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Thrombin Generation and Atherothrombosis: What Does the Evidence Indicate?

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Thrombin is a key enzyme in hemostasis and thrombosis, regulating pro- and anticoagulant reactions by interacting with other coagulation proteins and cellular receptors.[1] Thrombin also carries out a plethora of biologically relevant actions that link to other complex biological processes such as angiogenesis, inflammation, and cell proliferation.[2] Thrombin is therefore likely to be involved in cancer, chronic inflammatory diseases, atherosclerosis, and other diseases.

The capacity of blood to form thrombin is a critical determinant of hemostasis and according to Hemker's first law, a low amount of thrombin produced in clotting blood results in a bleeding risk, whereas high thrombin production translates into a risk of *venous* thrombosis.[3–5]

In case of *arterial* thrombosis, we distinguish thrombosis related to atherosclerosis (atherothrombosis) from arterial thromboembolism such as in atrial fibrillation. The role of the coagulation system in the latter follows from the efficacy of oral anticoagulants in preventing ischemic stroke. Here we want to focus on a less obvious question: What is the relation between thrombin generation and clinical manifestations of *atherothrombotic* disease?

The clinical manifestations of atherothrombosis evolve from ischemia and organ damage downstream of the thrombus, notably in the heart (myocardial infarction), the brain (ischemic stroke), and peripheral arteries (peripheral artery disease, PAD). That thrombin plays a role here is

strongly suggested by the observation that oral anticoagulation as well as heparin have a preventive action on the reoccurrence of myocardial reinfarction,[6,7] all the more as the effect superimposes on that of aspirin[8]; nevertheless, the role of thrombin remains rather equivocal. While straightforward risk associations between thrombin generation and thrombosis have been published, also by us, several other studies show inverse relationships (to be discussed below). The problem therefore merits reconsideration.

Before reviewing the available evidence, it is necessary to stress the fundamental difference between in vivo and in vitro thrombin generation.[9] In vivo thrombin generation (ie, the appearance of active thrombin inside the body) is to a limited degree a normal phenomenon; markers of thrombin activity, such as prothrombin fragment 1.2, thrombin-antithrombin (TAT) complexes, and forms of degraded fibrin such as D-dimers, are always detectable in blood.

In vitro thrombin generation (TG) is a test that probes the *capacity* of blood (plasma) to form thrombin. A completely normal person with a provoked thrombosis will show signs of in vivo increased thrombin generation while their capacity to form thrombin may be normal. A toddler with an antithrombin deficiency will have a high in vitro TG but no signs (yet) of excessive thrombin production in their body.

Atherothrombosis

Atherothrombosis forms on the basis of atherosclerotic lesions in 1 or more large arteries supplying the heart, brain, or other organs.[10] Rupture or erosion of an atherosclerotic lesion provokes a hemostatic reaction, which may occur repeatedly and which will not cause major damage to the affected organ unless an occlusive thrombus forms[11] that stops blood supply downstream and thus may result in myocardial infarction (MI), or ischemic stroke.

The plug forms through the interaction of platelets, plasmatic clotting factors, subendothelial proteins, and atheromatous debris, exposed upon the wounded atherosclerotic lesion. Platelets adhere to collagen and von Willebrand factor and the exposure of tissue factor (TF) in the lesion activates the plasma coagulation mechanism.[12,13]

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A role of thrombin on the progression, persistence, and stability of atherothrombosis is extremely likely (see above), but what is the precise role is an issue of long-lasting debate. Both Virchow and Rokitansky recognized the importance of both the fibrin-forming system and inflammation in atherosclerosis but they disagreed on the "primum movens," Virchow seeing inflammation as the first cause and Rokitansky clot formation (discussed in 14, 15). Current insights suggest that they were both correct in that atherosclerosis is to be recognized as a chronic inflammatory vascular disease in which coagulation proteases are actively involved, to become instrumental in its ultimate consequence: vascular occlusion due to the formation of thrombus.

In previous work and reviews, we and others have explored the molecular mechanisms that may lie at the basis of the interactions between atherosclerosis and thrombin formation.[16–18] Mendelian randomization studies as well as case–control studies show highly significant risk associations between different coagulation proteins and atherosclerosis as well as atherothrombotic complications. The effect size is small, however, and not always consistent.[16] The clinical relevance of these associations as well as the relevance of experimentally found causal effects remain under debate.

