Markers of hypercoagulation in cardiovascular disease in the general population

Chapter 6: Summary and general discussion

Thrombin generation: from research to bedside

Plasma TGA as a laboratory test was first introduced by Biggs and McFarlane in the 1950s.[1] Over the years, Hemker et al. implemented substantial improvements and by 1993 the continuous assessment of thrombin generation was established.[2] However, this method was still time-consuming and it was only after the introduction of the CAT assay in 2003 that the TGA became more widely used in thrombosis and hemostasis research.[3] In recent years, more attention has been drawn to the TGA as a research tool in arterial thrombosis, such as MI, ischemic stroke and PAD along with VTE. In brief, the obtained data suggest that an enhanced TGA profile identifies a greater risk for VTE, whereas the results on arterial thrombosis are conflicting.[4] TGA analysis may have potential in evaluation of the severity of a bleeding phenotype as well as in the management of bleeding (risk), eg in hemophilia. Finally, the TGA has been useful in exploring mechanisms of thrombogenesis in conditions or diseases like obesity, diabetes mellitus and Cushing's disease.[5-7] Although the TGA is widely used as a research tool, its applicability as a clinical tool to assess a subject's risk of arterial and/or venous thrombosis remains to be addressed.

Increased endogenous thrombin in CVD and mortality

To address the respective question, we conducted an analysis of the TGA in a subsample of 5,000 individuals of the large-scale population-based GHS, to obtain reference values for the TGA as well as to explore associations with CVRFs, CVD and overall mortality (**chapter 2**). One of the main findings was that a high ETP, a parameter that is a net reflection of pro- and anticoagulant forces, is associated with an increased mortality risk of 1.5 (HR), independent of age, sex, pre-existing CVRFs and CVD. Despite the limitation of mortality data not being cause-specific, we may presume, considering the high prevalence of CVD in the general population, that most deaths were related to a cardiovascular cause.

When comparing ETP as a prognostic marker for mortality to other hemostatic markers such as fibrinogen, the HR was similar (HR: 1.55, 95%CI(1.25-1.93)).[8] However, it was conducted on a population of angiographically-proven CAD patients. Additional research, focusing on CVD-related deaths in the general population and patient population, is required to further elucidate the value of ETP for risk assessment in subjects at risk of, or with CVD.

Prolonged lag time as a biomarker of endothelial damage

TGA analysis in the GHS revealed interesting results on the lag time, being the time needed for the first traces of thrombin to be generated (**chapter 2**). A prolonged lag time (above the 95th percentile of the reference) was linked to a 1.46 times higher risk of death, regardless of age, sex, use of VKA, CVRFs and CVD. This seemingly paradoxical association remains unexplained so far; it may be postulated that the demonstrated relation between the two variables may be mediated through elevated levels of TFPI, a significant determinant of the lag time.[9] The link through TFPI may have a biological significance. For instance, elevated TFPI levels have been associated with risk factor for vascular endothelial damage, such as impaired glucose tolerance and type 2 diabetes mellitus.[10] TFPI, when released from damaged vascular endothelium, one of the main sources of this anticoagulant protein, would inhibit the effect of TF triggered TGA, reflected by a prolonged lag time. In support, Smid and colleagues found that a prolongation in lag time may be due to release of TFPI in patients with previous MI.[11] A similar finding was reported by Loeffen and colleagues, describing an association between prolonged lag time and increased all-cause mortality; in this case the effect was lost after adjusting for inflammatory markers, such as IL-6 and CRP, suggesting an even stronger relation with inflammation.[12] Both findings may be compatible with a condition in which both vascular endothelium activation and inflammation occur, representing a thrombo-inflammatory state, and are linked to a higher risk of cardiovascular morbidity and mortality.

