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Atherothrombosis and Thromboembolism; position paper from the 2nd Maastricht Consensus Conference on Thrombosis

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Summary

Atherothrombosis is a leading cause of cardiovascular mortality and long term morbidity. Platelets and coagulation proteases, interacting with circulating cells and in different vascular beds, modify several complex pathologies including atherosclerosis. In the 2nd Maastricht Consensus Conference on Thrombosis this theme was addressed by diverse scientists from bench to bedside. All presentations were discussed with audience members and the results of these discussions were incorporated in the final document that presents a state-of-the-art reflection of expert opinions and consensus recommendations regarding the following five topics:

- *Risk factors, biomarkers and plaque instability:* in atherothrombosis research more focus on the contribution of specific risk factors like ectopic fat needs to be considered; definitions of atherothrombosis are important distinguishing different phases of disease, including plaque (in)stability; proteomic and metabolomics data are to be added to genetic information.
- *Circulating cells including platelets and atherothrombosis:* Mechanisms of leukocyte and macrophage plasticity, migration and transformation in murine atherosclerosis need to be considered; disease mechanism-based biomarkers need to be identified; experimental systems are needed that incorporate whole blood flow to understand how red blood cells (RBCs) influence thrombus formation and stability; knowledge on platelet heterogeneity and priming conditions needs to be translated towards the *in vivo* situation.
- *Coagulation proteases, fibrin(ogen) and thrombus formation:* The role of factor (F) XI in thrombosis including the lower margins of this factor related to safe and effective antithrombotic therapy need to be established; FXI is a key regulator in linking platelets, thrombin generation and inflammatory mechanisms in a renin-angiotensin dependent manner; however, the impact on thrombin dependent PAR signaling needs further study; the fundamental mechanisms in FXIII biology and biochemistry and its impact on thrombus biophysical characteristics need to be explored; the interactions of red cells and fibrin formation and its consequences for thrombus formation and lysis need to be addressed. Platelet fibrin interactions are pivotal determinants of clot formation and stability with potential therapeutic consequences
- *Preventive and acute treatment of atherothrombosis and arterial embolism; novel ways and tailoring?* The role of protease activated receptor (PAR)-4 vis à vis PAR-1 as target for antithrombotic therapy merits study; ongoing trials on platelet function test-based antiplatelet therapy adjustment, supports development of practically feasible tests; risk

scores for patients with atrial fibrillation need refinement, taking new biomarkers including for coagulation into account; risk scores that consider organ system differences in bleeding may have added value; all forms of oral anticoagulant treatment require better organization, including education and emergency access; laboratory testing still needs rapidly available sensitive tests with short turnaround time.

- *Pleiotropy of coagulation proteases, thrombus resolution and ischemia-reperfusion:* Biobanks specifically for thrombus storage and analysis are needed; further studies on novel modified activated protein C based agents are required including its cytoprotective properties; new avenues for optimizing treatment of patients with ischemic stroke are needed, also including novel agents that modify fibrinolytic activity (aimed at plasminogen activator inhibitor-1 (PAI-1) and thrombin activatable fibrinolysis inhibitor (TAFI).

Key words

Atherothrombosis, arterial thrombosis, coagulation, platelets, antiplatelet agents, anticoagulants, atherosclerosis, ischemic stroke, atrial fibrillation, myocardial infarction, endothelium, monocytes.

Introduction

During the second Maastricht Consensus Conference on Thrombosis (MCCT), atherothrombosis and cardiovascular disease (CVD) were tackled from different angles by basic and clinical scientists, younger as well as seasoned experts from an international background. Concise presentations on subtopics within a larger theme (www.mcct.eu), were held, followed by discussion sessions (presenters, students, other attendants) to outline targets for future research. Based on a plenary synthesis of all sessions, a final consensus document summarizing all topics and recommendations (Figure 1) was drafted based on the notes from all contributing authors and condensed into this position paper. This document does not intend to provide a comprehensive overview of the field, primarily it reflects the personal interests and opinions of the experts and input from the audience.

1. Risk factors, biomarkers and plaque instability.

Atherothrombosis occurs in the course of atherosclerosis, a condition driven by chronic inflammation (1,2). Current concepts on “thomboinflammation” (3) also fit in the context of atherothrombosis driven by the interplay between inflammation and coagulation as a critical mechanism (4,5). Obesity is one of the main drivers of chronic inflammation in atherogenesis.

Obesity, ectopic fat and thromboinflammation

Obesity is a worldwide health problem with increasing prevalence (6). Although obesity is heterogeneous, approximately 30% of obese individuals remain healthy into old age (7). Ectopic fat accumulation rather than overall obesity is responsible for increased cardiometabolic risk and is a better predictor for CVD, including atrial fibrillation and heart failure (8-10). Ectopic fat refers to fat that accumulates in or around specific organs or compartments and include abdominal viscera (visceral fat), liver (intrahepatic fat), heart (pericardial fat), and blood vessels (perivascular fat). Recent findings support an independent contribution of ectopic fat deposition to cardiovascular risk (11).

Strengthening research on the contribution of ectopic fat depots to cardiometabolic risk and risk of CVD is necessary. Adipose tissue is a dynamic endocrine organ consisting of adipocytes and non-adipocytes including immune cells. Excess ectopic fat may exert harmful effects by direct lipotoxicity, or by a chronic pro-inflammatory effect, at both the local and systemic level. The impact of ectopic fat on CVD might occur at different stages of disease progression. The growth of fat mass leads to changes in the adipocyte cell size and number as well as in the pattern of immune cells with an

exacerbated presence of macrophages and lymphocytes, with a pro-inflammatory phenotype. This leads to a deregulated expression of bioactive molecules including cytokines and adipokines, which contribute to inflammation, endothelium dysfunction, progression of atherogenesis and a prothrombotic state, all increasing cardiovascular risk (12,13). Further research should unravel differences between fat depots in cell composition, inflammatory response and cell-signalling pathways and ultimately how this translates into atherosclerosis.

Traditionally, obesity is determined through the Body Mass Index (BMI), but clearly, the pattern of fat distribution is more important than the absolute amount of stored fat; it is conceivable that ectopic fat becomes the diagnostic and therapeutic target in cardiometabolic disorders shifting the focus from quantity (obesity) to quality (dysfunctional fat) (14). Current methodologies for tracking ectopic fat distribution are limited to expensive imaging techniques (e.g. computed tomography, magnetic resonance imaging) (15).

Genetics and Cardiovascular Disease

For the advancement of prevention in cardiovascular disease, improving the accuracy of genetic risk stratification will be of major importance. Single nucleotide polymorphisms increasing the risk of cardiovascular disease via interference with inflammation, lipids, blood pressure, NO/cGMP signalling, vascular remodelling, and plaque formation have been detected (16). Several research groups have addressed the implementation of these genetic variants into genetic risk models for arterial thrombosis (17,18). Nevertheless, current guidelines only recommend standardized assessment of family history of premature cardiovascular disease and discourage the generalized use of DNA-based test for risk assessment (19). Since current treatment concepts predominantly focus on the therapy of disease phenotypes and our understanding of organ-specific gene translation is still limited, more efforts are needed to unravel gene-gene and gene-environment interactions in a systems-medicine oriented approach.

In order to improve current knowledge about the translation of genotype into phenotype, integrative data analysis and deep clinical phenotyping for subclinical disease will be required. Therefore, epigenetic, proteomic and metabolomic information should be analysed in conjunction with genomic data to improve the understanding of the pathophysiology of cardiovascular disease and to advance the prevention of cardiovascular disease. Considering the required sample size and the expertise in 'omics-technology' and bioinformatics needed to study the complex interplay of genetic variation and its functional role, combined efforts (e.g. in consortia) will be needed. These efforts should be accompanied by an interdisciplinary discussion on ethical aspects since

advancements in the field of genetics will entail novel ethical demands for regulatory authorities, health care providers and patients.

