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Pauline van Paridon

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DISSERTATION

to obtain the degree of Doctor at Maastricht University
on the authority of the Rector Magnificus,
Prof. Dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public
on Thursday, June 9th 2022, at 10:00

by

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Born on September 11th 1994 in Deventer

ISBN 978-90-9036115-4
Layout and cover design: Wieke Willemsen
Printed by Print&Bind, Amsterdam

Cover image: The image presented is based on a satellite photo of a river near the city of Abadan, southwest Iran.

The studies presented in this thesis were conducted at the Center of Thrombosis and Hemostasis of the Johannes Gutenberg University Medical Center Mainz and the department of Biochemistry at the Maastricht University Medical Center.

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The research described in this thesis was supported by a grant of the Dutch Heart Foundation (DHF grant number: 2017SB015). Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

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List of abbreviations

ACE2	Angiotensin-converting enzyme 2
AF	Atrial fibrillation
APC	Activated protein C
ATC	Anatomical therapeutic chemical
BMI	Body mass index
CAD	Coronary artery disease
CAT	Calibrated automated thrombogram
CHF	Congestive heart failure
CI	Confidence interval
CVD	Cardiovascular disease
CVRFs	Cardiovascular risk factors
DVT	Deep venous thrombosis
ELISA	Enzyme-linked immunosorbent assay
ETP	Endogenous thrombin potential
F-	Coagulation factor
GHS	Gutenberg health study
HDL	High-density lipoprotein
HF	Heart failure
HR	Hazard ratio
HRT	Hormonal replacement therapy
IQR	Interquartile range
LDL	Low-density lipoprotein
LMWH	Low-molecular-weight heparin
MACE	Major adverse cardiovascular events
MI	Myocardial infarction
PAD	Peripheral arterial disease
PE	Pulmonary embolism
PPP	Platelet-poor plasma
SD	Standard deviation
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
VKA	Vitamin K antagonists
VTE	Venous thromboembolism
VWD	Von Willebrand disease
VWF	Von Willebrand factor
WPBs	Weibel-Palade bodies

Chapter 1: General introduction and outline of the thesis

Introduction

Cardiovascular disease (CVD) is the number one cause of death and disability worldwide.[1] Even more worrying, the scope of the problem is still expanding due to an increasing prevalence of cardiovascular risk factors (CVRFs) such as obesity, dyslipidemia, diabetes and hypertension in middle- and low-income countries.[2,3] Needless to say, the identification of high risk individuals to ensure timely prevention of cardiovascular events is of eminent importance. In the general introduction of this thesis, I will first discuss the coagulation system and pathogenesis of CVD. Hereafter I will further delve into the outline and aims of this thesis.

Coagulation system, arterial and venous thrombosis

Hemostasis, the physiological process to stop bleeding, is traditionally divided into primary and secondary hemostasis, although both occur simultaneously and are intertwined. Primary hemostasis involves the activation and aggregation of platelets through multifaceted processes, in which von Willebrand factor (vWF) plays an important role.[4] Following vascular injury vWF binds to exposed fibrillar collagen type I and type III. Once vWF is immobilized on the collagen layer, it then mediates hemostasis through binding of circulating platelets via specific platelet receptors.[5] Secondary hemostasis encompasses the process of the formation of a fibrin clot. The intact vascular endothelium has anticoagulant properties through the membrane proteins thrombomodulin and heparan sulfate proteoglycans, as well as via released tissue factor pathway inhibitor (TFPI). Upon injury of the vascular wall due to trauma, or rupture or erosion of an atherosclerotic plaque, subendothelial tissue factor (TF) is exposed to blood; TF binds the serine protease factor (F)-VII, forming a catalytic complex which binds and activates both F-X and F-IX. In turn, F-Xa activates, in presence of its cofactor F-Va, prothrombin into thrombin. Thrombin generates a soluble form of fibrin which then, under the influence of F-XIIIa, precipitates into an insoluble fibrin mesh at the site of injury.[6] After the initial fibrin formation, amplification of thrombin generation occurs as a result of positive feedback mechanisms. Therefore, more than 95% of thrombin occurs after the initial fibrin formation. Ultimately, thrombin also down-regulates the coagulation cascade by binding to thrombomodulin on endothelial cells which enhances the activation of protein C, thereby generating the anticoagulant activated protein C (APC). APC proteolytically inactivates F-VIIIa and F-Va, a process which requires cofactor protein S and additionally F-V. Another inhibitor of the coagulation system is TFPI, which directly inhibits the main initiator of the hemostatic cascade, the TF/F-VIIa complex and F-Xa.[7]

The hemostatic cascade is further down regulated by serine protease inhibitors; most notably antithrombin. Antithrombin directly inactivates thrombin, F-Xa, F-IXa and F-XIa, a reaction that is markedly enhanced in presence of glycosaminoglycans including the endogenous heparan sulfate.

