bromberger T, Klapproth S, Rohwedder I, et al. Direct Rap1/Talin1 interaction regulates platelet and neutrophil integrin activity in mice. *Blood* 2018;132(26):2754–2762
- 57 Soehnlein O, Steffens S, Hidalgo A, Weber C. Neutrophils as protagonists and targets in chronic inflammation. *Nat Rev Immunol* 2017;17(04):248–261
- 58 Drechsler M, Megens RT, van Zandvoort M, Weber C, Soehnlein O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation* 2010;122(18):1837–1845
- 59 Döring Y, Drechsler M, Wantha S, et al. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ Res* 2012;110(08):1052–1056
- 60 Koenen RR, von Hundelshausen P, Nesmelova IV, et al. Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat Med* 2009;15(01):97–103
- 61 von Hundelshausen P, Agten SM, Eckardt V, et al. Chemokine interactome mapping enables tailored intervention in acute and chronic inflammation. *Sci Transl Med* 2017;9(384):eah6650
- 62 Alard JE, Ortega-Gomez A, Wichapong K, et al. Recruitment of classical monocytes can be inhibited by disturbing heteromers of neutrophil HNP1 and platelet CCL5. *Sci Transl Med* 2015;7(317):317ra196
- 63 Ortega-Gomez A, Salvermoser M, Rossaint J, et al. Cathepsin G controls arterial but not venular myeloid cell recruitment. *Circulation* 2016;134(16):1176–1188
- 64 Scheiermann C, Gibbs J, Ince L, Loudon A. Clocking in to immunity. *Nat Rev Immunol* 2018;18(07):423–437
- 65 Winter C, Silvestre-Roig C, Ortega-Gomez A, et al. Chronopharmacological targeting of the CCL2-CCR2 axis ameliorates atherosclerosis. *Cell Metab* 2018;28(01):175–182
- 66 Scheiermann C, Kunisaki Y, Lucas D, et al. Adrenergic nerves govern circadian leukocyte recruitment to tissues. *Immunity* 2012;37(02):290–301
- 67 He W, Holtkamp S, Hergenhan SM, et al. Circadian expression of migratory factors establishes lineage-specific signatures that guide the homing of leukocyte subsets to tissues. *Immunity* 2018;49(06):1175–1190.e7

- 68 Angkananard T, Anothaisintawee T, McEvoy M, Attia J, Thakkinian A. Neutrophil lymphocyte ratio and cardiovascular disease risk: a systematic review and meta-analysis. *BioMed Res Int* 2018;2018:2703518
- 69 Gaul DS, Stein S, Matter CM. Neutrophils in cardiovascular disease. *Eur Heart J* 2017;38(22):1702–1704
- 70 Megens RT, Vijayan S, Lievens D, et al. Presence of luminal neutrophil extracellular traps in atherosclerosis. *Thromb Haemost* 2012;107(03):597–598
- 71 Quillard T, Araújo HA, Franck G, Shvartz E, Sukhova G, Libby P. TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *Eur Heart J* 2015;36(22):1394–1404
- 72 Franck G, Mawson T, Sausen G, et al. Flow perturbation mediates neutrophil recruitment and potentiates endothelial injury via TLR2 in mice: implications for superficial erosion. *Circ Res* 2017;121(01):31–42
- 73 Franck G, Mawson TL, Folco EJ, et al. Roles of PAD4 and NETosis in experimental atherosclerosis and arterial injury: implications for superficial erosion. *Circ Res* 2018;123(01):33–42
- 74 Silvestre-Roig C, Braster Q, Wichapong K, et al. Externalized histone H4 orchestrates chronic inflammation by inducing lytic cell death. *Nature* 2019;569(7755):236–240
- 75 Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol* 2013;13(01):34–45
- 76 Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science* 2004;303(5663):1532–1535
- 77 Marsman G, Zeerleder S, Luken BM. Extracellular histones, cell-free DNA, or nucleosomes: differences in immunostimulation. *Cell Death Dis* 2016;7(12):e2518
- 78 Marsman G, von Richthofen H, Bulder I, et al. DNA and factor VII-activating protease protect against the cytotoxicity of histones. *Blood Adv* 2017;1(26):2491–2502
- 79 Xu J, Zhang X, Pelayo R, et al. Extracellular histones are major mediators of death in sepsis. *Nat Med* 2009;15(11):1318–1321
- 80 Chaaban H, Keshari RS, Silasi-Mansat R, et al. Inter- $\alpha$  inhibitor protein and its associated glycosaminoglycans protect against histone-induced injury. *Blood* 2015;125(14):2286–2296
- 81 Lee KH, Cavanaugh L, Leung H, et al. Quantification of NETs-associated markers by flow cytometry and serum assays in patients with thrombosis and sepsis. *Int J Lab Hematol* 2018;40(04):392–399
- 82 de Buhr N, von Köckritz-Blickwede M. Detection, visualization, and quantification of neutrophil extracellular traps (NETs) and NET markers. *Methods Mol Biol* 2020;2087:425–442
- 83 Yousefi S, Simon HU. NETosis - does it really represent nature's "suicide bomber"? *Front Immunol* 2016;7:328
- 84 Yipp BG, Petri B, Salina D, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med* 2012;18(09):1386–1393
- 85 Buchanan JT, Simpson AJ, Aziz RK, et al. DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr Biol* 2006;16(04):396–400
- 86 Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med* 2010;207(09):1853–1862
- 87 Martinod K, Demers M, Fuchs TA, et al. Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. *Proc Natl Acad Sci U S A* 2013;110(21):8674–8679