Phenotyping the atherothrombotic lesion

Tissue composition of plaques rather than the degree of luminal stenosis determines the vulnerability of an atherosclerotic plaque to develop disruption followed by thrombosis (20). A thin fibrous cap, large lipid-rich necrotic core, low amount of collagen and high inflammatory activity are major determinants of plaque vulnerability (2,21). Tissue characteristics of high risk plaques can now be visualized with the use of invasive vascular imaging techniques, which has created considerable progress in defining the composition of plaques in patients. Intravascular Ultrasound (IVUS) with virtual histology analysis (IVUS-VH) provides a cross-section view of the entire vessel wall and allows to detect the thickness of a fibrous cap and size of a lipid core (22). Optical Coherence Tomography (OCT) provides higher resolution imaging but limited depth penetrance, with more detailed information on the near lumen morphology such as fibrous cap structure, superficial macrophage accumulations or plaque rupture (23). However, imaging of the activity and specific location of inflammatory cells and their secretory products in plaques is less successful (24). Some progress has been made in experimental settings. For example, a critical step in the destabilization of plaques is the secretion of matrix-degrading metalloproteinases (MMPs) by inflammatory cells (25). With the use of SPECT/micro CT imaging with radiolabelled metalloproteinase inhibitors, MMP activity could be detected in animals (rabbits) in a non invasive manner (26). And in humans, application of Near Infrared Autofluorescence imaging (NIRAF) appeared successful to detect fluorescence of naturally occurring molecules in necrotic cores of high risk plaques, which provides some information about the biological activity inside a lesion (27). Such non-invasive imaging techniques describing plaque phenotype over time could elucidate the relation between inflammation, plaque vulnerability, and clinical events. Still, up to now it remains a challenge to predict plaque rupture in individual patients (28). Therefore, there is a call for new definitions and risk assessment strategies regarding atherothrombosis. Besides morphology of plaques, vulnerability of blood (e.g. hypercoagulability and inflammation) is a contributor to atherothrombosis (28).

There is a knowledge gap with respect to biomarkers of ongoing plaque destabilization. Pathologic analysis of thrombectomy specimens retrieved from acute myocardial infarction (AMI) patients has revealed huge variations in the propagation of thrombosis after onset of plaque rupture.

Stratification of so-called 'fresh' (<1 day) and 'old' (>5 days) thrombi should also be considered (29). Old thrombus is an independent predictor of mortality in AMI patients, which probably relates to their fragile structure and inherent risk of embolization (30). For example, MMP contents of such

thrombi is much higher than in the underlying plaques. Therefore, also thrombus instability appears a matter of concern and the cellular pathways leading to thrombus healing (stabilization) or otherwise embolization or progression to full luminal occlusion need to be further elucidated. Combining morphological characteristics with biomarkers of vulnerable blood, plaque vulnerability and thrombus instability should result in a pan-arterial approach in which atherothrombotic risk establishment becomes more accurate.

Utility of biomarkers

Meta-analyses of prospective studies have established positive associations of circulating levels of fibrinogen, von Willebrand factor (VWF), fibrin D-dimer, and tissue plasminogen activator with risk of coronary heart disease (CHD) and stroke. These data support the concept of coagulation activity as an important contributing mechanism in the processes of atherosclerosis as well as –thrombosis. The biomarker data are not yet comprehensive and in particular further studies are required to establish the associations of other coagulation proteins, including those from the contact system, with risk of CHD and CVD (31). Given their importance as potential therapeutic targets in CVD, additional biomarkers for vWF, factors VIII, IX, XI and XII merit further research (32).

D-dimer level is associated with risk of stroke in patients with atrial fibrillation (33), irrespective of anticoagulant drugs (34). Prospective studies are required to establish its potential role in risk stratification, its relationship to other coagulation activation markers (e.g. prothrombin F1+2) and other biomarkers (e.g. CRP, Troponin and pro-NT-BNP) (35). These may inform future management into two directions. One route concerns the optimized selection of patients that benefit most from anticoagulant treatment (eg the discussion in theme 4 on thye need for improved selection of patients with atrial fibrillation (AF)). A second direction would involve, if possible, dose titration in high risk subjects. An indication for its potential comes from studies showing that d-dimer level while on anticoagulation predict ischemic stroke risk in patients with AF (36).

Recommendations from theme 1:

- Future research should focus on identifying biomarkers of ectopic body fat content and distribution and on developing clinical algorithms including ectopic fat assessment for cardiovascular risk stratification.
- Genetic risk stratification, including family history, gene-gene and gene-environment interactions as well as comorbidity need to be explored; integrative data analysis and deep phenotyping are crucial. Proteomic and metabolomic data need to be implemented with genomic data in multi-center trials.

- Develop new definitions and risk assessment strategies for atherothrombosis, potentially combining morphological characteristics with biomarkers of “vulnerable blood” as well as for plaque destabilization to optimize atherothrombotic risk stratification.

2. Circulating cells including platelets and atherothrombosis

New insights in the diversity and functionality of monocytes (macrophages), leukocytes and platelets were discussed. The importance of microvesicles was recently discussed in this journal (37).

Leukocytes in atherothrombosis

The importance of leukocytes in the pathophysiology of atherosclerosis has been well recognized. T lymphocytes and monocytes/macrophages are abundantly present in atherosclerotic lesions and strategies that modulate the abundance of these cells show strong effects on the extent of atherosclerosis, at least in mouse models. Also B cells, divided in two main families, provide important protective effects against atherosclerosis (B1), while B2 cells are thought to be atherogenic (38,39). Mouse models have also revealed roles of polymorphonuclear cells, most notably neutrophils and mast cells, in the pathophysiology of atherosclerosis (40,41). Lineage tracing and specific labelling of leukocyte subtypes *in vivo*, along with innovative strategies to deplete certain leukocyte subsets in mice have led to the identification of important contributions of almost any leukocyte subtype tested (39,42-44). However, there is still no viable therapeutic or preventive strategy that directly targets leukocytes.

The puzzle is more complex than initially assumed. First, the cell content of the plaque is quite dynamic. Whereas influx of leukocytes is still believed to be crucial in plaque development, emigration of immune cells from plaques, e.g. to nearby lymphoid organs, also plays a role (45). Interestingly, mouse studies revealed that local proliferation outweighs infiltration and is the major determinant of macrophage numbers in the plaque (46). Smooth muscle cells (SMC) that are highly abundant in plaques, migrate from the media to the intima, where they proliferate. SMC can also turn into macrophage-like cells with phagocytic activity and the excessive ingestion of lipoproteins cause them to degenerate into foam cells, much like classical macrophages (47,48). In general, mouse models of atherosclerosis will remain to have utility but their importance in basic pathophysiologic research might decline, as the emergence of the "omics" class of technology, e.g. genome-wide association studies, proteomics and RNA sequencing, will gradually take over their roles in human cohorts. This might even lead to the identification of drug principles targeting leukocyte dynamics, once its role in plaque development is fully understood.

Macrophages and signalling effects

Monocytes and neutrophils contribute to venous thrombosis (49). Recent studies show that myeloid cell tissue factor (TF) activation in thrombosis is linked to complement activation (50). Remarkably, complement factor (C) 3 plays a pivotal role in platelet activation independent of full complement activation downstream of C5. C5 nevertheless is crucial for outer membrane phosphatidylserine exposure and activating TF prothrombotic activity, as previously also demonstrated for complement fixing antibodies causing thrombotic complications in stem cell therapy (51). Protein disulfide isomerase pathways are necessary for affinity maturation of TF and phosphatidylserine exposure but is not sufficient to render microvesicle (MV) associated TF fully prothrombotic (52). Complement-dependent release of MV from monocytes in LPS-stimulated whole blood produces TF that due to its high affinity is already in complex with its ligand FVIIa.

Venous thrombosis and atherothrombosis are likely driven by distinct but partially overlapping pathophysiological interactions of blood and vessel wall cells (53). Atherothrombosis is closely tied to inflammation and interleukin 1 β (IL1 β) is one of the inflammatory cytokines implicated as a risk factor for arterial occlusive events. Indeed, the recently published CANTOS trial provides proof of concept evidence in showing clinical efficacy of the administration of an anti-interleukin-1 β antibody in reducing cardiovascular events (54). Macrophages in atherosclerotic lesions are rich sources of TF, but macrophage-expressed TF is also largely in a non-coagulant state. Cell injury signals, specifically ATP triggering the prothrombotic P2X7 receptor (55), induces a thioredoxin-reductase-dependent inflammasome and caspase-1 activation in primed macrophages. Active caspase-1 simultaneously generates active IL1 β and is responsible for the terminal release step of highly prothrombotic MV carrying active TF (56). These data provide evidence for a direct mechanistic coupling of thrombo-inflammatory pathways. It will be of interest to further study whether this close coupling of inflammation and coagulation is common to other activators of the inflammasome. Since macrophages in atherosclerotic lesions express the P2X7 receptor (57), a possible role of this receptor in lesion thrombogenicity should be investigated.