To maintain adequate blood flow to the tissue, fibrinolysis occurs to proteolytically degrade the fibrin clot at the site of the injury. In this process, tissue plasminogen activator catalyzes the conversion of plasminogen into plasmin which cleaves and breaks down fibrin into fibrin degradation products including D-dimer fragments.

Hemostasis acts as protection from bleeding while maintaining blood flow. Normally, there is an equilibrium, while imbalances contribute to a risk of bleeding or thrombosis.[8] According to Virchow's triad, there are three mechanisms underlying thrombosis; stasis of blood, vessel injury and hypercoagulability.[9] Thrombosis is a condition which can be classified according to the vascular location into arterial and venous thrombosis and although traditionally seen as distinct conditions, there is overlap in both risk factors and underlying pathology.[10,11] Venous thromboembolism (VTE) most commonly manifests as deep venous thrombosis (DVT) in the lower extremities, complicated by pulmonary embolism (PE) (although the events may also occur independently). In terms of classification we speak of provoked VTE (risk factors consist of cancer, use of oral contraceptive, immobility, trauma, surgery, pregnancy and inherited or congenital thrombophilia), however most events are unprovoked.[12] As indicated, arterial thrombosis occurs in the arterial system, mostly as a result of atherosclerosis and clinically manifests as myocardial infarction (MI), ischemic stroke and/or peripheral arterial disease (PAD). Alternatively, arterial thromboembolism occurs in conditions including atrial fibrillation (AF), where altered atrial flow dynamics and endothelial changes precipitate thrombi that may dislodge and transfer to the brain: embolic stroke. Arterial thrombi predominantly consist of platelets, whereas venous thrombi are rich in red blood cells and fibrin. While fibrin also consist in arterial thrombi, it predominantly plays an important role in venous thrombi.[13]

Atherosclerosis and atherothrombosis

Many of the manifestations of CVD result from atherothrombosis due to erosion or rupture of an atherosclerotic plaque in the large arteries of the brain (stroke) or heart (MI). In specific settings, such as, PAD, impaired blood flow as a result of atherosclerotic stenosis of arteries in the extremities is a first clinical sign of systemic atherosclerosis with secondary atherothrombotic complications as main cause of morbidity and death.

Atherosclerosis is a chronic inflammatory process as a consequence of long-term endothelial dysfunction and damage inflicted by risk factors such as smoking, hypercholesterolemia and hypertension. Endothelial perturbation triggers inflammation and leukocyte migration into the arterial vessel wall. Subsets of leukocytes, including neutrophils and particularly mononuclear cells attract more leukocytes and monocytes through secretion of chemokines and cytokines. The monocytes that transmigrate into the vascular wall differentiate into macrophages which then engulf modified lipoproteins (oxidized LDL). These cells, commonly known as foam cells, produce chemo-attractants that promote the proliferation of smooth muscle cells which eventually forms a fibrous cap. The end product is either a stable fibrous fatty streak or an unstable plaque, depending on the inflammatory burden.[14]

Biomarkers for thrombotic risk assessment

As previously discussed, arterial and venous thrombotic disease are two different disease entities with distinct pathophysiology, but also with shared commonalities. Considering that this thesis mainly focuses on the risk assessment of arterial thrombotic disease, I will not further discuss the biomarkers that are investigated in the context of venous thrombotic risk assessment. Hitherto, risk assessment for arterial thrombotic disease relies for the majority on identifying individuals at risk by clinical risk factors, such as hypertension, diabetes and hypercholesterolemia; many subjects will have one or more CVRFs. Up until now, literature on biomarkers in CVD report conflicting findings: whereas some reported biomarkers aid in risk prediction, others show not only that the impact of systemic markers of inflammation and/or coagulation for the prediction of CVD risk is modest, but also that their use does not have therapeutic consequences. [15-22]

Thrombin generation assay

Thrombin generation is a pivotal process in clot formation and its analysis may be potentially beneficial in estimating bleeding or thrombosis risk. The continuous assessment of generation of thrombin was first introduced by Hemker et al. in 1993.[23] At that time, TG assays (TGA) were time-consuming: it took a skilled laboratory technician one hour to complete measuring one sample. The efforts from Hemker and colleagues led to the establishment of the calibrated automated thrombogram (CAT) in 2003.[24] The CAT employs a slow-reacting fluorogenic substrate that enables for the continuous measurement of TGA. Thrombin activity is calculated as a function of time

by comparing the fluorescent signal from the thrombin-generating sample to that from a known stable concentration of thrombin activity measured simultaneously in a parallel sample. TGA parameters are derived from the TGA curve and include lag time (time to minimum thrombin formed [min]), peak height (the maximum amount of thrombin formed [nM]) and endogenous thrombin potential (ETP or area under the curve [nM.min]). Several studies have shown that TGA is a potential indicator of thrombotic or hemorrhagic tendency.[25–28]