In a recent paper on the clinical relevance of TGA analysis, the authors simplify certain applications of commercial TGA procedures, in particular with regards to arterial vascular disease.[13] As discussed quite extensively by ten Cate and Hemker, there is indeed quite abundant evidence for an association between plasma hypercoagulability and severity of CAD, documented by elevated levels of prothrombin F1+2 fragment or thrombin-antithrombin complexes. However, associations between such markers and (recurrent) thrombotic outcomes were not always consistent or linear and sometimes U shaped (reviewed in JAHA).[13] Similarly, ex vivo TGA analysis, such as with CAT assay, does not yet produce consistent associations with clinical outcomes. In particular, the lag time or time to peak may be paradoxically prolonged, although peak levels may be higher with higher risk of atherothrombosis. This raises the question of what the net effect is that is measured: delayed TGA with a higher maximal amplitude? And how does this translate to risk for thrombosis when considered at an individual level?

Thrombin generation, (anti-)coagulant proteins and CV

Thrombin generation is a multifaceted process, which involves numerous proteases. To better comprehend the previous associations of the TGA, we further delved into the mechanism of the driving force of thrombin generation. Hence, we set up an analysis of the biochemical determinants (i.e. natural coagulant and anticoagulant factors) of TGA in the context of cardiovascular-healthy subjects versus those with a history of arterial or venous disease (**chapter 3**). Interestingly, the analysis demonstrated that the natural coagulant and anticoagulant factors contribute to a greater extent to the TGA profile in subjects with a history of arterial or venous disease as compared to cardiovascular-healthy subjects. First, in line with previous reports, these results illustrate that individuals with pre-existing CVD are in a "hypercoagulable" state which is picked up by the TGA.[14] Second, in keeping with prior data, it suggests that the "coagulome" might be tuned to a "hypersensitive" state and thereby increasing the risk for recurrence.[15,16] Hence, the present study demonstrates the importance of considering the levels of the natural coagulation factors upon assessing TGA results.

Older studies already suggested that the plasma factor composition is a relevant factor in the TGA profile in patients with CAD.[17] This is true for the plasma proteins that make up the thrombogram, mostly coagulation factors. However, from previous studies we learned that in subjects with CVD, factors like glucose, C-reactive protein, apo C-III (but not other lipid fractions) were also determinants of ex vivo TGA.[18,19] The total effects of all such elements makes simple applications of TGA difficult in individual subjects.

TGA and personalized preventive treatment in thrombosis

From the previous paragraphs we can conclude that the TGA parameters ETP and lag time are of interest to further investigate in the context of CVD. The question remains how to approach this. Current literature on applications of TGA is substantial but mostly focused on comparison of groups of patients with controls or on following cohorts of patients in time. The latter approach may provide a feasible approach in individual patients. Ideally, one would obtain a thrombogram of a subject that is still healthy, as addressed in part of the GHS population. Following up TGA in these subjects, as soon as life changing events like MI or stroke occur, could give an indication of the TGA profile during disease, in the specific individual. Application of secondary preventive medication could show signs of "normalization" in such a person. That way more individualized profiles could be obtained. This approach may be comparable to what is implemented in some clinics for hemophilia patients. In correcting a bleeding diathesis, TGA may support the degree of correction of plasma factor product,

to such extent that normalization is achieved. Specifically in patients with high factor inhibitor levels such laboratory support is a promising tool.[20,21]

Our preliminary data suggest that TFPI is a main determinant of the lag time in TGA and as such, could be a surrogate marker for vascular endothelium activation and inflammation. If so, therapeutic intervention to decrease endothelial damage, targeted at a subset of subjects with a prolonged lag time, or an elevated TFPI level in plasma, could have value for reducing the risk of cardiovascular events. One way to investigate this hypothesis could be by organizing a proof of concept study in patients at risk for arterial thrombosis and with a prolonged lag time (as compared to age and sex matched healthy persons). Such patients could be randomized to receive an antiinflammatory drug (e.g. statin, colchicine), to dampen endothelial inflammation, or placebo, in a cross over design that may be sufficiently sensitive to detect subtle changes in individuals correcting also for time dependent effects. Such design has been applied in the past to assess effects of pravastatin on markers of activated clotting.[22] Whether the TGA analysis would have added value over simply measuring TFPI levels is another question and most likely the answer could only be obtained by correlating changes in TGA or TFPI in time with any changes in endothelial "fitness" probed by more or less specific proteins like vWF, soluble thrombomodulin or endothelin, and/or by functional analysis of endothelial function (e.g. induced vasodilation).