- 88 Martinod K, Fuchs TA, Zitomersky NL, et al. PAD4-deficiency does not affect bacteremia in polymicrobial sepsis and ameliorates endotoxemic shock. *Blood* 2015;125(12):1948–1956
- 89 Biron BM, Chung CS, O'Brien XM, Chen Y, Reichner JS, Ayala A. Cl-amidine prevents histone 3 citrullination and neutrophil extracellular trap formation, and improves survival in a murine sepsis model. *J Innate Immun* 2017;9(01):22–32
- 90 Liang Y, Pan B, Alam HB, et al. Inhibition of peptidylarginine deiminase alleviates LPS-induced pulmonary dysfunction and improves survival in a mouse model of lethal endotoxemia. *Eur J Pharmacol* 2018;833:432–440
- 91 Mackman N. The role of tissue factor and factor VIII in hemostasis. *Anesth Analg* 2009;108(05):1447–1452
- 92 Chen VM, Hogg PJ. Encryption and decryption of tissue factor. *J Thromb Haemost* 2013;11(Suppl 1):277–284
- 93 Ansari SA, Pendurthi UR, Rao LVM. Role of cell surface lipids and thiol-disulphide exchange pathways in regulating the encryption and decryption of tissue factor. *Thromb Haemost* 2019;119(06):860–870
- 94 Mackman N. Triggers, targets and treatments for thrombosis. *Nature* 2008;451(7181):914–918
- 95 van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev* 2012;64(03):676–705
- 96 Hisada Y, Alexander W, Kasthuri R, et al. Measurement of microparticle tissue factor activity in clinical samples: a summary of two tissue factor-dependent FXa generation assays. *Thromb Res* 2016;139:90–97
- 97 Key NS, Mackman N. Tissue factor and its measurement in whole blood, plasma, and microparticles. *Semin Thromb Hemost* 2010;36(08):865–875
- 98 Kuijpers MJ, van der Meijden PE, Feijge MA, et al. Factor XII regulates the pathological process of thrombus formation on ruptured plaques. *Arterioscler Thromb Vasc Biol* 2014;34(08):1674–1680
- 99 Wolberg AS, Rosendaal FR, Weitz JI, et al. Venous thrombosis. *Nat Rev Dis Primers* 2015;1:15006
- 100 Timp JF, Braekkan SK, Versteeg HH, Cannegieter SC. Epidemiology of cancer-associated venous thrombosis. *Blood* 2013;122(10):1712–1723
- 101 Khorana AA, Francis CW, Menzies KE, et al. Plasma tissue factor may be predictive of venous thromboembolism in pancreatic cancer. *J Thromb Haemost* 2008;6(11):1983–1985
- 102 Hisada Y, Ay C, Auriemma AC, Cooley BC, Mackman N. Human pancreatic tumors grown in mice release tissue factor-positive microvesicles that increase venous clot size. *J Thromb Haemost* 2017;15(11):2208–2217
- 103 Sacco RL, Diener HC, Yusuf S, et al; PROFESS Study Group. Aspirin and extended-release dipyridamole versus clopidogrel for recurrent stroke. *N Engl J Med* 2008;359(12):1238–1251
- 104 Johnston SC, Amarenco P, Albers GW, et al; SOCRATES Steering Committee and Investigators. Ticagrelor versus aspirin in acute stroke or transient ischemic attack. *N Engl J Med* 2016;375(01):35–43
- 105 Ornello R, Degan D, Tiseo C, et al. Distribution and temporal trends from 1993 to 2015 of ischemic stroke subtypes: a systematic review and meta-analysis. *Stroke* 2018;49(04):814–819
- 106 Diener HC, Bogousslavsky J, Brass LM, et al; MATCH investigators. Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomised, double-blind, placebo-controlled trial. *Lancet* 2004;364(9431):331–337
- 107 Benavente OR, Hart RG, McClure LA, Szychowski JM, Coffey CS, Pearce LA; SPS3 Investigators. Effects of clopidogrel added to aspirin in patients with recent lacunar stroke. *N Engl J Med* 2012;367(09):817–825
- 108 Bath PM, Woodhouse LJ, Appleton JP, et al; TARDIS Investigators. Antiplatelet therapy with aspirin, clopidogrel, and dipyridamole versus clopidogrel alone or aspirin and dipyridamole in patients with acute cerebral ischaemia (TARDIS): a randomised, open-label, phase 3 superiority trial. *Lancet* 2018;391(10123):850–859
- 109 Wang Y, Wang Y, Zhao X, et al; CHANCE Investigators. Clopidogrel with aspirin in acute minor stroke or transient ischemic attack. *N Engl J Med* 2013;369(01):11–19

- 110 Johnston SC, Easton JD, Farrant M, et al; Clinical Research Collaboration, Neurological Emergencies Treatment Trials Network, and the POINT Investigators. Clopidogrel and aspirin in acute ischemic stroke and high-risk TIA. *N Engl J Med* 2018;379(03):215–225
- 111 Donkel SJ, Benaddi B, Dippel DWJ, Ten Cate H, de Maat MPM. Prognostic hemostasis biomarkers in acute ischemic stroke. *Arterioscler Thromb Vasc Biol* 2019;39(03):360–372
- 112 A.L. Platelet function and thrombin generation in ischemic stroke – clinical correlates and prognostic importance' Dissertation 2018. Available at: [https://openarchive.ki.se/xmlui/bitstream/handle/10616/46302/Thesis\\_%20Annika\\_Lundstr%c3%b6m.pdf?sequence=3&isAllowed=y](https://openarchive.ki.se/xmlui/bitstream/handle/10616/46302/Thesis_%20Annika_Lundstr%c3%b6m.pdf?sequence=3&isAllowed=y). Accessed February 14, 2020
- 113 De Luca C, Virtuoso A, Maggio N, Papa M. Neuro-coagulopathy: blood coagulation factors in central nervous system diseases. *Int J Mol Sci* 2017;18(10):E2128