Despite its potential in a diagnostic setting, the clinical validation and implementation of the TGA are still pending. One missing element is the analysis of reference ranges in a larger population and this is one of the reasons for testing the CAT assay in the Gutenberg Health Study (GHS).

Gutenberg Health Study

The GHS is a prospective population-based study which started in 2007 in the area of the Rhein-Main in mid-west Germany. It was designed primarily to improve the individual cardiovascular risk evaluation by taking into account traditional, psycho-social, socio-economic, environmental and life style risk factors. In addition, GHS aimed to investigate development and progression of CVD, but also metabolic disorders, pulmonary, psychosomatic, ophthalmological, auditory, and dermatological diseases, cancer as well as disorders of the immune and coagulation system and to improve diagnosis, prognosis, therapy and prevention of these diseases.[29] At baseline, 15,010 individuals between the age of 35-74 years underwent a standardized 5-hour clinical examination to collect data on the participant's (medical) background, psychosocial factors, laboratory parameters and to investigate the extent of subclinical disease by using standardized medical measurements of systems. Moreover, a biobank was established to perform future laboratory and genetic analyses. After 2.5 years, participants are contacted for a computer-assisted standardized telephone interview with assessment of endpoints. After 5 years, participants are invited for a detailed follow-up examination comparable to the visit at study inclusion in the study center. Primary endpoints of the GHS include MI and cardiac mortality. Secondary endpoints include the occurrence of ischemic stroke, diabetes mellitus, heart failure (HF), AF and all-cause mortality.

One of the main aims of the GHS is to identify new clinically relevant risk factors for CVD. Therefore, blood samples are taken from the study participants to measure a variety of components of the coagulation system in addition to standard laboratory measurements.

Outline of the thesis

This thesis focuses on the coagulation markers and assays as markers for risk of CVD. Hence it is divided into 2 parts, each focusing on a specific part of the coagulation cascade. The first two chapters centre around the TGA measured in the first 5,000 participants of the GHS. **Chapter 2** discusses the sex-specific reference values of the TGA parameters derived from a cardiovascular healthy subpopulation. In addition, this chapter reports on the relation between TGA parameters and CVRFs, CVD and all-cause mortality. **Chapter 3** addresses the biochemical (i.e. natural coagulation and anticoagulant factors) determinants of the TGA within cardiovascular healthy individuals as compared to individuals with a history of arterial or venous thrombosis. The second part of this thesis, chapter 4 and 5, focuses on TFPI, a natural anticoagulant, and vWF, a key protein in the haemostatic process. **Chapter 4** explores the relation between TFPI activity levels and CVRFs and CVD. **Chapter 5** addresses whether low vWF antigen and activity levels protect against CVD.

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Part 1: Thrombin Generation

Chapter 2: Thrombin Generation in Cardiovascular Disease and Mortality - Results from the Gutenberg Health Study

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Haematologica, 2020

Abstract

Background

TGA may be a potential tool to improve risk stratification for CVD. This study aims to explore the relation between TGA and CVRFs, CVD and total mortality. For this study, N=5000 subjects from the population-based GHS were analyzed in a highly standardized setting.

Methods

TGA was assessed by the CAT method at 1 and 5 pM TF trigger in platelet-poor plasma (PPP). Lag time, ETP and peak height were derived from the TGA curve. Sex-specific multivariable linear regression analysis adjusted for age, CVRFs, CVD and therapy, was used to assess clinical determinants of TGA. Cox regression models adjusted for age, sex, CVRFs and vitamin K antagonists (VKA) investigated the association between TGA parameters and total mortality.

Results

Lag time was positively associated with obesity and dyslipidemia for both sexes ($p<0.0001$). Obesity was also positively associated with ETP in both sexes ($p<0.0001$) and peak height in males (1 pM TF, $p=0.0048$) and females ($p<0.0001$). Cox regression models showed an increased mortality in individuals with lag time (1 pM TF, hazard ratio (HR)=1.46, [95% CI: 1.07; 2.00], $p=0.018$) and ETP (5 pM TF, HR = 1.50, [1.06; 2.13], $p=0.023$) above the 95th percentile of the reference group, independent of the cardiovascular risk profile.