Biomarkers of coagulation in CVD risk assessment

CVD is the leading cause of death and disability globally and the (re)search for improving (therapeutic) prevention and individual risk assessment is gaining interest.[23] Currently, clinical risk factors are the basis of cardiovascular risk assessment. The Framingham Heart Study, which launched in 1948, started to identify common factors in CVD and resulted in the gender-specific algorithm 'Framingham Risk Score' or the adapted 'SCORE chart' in Europe, to assess the risk of (recurrent) cardiovascular risk[24,25], which is nowadays widely used in the outpatient clinic. Nonetheless, the current risk assessment is not optimal, as not all patients share a common pathophenotype and, therefore, should not be managed similarly.[26] Notably, inflammatory cytokines have been of interest as biomarkers in cardiovascular risk assessment. In an extensive meta-analysis of 29 population-based cohort studies, Kaptoge and colleagues found an adjusted relative risk for non-fatal MI and CAD of 1.25 (1.19-1.32) for IL-6, 1.13 (1.05-1.20) for IL-18 and 1.17 (1.05-1.25) for TNF-alpha.[27] In a randomized controlled trial, Lindholm et al. extracted a total of five readily available biomarkers and their prognostic value in CVD-(related mortality), most notably N-terminal pro-B-type natriuretic peptide (NT-proBNP), high sensitivity cardiac troponin T (hs-cTnT) and LDL. NT-proBNP and hs-cTnT had a greater prognostic value

than any other clinical parameter or biomarker, on the cardiovascular outcomes such as cardiovascular related death, non-fatal MI, non-fatal stroke and hospitalization for HF. Based on these results a biomarker-based model for prediction of cardiovascular death in patients with stable CAD was developed and included age (A), NT-proBNP, hs-cTnT and LDL-c (B), and the clinical variables (C) smoking, diabetes mellitus and PAD. Applying this biomarker-based ABC model was substantially better in identifying individuals at risk of cardiovascular death than a clinical variable-derived prediction model.[28] Thus, this novel prediction model, based on a small number of readily available biomarkers, can be widely applied in clinical risk assessment. In the Heart and Soul study, 1130 proteins were quantified from two cohorts of individuals with stable CAD, to identify prognostic proteins that improve cardiovascular risk assessment. In this study, the investigators identified 200 prognostic proteins for cardiovascular events, of which the majority were novel biomarkers. In addition, a 9-protein risk prediction model was established for the composite end points of MI, heart failure, stroke, and death, including troponin I (HR: 1.27 [95%CI: 1.18-1.37]), angiopoietin-2 (HR: 1.67 [95%CI: 1.53-1.82]), and matrix-metalloproteinase-12 (HR: 1.65 [95%CI: 1.50-1.80]). The results demonstrated that both the 9-Protein Model in itself as well as the combination of the 9-Protein Model with the standard Refit Framingham Models outperformed the traditional CVRFs and the Refit Framingham Model alone (c-indices, 0.71 vs. 0.64 in the validation cohort) in predicting a patient's risk.[29] In another study, the CASABLANCA study, a prediction model for major adverse cardiovascular events (MACE; death, MI and stroke) was developed in a high risk population (consisting of 649 participants in the derivation cohort and 278 in the validation cohort) referred for coronary angiography. The final model, including the four strong prognostic biomarkers NT-proBNP, kidney injury molecule-1, osteopontin, and tissue inhibitor of metalloproteinase-1, had a significantly better performance than the model with clinical variables alone, with an area under the receiver operating characteristic curve (AUC) of 0.79 ($p < 0.001$) in comparison to 0.75.[30] More recently, novel biomarkers such as angiotensin-converting enzyme 2 (ACE2) have come to the attention in the light of the COVID-19 pandemic. In general, the receptor for ACE2 is responsible for cardiac function and in addition, facilitates entry of the SARS-CoV-2 virus into the cell, which results in more severe COVID-19 infection with subsequently a poorer prognosis.[31] In a long-term large-scale prospective study, plasma concentrations of ACE2 were measured in 10753 individuals from five continents (Africa, Asia, Europe, North America, South America). Results showed that higher plasma concentrations of ACE2 were associated with higher risk of death (cardiovascular and non-cardiovascular), MI, stroke and heart failure, independent of age, sex, traditional CVRFs and NT-proBNP. However, prognostic implications and usefulness in risk assessment scores needs to be further addressed.[32] In conclusion, not only