Coagulation also directly influences atherosclerotic lesion progression through effects on leukocytes (58). It is notable that expression of coagulation factors by macrophages is not restricted to TF. Lung and peritoneal macrophages express FVII in mouse and man (59,60) and tumor macrophages produce FVII and FX (61). It is an intriguing possibility that macrophage phenotypes in atherosclerosis are directly regulated by signaling through protease activated receptors (PARs). PAR2 in particular has been implicated in the regulation of macrophage activation (62) and chronic inflammation in obesity (63). It will be of interest to determine whether coagulation factors synthesized by macrophages have effects on lesions progression, stabilize plaque structure or

contribute to lesion thrombogenicity. While coagulation inhibitors may broadly interfere with thrombosis-related pathways, experimental studies can more precisely define the contributions of intravascular and vessel wall compartments to lesions progression. Translating experimental findings into the clinic will require the development of new diagnostic, biomarker and imaging approaches based on these mechanistic studies in thrombo-inflammation.

Red blood cells

In healthy humans, red blood cells (RBCs) occupy ~35-45% of the blood volume and elevated hematocrit has been associated with increased risk of both arterial and venous thrombosis (reviewed in (64)). RBCs have biophysical and biochemical properties that contribute to thrombus formation. For example, RBCs are major determinants of blood viscosity, and increased viscosity has been associated with risk of cardiovascular events (65). In vitro studies suggest RBCs promote platelet interactions with the vessel wall (66,67), expose phosphatidylserine, support thrombin generation (68-72), bind fibrinogen and fibrin, and decrease clot permeability (73) and susceptibility to fibrinolysis (74). Studies are needed to determine whether RBC's are causative in thrombus formation.

Although RBCs are present in both arterial and venous thrombi, their local function as well as their beneficial or pathological effects during thrombus evolution, remain unknown. For example, heme deposited by decomposing or phagocytized RBCs may alter recruitment or function of other blood cells within the clot. Compaction of RBCs by platelet-mediated contractile force may stabilize thrombi and prevent embolization, but prolong thrombus resolution. Experimental systems that incorporate blood flow, RBCs, platelet-mediated contraction, thrombin generation, and fibrinolysis are needed to understand how RBCs influence thrombus formation and stability.

Platelet pleiotropy: different platelets, different functions?

The concept of inter-platelet heterogeneity (75) is receiving increased interest. Platelets from an individual donor can differ in glycoprotein receptors expression levels and activation. Circulating platelets differ in mRNA content and capacity to translation, depending on their life time (76). Further, platelets undergoing secretion can differ in the process of secretory granule fusion and exocytosis (77). On the other hand, platelets assembling in a growing thrombus diverge into (i) aggregated and contracted platelets of the thrombus core, (ii) transiently adhered platelets of the outer thrombus shell, and (iii) highly activated, procoagulant platelets in thrombus patches (75). This raises the intriguing question if the known pleiotropy in platelet functions implies that different

populations of platelets are 'specialized' in carrying out different functions. Several key elements in the life of platelets that are worth further investigation, can be identified:

i. *Difference between individual megakaryocytes and platelets.* Despite visualization of (pro)platelet production in the bone marrow (78), it is unknown how megakaryocytes regulate the numbers of receptors per formed platelet. A current assumption is that a certain megakaryocyte produces similar (pro)platelets, but cultured megakaryocytes, even when produced from a single-cell clone of hematopoietic precursors, show a remarkably large inter-cellular variability in surface receptor expression (79). This raises the possibility that different megakaryocytes in the bone marrow can form different 'types' of platelets.

ii. *Priming of platelets (macro-environment).* Due to the presence of circulating hormones or other biomolecules, platelets can be primed to become more or less responsive to agonists (80). Priming can occur rapidly, and be followed by a prolonged period of sensitization or desensitization (81). Given the differences in makeup of individual platelets, some platelets may be more prone to priming than others. Both acute and chronic disease conditions, may acts as primers, either through exposure to tissue damage, or through inflammatory mediators or pathogens.

iii. *Platelet populations in a thrombus (micro-environment).* Densely aggregated and contracted platelets have been observed in the thrombus core, surrounded by an outer shell of more loosely attached platelets (82). Surrounding the aggregated platelets, patches of highly activated platelets are seen with a specialized function in coagulation (75). Likely, the micro-environment - e.g. the direct exposure of platelets to collagen and thrombin - plays a role in this platelet heterogeneity.

iv. *Platelet age and size.* Platelets circulate in the blood stream for 7 to 11 days. There is debate whether newly formed platelets are larger in size and more active than aged platelets. Such associations have been measured, but these are weak. Increased mRNA content of the younger platelets may direct platelet function and fate and even the vesiculation of platelets.

v. *Platelet heterogeneity in vivo versus in vitro.* The properties and even the populations of isolated platelets can differ - e.g. with respect to priming - from the properties of platelets circulating in the blood. Moreover, in vivo pre-activated platelets will be more prone to being cleared than inhibited platelets, whereas both can stay in suspension after isolation (81). This brings about a need for new

technologies to study single-cell activation properties of platelets in vivo and in vitro, and a requirement for standardized isolation of platelets without affecting their priming state.

Recommendations from theme 2:

- Address mechanisms of leukocyte plasticity, migration and transformation in murine atherosclerosis; various "omics" technologies have the potential to take over the role of mouse models in human cohorts.
- Characterize the role of hemostatic factors in macrophages related to inflammation and atherothrombosis; develop mechanism-based biomarkers.
- Investigate the causal contribution of RBCs in thrombus formation.
- Incorporate whole blood flow in experimental systems to assess how RBCs influence thrombus formation and stability.
- Improve knowledge on platelet heterogeneity, how this translates into the formation of platelet populations by megakaryocytes, how to translate from single-cell (platelet, megakaryocyte) assays to the in vivo situation? Investigate how platelet priming fits into the pathogenesis of thrombotic disease (cause or consequence?).

3. Coagulation proteases, fibrin(ogen) and thrombus formation

Factor XI

Factor XI (FXI) is a pivotal protein in the coagulation cascade as it links the contact system with thrombin and fibrin formation, and it also determines the flux through the coagulation cascade after tissue factor exposure via thrombin-mediated FXI activation (83). Drugs targeting FXI can be effective for thrombosis prevention, but there are no data showing that FXI targeting is useful in the treatment of thrombosis. An intervention study in humans who underwent total knee replacement demonstrated that reducing FXI levels by antisense oligonucleotides was effective and safe (84). This study was carefully chosen, since knee replacement introduces a medical device, which likely activates the contact system. Furthermore, the tissue factor concentration in knees is very low, meaning that the amplification of thrombin formation via FXI becomes relatively important (85). The bleeding tendency in FXI deficiency is most often trauma-related especially in tissues with high local fibrinolytic activity, while spontaneous bleeding is rare, and therefore low levels of FXI appear to be safe. Preclinical and clinical studies suggest that complete deficiency is not necessary to

effectively prevent thrombosis; a level of 20-30% FXI is effective and expected to result in less bleeding compared to complete inhibition.

The optimal approach to target FXI is still debated (86). Vaccination (probably not reversible) and antisense oligonucleotides have the disadvantage of a slow onset of action. The use of small molecules and (monoclonal) antibodies requires additional studies, also considering potential off-target effects of these approaches.

Coagulation (factor XI) and its link with platelets and hypertension; a novel risk mechanism?

Recent experimental and early clinical data suggest that targeting FXI could be effective in treating vascular inflammatory conditions that go beyond treating clotting disorders. FXI deficiency improved survival in polymicrobial sepsis (87) and protected from disease progression in atherosclerotic mice (88). Individuals with congenital FXI deficiency are partially protected from myocardial infarction and stroke, both sequels of chronically increased blood pressure (89). FXI antisense oligonucleotide (ASO) application prevented arterial hypertension and reduced established hypertension in animal models. This effect was connected to interruption of the thrombin-FXI amplification loop involving glycoprotein (GP)Ib α on platelets, attenuating the proinflammatory state of platelets and reducing vascular accumulation of inflammatory leukocytes, endothelial dysfunction and tissue remodeling in response to high blood pressure. The FXI dependent platelet-localized amplification of FII was validated in humans with uncontrolled hypertension (90).