The interpretation of the beneficial effect of vitamin K antagonists is not necessarily unequivocal because these drugs have multiple effects: in the first place on the coagulation system itself, where they not only impair the function of procoagulant factors (II, VII, IX, X) but also that of the delimiters of thrombin generation proteins C and S.[19] Furthermore, they inhibit vascular vitamin K–dependent proteins such as Matrix Gla protein and osteocalcin, which, in experimental animals, results in rapid calcification of arteries. Recently the clinical analogue of this process has been recognized.[20] Last but not least, pathology studies suggest that the use of warfarin is an independent risk factor for plaque instability.[21]

In spite of all this, there is no evidence whatsoever that warfarin would actually increase the risk of myocardial infarction (MI) or stroke, on the contrary. Likely the effect of inhibition of thrombin generation outweighs the unfavorable effects of vitamin K antagonists, at least when stable and therapeutic intensity anticoagulation is achieved. This is corroborated by the observation that parenteral anticoagulation (12.500 IU unfractionated heparin once daily) with heparin also protects against reinfarction.[7] Different experimental studies show that application of the selective thrombin inhibitor dabigatran markedly reduces atherogenesis in susceptible mice (apoE−/− background) and beneficially affects the plaque phenotype.[22–25] Collectively, these studies strongly suggest that there exists a link between atherosclerosis and atherothrombosis on the one hand and thrombin on the other, a link that we thought timely to explore from a clinical perspective.

Literature Studies

We have not performed a formal systematic review, but we have sought papers that focused on in vivo thrombin generation via determination of fragment 1.2 or TAT complexes and/or on in vitro thrombin-generating capacity and their relation to atherothrombotic disease. We used the search terms "thrombin generation" and (arterial or atherosclerosis or thrombosis or atherothrombosis) in PubMed, updated until March 7, 2016. After manual screening on title and subsequent on abstract, the search narrowed down to 57 papers from a period starting in 1990. Although fibrinopeptide A and D-dimer indirectly reflect the presence of thrombin, we confined the search to direct measures of "thrombin activity" or methods of thrombin generation so as to avoid data influenced by fibrin and the fibrinolytic system.

Coronary Artery Disease

In vivo thrombin generation as judged by TAT, or F1.2 assay has been found to be increased in symptomatic coronary artery disease (CAD),[26–33] lasting for up to 2 years after the event,[32,34] as well as in patients with recurrent coronary events.[35] Some studies find it increased in unstable angina;[36] however, Cooper et al did not find significant associations between F1.2 and a first ischemic event.[37] van der Bom found no relation between TG markers and a history of CAD, although they observed a significant increase of D-dimer and a positive association between thrombin markers and D-dimer.[38]

An association between in vivo thrombin generation and severity of coronary vessel disease on the angiogram[29] or coronary calcification on the computed tomography scan[39] has been reported. Granger et al found that baseline F1.2 was related to risk of death or re-infarction at 30 days following acute MI.[31] Ardissino et al showed that both high and low F1+2 levels were associated with cardiac death or re-infarction in acute coronary syndrome patients.[40] Elevated TAT at baseline predicted death or re-percutaneous coronary intervention in ST-segment elevation myocardial infarction patients, whereas F1.2 only predicted events at 24 hours[41]; the data also tended to present a U-shaped relation between markers and major coronary events.

Several studies have shown a relation between age and sex and in vivo thrombin generation. Of the cardiovascular risk factors, F1.2 was associated with fibrinogen for both sexes and with factor VIIc in women in this population-based study.[42] TAT was associated with Lp(a) in a subgroup of patients experiencing acute MI in the early morning as compared to those with acute MI at other times.[43]

In summary, although it is evident from most studies that CAD is accompanied by thrombin activity in vivo, there is no certainty on the precise relations between the clinical picture and the extent of the phenomenon.

Ex vivo TG analysis was first reported in 2008, showing that it was faster, earlier, and higher in its peak in subjects with a history of acute coronary syndrome as compared to patients with stable CAD.[44] Positive correlations were found between peak of TG and fibrinogen, glucose, and C-reactive protein, but only in those with stable CAD, not in those with previous MI.[44] Differences between the populations, for instance, in the degree of hyperlipidemia, were suggested to be involved in some of these risk associations. Indeed, Olivieri et al found that Apo C-III was an independent risk factor for CAD and that it was associated with endogenous thrombin potential (ETP) and peak thrombin activity.[45] This was not the case for total cholesterol, low-density lipoprotein, triglycerides, and apoE, suggesting that Apo C-III may play a functional role in thrombin generation.