identifying novel biomarkers but also implementing established, inexpensive and readily available biomarkers into prediction models may allow more accurate risk-based stratification and basis towards precision medicine. In this context, TFPI and vWF were studied in the current thesis.

TFPI: marker of endothelial damage

TFPI is a potent anticoagulant protein that mainly originates from vascular endothelial cells and partly also from platelets. The majority of TFPI is lipid-bound, only 10-20% of plasma TFPI circulates in free full-length form and has previously been recognized as the active, and biologically more important, anticoagulant in vitro.[33,34] In a large observational study, a lower functional TFPI level in patients with an unprovoked first time DVT was associated with a 10-fold increased risk for recurrent VTE.[35] Prior studies have documented positive associations of TFPI activity and incidence of MI in young women.[36] Given the apparent diverging risk associations, we aimed to clarify the link between total TFPI activity and CVRFs, CVD and mortality in **chapter 4**.
We found that a high total TFPI activity correlated with a higher risk of death, independent of age, sex, and cardiovascular risk profile. In addition, total TFPI activity was associated with CAD, despite age, sex and traditional CVRFs. However, after adjusting for lipid profile, the association was lost. Smoking was additionally associated with high total TFPI activity levels. These data suggest that the association of CAD with TFPI, a lipoprotein-associated anticoagulant, is mediated through low-density lipoproteins. This is the first study to discuss the total TFPI activity in relation to CVD and cardiovascular mortality, where increased levels of total TFPI activity may be a surrogate for endothelial cell damage.

Prior to this study, one might have anticipated that TFPI, an anticoagulant protein, would have been negatively correlated with CVD; the more coagulation would be inhibited, the lower the risk for cardiovascular events. However, the positive association may represent a proxy for endothelial dysfunction and/or dyslipidemia, rather than a primary risk factor for cardiovascular incidents. In case of atherosclerosis related to dyslipidemia, endothelial cell perturbation may contribute to release of TFPI from the extracellular matrix, which will increase the plasma levels of circulating total TFPI.[37] Endothelial dysfunction is a key mediator in atherosclerosis and may be precipitated by factors causing damage to the vascular endothelium, such as smoking, dyslipidemia and diabetes. Notably, endothelial dysfunction is an important prognostic factor in cardiovascular events.[38]

How could one translate these findings to the clinic? First, it is important to note that the results presented in the thesis mainly relate to arterial thrombotic diseases, rather than venous thrombosis. Second, TFPI was measured weeks, if

not years, after the acute cardiovascular event. Therefore, translating the use of TFPI measurements to the clinic would be appropriate in the chronic phase (outpatient clinic) rather than the acute phase (emergency department). One way one could visualize this, would be in the outpatient clinic of the internal medicine specialist, cardiologist or even the general practitioner. Usually, TFPI, as a marker of endothelial dysfunction, could be used to assess an individual's risk for (recurrent) cardiovascular incident, combined with the traditional CVRFs. However, given the usual substantial overlap in plasma TFPI levels between patients and controls determination of cut off levels for such biomarkers would be needed prior to implementation. In the routine laboratory.