- 114 Aikawa E, Nahrendorf M, Figueiredo JL, et al. Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo. *Circulation* 2007;116(24):2841–2850
- 115 Wasilewski GB, Vervloet MG, Schurgers LJ. The bone-vasculature axis: calcium supplementation and the role of vitamin K. *Front Cardiovasc Med* 2019;6:6
- 116 Vajen T, Benedikter BJ, Heinzmann ACA, et al. Platelet extracellular vesicles induce a pro-inflammatory smooth muscle cell phenotype. *J Extracell Vesicles* 2017;6(01):1322454
- 117 Petsophonsakul P, Furmanik M, Forsythe R, et al. Role of vascular smooth muscle cell phenotypic switching and calcification in aortic aneurysm formation. *Arterioscler Thromb Vasc Biol* 2019;39(07):1351–1368
- 118 Kapustin AN, Schoppet M, Schurgers LJ, et al. prothrombin loading of vascular smooth muscle cell-derived exosomes regulates coagulation and calcification. *Arterioscler Thromb Vasc Biol* 2017;37(03):e22–e32
- 119 Brandenburg VM, Schurgers LJ, Kaesler N, et al. Prevention of vasculopathy by vitamin K supplementation: can we turn fiction into fact? *Atherosclerosis* 2015;240(01):10–16
- 120 Borissoff JJ, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* 2011;364(18):1746–1760
- 121 Posma JJ, Posthuma JJ, Spronk HM. Coagulation and non-coagulation effects of thrombin. *J Thromb Haemost* 2016;14(10):1908–1916
- 122 Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C: biased for translation. *Blood* 2015;125(19):2898–2907
- 123 Sinha RK, Wang Y, Zhao Z, et al. PAR1 biased signaling is required for activated protein C in vivo benefits in sepsis and stroke. *Blood* 2018;131(11):1163–1171
- 124 Borissoff JJ, Heeneman S, Kiliç E, et al. Early atherosclerosis exhibits an enhanced procoagulant state. *Circulation* 2010;122(08):821–830
- 125 Quillard T, Franck G, Mawson T, Folco E, Libby P. Mechanisms of erosion of atherosclerotic plaques. *Curr Opin Lipidol* 2017;28(05):434–441
- 126 Libby P, Pasterkamp G, Crea F, Jang IK. Reassessing the mechanisms of acute coronary syndromes. *Circ Res* 2019;124(01):150–160
- 127 Saia F, Komukai K, Capodanno D, et al; OCTAVIA Investigators. Eroded versus ruptured plaques at the culprit site of STEMI: in vivo pathophysiological features and response to primary PCI. *JACC Cardiovasc Imaging* 2015;8(05):566–575
- 128 Wang Z, Jia H, Tian J, et al. Computer-aided image analysis algorithm to enhance in vivo diagnosis of plaque erosion by intravascular optical coherence tomography. *Circ Cardiovasc Imaging* 2014;7(05):805–810
- 129 Marchesseau S, Seneviratna A, Sjöholm AT, et al. Hybrid PET/CT and PET/MRI imaging of vulnerable coronary plaque and myocardial scar tissue in acute myocardial infarction. *J Nucl Cardiol* 2018;25(06):2001–2011
- 130 Ten Cate H, Meade T. The Northwick Park Heart Study: evidence from the laboratory. *J Thromb Haemost* 2014;12(05):587–592
- 131 Lowe G, Rumley A. The relevance of coagulation in cardiovascular disease: what do the biomarkers tell us? *Thromb Haemost* 2014;112(05):860–867
- 132 Mackman N, Spronk HMH, Stouffer GA, Ten Cate H. Dual anticoagulant and antiplatelet therapy for coronary artery disease and peripheral artery disease patients. *Arterioscler Thromb Vasc Biol* 2018;38(04):726–732
- 133 Posthuma JJ, Posma JJN, van Oerle R, et al. Targeting coagulation factor Xa promotes regression of advanced atherosclerosis in apolipoprotein-e deficient mice. *Sci Rep* 2019;9(01):3909
- 134 Gurbel PA, Fox KAA, Tantry US, Ten Cate H, Weitz JI. Combination antiplatelet and oral anticoagulant therapy in patients with coronary and peripheral artery disease. *Circulation* 2019;139(18):2170–2185
- 135 Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C, protease activated receptor 1, and neuroprotection. *Blood* 2018;132(02):159–169
- 136 Lyden P, Pryor KE, Coffey CS, et al; NeuroNEXT Clinical Trials Network NN104 Investigators. Final results of the RHAPSODY trial: a multi-center, phase 2 trial using a continual reassessment method to determine the safety and tolerability of 3K3A-APC, a recombinant variant of human activated protein C, in combination with tissue plasminogen activator, mechanical thrombectomy or both in moderate to severe acute ischemic stroke. *Ann Neurol* 2019;85(01):125–136
- 137 Jennings RB. Historical perspective on the pathology of myocardial ischemia/reperfusion injury. *Circ Res* 2013;113(04):428–438
- 138 Wu MY, Yiang GT, Liao WT, et al. Current mechanistic concepts in ischemia and reperfusion injury. *Cell Physiol Biochem* 2018;46(04):1650–1667
- 139 Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *J Clin Invest* 2013;123(01):92–100
- 140 Russo I, Penna C, Musso T, et al. Platelets, diabetes and myocardial ischemia/reperfusion injury. *Cardiovasc Diabetol* 2017;16(01):71
- 141 Warner TD, Nylander S, Whatling C. Anti-platelet therapy: cyclooxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy. *Br J Clin Pharmacol* 2011;72(04):619–633