Since thrombin propagation is essential in the observed not clotting-related effects, thrombin signalling, which is likely mediated through protease activated receptors (PARs), must be further explored. Since PAR-1 is expressed on monocytes, endothelial cells and smooth muscle cells alike, the thrombin-signaling through PAR-1 needs to be investigated in greater detail to understand possible beneficial off-target and/or disease modifying effects of direct thrombin inhibitors like dabigatran. However, RELY investigators (91) did so far not report on alterations in blood pressure in patients on dabigatran treatment.

If the FXI mediated effects were all related to propagation of thrombin formation, patients with hemophilia A or B should also have lower blood pressure. However, the contrary is the case; this may be due to inflammatory responses associated with repeated episodes of severe bleeding that might potentially override a putative "antihypertensive" effect of FIX or FVIII deficiency. An additional potential target is the GPIb α , the receptor on platelets to which thrombin as well as FXI, but also the MAC-1 integrin on leukocytes and von Willebrand factor can bind.

FXI has a striking homology to pre-kallikrein (PK). Depletion of FXI (e.g., by an ASO approach) might cause counter-regulatory upregulation of PK with ensuing activation of bradykinin, which might

directly cause vasodilation and blood pressure lowering in a nitric oxide dependent manner. It should be evaluated whether targeting of C1-esterase-inhibitor, a natural FXIa inhibitor, would affect blood pressure. Furthermore, using small molecules targeting FXIa (92) should be considered to evaluate whether inactivation of the active protease is equally effective in influencing blood pressure as the ASO approach. This is of interest, since nonspecific effects of ASOs on platelet count and function (93) could impact on vascular function independently of the protease activity of FXI. Datasets of the aforementioned FXI ASO clinical trial and population based cohorts (e.g. Clalit Health Service database (89), Gutenberg Health Study (94) should be explored for associations of FXI activity or single nucleotide polymorphisms in the FXI gene with vascular function, hypertension or incident CVD.

Factor XII, polyphosphates, prekallikrein and high molecular weight kininogen

The FXII-driven contact system and its endogenous activator polyphosphate (polyP) play important roles in thrombosis (95). In contrast to FXI deficiency, FXII-deficient humans do not suffer from any abnormal bleeding. Targeting the contact system or polyP represent promising therapeutic targets for prevention of, or interference with thrombotic disorders (96,97). In addition, activated FXII induces potent inflammatory responses via activation of the kallikrein-kinin system resulting in the formation of the inflammatory mediator bradykinin (98). The dual role of FXII in thrombosis and inflammation makes it an attractive therapeutic drug target for anticoagulation without any bleeding risks and additional anti-inflammatory activities (99,100).

Prekallikrein circulates in plasma bound to high molecular weight kininogen (HK), the bradykinin precursor. Upon activation by FXIIa, prekallikrein is converted to plasma kallikrein and then reciprocally amplifies further FXII cleavage that in turn produces additional plasma kallikrein (101,102). Similarly to FXII, plasma kallikrein-deficient mice have an antithrombotic phenotype and do not bleed excessively (103). As plasma kallikrein functions mostly as an amplifier of the coagulation cascade, its deficiency can be overcome by strong contact activators which limits its potential use as target for anticoagulation (98). However, targeting plasma kallikrein has proven beneficial effects for interference with bradykinin-driven inflammation such as hereditary angioedema (104,105).

Inhibition of HK seems to be another promising strategy for safe thromboprotection as suggested from mouse models. In contrast to humans, mice have two kininogen genes and ablation of kininogen 1 (*mkng1*^{-/-} mice) protects from experimental arterial thrombosis without causing bleeding (106). Consistently, a patient with plasma kininogen activity of < 1% did not bleed (106). While serine

proteases are targetable by small molecule inhibitors that bind into the enzymatic pocket, strategies for interference with HK or HK expression should be further investigated.

PolyP, a linear polymer consisting of a few to several hundred residues of orthophosphate linked by phosphoanhydride bonds, initiates coagulation in a FXII-dependent manner (107). Platelets contain two pools of polyphosphate. Short chain soluble polyP with low FXII activating capacity that are released from platelet dense granules. The majority of platelet polyP however forms nanoparticles (similarly to other FXII activators such as kaolin or ellagic acid) with divalent metal ions (mostly as Ca^{2+}) that are retained on the surface of platelets and potently activate FXII (107). The role, structure and composition of these nanoparticles in thrombotic conditions warrants further investigation.

Factor XIII

Factor XIII (FXIII) is unlike other coagulation enzymes in its ability to crosslink, rather than cleave, proteins within the clot. Activated FXIII (FXIIIa) activity is essential for clot biochemical and mechanical stability (108). Several FXIII polymorphisms appear to modify thrombosis risk (109,110); these function in part, through complex interactions that mediate the kinetics of FXIII activation and fibrin crosslinking (111,112). FXIII activity also promotes retention of RBCs within clots during platelet-mediated clot contraction (113). However, fundamental mechanisms in FXIII biology and biochemistry and its impact on thrombus biophysical characteristics remain unclear.

Although studies suggest FXIIIa can be proteolytically inactivated (114), endogenous inhibitors of FXIIIa have not been identified. Importantly, currently available FXIIIa inhibitors lack the pharmacological properties necessary for in vivo investigations.

FXIII is found in both plasma and cellular compartments, but the relative roles of plasma and platelet FXIII in platelet function, fibrin crosslinking, and thrombus composition and stability are poorly understood.

Differences in FXIII expression between humans and mice should be considered when using animal models.

Fibrin network structure

Fibrinogen conversion to fibrin is essential for hemostasis. Formation of clots with abnormal structure and stability is associated with hemostatic and thrombotic disorders, including both arterial and venous thrombosis/thromboembolism (31). Clots with overly dense fibrin network structure have been associated with enhanced mechanical and biochemical stability and increased risk of thrombosis (65-68). These observations indicate that the clot structure may be a biomarker of hemostatic and thrombotic potential.

Most studies use *in vitro* methods to assess plasma clot structure in the absence of cells, but this approach fails to reveal contributions of vascular and blood cells to clot formation and function; these include both procoagulant and fibrinolytic activities produced by local blood and vascular cells, and mechanical forces imparted by platelets during clot contraction.

Platelet-fibrin interactions and clot architecture

Knowledge of fibrin interactions with platelets and their respective interactions with other blood cells is incomplete. Platelets bind fibrin through activated $\alpha_{IIb}\beta_3$, which also binds fibrinogen. However, recent studies show that fibrin but not fibrinogen binds GPVI and activates platelets (115,116). The thrombus is not homogeneous, but composed of a core of strongly activated platelets and a periphery of more loosely activated platelets (117). It is possible that combined collagen and fibrin interactions with platelets drive thrombus core formation, while the interaction of fibrin with platelets plays a role in determining the thrombus periphery.

i. *Filling the void*: there is a need for novel *in vitro* and *in vivo* models to probe interactions between platelets and coagulation. However, studying platelet-fibrin interactions is practically challenging as the fibrin mesh that is formed eliminates a number of techniques. Vice versa, platelet aggregates interfere with clotting assays. An intermediate stage of fibrin protofibrils and oligomers precede the gelation point (118). Further studies are required to determine platelet interactions with these fibrin species.

ii. *Novel GPVI – fibrin interactions* shed new light and should be investigated in diseases of thrombosis and haemostasis. Recent data indicate that collagen and fibrin bind distinct GPVI epitopes (Onselaer MB et al. unpublished), suggesting that it is possible to specifically target each ligand interaction. GPVI polymorphisms have also been associated with venous thromboembolism in GWAS studies (119). It would be interesting to investigate whether GPVI polymorphisms that result in less active GPVI signalling are also associated with decreased fibrin-GPVI interactions.

iii. *There could be other coagulation and platelet interactions that are currently unknown*. Proteins such as vWF (120), fibronectin (121), FXIII (122,123) and α_2 -antiplasmin (124) are incorporated into the fibrin clot and their role in platelet interactions with the clot in the context of the clot network are incompletely understood. Furthermore, platelets express a number of fibrinolytic proteins and FXIII on their surface (125,126), which may interact with the surrounding fibrin network.

iv. *Interactions with red and white cells.* There is evidence for the binding of red cells to fibrinogen (127,128) and platelets (129,130). The implications of these interactions deserve further study. Neutrophils contribute to thrombosis through the production of NETs (131). DNA and histones have been shown to impart resistance to fibrinolysis (132). How DNA and histones interact with fibrin at the fibre level is poorly understood. White cell incorporation or invasion into thrombi may also contribute to thrombus resolution through the production of elastase, which has been shown to degrade fibrin and FXIII (133,134).