Conclusion

This large-scale study demonstrates traditional CVRFs, particularly obesity, as relevant determinants of TGA. Lag time and ETP were found as potentially relevant predictors of increased total mortality, which deserves further investigation.

Introduction

Thrombin generation is one of the key enzymatic processes that direct the activity of the hemostatic system and coagulation cascade up to and including the formation of a fibrin clot.[1] Physiologically, thrombin formation is essential to maintain hemostasis and bleeding tendencies are associated with reduced thrombin (and hence fibrin) formation.[2] An enhanced plasma potential to generate thrombin has been linked to an increased risk of VTE, while the associations with arterial vascular disease are still inconsistent.[3-8] The TGA is an important method addressing the overall potential of a plasma sample to form thrombin. More than 95% of TG occurs after initial formation of fibrin, so routine diagnostic coagulation tests, such as prothrombin time and activated partial thromboplastin time fail to reproduce this overall potential. Hence, there is a strong research interest in TGA as a promising diagnostic tool for hypo- and particularly hypercoagulability phenotypes.[9]

In a study of healthy individuals, fibrinogen, F-XII, free TFPI and antithrombin have been identified as major determinants of TGA parameters.[10] Relation to demographic characteristics, such as age and sex, has been previously addressed but the studies have been small and results are not entirely consistent.[11,12] As TGA analysis is a promising tool to estimate a subject's risk for thrombosis or more broadly CVD, it is of eminent importance to fully understand the nature and direction of effects of CVRFs.

Hence, we undertook the present investigation in the first 5,000 participants of the population-based GHS. The primary aim of this study was to investigate CVRFs and CVD as major clinical determinants of increased TGA in a large population-based sample. Additional aims were to obtain age- and sex-related reference values for TGA parameters in a representative subsample of adults who were healthy from a cardiovascular point of view. Finally, having prospective data on total mortality allowed us to investigate the relation between TGA parameters and all-cause mortality.

Methods

Research design

The GHS, a population-based, prospective, observational, single-center cohort study in the Rhine-Main region in Western Mid-Germany, was designed to improve the individual risk prediction of CVD. At baseline examination, the study included a total of 15,010 individuals. The sample was drawn randomly from governmental local registry offices in the City of Mainz and the district of Mainz-Bingen and was stratified 1:1 for sex and residence (urban and rural) and in equal strata for decades of age. Individuals between 35 and 74 years of age were enrolled, and written informed consent was obtained from all participants for clinical examinations, laboratory analyses, sampling of biomaterial and use of data records for research purposes. As part of the study, every participant underwent a comprehensive, highly standardized 5-hour clinical examination program. In addition to the clinical assessment, a large biobank has been established for biochemical and genetic analyses. Further details of the study protocol and purpose are discussed elsewhere.[13]

The study was designed in accordance with the tenets of the revised Helsinki protocol, and the protocol and sampling design were approved by the local ethics committee. The sampling design was additionally approved by local and state data safety commissioners.

Study sample and reference sample

The study sample consisted of the first 5,000 subjects enrolled into the GHS between April 2007 and October 2008. After excluding subjects without biomaterial available or without complete TGA assessment (one or several TGA parameters missing), 4,843 individuals were successfully included in the overall study sample for the present analysis.

The reference group was defined as subjects apparently healthy from a cardiovascular point of view, without a history of CVD (MI, congestive heart failure (CHF), coronary artery disease (CAD), VTE, AF or PAD), presence of CVRFs (obesity, dyslipidemia, arterial hypertension, diabetes mellitus) or use of antithrombotic agents, oral contraceptives or hormonal replacement therapy. In addition, individuals with a self-reported history of inherited coagulation abnormalities were excluded from the reference sample. A detailed definition of traditional CVRFs and categorization of medications are provided in **Appendix: Supplemental Material**.