- 142 Ye Y, Long B, Qian J, Perez-Polo JR, Birnbaum Y. Dipyridamole with low-dose aspirin augments the infarct size-limiting effects of simvastatin. *Cardiovasc Drugs Ther* 2010;24(5-6):391–399
- 143 Birnbaum Y, Lin Y, Ye Y, et al. Aspirin before reperfusion blunts the infarct size limiting effect of atorvastatin. *Am J Physiol Heart Circ Physiol* 2007;292(06):H2891–H2897
- 144 Yang XM, Liu Y, Cui L, et al. Two classes of anti-platelet drugs reduce anatomical infarct size in monkey hearts. *Cardiovasc Drugs Ther* 2013;27(02):109–115
- 145 Wallentin L, Becker RC, Budaj A, et al; PLATO Investigators. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med* 2009;361(11):1045–1057
- 146 Ye Y, Perez-Polo JR, Birnbaum Y. Protecting against ischemia-reperfusion injury: antiplatelet drugs, statins, and their potential interactions. *Ann N Y Acad Sci* 2010;1207:76–82
- 147 Kingma JG. Inhibition of Na<sup>+</sup>/H<sup>+</sup> exchanger with EMD 87580 does not confer greater cardioprotection beyond preconditioning on ischemia-reperfusion injury in normal dogs. *J Cardiovasc Pharmacol Ther* 2018;23(03):254–269
- 148 Yang XM, Cui L, White J, et al. Mitochondrially targeted endonuclease III has a powerful anti-infarct effect in an in vivo rat model of myocardial ischemia/reperfusion. *Basic Res Cardiol* 2015;110(02):3

- 149 Sivaraman V, Yellon DM. Pharmacologic therapy that simulates conditioning for cardiac ischemic/reperfusion injury. *J Cardiovasc Pharmacol Ther* 2014;19(01):83–96
- 150 Ras M, Reitsma JB, Hoes AW, Six AJ, Poldervaart JM. Secondary analysis of frequency, circumstances and consequences of calculation errors of the HEART (history, ECG, age, risk factors and troponin) score at the emergency departments of nine hospitals in the Netherlands. *BMJ Open* 2017;7(10):e017259
- 151 Ma H, Campbell BCV, Parsons MW, et al; EXTEND Investigators. Thrombolysis guided by perfusion imaging up to 9 hours after onset of stroke. *N Engl J Med* 2019;380(19):1795–1803
- 152 Schellinger PD, Demaerschalk BM. Endovascular stroke therapy in the late time window. *Stroke* 2018;49(10):2559–2561
- 153 Ebinger M, Winter B, Wendt M, et al; STEMO Consortium. Effect of the use of ambulance-based thrombolysis on time to thrombolysis in acute ischemic stroke: a randomized clinical trial. *JAMA* 2014;311(16):1622–1631
- 154 Calderon VJ, Kasturiarachi BM, Lin E, Bansal V, Zaidat OO. Review of the mobile stroke unit experience worldwide. *Intervent Neurol* 2018;7(06):347–358
- 155 Ebinger M, Harmel P, Nolte CH, Grittner U, Siegerink B, Audebert HJ. Berlin prehospital or usual delivery of acute stroke care - study protocol. *Int J Stroke* 2017;12(06):653–658
- 156 Harmel P, Ebinger M, Freitag E, et al. Functional stroke outcomes after mobile stroke unit deployment – the revised protocol for the Berlin Prehospital Or Usual Delivery of acute stroke care (B\_PROUD) part 2 study. *Neurol Res Pract* 2019;1(01):18
- 157 Klok FA, Barco S, Siegerink B. Measuring functional limitations after venous thromboembolism: A call to action. *Thromb Res* 2019;178:59–62
- 158 Maino A, Rosendaal FR, Algra A, Peyvandi F, Siegerink B. Hypercoagulability is a stronger risk factor for ischaemic stroke than for myocardial infarction: a systematic review. *PLoS One* 2015;10(08):e0133523
- 159 Siegerink B, Maino A, Algra A, Rosendaal FR. Hypercoagulability and the risk of myocardial infarction and ischemic stroke in young women. *J Thromb Haemost* 2015;13(09):1568–1575
- 160 Al-Horani RA, Desai UR. Factor XIa inhibitors: a review of the patent literature. *Expert Opin Ther Pat* 2016;26(03):323–345
- 161 Ducroux C, Di Meglio L, Loyau S, et al. thrombus neutrophil extracellular traps content impair tpa-induced thrombolysis in acute ischemic stroke. *Stroke* 2018;49(03):754–757
- 162 Aboyans V, Ricco JB, Bartelink MEL, et al; ESC Scientific Document Group. 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with the European Society for Vascular Surgery (ESVS): Document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteries Endorsed by: the European Stroke Organization (ESO)The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS). *Eur Heart J* 2018;39(09):763–816
- 163 Espinola-Klein C, Rupprecht HJ, Blankenberg S, et al; AtheroGene Investigators. Impact of infectious burden on extent and long-term prognosis of atherosclerosis. *Circulation* 2002;105(01):15–21
- 164 CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet* 1996;348(9038):1329–1339
- 165 Hiatt WR, Fowkes FG, Heizer G, et al; EUCLID Trial Steering Committee and Investigators. Ticagrelor versus clopidogrel in symptomatic peripheral artery disease. *N Engl J Med* 2017;376(01):32–40
- 166 Fowkes FG, Price JF, Stewart MC, et al; Aspirin for Asymptomatic Atherosclerosis Trialists. Aspirin for prevention of cardiovascular events in a general population screened for a low ankle brachial index: a randomized controlled trial. *JAMA* 2010;303(09):841–848