Recommendations from theme 3:

- Address the relevance of FXI for treatment of thrombosis and thromboinflammatory disorders; a “safe” lower level of FXI targeting thrombotic complications remains to be determined. Off target effects of inhibiting FXI need to be considered.
- Undertake further studies on FXI induced increased thrombin production and PAR mediated cell signalling; these should consider upregulation of PK mediated activation of the bradykinin pathways; population based cohorts or large trials with anticoagulants, should be explored for signals of blood pressure lowering effects or changes in incident CVD linked to FXI or other clotting protease activity.
- Address the dual role of FXII in thrombosis and inflammation in different disorders; investigate strategies for interference with HK or HK expression; investigate the role of PolyP nanoparticles on platelets.
- Explore the fundamental mechanisms in FXIII biology and biochemistry and its impact on thrombus biophysical characteristics; development of FXIII(a) inhibitors would facilitate studies to investigate FXIII(a) function in hemostasis and thrombosis.
- Development of technologies to image fibrin structure and biophysical characteristics in the presence of blood cells and in thrombi formed in vivo would yield important insights; differences in fibrin network structure between species (e.g., humans versus mice) should be considered in studies of fibrin structure and function in health and disease.
- Study the relationships between abnormal fibrin structure and functional consequences, including susceptibility to fibrinolysis and mechanical deformability, which should involve new technologies to image fibrin structure and biophysical characteristics in the presence of blood cells and in thrombi formed in vivo.
- Investigate whether there are additional aspects of the interaction between platelets, fibrin (e.g. fibrin-GPVI interactions), red and white blood cells that play key roles in thrombosis and haemostasis.

4. Preventive and acute treatment of atherothrombosis and arterial embolism; novel ways and tailoring?

Inhibition of protease activated receptor mediated cell signaling as antithrombotic strategy

Despite the prematurely halted TRACER study due to increases in the risk of moderate and severe bleeding events in stroke patients, pharmacologic blockade of protease activated receptor-1 (PAR1) is still potentially an attractive target for the prevention of several cardiovascular pathologies, including acute coronary syndrome, chronic secondary prevention of ischemic events and peripheral artery disease (PAD) (135,136). In the TRA2°P-TIMI 50 study, the PAR1 antagonist, vorapaxar, did meet primary endpoints with significant reductions in major adverse coronary events (MACE) in secondary prevention in non-stroke patients with prior myocardial infarction and PAD. In particular, the subgroup of patients with prior MI also demonstrated a significant reduction in cardiovascular death and ischemic events (137). Despite approval by FDA, vorapaxar has not seen widespread use despite these documented improvements in MACE reduction, especially in the subjects with prior MI, due to the perceived elevated bleeding risk, especially intracerebral hemorrhage. The use of vorapaxar on top of dual antiplatelet therapy (e.g. triple antiplatelet therapy) may likely have contributed to the increase in incidence of bleeding events. Studying the effects of Vorapaxar as monotherapy or dual therapy might reveal reduced bleeding risk and would be worth testing especially in subjects with prior MI.

One potential explanation of these increased intracranial bleeds on triple therapy might be that akin to tissue factor and thrombomodulin, PAR1 is differentially expressed in endothelial cells of the brain (138), thereby giving rise to different integrin/leukocyte adhesion, regulation and hemostatic functions. Another issue with vorapaxar is that it displays functionally irreversible binding to PAR1 with pharmacodynamic anti-platelet effects of 4-8 weeks (139). As reversibility is important to mitigate bleeding risk, vorapaxar may not be practical to use in patients with heightened risk of bleeding, e.g. older patients, those with low body weight, or uncontrolled hypertension. Another interesting pharmacologic effect to be addressed is why a single dose of vorapaxar exhibits such a long lasting anti-PAR1 platelet effect well after replenishment with fresh platelets 8-10 days after drug administration. Does this imply that vorapaxar binds to megakaryocyte PAR1 and somehow changes the megakaryocyte behavior leading to a 'memory' effect in the megakaryocyte due to phenotypic switching or imprinting? Does vorapaxar alter PAR1 expression or are other critical platelet signaling components down-regulated or altered to suppress long-term PAR1 function in the

nascent platelets? The new PAR1 peptidic inhibitor, PZ-128, which has a fast on-rate and improved reversibility of recovery of PAR1 platelet function (140), and may provide a more favourable balance of efficacy and safety, is currently being evaluated in the phase 2 TRIP-PCI clinical trial in the US. Aside from PAR1, is the lower affinity PAR4 thrombin receptor inherently a better target than PAR1 because PAR4 blockade causes less bleeding, or is PAR4 a poor target because PAR1 is the high affinity thrombin receptor (141)? Success in targeting PAR4 or the PAR1-PAR4 heterodimer (142) will require achieving efficacy even if safety concerns are not an issue. Recent identification of a super-active PAR4 polymorphism in transmembrane domain-2 (TM2) in Blacks would suggest enriching clinical trials with appropriate populations to fully understand the pharmacogenomics of PAR4 inhibitors (143).

Optimizing antiplatelet therapy by platelet function monitoring?

Dual antiplatelet therapy, consisting of aspirin and a P2Y₁₂ inhibitor, is the mainstay of treatment for patients with coronary artery disease undergoing percutaneous intervention, to prevent stent thrombosis (144,145). However, some patients continue to have thrombotic events while on antiplatelet therapy, while others suffer from bleeding (146). The degree of P2Y₁₂ inhibition as measured by platelet function tests (PFTs) has been related to the risk of both thrombotic and bleeding events and has led to the concept of a therapeutic window for optimizing antiplatelet therapy (146-148). Several large clinical trials have investigated the value of PFT-based tailoring of antiplatelet therapy. These trials, such as GRAVITAS, TRIGGER-PCI and ARCTIC, all failed to prove that intensifying P2Y₁₂ inhibitor therapy prevents thrombotic events in patients with a low response to P2Y₁₂ inhibitors (high on-treatment platelet reactivity – HTPR) (149-151). The ANTARCTIC trial was the first to also include a de-escalation arm of antiplatelet therapy in cases of low on-treatment platelet reactivity (LTPR) to prevent bleeding in the elderly, but this also appeared ineffective (152). Results of the second de-escalating trial, TROPICAL-ACS, are expected in the third quarter of 2017 (153).

So, have any of these studies actually proven that the concept of individualising antiplatelet therapy based on PFTs does not improve clinical outcome? Members of the workshop felt that this cannot be concluded, due to limitations of both the trial designs and PFTs. Recognised drawbacks of the trial designs include: 1) the study protocols regarding intensification of P2Y₁₂ inhibition mainly allowed a singular switch to another drug or dose, 2) the studies had low statistical power because of a low incidence of primary endpoints, and 3) patients with a higher risk of recurrent events were mostly excluded (e.g. STEMI) (147). The importance of this last limitation is underpinned by two recent meta-analyses indicating that both the predictive value of platelet reactivity for thrombotic events,

and the benefit of PFT-based tailoring of therapy is dependent upon the patient's cardiovascular risk profile (154,155).

Limitations of PFTs also significantly hinder the measurement of platelet reactivity and optimization of treatment. Widely used PFTs include Light transmission aggregometry (LTA), PFA-200, Multiplate and the only true point of care test in use the VerifyNow. Most importantly, these assays do not reflect normal physiological platelet function. Platelets tend to be activated with single agonists while in vivo multiple stimulation occurs, the influence of coagulation or fibrinolysis is not usually assessed, pleiotropic effects of the P2Y₁₂-inhibitors cannot be measured, and with the exception of PFA-200 platelet aggregation is measured under low shear conditions (156). Another limitation of the widely used PFTs concerns the large influence of pre-analytical variables on the test results, including the type of anticoagulant used, delay time from blood withdrawal to measurement, and concentrations of agonists used (156). Finally, current PFTs lack assay standardisation and consensus regarding optimal cut-offs for HTPR and LTPR (147). One of the ongoing activities of the ISTH Platelet Physiology SSC is to publish new guidelines for monitoring of P2Y₁₂-inhibition (<http://www.isth.org/members/group.aspx?id=100371>).