Clinical assessment and laboratory measurements

Clinical examination and determination of CVRFs were performed as published elsewhere.[14,15] Standard laboratory measurements were carried out at the Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Mainz, Germany. Venous blood sampling was performed according to standard operating procedures in lying position and the blood was collected in trisodium citrate (0.109 M, 1:9 vol:vol) monovette plastic tubes, while the subject was in fasting state (i.e. overnight fast, if subject was examined before 12 p.m. and 5 hour fast, if subject was examined after 12 p.m.). PPP was prepared by 10 minutes centrifugation at 2,000 x *g* at room temperature, aliquoted and immediately stored at -80 °C. . TGA was assessed according to the recommendations[16] for the CAT assay (Thrombinoscope BV, Maastricht, the Netherlands) in PPP. The TGA was triggered by 1 pM TF with 4 µM phospholipids at 20:20:60 mol% hosphatidylserine/phosphatidylethanolamine/ phosphatidylcholine, or 5 pM TF with 4 µM phospholipids. Trigger reagents were selected for commercial availability, e.g. PPP Reagent and PPP Low Reagent. The CAT method employs a low affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC) in order to monitor thrombin activity continuously in clotting plasma. TGA measurements were calibrated against the fluorescence curve obtained in a sample from the same plasma (80 µL), supplemented with a fixed amount of thrombin-a 2-macroglobulin complex (20 µL of Thrombin Calibrator; Thrombinoscope BV, Maastricht, the Netherlands) and 20 µL of the fluorogenic substrate and calcium chloride mixture.[16] TGA parameters were derived from the TGA curve and include lag time (time to minimum thrombin formed, in min), peak height (the maximum amount of thrombin formed, in nM) and ETP (area under the curve, in nM.min). All samples were tested as one batch using one batch of reagents within a period of 6 months. Two technicians performed the analyses on three validated systems and normal pooled plasma was included in each assay run for in-house quality control according to our ISO9001 certification (Coagulation Profile BV, Maastricht, the Netherlands).

Data management and statistical analysis

A central data management unit conducted quality control on all data. For presenting the distribution of TGA parameters, means with standard deviation (SD) were calculated for ETP and peak height whereas medians with the interquartile range were used for the lag time, due to its skewed distribution. P-values for means were calculated by t-test and for medians by Mann-Whitney test. Linear regression analysis performed on the overall study sample, exploring clinical determinants of TGA parameters, was stratified by sex and adjusted for age, CVRFs, CVD and use of the following medications: VKA, oral

contraceptives and hormone replacement therapy (HRT). Due to a skewed distribution, lag time, as a dependent variable, was log-transformed prior to further analysis. Estimated regression coefficients are presented with corresponding 95% confidence interval (CI). Considering the explorative character of the analysis, a significant threshold was not defined for p-values. P-values were interpreted as continuous measure of statistical evidence. Multiple linear regression models, exploring associations of TGA parameters and medications in the study sample, were adjusted for age, sex, CVRFs and CVD. Analysis of prospective mortality data with censoring, performed on the overall study sample, is presented by Kaplan-Meier curves and by three Cox proportional hazards models, excluding individuals using oral contraceptives or HRT from the latter. The first Cox regression model was adjusted for age, sex and VKA, where the second and third models were additionally adjusted for CVRFs and CVD, respectively. Statistical analysis was performed with software program R, version 3.3.1 (http://www.R-project.org).

Results

Study sample and reference subsample characteristics

While there was a balanced sex ratio in the overall study sample, there was a slight preponderance of women (55.3%) in the reference subsample. The median and interquartile range (IQR) of age of the study sample was 56 years (IQR, 46-66) in males and 55 years (IQR, 45-64) in females. The reference sample included 1,210 subjects, of whom 541 (44.7%) were male and 669 (55.3%) female.

The median age of the reference sample was 47 years (IQR, 42-55) in males and 48 years (IQR, 41-54) in females. In the study population, hypertension was the most prevalent CVRFs, being present in 56.6% of the male population and 46.1% of the female population, followed by dyslipidemia. Antithrombotic agents were taken by 15.9% of males and 9.4% of females. Among females, 6.4% were taking oral contraceptives and 12.3% hormone replacement therapy. Detailed characteristics of the study population and reference sample are presented in **Supplementary Table S1**.

TGA reference values and parameters in the overall study sample

The results of the TGA parameters in males and females from the study sample and reference subsample (reference values) are shown in **Table 1**. Females presented with a shorter lag time at 1 pM TF and 5 pM TF ($p<0.0001$ for both), higher ETP at 1 pM TF ($p<0.0001$) and higher peak height at 1 pM TF ($p=0.014$) and at 5 pM TF ($p<0.0001$) compared to males from the reference subsample. In the study sample, females presented with a shorter lag time at 1 pM TF ($p<0.0001$), as well as higher ETP and peak height at both 1 pM TF and 5 pM TF ($p<0.0001$ for both), compared to males.