- 167 Hess CN, Norgren L, Ansel GM, et al. A structured review of antithrombotic therapy in peripheral artery disease with a focus on revascularization: a TASC (InterSociety Consensus for the Management of Peripheral Artery Disease) initiative. *Circulation* 2017;135(25):2534–2555
- 168 Efficacy of oral anticoagulants compared with aspirin after infrainguinal bypass surgery (The Dutch Bypass Oral Anticoagulants or Aspirin Study): a randomised trial. *Lancet* 2000;355(9201):346–351
- 169 Eikelboom JW, Connolly SJ, Bosch J, et al; COMPASS Investigators. Rivaroxaban with or without aspirin in stable cardiovascular disease. *N Engl J Med* 2017;377(14):1319–1330
- 170 Steven S, Daiber A, Doppeide JF, Münzel T, Espinola-Klein C. Peripheral artery disease, redox signaling, oxidative stress - Basic and clinical aspects. *Redox Biol* 2017;12:787–797
- 171 Doppeide JF, Scheer M, Doppler C, et al. Change of walking distance in intermittent claudication: impact on inflammation, oxidative stress and mononuclear cells: a pilot study. *Clin Res Cardiol* 2015;104(09):751–763
- 172 Kaptoge S, Seshasai SRK, Gao P, et al. Inflammatory cytokines and risk of coronary heart disease: new prospective study and updated meta-analysis. *Eur Heart J* 2014;35(09):578–589
- 173 Hagström E, Held C, Stewart RA, et al; STABILITY Investigators. Growth differentiation factor 15 predicts all-cause morbidity and mortality in stable coronary heart disease. *Clin Chem* 2017;63(01):325–333
- 174 Held C, White HD, Stewart RAH, et al; STABILITY Investigators. Inflammatory biomarkers interleukin-6 and C-reactive protein and outcomes in stable coronary heart disease: experiences from the STABILITY (stabilization of atherosclerotic plaque by initiation of darapladib therapy) trial. *J Am Heart Assoc* 2017;6(10):e005077
- 175 Lindholm D, Lindbäck J, Armstrong PW, et al. Biomarker-based risk model to predict cardiovascular mortality in patients with stable coronary disease. *J Am Coll Cardiol* 2017;70(07):813–826
- 176 Gold L, Ayers D, Bertino J, et al. Aptamer-based multiplexed proteomic technology for biomarker discovery. *PLoS One* 2010;5(12):e15004
- 177 Assarsson E, Lundberg M, Holmquist G, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PLoS One* 2014;9(04):e95192
- 178 Ganz P, Heidecker B, Hveem K, et al. Development and validation of a protein-based risk score for cardiovascular outcomes among patients with stable coronary heart disease. *JAMA* 2016;315(23):2532–2541
- 179 McCarthy CP, van Kimmenade RRJ, Gaggin HK, et al. Usefulness of multiple biomarkers for predicting incident major adverse cardiac events in patients who underwent diagnostic coronary angiography (from the catheter sampled blood archive in cardiovascular diseases [CASABLANCA] STUDY). *Am J Cardiol* 2017;120(01):25–32
- 180 Ridker PM, Everett BM, Thuren T, et al; CANTOS Trial Group. antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med* 2017;377(12):1119–1131
- 181 Aday AW, Ridker PM. Antiinflammatory therapy in clinical care: the CANTOS Trial and beyond. *Front Cardiovasc Med* 2018;5:62
- 182 Ridker PM. Anticytokine agents: targeting interleukin signaling pathways for the treatment of atherothrombosis. *Circ Res* 2019;124(03):437–450
- 183 Tardif JC, Kouz S, Waters DD, et al. Efficacy and safety of low-dose colchicine after myocardial infarction. *N Engl J Med* 2019;381(26):2497–2505
- 184 Hijazi Z, Oldgren J, Siegbahn A, Wallentin L. Application of biomarkers for risk stratification in patients with atrial fibrillation. *Clin Chem* 2017;63(01):152–164



- 185 Hijazi Z, Aulin J, Andersson U, et al; ARISTOTLE Investigators. Biomarkers of inflammation and risk of cardiovascular events in anticoagulated patients with atrial fibrillation. *Heart* 2016;102(07):508–517
- 186 Hijazi Z, Lindbäck J, Alexander JH, et al; ARISTOTLE and STABILITY Investigators. The ABC (age, biomarkers, clinical history) stroke risk score: a biomarker-based risk score for predicting stroke in atrial fibrillation. *Eur Heart J* 2016;37(20):1582–1590
- 187 Hijazi Z, Oldgren J, Lindbäck J, et al; ARISTOTLE and RE-LY Investigators. The novel biomarker-based ABC (age, biomarkers, clinical history)-bleeding risk score for patients with atrial fibrillation: a derivation and validation study. *Lancet* 2016;387(10035):2302–2311
- 188 Berg DD, Ruff CT, Jarolim P, et al. Performance of the ABC scores for assessing the risk of stroke or systemic embolism and bleeding in patients with atrial fibrillation in ENGAGE AF-TIMI 48. *Circulation* 2019;139(06):760–771
- 189 Esteve-Pastor MA, Roldán V, Rivera-Caravaca JM, Ramírez-Macías I, Lip GYH, Marín F. The use of biomarkers in clinical management guidelines: a critical appraisal. *Thromb Haemost* 2019;119(12):1901–1919
- 190 Schwartz GG, Steg PG, Szarek M, et al; ODYSSEY OUTCOMES Committees and Investigators. Alirocumab and cardiovascular outcomes after acute coronary syndrome. *n engl j med* 2018;379(22):2097–2107