Anticoagulation in atrial fibrillation (AF): to treat or not to treat, that remains the question!

Patients with atrial fibrillation (AF) should ideally be treated by a team of cardiologists and specialized nurses (157) – which treats comorbidities and decides on anticoagulation therapy. Current ESC guidelines recommend using the CHA₂DS₂-VASc-score to guide initiation of anticoagulation therapy in patients with AF (158). However, there is room for improvement in the identification of patients who need and tolerate anticoagulation, to prevent overtreatment and reduce bleeding events, respectively.

The risk factors that are part of the CHA₂DS₂-VASc-score should be further specified: for example, currently it is not defined whether left ventricular hypertrophy on echocardiography should be counted as 'hypertension' and whether coronary artery disease on CT angiography should count as 'vascular disease'.

There is no consensus on the use of anticoagulation in patients with one risk factor (CHA₂DS₂-VASc-score of 1 in men, 2 in women). The ESC guidelines suggest considering starting anticoagulation (158), while the AHA/ACC suggests considering not to start anticoagulation (159). Recent large scale reports show differing results: there are high stroke rates in patients with one risk factor not on anticoagulation (160), while the bleeding risk on warfarin is exceptionally high and probably higher than the stroke risk (161). Additionally, AF pattern (ie paroxysmal or persistent) may be considered

in the decision, as it may reflect underlying pathology and hence associate with stroke and bleeding risk (162).

Biomarkers may be used to improve risk stratification, e.g. the Age Biomarkers Clinical History (ABC)-score (163), in which NT-proBNP and troponin are incorporated. However, it is currently unknown how this score should be implemented in daily practice.

Both the CHA₂DS₂-VAsc-score and the ABC-score identify those patients that are susceptible to vascular disease, such as stroke, rather than the patient's clotting tendency. However, it has been shown that hypercoagulability may be the underlying process leading to AF (164), even in vascularly healthy patients (165). Future risk stratifying strategies may need to include measurements of hypercoagulability.

There are several imaging strategies that may enhance identification of patients with a high stroke risk. With transoesophageal echocardiography, the presence of high-risk echocardiographic features can be assessed (166), while echocardiography, cardiac CT or MRI can be used to assess left atrial appendage morphology (167), all of which may enhance the identification of those patients with a phenotype prone to stroke.

Current risk scores are only validated for patients with AF which is recorded on ECG or Holter. Cardiac implantable electronic devices (CIEDs) continuously monitor the (atrial) heart rhythm. In case a CIED registers an atrial high rate event, it is currently unknown whether anticoagulation should be started. The ongoing randomized NOAH (NCT02618577) and ARTESiA (NCT01938248) trials will provide valuable answers for this category.

How to handle the risk of anticoagulant therapy?

For many years anticoagulation was based on vitamin-K-antagonists (VKA). Monitoring of anticoagulant treatment is done via regular measurements of the international normalized ratio (INR) in a secondary care setting, at least in a number of countries. These specialized anticoagulation centres in which knowledge and experience with VKA treatment is present, play a key role as coordinator of anticoagulation therapy.

The field of anticoagulation therapy is evolving. Non-VKA oral anticoagulants (NOAC) have shown to be at least as effective and safer as VKA in large trials (91,168-170), and are now changing clinical practice. Dabigatran, Rivaroxaban, Apixaban and Edoxaban are replacing VKA as first choice treatment for several indications. The possibility to prescribe different types of oral anticoagulants for similar indications is making anticoagulant treatment more complex.

The balance between the risk of thrombosis and the risk of bleeding remains an important concern in patients receiving anticoagulant therapy. Due to a more predictable pharmacological profile,

routine monitoring is not required for NOACs. However, this should not preclude regular follow-up to oversee safety and efficacy of treatment (171). Currently there is no standardized follow-up available for patients on NOAC therapy. The question rises how this follow-up should be implemented in existing healthcare systems that are currently based on VKA management (171,172). We propose a solution for NOAC follow-up, designed to increase treatment efficacy and safety, based on expanding the capacity and capability of current anticoagulation centres, without a big increase in costs.

The current anticoagulation centres are well-trained and equipped to adequately monitor and guide patients on VKA, reaching high TTRs (Time in Therapeutic Range) (173). These centres are local and give centralized coagulation care to a small population. By educating specialist coagulation nurses and physicians, their knowledge on NOACs can be increased. Specific software tools and checklists can help nurses with systematic assessment of patients on NOACs. A physician should be available for consultation, but overall the anticoagulant care should, ideally, remain nurse-based. Nurses should monitor several aspects, i.e.:

- Kidney and hepatic function;
- Compliance to drugs, which can also be checked via information from the pharmacy;
- Weight;
- Blood pressure;
- Adverse events during treatment, i.e. bleeding events;
- Haemoglobin and platelet counts;
- Reassessing the indication for anticoagulation.

Furthermore, haemostasis centres are needed, which should be regional and should be located in a medical centre with a multi-disciplinary team, for example consisting of a haematologist, cardiologist, neurologist and a gastro-enterologist.

Organ specificity of anticoagulant effects?

The pattern of bleeding with oral anticoagulants differs by agent (174). Compared with warfarin, all of the NOACs reduce intracranial bleeding, some NOACs are associated with increased gastrointestinal bleeding, and some are associated with abnormal uterine bleeding (175). Several mechanisms have been proposed to explain the organ-specific effects of different oral anticoagulants.

First, the mechanisms of action of VKA compared with NOACs; VKA inhibit the production of vitamin K dependent coagulation proteins, whereas NOACs selectively target coagulation factors Xa or IIa (176,177). By targeting multiple coagulation factors, VKAs appear to produce greater inhibition of

thrombin generation than the NOACs (178), which may in part explain why they are associated with a higher risk of bleeding. However, this does not explain organ-specific differences in bleeding. The second mechanism is related to physiologic differences between vascular beds of the brain, gastro-intestinal tract and uterus. The brain is rich in tissue factor (TF) and hemostasis is dependent both on expression of TF on sub-endothelial membranes, and on low levels of tissue factor pathway inhibitor (TFPI) (179), an inhibitor of the TF/VIIa complex. By inhibiting factor VII, VKAs prevent the formation of the TF/VII complex and thereby suppress hemostatic mechanisms in the brain, whereas this mechanism is unaffected by the NOACs. This may explain the reduction in intracranial hemorrhage with NOACs compared with warfarin. In the uterus, animal studies suggest that factor Xa is important in the regulation of hemostasis. This may explain why factor Xa inhibition by Rivaroxaban is associated with an increase in abnormal uterine bleeding compared with warfarin (180).

The third mechanism is relevant to excess GI bleeding seen with several NOACs. The integrity of the GI mucosa can be disrupted by intra-luminal factors (181), and this allows uptake of drugs from the lumen. VKAs exert their anticoagulant effect by inhibiting vitamin K epoxide reductase, which requires absorption from the gut into the systemic circulation, whereas NOACs are only partially absorbed from the gut and residual drug can exert effects locally on the GI mucosa leading to bleeding (174,179,182).

The ESC guidelines (158) for stroke prevention in atrial fibrillation recommend use of the HAS-BLED score to predict bleeding but this score is only weakly predictive. One possible contributor to its poor predictive value is that it fails to take into account organ-specific differences in bleeding risk. The future development of separate risk scores for intracranial, GI and uterine bleeding may improve risk prediction but might also increase complexity because of difficulties in integrating the information for individual patients.

Towards individualized optimized NOAC therapy?

Although routine coagulation monitoring is not required in patients taking NOAC, certain clinical situations require guidance on the intensity of anticoagulation. Currently, there is no consensus on which clinical situations would benefit from such occasional monitoring. Some experts propose specific cut-offs in plasma concentration equivalent to reduce the risk of hemorrhagic complications in the perioperative setting or to guide the administration of reversal agents (183-185).

Importantly, these different clinical situations require different test characteristics. For emergency situations, the question is whether any remaining anticoagulant effect is still present and a sensitive test with short turn-around time and a 24-hour availability is essential. Classical coagulation tests,

like PT, aPTT and thrombin time, have important limitations (186-188). For more robust and quantitative purposes, specific assays are available, including diluted thrombin time (dTT) or ecarin chromogenic assay (ECA) for dabigatran, and NOAC calibrated chromogenic anti-Xa assays for FXa inhibitors (172175). The turnaround time of these specialized assays is similar to the one of routine tests making them preferable (189). However, rapid, point-of-care, whole blood tests for use in emergency situations would be desirable while other specialized assays such the thrombin generation test, the thromboelastography or ROTEM® and other microfluidic assays might offer potential in the near future (190).