Table 1. Parameters of thrombin generation in the reference subsample and the study sample

	Reference subsample (n=1,210)				Study sample (n=4,843)				
	Male (n=541)	Female (n=669)	p-value	Male (n=2,471)	Females without OC (n=2,218)	Females with OC (n=151)	p-value
Lag Time 1 pM TF [min]	5.07 (4.67/5.67)	4.67 (4.33/5.33)	<0.0001	5.33 (4.74/6.07)	5 (4.40/5.67)	4 (3.40/4.33)	<0.0001
ETP 1 pM TF [nM.min]	1047 (216)	1099 (203)	<0.0001	1068 (267)	1115 (260)	1491 (308)	<0.0001
Peak Height 1 pM TF [nM]	108 (51)	115 (48.7)	0.014	113 (51.9)	117 (51)	201 (63)	<0.0001
Lag Time 5 pM TF [min]	2.67 (2.33/3.00)	2.39 (2.33/2.67)	<0.0001	2.67* (2.40/3.00)	2.67* (2.33/3.00)	2.06 (2/2.33)	<0.0001
ETP 5 pM TF [nM.min]	1322 (196)	1318 (212)	0.71	1352 (267)	1370 (266)	1661 (350)	<0.0001
Peak Height 5 pM TF [nM]	236 (52.2)	259 (53.3)	<0.0001	238 (59.3)	257 (61)	365 (71)	<0.0001

Presented are thrombin generation parameters at 1 pM and 5 pM tissue factor (TF) in the reference subsample and study sample. Medians (interquartile range) of lag time and means (standard deviation) of ETP and peak height are presented. *Due to equal ties, the median values in males and females are the same, however, the distribution of the lag time values is different in males and females.

Clinical determinants of TGA in the overall study sample

As shown in **Table 2**, age was associated with longer lag time, both in males (**Table 2A**) and females (**Table 2B**), at 1 pM TF (males: p=0.014; females: p<0.0001) and at 5 pM TF (**Supplementary Table S2A, B**). In males, age was positively associated with ETP at 1 pM TF (**Table 2A**) and peak height at 1 pM TF (**Table 2A**) and at 5 pM TF (**Supplementary Table S2A**). In contrast, in females, age was associated with lower ETP at 1 pM TF (p=0.015) and lower peak height at 5 pM TF (**Supplementary Table S2B**).

Of the various CVRFs considered, obesity showed a positive association with lag time (males: p<0.0001; females: p<0.0001), ETP (males: p<0.0001; females: p<0.0001) and peak height (males: p=0.0048; females: p<0.0001) at 1 pM TF. Dyslipidemia was positively associated with lag time in both males (p<0.0001) and females (p<0.0001) and with ETP in males only (p=0.0057) at 1 pM TF. Similar findings for both obesity and dyslipidemia were observed at 5 pM TF as shown in **Supplementary Table S2A, B**. No associations were found for TGA and history of CVD.

Therapeutic agents and TGA parameters in the overall study sample

Females using oral contraceptives or hormone replacement therapy presented with shorter lag time, higher ETP and peak height at both 1 pM TF (**Table 2B**) and 5 pM TF (**Supplementary Table S2B**).

Use of VKA reduced TGA as shown by prolonged lag time, and reduced ETP and peak height (**Table 2A, B**). These effects were detectable at 1 pM TF in both males (p<0.0001; ETP: p<0.0001; peak height: p<0.0001) and females (p<0.0001; ETP: p<0.0001; peak height: p<0.0001;). Similar results were obtained at 5 pM TF (**Supplementary Table S2A, B**).

Results from multiple linear regression analysis for TGA parameters at 1 pM TF and at 5 pM TF, demonstrated associations between other medications, in addition to VKA, and TGA as shown in **Table 3A, B**. The lag time at 1 pM TF was positively associated with intake of cardiac drugs (p<0.0001), diuretics (p=0.00043), anti-gout preparations (p=0.00038) and immunosuppressants (p=0.00021), and inversely associated with hormone- containing drugs (i.e., hormone replacement therapy and oral contraceptives, p<0.0001). Differently, ETP was inversely associated with cardiac drugs, ATC code C01 (p<0.0001) at 5 pM TF and positively with hormone-containing drugs (p<0.0001). Peak height showed a positive association with hormone-containing drugs (p<0.0001). The results at 5 pM TF were comparable to the results at 1 pM TF.

Table 2B. Multivariable linear regression in the overall study sample for parameters of thrombin generation in females at 1 pM tissue factor (TF)