- 191 Zinman B, Wanner C, Lachin JM, et al; EMPA-REG OUTCOME Investigators. Empagliflozin, cardiovascular outcomes, and mortality in type 2 diabetes. *N Engl J Med* 2015;373(22):2117–2128
- 192 Bauer T, Bouman HJ, van Werkum JW, Ford NF, ten Berg JM, Taubert D. Impact of CYP2C19 variant genotypes on clinical efficacy of antiplatelet treatment with clopidogrel: systematic review and meta-analysis. *BMJ* 2011;343:d4588
- 193 Claassens DMF, Vos GJA, Bergmeijer TO, et al. A genotype-guided strategy for oral P2Y<sub>12</sub> inhibitors in primary PCI. *N Engl J Med* 2019;381(17):1621–1631
- 194 Makkar RR, Fontana G, Jilalawi H, et al. Possible subclinical leaflet thrombosis in bioprosthetic aortic valves. *N Engl J Med* 2015;373(21):2015–2024
- 195 Yanagisawa R, Fetterly KA, Johnson GB, et al. Integrated use of perfusion SPECT/CTA fusion imaging and pulmonary balloon angioplasty for chronic pulmonary thromboembolism. *JACC Cardiovasc Interv* 2017;10(05):532–534
- 196 Ruile P, Jander N, Blanke P, et al. Course of early subclinical leaflet thrombosis after transcatheter aortic valve implantation with or without oral anticoagulation. *Clin Res Cardiol* 2017;106(02):85–95
- 197 Chakravarty T, Søndergaard L, Friedman J, et al; RESOLVE; SAVORY Investigators. Subclinical leaflet thrombosis in surgical and transcatheter bioprosthetic aortic valves: an observational study. *Lancet* 2017;389(10087):2383–2392
- 198 Dangas GD, Tijssen JGP, Wöhrle J, et al; GALILEO Investigators. A controlled trial of rivaroxaban after transcatheter aortic-valve replacement. *N Engl J Med* 2020;382(02):120–129
- 199 Büller HR, Bethune C, Bhanot S, et al; FXI-ASO TKA Investigators. Factor XI antisense oligonucleotide for prevention of venous thrombosis. *N Engl J Med* 2015;372(03):232–240
- 200 Povsic TJ, Vavalle JP, Aberle LH, et al; RADAR Investigators. A Phase 2, randomized, partially blinded, active-controlled study assessing the efficacy and safety of variable anticoagulation reversal using the REG1 system in patients with acute coronary syndromes: results of the RADAR trial. *Eur Heart J* 2013;34(31):2481–2489
- 201 Peeters A, Mamun AA, Willekens F, Bonneux L. A cardiovascular life history. A life course analysis of the original Framingham Heart Study cohort. *Eur Heart J* 2002;23(06):458–466
- 202 Rioufol G, Finet G, Ginon I, et al. Multiple atherosclerotic plaque rupture in acute coronary syndrome: a three-vessel intravascular ultrasound study. *Circulation* 2002;106(07):804–808
- 203 Storey RF, Parker WA. Choices for potent platelet inhibition in patients with diabetes mellitus. *Circulation* 2016;134(11):793–796
- 204 Storey RF, Angiolillo DJ, Bonaca MP, et al. Platelet inhibition with ticagrelor 60 mg versus 90 mg twice daily in the PEGASUS-TIMI 54 trial. *J Am Coll Cardiol* 2016;67(10):1145–1154
- 205 Orme RC, Parker WAE, Thomas MR, et al. Study of two dose regimens of ticagrelor compared with clopidogrel in patients undergoing percutaneous coronary intervention for stable coronary artery disease (STEEL-PCI). *Circulation* 2018;CIRCULATIONAHA.118.034790
- 206 Steg PG, Harrington RA, Emanuelsson H, et al; PLATO Study Group. Stent thrombosis with ticagrelor versus clopidogrel in patients with acute coronary syndromes: an analysis from the prospective, randomized PLATO trial. *Circulation* 2013;128(10):1055–1065
- 207 Gosling R, Yazdani M, Parviz Y, et al. Comparison of P2Y<sub>12</sub> inhibitors for mortality and stent thrombosis in patients with acute coronary syndromes: Single center study of 10 793 consecutive 'real-world' patients. *Platelets* 2017;28(08):767–773
- 208 Bonaca MP, Bhatt DL, Cohen M, et al; PEGASUS-TIMI 54 Steering Committee and Investigators. Long-term use of ticagrelor in patients with prior myocardial infarction. *N Engl J Med* 2015;372(19):1791–1800
- 209 Sumaya W, Geisler T, Kristensen SD, Storey RF. Dual antiplatelet or dual antithrombotic therapy for secondary prevention in high-risk patients with stable coronary artery disease? *Thromb Haemost* 2019;19(10):1583–1589
- 210 Hagström E, James SK, Bertilsson M, et al; PLATO Investigators. Growth differentiation factor-15 level predicts major bleeding and cardiovascular events in patients with acute coronary syndromes: results from the PLATO study. *Eur Heart J* 2016;37(16):1325–1333
- 211 Bhatt DL, Bonaca MP, Bansilal S, et al. Reduction in ischemic events with ticagrelor in diabetic patients with prior myocardial infarction in PEGASUS-TIMI 54. *J Am Coll Cardiol* 2016;67(23):2732–2740
- 212 Sumaya W, Wallentin L, James SK, et al. Fibrin clot properties independently predict adverse clinical outcome following acute coronary syndrome: a PLATO substudy. *Eur Heart J* 2018;39(13):1078–1085
- 213 Storey RF, James SK, Siegbahn A, et al. Lower mortality following pulmonary adverse events and sepsis with ticagrelor compared to clopidogrel in the PLATO study. *Platelets* 2014;25(07):517–525