Laboratory measurement is not only useful in emergency situations, but also for eventual dose adjustment purposes. Although the clinical trials with NOACs proved the safety of the fixed dose policy (91,168-170,191-195), the observed considerable inter-individual variation in drug levels in pharmacokinetic studies and clinical trials, suggests that dose adjustment guided by laboratory assays may be appropriate in some circumstances (provided that registered doses are used) (196-205)*.

At present, however, there are neither established, therapeutic ranges, nor is there evidence-based data supporting the laboratory approach. At the same time, on-therapy ranges are available from the large phase-2/3 studies (201,206-208)[†], which can be considered in daily practice.

Although this proposal did not reach consensus, we propose that future studies should focus on the definition of a 'therapeutic window', and explore the possibilities of tailoring the dose based on these safety margins. This approach may improve the benefit risk ratio in elderly/frail patients.

Other new anticoagulants?

* Food and Drug Administration. Eliquis - Clinical Pharmacology and Biopharmaceutics Review(s) 2012 [updated 2016 Oct 25. Available from: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2012/202155Orig1s000ClinPharmR.pdf. European Medicines Agency. Eliquis - EMEA/H/C/002148/X/04/G 2012 [Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Assessment_Report_-_Variation/human/002148/WC500136575.pdf)

[†] (European Medicines Agency. Xarelto - Summary of Product Characteristics 2016 [updated 2016 Oct 14. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000944/WC500057108.pdf, European Medicines Agency. Eliquis - Summary of Product Characteristics 2017 [updated 2017 Feb 22. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002148/WC500107728.pdf, European Medicines Agency. Pradaxa - Summary of Product Characteristics 2017 [updated 2017 Jan 12. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000829/WC500041059.pdf)

The ideal anticoagulant should prevent thrombosis without increasing bleeding risk. Additional features could include low renal clearance, minimal drug-drug/drug-food interactions, good oral bioavailability, and an effective reversal agent, if required. Importantly, the residual major bleeding risk with current anticoagulant drugs is of the order of 2-3% per year in patients with AF (209-211) and 1% in patients with VTE (212). Moreover, the number needed to treat with a NOAC for preventing one major bleeding event, compared to VKA remains high (>100) (211,212). Until recently, clinical anticoagulation has been oversimplified, without addressing organ-specific processes of bleeding and thrombosis and the differences in pathogenesis across various clinical scenarios, as exemplified by bleeding patterns with NOACs (211,212). Thus, during drug development, indication-specific targeting should be considered. This includes, for example, researching contact pathway inhibition for indwelling artificial surfaces such as catheters or mechanical heart valves, and taking into account oscillation of drug levels and lack of protective mechanisms present in normal vessels walls.

NOACs have yet to challenge VKA treatment in rarer sites of thrombosis, such as cerebral venous sinus thrombosis and chronic portal vein thrombosis. Patients with mechanical heart valves are a group in need of new anticoagulants. The optimal anticoagulant for patients with cancer requires further evaluation. Some of these issues may be addressed by clinical trials with contemporary anticoagulants already approved for other indications rather than by development of new anticoagulants.

Hemostasis and thrombosis are generally considered to be manifestations of the same enzymatic and cellular processes. Consequently, dissociation of antithrombotic and bleeding effects might not be achievable, except perhaps for the use of anti-factor XIIa targeted therapy that should at least from a theoretical point of view, be unaccompanied by a bleeding risk (100).

Recent studies indicate that the 'anatomy of a clot' is not homogeneous with compartmentalization of a clot into a core and a shell (82). If the anatomy of hemostatic clot as opposed to a thrombotic clot is different then it is possible that novel therapeutic molecules that are able to exploit such a difference might have a more favourable profile. The recent report of a patient with a monoclonal IgA paraprotein targeting thrombin who did not have an associated severe bleeding phenotype suggests that it may be possible to target components of coagulation with a resultant antithrombotic effect but little if any bleeding effect (213). In this patient the antibody bound to exosite 1 and not the active site of thrombin and so there may also be a kinetic basis for an apparent dissociation of effects.

It is important to understand the animal models used to evaluate new anticoagulants. For bleeding, the tail transection model (214) is frequently used in the pre-clinical evaluation of candidate

antithrombotic molecules but it is problematic because of the effect of vasoconstriction and that it is an open rather than a closed bleeding model. The saphenous vein bleeding model (215) has been suggested as an alternative that may correlate more closely with clinical bleeding phenotype (216). Skin bleeding time is more suited to evaluating primary hemostasis (e.g. evaluating antiplatelet medication) but less suited to assessing effects of anticoagulants.

To assess effects on arterial thrombosis, ultrasound induced plaque rupture in the APO-E KO mouse has been used as a pre-clinical model (217). The frequently used ferric chloride-induced thrombosis model (215) recreates a predictive and reproducible thrombus which can be subjected to inhibition by various drugs. This can be viewed as a screening procedure, selecting drugs only with potential inhibitory activity. However, this model does not indicate how a particular drug will work in humans or enable ranking of drugs.

Recommendations theme 4:

- Study the role of lower affinity PAR4 thrombin receptor vis a vis PAR1 to establish whether PAR4 is a better target than PAR1, because PAR4 blockade causes less bleeding.
- Develop a practically feasible PFT that reflects in vivo platelet function and thrombus formation under physiologic conditions; Standardize monitoring of P2Y₁₂-inhibition; trials in high risk patients, using clinically relevant physiological PFTs, should be performed to assess the relevance of PFT-based tailoring of therapy.
- Refine current risk scores in patients with AF, in particular the decision to start anticoagulation in those with low scores; revise CHA₂DS₂-VASc-score and the ABC-score that includes biomarkers, also taking into account hypercoagulability markers; imaging studies may provide additional information in stroke risk.
- Organize regular follow-up of patients on NOAC treatment in a nurse-based manner involving anticoagulation clinics. Regional haemostasis centres can manage complex patients in a multi-disciplinary manner.
- Develop separate risk scores for intracranial, GI and uterine bleeding in patients on oral anticoagulants to improve risk prediction; this may also increase complexity.
- Develop In those on NOACs, a sensitive test with short turn-around time and 24-hour availability for emergency situations; dose adjustment guided by laboratory assays may be appropriate in some circumstances; the 'therapeutic window' should be defined, in order to improve dose finetuning.

- Try to target components of coagulation with a resultant antithrombotic effect but little if any bleeding effect; optimize pre-clinical testing including a better choice in animal models, based on the model's characteristics related to thrombosis or bleeding.

5. Pleiotropy of coagulation proteases, thrombus resolution and ischemia-reperfusion

Clot resolution and ischemia-reperfusion injury

Until recently, thrombectomy was considered an integral part of the treatment strategy for acute MI. Recent meta-analyses indicate that this is no longer tenable; only in case of heavy thrombus burden may thrombectomy be advantageous albeit at a potential cost of increased risk of stroke (218). For acute ischemic stroke however, the MRCLEAN study has shown that intra-arterial therapy, using for instance stent-retrievers, significantly improved patient outcome and has since gained status of preferred treatment (219). Thrombectomy results in biobanks containing this material and allows analysis of this unique material to further our understanding of the thrombotic event and the processes involved in “successful” thrombectomy and its sequellae. The following aspects deserve further attention:

Thrombus fragmentation and embolization. It was deemed important to develop new and relevant ways to phenotype the thrombus and determine its source, as current strategies seem inadequate. Furthermore, it was considered relevant to study the role of local fibrinolysis in embolization and fragmentation and the composition and disease state of the atherosclerotic vessel wall.

Stent-thrombus interaction. We discussed which processes might play a role in the interaction between stent and thrombus (220); two issues were specified: the status of the local vasculature and of the coagulation system. With regards to the vasculature at the site of thrombectomy, tissue factor content and the depth of injury were considered possible determinants of stent-thrombus interaction. Activation of the coagulation system was deemed equally important, considering that both “erythrocyte rich” and “platelet-fibrin rich” thrombi could grow fast, within minutes, while not reflecting thrombus age. Potentially, phenotyping based on the relative contribution of the different leukocyte subtypes adds relevant information.