Variable	Females (N)	Log(lag time [min])			ETP [nM.min]			Peak height [nM]		
		beta	95% CI	p-value	beta	95% CI	p-value	beta	95% CI	p-value
Age [10y]		0.0410	(0.0332/0.0488)	<0.0001	-15.5	(-28.0/-2.99)	0.015	0.657	(-1.90/3.22)	0.62
Diabetes	123	-0.00149	(-0.0342/0.0312)	0.93	-34.2	(86.8/18.3)	0.20	2.24	(-8.54/13.0)	0.68
Obesity	542	0.0587	(0.0411/0.0763)	<0.0001	110	(81.9/139)	<0.0001	11.7	(5.86/17.5)	<0.0001
Smoking	415	0.0222	(0.00372/0.0408)	0.019	-17.3	(-47.1/12.4)	0.25	-7.23	(-13.3/-1.12)	0.020
Hypertension	1,092	0.0214	(0.00582/0.0370)	0.0072	-14.7	(-39.8/10.4)	0.25	-4.76	(-9.90/0.390)	0.070
Dyslipidemia	517	0.0429	(0.0254/0.0604)	<0.0001	24.5	(-3.63/52.6)	0.088	-2.02	(-7.80/3.75)	0.49
FH of MI/stroke	924	-0.0089	(-0.0233/0.00546)	0.22	5.08	(-18.0/28.1)	0.67	1.50	(-3.24/6.23)	0.54
History of MI	35	-0.0273	(-0.099/0.0445)	0.46	-19.2	(-135/96.2)	0.74	8.04	(-15.6/31.7)	0.51
History of stroke	35	-0.0826	(-0.149/-0.0159)	0.015	105	(-2.54/212)	0.056	21.2	(-0.799/43.2)	0.059
History of CAD	50	0.0336	(-0.0178/0.0850)	0.20	-46.4	(-129/36.1)	0.27	3.34	(-13.6/20.3)	0.70
History of AF	35	-0.0320	(-0.106/0.0417)	0.39	104	(-14.8/222)	0.086	20.3	(-3.95/44.6)	0.10
History of PAD	93	0.0390	(0.00303/0.0749)	0.034	-25.1	(-82.9/32.6)	0.39	-3.96	(-15.8/7.89)	0.51
History of VTE	124	-0.0348	(-0.0687/-0.000971)	0.044	44.4	(-10.0/98.8)	0.11	1.70	(-9.47/12.9)	0.77
History of CHF	36	-0.0588	(-0.13/0.0122)	0.10	-3.77	(-118/110)	0.95	-9.50	(32.9/13.9)	0.43
History of cancer	236	0.00886	(-0.015/0.0327)	0.47	-33.6	(-71.9/4.75)	0.086	-3.68	(-11.5/4.19)	0.36
Hormone replacement therapy	223	-0.0457	(-0.0669/-0.0246)	<0.0001	60.1	(26.2/94.1)	0.00053	14.3	(7.28/21.2)	<0.0001
Oral contraceptives	151	-0.145	(-0.175/-0.116)	<0.0001	379	(331/427)	<0.0001	91.1	(81.3/101)	<0.0001
Vitamin K antagonists	292	0.816	(0.743/0.889)	<0.0001	-751	(-869/-633)	<0.0001	-92.1	(-116/-67.9)	<0.0001

Multivariable linear regression models were calculated in the overall study sample for each parameter of thrombin generation as dependent variable separately. The analysis was adjusted for age, vitamin K antagonists, oral contraceptives, hormone replacement therapy, CVRFs and CVD.

Table 2A. Multivariable linear regression in the overall study sample for parameters of thrombin generation in males at 1 pM tissue factor (TF)

Variable	Males (N)	Log(lag time [min])			ETP [nM.min]			Peak height [nM]		
		beta	95% CI	p-value	beta	95% CI	p-value	beta	95% CI	p-value
Age [10y]		0.0098	(0.00199/0.0175)	0.014	28.9	(17.9/39.9)	<0.0001	7.60	(5.23/9.97)	<0.0001
Diabetes	242	-0.0318	(-0.0593/-0.00441)	0.023	-6.00	(-45.0/33.0)	0.76	5.16	(-3.21/13.5)	0.23
Obesity	628	0.0392	(0.0214/0.0570)	<0.0001	54.7	(29.5/80.0)	<0.0001	7.83	(2.40/13.3)	0.0048
Smoking	515	0.0151	(-0.00328/0.0334)	0.11	18.2	(-7.82/44.3)	0.17	-1.64	(7.24/3.96)	0.57
Hypertension	1,395	0.0135	(-0.00263/0.0297)	0.10	-6.70	(29.7/16.3)	0.57	-3.22	(-8.15/1.72)	0.20
Dyslipidemia	917	0.0428	(0.0275/0.0580)	<0.0001	30.6	(8.96/52.3)	0.0057	1.39	(-3.27/6.04)	0.56
FH of MI/stroke	853	-0.00624	(-0.0217/0.00919)	0.43	17.1	(-4.84/39.0)	0.13	-0.283	(-4.99/4.42)	0.91
History of MI	116	0.0263	(-0.0193/0.0719)	0.26	-57.5	(-122/7.32)	0.082	-8.36	(-22.3/5.56)	0.24
History of stroke	57	0.0352	(-0.0169/0.0874)	0.19	-39.7	(-114/34.4)	0.29	-2.67	(-18.6/13.2)	0.74
History of CAD	167	-0.0306	(-0.0674/0.00617)	0.10	25.7	(-26.6/77.9)	0.34	1.21	(-10.0/12.4)	0.83
History of AF	93	0.0418	(-0.00183/0.0854)	0.061	-19.5	(-81.4/42.4)	0.54	-6.31	(-19.6/6.99)	0.35
History of PAD	107	0.0367	(-0.00267/0.0761)	0.068	-53.0	(-109/2.95)	0.064	-10.6	(-22.6/1.40)	0.083
History of VTE	71	-0.0342	(-0.0794/0.0111)	0.14	-4.91	(-69.2/59.4)	0.88	-1.44	(-15.3/12.4)	0.84
History of CHF	39	-0.00240	(-0.0652/0.0604)	0.94	-15.1	(-104/74.0)	0.74	5.46	(-13.7/24.6)	0.58
History of cancer	194	-0.000508	(-0.0284/0.0273)	0.11	0.764	(-38.8/40.3)	0.97	-2.11	(-10.6/6.39)	0.63
Vitamin K antagonists	391	0.646	(0.0589/0.702)	<0.0001	-716	(-796/-637)	<0.0001	-66.3	(-83.5/-49.2)	<0.0001