- 214 Thomas MR, Outteridge SN, Ajjan RA, et al. Platelet P2Y<sub>12</sub> inhibitors reduce systemic inflammation and its prothrombotic effects in an experimental human model. *Arterioscler Thromb Vasc Biol* 2015;35(12):2562–2570
- 215 Kiers D, van der Heijden WA, van Ede L, et al. A randomised trial on the effect of anti-platelet therapy on the systemic inflammatory response in human endotoxaemia. *Thromb Haemost* 2017;117(09):1798–1807
- 216 Weitz JI. Insights into the role of thrombin in the pathogenesis of recurrent ischaemia after acute coronary syndrome. *Thromb Haemost* 2014;112(05):924–931
- 217 Tello-Montoliu A, Tomasello SD, Ueno M, Angiolillo DJ. Antiplatelet therapy: thrombin receptor antagonists. *Br J Clin Pharmacol* 2011;72(04):658–671
- 218 Franchi F, Angiolillo DJ. Novel antiplatelet agents in acute coronary syndrome. *Nat Rev Cardiol* 2015;12(01):30–47
- 219 Mega JL, Braunwald E, Wiviott SD, et al; ATLAS ACS 2-TIMI 51 Investigators. Rivaroxaban in patients with a recent acute coronary syndrome. *N Engl J Med* 2012;366(01):9–19
- 220 Alexander JH, Lopes RD, James S, et al; APPRAISE-2 Investigators. Apixaban with antiplatelet therapy after acute coronary syndrome. *N Engl J Med* 2011;365(08):699–708

- 221 Ridker PM, Everett BM, Pradhan A, et al; CIRT Investigators. Low-dose methotrexate for the prevention of atherosclerotic events. *N Engl J Med* 2019;380(08):752–762
- 222 DeLoughery EP, Olson SR, Puy C, McCarty OJT, Shatzel JJ. The safety and efficacy of novel agents targeting factors XI and XII in early phase human trials. *Semin Thromb Hemost* 2019;45(05):502–508
- 223 Chan NC, Weitz JL. Antithrombotic agents. *Circ Res* 2019;124(03):426–436
- 224 von Brühl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med* 2012;209(04):819–835
- 225 Ponomaryov T, Payne H, Fabritz L, Wagner DD, Brill A. Mast cells granular contents are crucial for deep vein thrombosis in mice. *Circ Res* 2017;121(08):941–950
- 226 Bertin FR, Rys RN, Mathieu C, Laurance S, Lemarié CA, Blostein MD. Natural killer cells induce neutrophil extracellular trap formation in venous thrombosis. *J Thromb Haemost* 2019;17(02):403–414
- 227 Penn MS, Igwe C. Role of inflammation in modulating thrombotic-fibrinolytic balance in venous thrombosis. *Circ Res* 2016;119(12):1256–1257
- 228 Luther N, Shahneh F, Brähler M, et al. Innate effector-memory T-cell activation regulates post-thrombotic vein wall inflammation and thrombus resolution. *Circ Res* 2016;119(12):1286–1295
- 229 Heestermans M, Salloum-Asfar S, Streef T, et al. Mouse venous thrombosis upon silencing of anticoagulants depends on tissue factor and platelets, not FXII or neutrophils. *Blood* 2019;133(19):2090–2099
- 230 Simon MM, Greenaway S, White JK, et al. A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. *Genome Biol* 2013;14(07):R82
- 231 Wilkerson WR, Sane DC. Aging and thrombosis. *Semin Thromb Hemost* 2002;28(06):555–568
- 232 Enden T, Haig Y, Kløw NE, et al; CaVenT Study Group. Long-term outcome after additional catheter-directed thrombolysis versus standard treatment for acute iliofemoral deep vein thrombosis (the CaVenT study): a randomised controlled trial. *Lancet* 2012;379(9810):31–38
- 233 Vedantham S, Goldhaber SZ, Julian JA, et al; ATTRACT Trial Investigators. Pharmacomechanical catheter-directed thrombolysis for deep-vein thrombosis. *N Engl J Med* 2017;377(23):2240–2252
- 234 Comerota AJ, Kearon C, Gu CS, et al; ATTRACT Trial Investigators. Endovascular thrombus removal for acute iliofemoral deep vein thrombosis. *Circulation* 2019;139(09):1162–1173
- 235 Notten P, Ten Cate-Hoek AJ, Arnoldussen CWKP, et al. Ultrasound-accelerated catheter-directed thrombolysis versus anticoagulation for the prevention of post-thrombotic syndrome (CAVA): a single-blind, multicentre, randomised trial. *Lancet Haematol* 2020;7(01):e40–e49
- 236 Brill A, Fuchs TA, Chauhan AK, et al. von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood* 2011;117(04):1400–1407
- 237 Simes J, Becattini C, Agnelli G, et al; INSPIRE Study Investigators (International Collaboration of Aspirin Trials for Recurrent Venous Thromboembolism). Aspirin for the prevention of recurrent venous thromboembolism: the INSPIRE collaboration. *Circulation* 2014;130(13):1062–1071
- 238 Becattini C, Agnelli G, Schenone A, et al; WARFASA Investigators. Aspirin for preventing the recurrence of venous thromboembolism. *N Engl J Med* 2012;366(21):1959–1967
- 239 Cheung YW, Middeldorp S, Prins MH, et al; Einstein PTS Investigators Group. Post-thrombotic syndrome in patients treated with rivaroxaban or enoxaparin/vitamin K antagonists for acute deep-vein thrombosis. A post-hoc analysis. *Thromb Haemost* 2016;116(04):733–738
- 240 Prandoni P, Ageno W, Mumoli N, et al. Recanalization rate in patients with proximal vein thrombosis treated with the direct oral anticoagulants. *Thromb Res* 2017;153:97–100