Biobanking initiatives. There are many biobanking initiatives but not many specifically geared towards arterial thrombosis. Prerequisites for successful biobanks, aside from regular ethical considerations, were considered (inter)national collaboration, thorough documentation of the clinical background of thrombus and patient; procedural aspects of thrombectomy; standardized and documented protocols for proper handling and storage of relevant materials.

The role of activated protein C in limiting brain damage in ischemic stroke

Despite extensive efforts, no novel drug for ischemic stroke therapy has been approved in the past decade. One promising drug candidate currently in phase II clinical studies for treatment of ischemic stroke is the activated protein C (APC) variant 3K3A-APC (221). APC provides natural anticoagulant function by inactivating the coagulation cofactors Va and VIIIa. In addition, APC conveys direct effects on cells that involve multiple receptors including protease-activated receptor (PAR) 1, PAR3, and the endothelial protein C receptor (EPCR) (222). Depending on the cell type and cell stress involved, these cellular activities of APC, collectively referred to as “APC’s cytoprotective activities,” can be characterized as anti-apoptotic and anti-inflammatory activity, beneficial alterations of gene expression profiles, protection of endothelial barrier function, and regenerative activities.

Studies have shown beneficial effects of APC in rodent stroke models (223). The use of molecularly engineered APC variants with altered selectivity profiles to rodent stroke models, such as the 3K3A-APC variant with minimal anticoagulant activity but normal cytoprotective activities, demonstrated that the beneficial effects of APC primarily require its cytoprotective activities but not its anticoagulant activities.

Recent insights into the molecular mechanisms for APC’s cytoprotective activities identified non-canonical PAR1 and PAR3 activation by APC that give rise to novel tethered-ligands capable of inducing biased cytoprotective signaling as a key distinction from the traditional canonical thrombin-mediated PAR1 and PAR3 signaling that generally results in prohemostatic and proinflammatory effects (224). Based on the available data, the consensus model for APC’s cytoprotective activities involves the binding of APC to the endothelial protein C receptor (EPCR) which permits APC-mediated non-canonical activation of PAR1 at Arg46 and PAR3 at Arg41 resulting in the initiation of biased signalling and the corresponding cytoprotective effects. In addition, it has become clear that additional receptors including ApoER2, MAC1, PAR2, Tie2, factor V, and protein S are involved to provide cell-type specific variations to this model, but their integration into the overall precipitation of APC’s cytoprotective activities remains incompletely understood.

Based on the new mechanistic insights, the following research avenues were identified:

1. To determine the effects of anticoagulant drugs on the endogenous protein C system and in particular the ability of the endogenous cytoprotective protein C pathway to continue to provide beneficial effects on cells and tissues. The (patho)physiological generation of APC by thrombin/thrombomodulin may be affected by anticoagulant drugs that aim to limit thrombin generation. With the increasing use of direct oral anticoagulants (NOACs), questions arise if and how these drugs affect normal APC generation and the ability of APC

to provide normal cytoprotective effects. Protein C is also sensitive to Vitamin K antagonists (VKA) as it contains a GLA domain that is involved in binding of negatively charged phospholipid vesicles required for APC's anticoagulant activity, binding to EPCR required for efficient protein C activation and APC's cytoprotective effects on cells. Thus, questions arise how potential effects of NOACs compare to VKA for protein C activation and APC's cytoprotective effects.

2. There is an urgent need for the development of tools, assays, and the identification of biomarkers to evaluate the endogenous cytoprotective protein C pathway in patient samples. Assays and tools are available for analysis of the anticoagulant protein C pathway but these cannot be readily extrapolated to analysis of the cytoprotective protein C pathway.
3. Basic research is needed to advance our understanding of the molecular mechanisms responsible for APC's cytoprotective activities. With the notion that APC induces non-canonical activation of PAR1 and PAR3, biased signaling, and the integration of PAR signaling based on homo- and hetero-dimer formation, the realm of possibilities for specialized signaling variations has grown exponentially.

New Concept of Thrombolysis and how to Improve Clot lysis in Ischemic Stroke

The clinical manifestations of ischemic stroke are heterogeneous and successful treatment depends on many variables involved in thrombus formation such as physical location in the brain, time from onset, and patient specific variables (age, gender, comorbidities, etc.). The standard of care for acute ischemic stroke now involves IV alteplase (recombinant tissue-type plasminogen activator, tPA) within 4.5 hours, followed by antiplatelet treatment (acetylsalicylic acid or clopidogrel) 24 hours after infusion of thrombolytics, in order to avoid excess risk of intracerebral haemorrhage (225,226). The major goal of thrombolytic treatment is to rapidly establish oxygenated blood flow to limit brain damage. This can be achieved with thrombolytic drugs, such as tPA, or by mechanical interventions such as endovascular thrombectomy. Mechanical thrombectomy by means of retrievable stents has been proven a very effective treatment for acute ischemic stroke caused by large artery thromboembolic occlusion (219,227-229) and is now accepted as standard treatment (230,231), and incorporated in major guidelines (232,233). Regarding the use of thrombolytic drugs the MRCLEAN initiative reported that clots could only be lysed in 1/3 of the patients who were treated with recombinant tPA (219). This relatively low success rate has been partly attributed to the fact that tPA only accomplishes its thrombolytic effect in small clots because then the core of the thrombus

can be reached (234). Infusion during a longer period of time might improve the impact of tPA, however it may increase the risk of massive brain damage caused by bleeding. Meanwhile, proper reperfusion of the brain after endovascular thrombectomy that is usually performed on patients with large thrombi, is often not achieved, for unknown reasons. It has been suggested that the formation/release of microthrombi that may diminish downstream microvascular perfusion may play a role (235,236). Nonetheless, reperfusion can only be visualized and analyzed in the larger vessels. These uncertainties and side effects of current treatments for ischemic stroke highlight the importance of improving drug treatment. The ideal treatment should comprise drugs that have immediate effects to minimize brain damage, to safely enhance fibrinolysis and simultaneously do not cause bleeding. This kind of treatment could be combined with thrombectomy resulting in fast mechanical removal of most of the thrombus and fibrinolytic removal of microthrombi.

One novel research avenue is directed at the interaction between von Willebrandfactor (vWF) and glycoprotein 1b, another approach is to infuse ADAMTS13 and both approaches appear to reduce clot stability in models of ischemic stroke (237,238).

Another concept of safe thrombolysis combines pro-fibrinolytic approaches such as anti-PAI-1, anti-TAFI and anti- α 2-antiplasmin (239). One promising strategy, for which the proof of concept has been demonstrated in mouse models, is based on a bispecific diabody against TAFI and PAI-1 (240). It simultaneously blocks the two anti-fibrinolytic proteins resulting in a significantly enhanced activity of endogenous t-PA. Compared to a monoclonal antibody (150 kDa), it is much smaller (50 kDa) and thus might be able to reach the core of the thrombus more easily. Additionally, it has a half-life of approximately 120 min instead of 2-3 weeks compared to monoclonal antibodies. Therefore, the diabody might be beneficial as a safe fibrinolytic with a reduced bleeding risk. Additionally, the possibilities of combining other fibrinolytics, such as anti- α 2-antiplasmin, with PAI-1 or TAFI still needs to be investigated, as well as combining such pro-fibrinolytics with thrombectomy. When designing a factorial 'cocktail' study, the challenge is to determine the right doses (beneficial effect with minimal harmful side effects) of the right combination treatments.

Recommendations theme 5

- Organize structured, focussed biobanking initiatives to support relevant ways of thrombus phenotyping; develop studies to identify vessel wall-stent-coagulation/fibrinolysis interactions;
- Determine the effects of anticoagulant drugs on the endogenous protein C system including its cytoprotective effects on cells and tissues; new tools needed to evaluate the endogenous

cytoprotective protein C pathway in patient samples; basic research to advance our understanding of the mechanisms of APC's cytoprotective activities;

- Improve drug treatment as well as management of patients suffering from ischemic stroke; the possibilities of combining fibrinolytics, such as anti- α 2-antiplasmin, with PAI-1 or TAFI needs to be investigated, as well as combining such pro-fibrinolytics with thrombectomy.

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Figure 1

Visual representation of the consensus recommendations regarding 1: Risk factors, biomarkers and plaque instability, 2: Circulating cells including platelets and atherothrombosis, 3: Coagulation proteases, fibrin(ogen) and thrombus formation, and 4: Preventive and acute treatment of atherothrombosis and arterial embolism; novel ways and tailoring?

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