In a study by Smid et al, there was a 7% higher ETP and a 15% higher peak thrombin generation in acute MI patients than in controls. This could not be attributed to a lower activity of the activated protein C (APC) system but rather to higher levels of the procoagulant factors VIII and prothrombin.[46] A more or less U-shaped risk association between ETP and CAD was observed by Borissoff et al[39]; the lowest levels in ETP were found in those with mild CAD (115%) when severity was divided in quartiles based on degree of luminal stenosis. Interestingly, only subjects with severe CAD had a significantly increased ETP (average 130% of normal), whereas the increase in low-grade and moderate CAD did not reach statistical significance (averages 123% and 121%, respectively).[39]

Carcaillon et al did not find an association between history of CAD and increased TG, but they did find an association with acute ischemic coronary disease and high TG, more apparent in women than in men.[47] While ETP and peak were elevated, the lag time was slightly delayed in patients with a history of MI as compared to those without such history; the inhibitory effect of thrombomodulin, indicative of APC resistance, was not significantly different in these 2 subgroups. In those with symptomatic CAD, a low ETP (+ high D-dimer level) versus a high ETP (+ low D-dimer level) was predictive for recurrence (odds ratio 5.8 [95% CI 1.1–30.7]).[46] In young women with MI, an increased APC sensitivity was associated with risk (odds ratio 1.7); this apparent paradoxical effect was attributed to negative effects of tissue factor pathway inhibitor and/or protein S, but this remains speculative at this stage.[48]

A prolonged lag time and time to peak in patients with diabetes after acute coronary syndrome were related to recurrent ischemic events at 6 months.[49] In the LURIC study, the event-free survival in the quartile of individuals with the lowest ETP was much lower than those with the highest ETP (P=0.004).[50] In elderly subjects from the PROSPER study (on the effects of pravastatin), only an increased normalized peak height was significantly associated with incident coronary heart disease (hazard ratio 1.17 [95% CI: 1.06–1.28],

P=0.002).[51] In the selected population of survivors from in-stent thrombosis, TG was significantly increased with reduced APC sensitivity, as compared to those without recurrent stent thrombosis.[52] In this study there was evidence of increased contact activation as a contributing procoagulant factor.

In general, formation of thrombin and the potential to generate thrombin are altered in patients with CAD. As seen for in vivo thrombin formed, also in vitro TG has a tendency to be increased but the effects are not large and the precise patterns of changes remain enigmatic. In fact it is not clear whether CAD and high TG have a common cause (ie, atherosclerosis), or whether there is causal relationship between high TG and developing atherothrombosis and/or its complications. In view of the important role of platelets in thrombin generation and their well-recognized involvement in arterial thrombosis, it may be expected that studies on TG in platelet-rich plasma (or whole blood) may solve some of the abovementioned riddles.

Ischemic Stroke

In the studies considered for this review, it was not always clear whether ischemic stroke was due to atherosclerosis, small-vessel disease, a cardiac source, or another cause,[53] so we cannot provide any reliable information on the origin. In the early 1990s, oral anticoagulants were not yet routinely applied in patients with atrial fibrillation, such that the absence of anticoagulation does not indicate that the stroke was nonembolic. Taking these limitations into account, what do the available studies on patients with ischemic stroke tell us?

In 1993 Yamazuki et al established that TAT levels were elevated in patients with cardioembolic stroke versus patients with lacunar infarcts or controls.[54] Fibrinopeptide A was elevated in the acute and subacute phase of stroke, while only protein C antigen was lower in the acute phase, possibly associated with consumption of this inhibitor. Feinberg and colleagues tested coagulation activity in 1531 patients with atrial fibrillation on aspirin and observed that F1.2 was associated with age, female sex, systolic blood pressure, and heart failure but not with stroke.[55] In those with cardioembolic stroke, it appeared that F1.2 was minimally elevated (P=0.03). A subsequent study by Barber et al showed that F1.2 and TAT were elevated in progressive stroke but only D-dimer and arterial blood pressure appeared to be independent predictors of stroke.[56] Furie et al did not find a correlation between the level of F1.2 and type of stroke.[57] F1.2 was associated with large carotid artery plaques in stroke patients, not in controls, and those with large plaques and high F1.2 levels were at greater risk of recurrent stroke and death than those with larger plaques and lower F1.2 levels.[58] These data suggest that severity of atherosclerosis as well as its thrombogenic activity may have an impact on the risk of recurrent thrombotic