In addition, TFPI -linking coagulation to endothelial activation- which is influenced by dyslipidemia, could play a role as a biomarker for treatment of CVRFs including systemic inflammation and dyslipidemia. The potential of reversing vascular inflammation was demonstrated by Ridker and colleagues.[39,40] Whereas the JUPITER trial demonstrated that the ‘‘residual inflammatory risk’’ was a more potent indicator for statin treatment, the CANTOS trial confirmed that by dampening chronic systemic inflammation by inhibiting the major inducer of the interleukin-6 pathway (interleukin-1beta) through Canakinumab, the risk of cardiovascular events decreased (HR: 0.85). Ultimately, similar research is required to elucidate the role of total TFPI in endothelial dysfunction or ‘‘residual inflammatory risk’’, by, for example, lowering LDL levels through a statin according to high total TFPI activity levels, rather than the actual cholesterol levels. Previous studies have demonstrated that lipid-modifying agents such as statins decrease the TFPI activity levels, caused by reduction of LDL-TFPI complexes.[40-42] These results likely represent normalization of the endothelium as a result of decreased TFPI levels, however it is debatable whether this affects the overall anticoagulant potency, since free TFPI was not affected by statin therapy.

VWF and blood coagulation

VWF, a multimeric glycoprotein in plasma, acts on hemostasis by facilitating platelet adhesion and by stabilizing coagulation F-VIII thereby optimizing its function to enhance fibrin formation. A deficiency in vWF contributes to a bleeding tendency (VWD; bleeding symptoms); in contrast, persistently elevated levels could theoretically drive a thrombotic tendency (atherothrombosis, venous thrombosis).[43] Conversely, the question whether low levels of vWF reduce the risk of CVD has been hardly addressed. Most of these studies investigating the latter question were set in a patient population, however, considering the wide variation of vWF within the population and the mild symptoms of VWD, exploring vWF as a continuous variable might result in different findings.[44,45] In short, the results in **chapter 5** revealed that relatively

low levels of vWF activity (<76.2%) resulted in a 0.6-fold lower risk of CVD, independent of age, sex, CVRFs and a history of CVD. After adjusting for levels of F-VIII, the association persisted. Whereas the link between blood group O and vWF has long been recognized, the exact mechanism is not known. Emerging recent data suggest that there is an enhanced clearance of vWF in subjects with blood group O, rather than a decreased synthesis.[46,47]

Although the current data shine new light on vWF as a potential target for novel therapeutic prevention of CVD or recurrence of cardiovascular events, we need to consider the adverse clinical consequences of lowering vWF levels. On one side, reducing levels of vWF will also diminish F-VIII levels thereby inducing a bleeding tendency. On the other side, reducing F-VIII levels to approximately 50% in those with levels above 100% and high CVD risk could be beneficial without apparent bleeding risk. Whether modification of concentrations or activity of vWF and its related impact on FVIII levels, is a feasible therapeutic strategy, remains questionable. Since the 90s, there has been an interest in anti-vWF therapy as antithrombotic therapy.[48] Recently, there have been studies investigating the effects of reducing levels of vWF through an anti-vWF aptamer after arterial thrombosis (acute MI and ischemic stroke), however, the effects of these agents in a setting of secondary prevention in a population at large needs to be addressed.[49,50]

General conclusion and future studies

In general, the results from the current thesis demonstrate that a higher ETP and a prolonged lag time are linked to an increased risk of death, most presumably cardiovascular related death. In addition, the results from this thesis show that increased TFPI levels were related to increased mortality as well as the prevalence of CAD, in which TFPI mag represent a proxy for endothelial dysfunction and/or dyslipidemia. Finally, the results pointed put an association between low levels of vWF and decreased risk of CVD, despite age, sex, CVRFs and levels of F-VIII. From this thesis and in particular the discussion of the thesis, new questions arise for which novel studies need to be undertaken, summarized as follows:

1. Are specific parameters of TGA analysis including lag time and ETP useful prognostic markers of CVD and CVD-related mortality? Can we use these parameters to improve cardiovascular risk stratification, tailoring preventive treatment, such as by anticoagulant or anti-inflammatory therapy?

2. Can we tailor lipid-modifying treatment to prevent CVD based on total TFPI activity levels, rather than only based on LDL levels?

3. Would integration of blood group into the cardiovascular risk model improve their diagnostic efficacy?

4. Would reducing vWF levels prevent or diminish rates of cardiovascular events? Or would it cause more harm by inducing bleeding tendency, by reducing F-VIII levels?