- 241 Wik HS, Eenden TR, Ghanima W, Engeseth M, Kahn SR, Sandset PM. Diagnostic scales for the post-thrombotic syndrome. *Thromb Res* 2018;164:110–115
- 242 Myers DD Jr, Henke PK, Bedard PW, et al. Treatment with an oral small molecule inhibitor of P selectin (PSI-697) decreases vein wall injury in a rat stenosis model of venous thrombosis. *J Vasc Surg* 2006;44(03):625–632
- 243 Hull RD, Pineo GF, Brant R, et al; LITE Trial Investigators. Home therapy of venous thrombosis with long-term LMWH versus usual care: patient satisfaction and post-thrombotic syndrome. *Am J Med* 2009;122(08):762–769.e3
- 244 Chitsike RS, Rodger MA, Kovacs MJ, et al. Risk of post-thrombotic syndrome after subtherapeutic warfarin anticoagulation for a first unprovoked deep vein thrombosis: results from the REVERSE study. *J Thromb Haemost* 2012;10(10):2039–2044
- 245 Ziegler S, Schillinger M, Maca TH, Minar E. Post-thrombotic syndrome after primary event of deep venous thrombosis 10 to 20 years ago. *Thromb Res* 2001;101(02):23–33
- 246 Diaz JA, Wroblewski SK, Alvarado CM, et al. P-selectin inhibition therapeutically promotes thrombus resolution and prevents vein wall fibrosis better than enoxaparin and an inhibitor to von Willebrand factor. *Arterioscler Thromb Vasc Biol* 2015;35(04):829–837
- 247 Obi AT, Diaz JA, Ballard-Lipka NL, et al. Plasminogen activator-1 overexpression decreases experimental postthrombotic vein wall fibrosis by a non-vitronectin-dependent mechanism. *J Thromb Haemost* 2014;12(08):1353–1363
- 248 Ripplinger CM, Kessinger CW, Li C, et al. Inflammation modulates murine venous thrombosis resolution in vivo: assessment by multimodal fluorescence molecular imaging. *Arterioscler Thromb Vasc Biol* 2012;32(11):2616–2624
- 249 Deatrick KB, Luke CE, Elfline MA, et al. The effect of matrix metalloproteinase 2 and matrix metalloproteinase 2/9 deletion in experimental post-thrombotic vein wall remodeling. *J Vasc Surg* 2013;58(05):1375–1384.e2
- 250 Metz AK, Diaz JA, Obi AT, Wakefield TW, Myers DD, Henke PK. Venous thrombosis and post-thrombotic syndrome: from novel biomarkers to biology. *Methodist DeBakey Cardiovasc J* 2018;14(03):173–181
- 251 Andraska EA, Luke CE, Elfline MA, et al. Pre-clinical model to study recurrent venous thrombosis in the inferior vena cava. *Thromb Haemost* 2018;118(06):1048–1057
- 252 Mukhopadhyay S, Johnson TA, Duru N, et al. Fibrinolysis and inflammation in venous thrombus resolution. *Front Immunol* 2019;10:1348
- 253 Kimball AS, Obi AT, Luke CE, et al. Ly6C<sup>Lo</sup> monocyte/macrophages are essential for thrombus resolution in a murine model of venous thrombosis. *Thromb Haemost* 2019
- 254 Appelen D, van Loo E, Prins MH, Neumann MH, Kolbach DN. Compression therapy for prevention of post-thrombotic syndrome. *Cochrane Database Syst Rev* 2017;9:CD004174
- 255 Klok FA, Zondag W, van Kralingen KW, et al. Patient outcomes after acute pulmonary embolism. A pooled survival analysis of different adverse events. *Am J Respir Crit Care Med* 2010;181(05):501–506
- 256 Klok FA, van der Hulle T, den Exter PL, Lankeit M, Huisman MV, Konstantinides S. The post-PE syndrome: a new concept for chronic complications of pulmonary embolism. *Blood Rev* 2014;28(06):221–226
- 257 Sista AK, Miller LE, Kahn SR, Kline JA. Persistent right ventricular dysfunction, functional capacity limitation, exercise intolerance, and quality of life impairment following pulmonary embolism: Systematic review with meta-analysis. *Vasc Med* 2017;22(01):37–43

- 258 Sista AK, Klok FA. Late outcomes of pulmonary embolism: the post-PE syndrome. *Thromb Res* 2018;164:157–162
- 259 Delcroix M, Lang I, Pepke-Zaba J, et al. Long-term outcome of patients with chronic thromboembolic pulmonary hypertension: results from an international prospective registry. *Circulation* 2016;133(09):859–871
- 260 Pepke-Zaba J, Delcroix M, Lang I, et al. Chronic thromboembolic pulmonary hypertension (CTEPH): results from an international prospective registry. *Circulation* 2011;124(18):1973–1981
- 261 Klok FA, Barco S, Konstantinides SV, et al. Determinants of diagnostic delay in chronic thromboembolic pulmonary hypertension: results from the European CTEPH Registry. *Eur Respir J* 2018;52(06):1801687
- 262 Ende-Verhaar YM, Meijboom LJ, Kroft LJM, et al. Usefulness of standard computed tomography pulmonary angiography performed for acute pulmonary embolism for identification of chronic thromboembolic pulmonary hypertension: results of the InShape III study. *J Heart Lung Transplant* 2019;38(07):731–738
- 263 Ende-Verhaar YM, van den Hout WB, Bogaard HJ, et al. Healthcare utilization in chronic thromboembolic pulmonary hypertension after acute pulmonary embolism. *J Thromb Haemost* 2018;16(11):2168–2174
- 264 Klok FA, Delcroix M, Bogaard HJ. Chronic thromboembolic pulmonary hypertension from the perspective of patients with pulmonary embolism. *J Thromb Haemost* 2018;16(06):1040–1051