occlusions. Interestingly, F1.2 levels were associated with cognitive decline in a study by Stott et al, suggesting that not only stroke itself but also its consequences may in part depend on thrombin-mediated processes.[59] In summary, as in MI, there seems to be some association between in vivo thrombin generation and ischemic stroke.

In vitro thrombin generation analysis was applied in an older study where Faber et al found a significantly increased thrombin generation potential in the plasma of a subgroup of young stroke patients.[60] In more recent studies, after the clinical introduction of semi-automated methods, Carcaillon et al observed that ETP and peak height were positively associated with risk of acute stroke (hazard ratio 1.16 and 1.31, respectively, for 1 SD increase), more obvious in women.[47] In contrast, Loeffen et al[51] found a highly statistically significant but inverse association between ETP as well as peak height in a similar cohort study, albeit in older subjects than in the study by Carcaillon et al.

In the acute phase of stroke, peak height was elevated both in cardioembolic and nonembolic stroke subtypes[61] and peak and ETP were elevated in early symptomatic versus asymptomatic patients with carotid artery stenosis.

Among patients who had detectable microembolic signals, those with early symptoms had a shorter "time to peak" than the asymptomatic ones, which suggests that thrombogenicity enhances the symptoms caused by microemboli.[62] This important study suggests that the microemboli (or maybe smaller microvesicular material) from the diseased vessel wall do more harm when present in a thrombogenic phenotype. In this context, studies that show a link between intima-media thickness, markers of thrombin (F1.2), and risk of stroke are also of importance.[63,64]

In general, hypercoagulability, as witnessed by thrombin formation in vitro or in vivo, is likely to be one of the factors that link carotid artery plaque lesions to recurrent vascular complications (ischemic stroke). Risk associations between TG and microembolic signals as well as cognitive decline deserve more study in order to better appreciate these potentially important interactions.

Systemic Atherosclerosis in Peripheral Artery Disease (PAD)

Two studies published in 1993 addressed markers of thrombin in patients with PAD, showing a positive association between TAT and PAD[65] and elevations of both F1.2 and TAT in patients with PAD, however, not related to severity of atherosclerosis.[66] Heinrich et al and van der Bom and colleagues did not find significant associations of F1.2,[67] or F1.2 or TAT[38] with prevalent PAD, respectively. One published report shows that thrombin generation had a negative

association with patients that reached an atherosclerotic clinical end point (low ETP and future thrombotic event, odds ratio 11.7). These authors suggested that consumption of coagulation proteins in more severely affected patients could explain the negative risk association seen for thrombin generation,[68] but evidence supporting this assumption is lacking.

Biochemical Mechanisms Linking Thrombin to Atherothrombosis

Thrombin is the pivotal enzyme of hemostasis. It originates in a complicated network of reactions, full of checks and balances and once formed it displays a plethora of actions primarily leading to hemostasis and wound repair but with important interactions with inflammation and cell proliferation. Recent research from, for example, the Furie group has clearly shown that thrombin plays a role in the earliest phases of hemostasis. Within 12 s after a lesion has been made, fibrin can be seen, so that thrombin must have appeared even earlier.[69,70] This challenges the old dogma of primary and secondary hemostasis and makes us believe that it is not bizarre that thrombin plays a role in arterial thrombosis and primary plug formation. The same work shows, however, that a role is played by numerous other elements, notably platelet- and microparticle-related but also from endothelial- and subendothelial tissues. It may therefore not be expected that the capacity of the plasma to form thrombin is the only parameter that governs the process.

Among the multiple functions of thrombin, there are those that are immediately related to further thrombin production, such as activation of factors V, VIII, and XI. In addition, thrombin activates platelets through protease activated receptor (PAR) receptors and this provides critical input of activated platelets as a source of phospholipids to drive coagulation reactions, as well as release of additional procoagulant proteins (eg, factor V) and enzymes. Even fibrin, the main thrombin product, by activating platelets via von Willebrand factor, fosters further thrombin formation.[71,72]

Although the net effect of thrombin is prothrombotic, we should not forget that it has antithrombotic actions as well. An intriguing anticoagulant action of thrombin is the activation of protein C, via binding of thrombin to thrombomodulin at cell surfaces (endothelial cells and others). Theoretically, given the density of receptors, the microvascular bed is an important reservoir of functional thrombomodulin and it may also be the most potent source of APC generation to control local but also systemic pro-inflammatory (and procoagulant) forces. APC can subsequently act on PAR-1 in concert with endothelial protein C receptor, to protect the vasculature against inflammatory stimuli. Thrombin also acts on PAR-1 to

mediate more offensive actions, and the regulation of this delicate balance has been studied in detail over the past decades.[73] As a further example of an anticoagulant action of thrombin, we mention that the presence of minute amounts of thrombin, too low to make plasma clot, can lead to the formation of free factor Va (ie, not lipid bound), which is a potent inhibitor of the VII-TF complex.[74]

This regulated cellular network of pro- and anticoagulant thrombin-driven actions makes it imaginable that both low and high thrombin concentrations can be relevant in the pathophysiology of arterial vascular disease and atherothrombosis. Low amounts of thrombin would theoretically provide insufficient drive for PC activation, to protect against inflammation. Relatively high thrombin concentrations could overcome locally protective effects of APC to drive PAR-mediated cell signaling functions.

Intriguingly, the presumed cellular actions of thrombin assume that sufficient free thrombin is available in plasma. The coagulation mechanism is a potent force driving fibrin formation when needed (ie, in situations of bleeding when, locally, high concentrations of thrombin and fibrin are required). Systemically, any excess free thrombin will be rapidly quenched by AT and other inhibitors. Nevertheless, the reaction with AT takes place in free solution, so there must be a finite concentration of free thrombin in the plasma. On basis of the half-life time of the TAT complex and the reaction constant of its formation and the plasma concentration of AT, it can be calculated that, in order to maintain a given concentration of TAT, 0.025 times that concentration of free thrombin must be present in plasma. If this concentration is available for AT, it is also available for thrombomodulin and for making anticoagulant free factor Va (see above; 73). In real life, the situation will be more complicated than just chemical reactions because flow and diffusion will play their roles; nonetheless, there is no interaction of thrombin with any ligand without free thrombin. In the context of atherogenesis and atherosclerosis, TG is linked to early atherosclerosis, likely one of the drivers of this complex process.[75] This model is strongly supported by experimental studies.[22–25]

It should also be kept in mind that there is a form of thrombin, meizothrombin, that is enzymatically active but that remains bound to phospholipid surfaces and therefore can be carried by lipid membranes, on microvesicles or otherwise and in this form "escape" inhibitors. A second thrombin "protection" mechanism may simply be related to the localization of thrombin formation; when accelerated TG and fibrin formation are induced, such as at sites of bleeding or plaque rupture, the process of TG takes places in situ and the concentration at phospholipid surfaces may facilitate TG to proceed in a way where inhibitors such as AT do not easily get access. This protection has been reported a long time ago for the tenase and prothrombinase complexes (reviewed in 76),

but perhaps most of the formed thrombin also stays locally active. The reason that thrombin may not be scavenged immediately may be that atherothrombosis is a process that takes place in the context of a damaged atherosclerotic lesion, in which besides proteins many cells and microvesicles provide a tight cell–fibrin network that does not allow much free protein transfer in the first instance.

A third mechanism mediating TG reactions involves lipid components. The idea of connections between lipids and coagulation goes back to the work by Meade and colleagues showing elevated factor VII clotting levels in patients with CAD.[77] In particular, effects of a high-fat diet on lipids and coagulation activity have since been explored, showing increased factor VII postprandial activity, linked to total fat intake rather than triglycerides (summarized in 78). Whether or not the effects on factor VII activity accelerate TG remains questionable, also since factor VII does not emerge as an important determinant of TG in subsequent studies. From the present analysis, Apo-CIII and Lp(a) emerge as determinants of TG. Olivieri et al provide an explanation for the effect of Apo-CIII showing that elevated concentrations of Apo CIII are associated with an increase in thrombin activity to an extent comparable to the carriership of G20210A gene variant and modulating TG in subjects with or without CAD.[45] Direct effects of Lp(a) on TG have not been reported. However, Lp(a) is an independent risk factor for cardiovascular disease and is probably involved in many steps of atherogenesis. The pleiotropy of this lipoprotein extends its influence toward several serine proteases causing disturbances in blood coagulation.[79] The best known are probably linked to inhibition of fibrinolysis. Other lipid-related effects on TG may be mediated by high-density lipoprotein particles, which stimulate anticoagulant effects of APC and protein S.[80] Finally, phospholipid transfer protein transfers lipids between donors and acceptors (eg, from high-density lipoprotein to very low-density lipoprotein) and provides an anticoagulant action in plasma, by inhibiting generation of factor XIIa in the presence of very low-density lipoprotein.[81]

All these mechanisms acting together are certainly complex enough to form a "nonlinear system" (ie, a system in which, on a theoretical basis, it can be assumed that the relation between 1 concentration and the other of whatever pair of reactants may show jumps and irregularities that are dependent upon a third reactant in a manner that is not easy to predict). Notably, it is not surprising that the actions of thrombin can switch from protective (generating APC) toward more offensive functions that act in a prothrombotic and pro-atherogenic manner.

Finally, recent work suggests that the contact system (factor XII, bradykinin, and prekallikrein) may be a more important determinant of atherothrombosis than previously thought.[82] Several in vivo studies support the concept that the contact system, either directly or through factor

XI-mediated thrombin generation, boosts fibrin formation and the formation of a tight fibrin clot.[83–85] On the other hand, factor XIIa also drives part of the fibrinolytic pathway. The current development of specific inhibitors against factors XIIa, XIa, and maybe other contact proteins may help to unravel the role of these molecules in the near future.

Conclusions and Suggested Avenues for Research

In contrast to studies in venous thromboembolism, where the author's (Hemker) concept of "more thrombin is greater thrombosis risk" is readily confirmed (summarized in 86), the precise role of thrombin in atherothrombosis—though undoubtedly present—is hard to derive from clinical data. In the settings of arterial thrombosis, several issues must be considered. We tried to cope with the heterogeneity of the process by focusing on atherothrombosis. However, atherothrombosis also occurs in different vascular beds, and the differences in organ-related effects may be substantial. Some of the studies on stroke we reviewed include patients with cardioembolic stroke, clearly a distinct entity, also in terms of risk factors.

While the role of platelets and other cellular elements in atherogenesis and thrombosis goes undisputed, a role of coagulation proteins and in particular thrombin has long been doubted but is evident from the clinical and experimental body of evidence that we reviewed. However, for a better understanding of the precise mechanisms by which thrombin displays its many functions, further clinical and experimental studies will be necessary. Automated in vitro thrombin generation is a relatively new technique that can be expected to yield more information in the years to come. Thrombin generation in platelet-rich plasma—a very useful technique for the study of the interplay between platelets and clotting factors[87]—has not as yet been clinically explored to any significant extent. This seems pertinent now that millions of patients are being exposed to direct oral thrombin (formation) inhibitors (non–vitamin K oral anticoagulants)! The association between thrombin and atherothrombosis risk does not show a linear relationship, and this aspect alone makes it quite important to carefully observe what we are actually achieving with long-term inhibition of thrombin in vivo. Is this inhibitory effect similar for men and women, is it age dependent, dependent on cardiovascular disease risk factors, on lipid profiles, etc? Short- and medium-term follow-up did not reveal any unexpected side effects of non–vitamin K oral anticoagulants, but vascular effects may take a decade or so to be detected (see the history of warfarin-associated calcification). Given the limited knowledge of all biochemical interactions that thrombin may be engaged in, building a strong knowledge base on thrombin and cardiovascular disease seems of eminent importance.

Disclosures

ten Cate and Hemker are consultants to Diagnostica Stago. ten Cate received fees for lectures and ad hoc consultations from various diagnostic and pharmaceutical industries and is Chair of the board of the Dutch Federation of Anticoagulation clinics. He is a Fellow of the Gutenberg Research Foundation, Center for Thrombosis and Haemostasis, Gutenberg University, Mainz, Germany.

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