Multivariable linear regression models were calculated in the overall study sample for each parameter of thrombin generation as dependent variable separately. The analysis was adjusted for age, vitamin K antagonists, CVRFs and CVD.

Table 3B. Multiple linear regression analysis of drugs in study sample at 5 pM TF

	Log(lag time [min])			ETP [nM.min]			Peak height [nM]		
Drug	**beta**	**95% CI**	**p-value**	**beta**	**95% CI**	**p-value**	**beta**	**95% CI**	**p-value**
sex hormones and modulators of the genital system	-0.0558	(-0.0706/-0.0411)	**<0.000001**	120	(93.6/147)	**<0.000001**	44.9	(38.7/51.1)	**<0.000001**
antithrombotic agents	0.0656	(0.0495/0.0817)	**<0.000001**	-122	(-151/-93.4)	**<0.000001**	-19.4	(-26.3/-12.6)	**<0.000001**
cardiac therapy	0.0916	(0.0633/0.120)	**<0.000001**	-122	(-173/-71.1)	**0.0000028**	-16.0	(-28.0/-3.89)	0.0096
Diuretics	0.0354	(0.0157/0.0522)	**0.00043**	-37.0	(-72.5/-1.45)	0.041	-4.55	(-12.9/3.84)	0.29

The analysis was adjusted for age, sex, cardiovascular risk factor and cardiovascular diseases. Bonferroni corrected p-value (0.00079) is used. For categorization of medication groups see appendix.

Table 3A. Multiple linear regression analysis of drugs in study sample at 1 pM TF

	Log(lag time [min])			ETP [nM.min]			Peak height [nM]		
drug	**Beta**	**95% CI**	**p-value**	**beta**	**95% CI**	**p-value**	**beta**	**95% CI**	**p-value**
sex hormones and modulators of the genital system	-0.0795	(-0.0979/-0.0611)	**<0.000001**	151	(124/178)	**<0.000001**	35.1	(29.7/40.4)	**<0.000001**
antithrombotic agents	0.0862	(0.0661/0.106)	**<0.000001**	-92.8	(-122/-63.4)	**<0.000001**	-5.01	(-11.0/0.952)	0.10
cardiac therapy	0.112	(0.0763/0.147)	**<0.000001**	-83.3	(-135/-31.5)	0.0016	-3.94	(-14.4/6.54)	0.46
immunosuppressants	0.114	(0.0537/0.174)	**0.00021**	-28.6	(-116/59.0)	0.52	-1.37	(-19.1/16.3)	0.88
antigout preparations	0.0552	(0.0248/0.0856)	**0.00038**	0.270	(-44.1/44.6)	0.99	3.76	(-5.20/12.7)	0.41

The analysis was adjusted for age, sex, cardiovascular risk factor and cardiovascular diseases. Bonferroni corrected p-value (0.00079) is used. For categorization of medication groups see appendix.

Kaplan-Meier survival curves and Cox-regression models

During the follow-up period until April 2017, with a median follow-up of 9.21 (8.83-9.65) years, a total of 308 deaths were registered. As presented in **Figure 1A-F**, a longer lag time above the 90th percentile of the reference, at both 1 pM TF (**Figure 1A**) and 5 pM TF (**Figure 1B**), was significantly associated with worse survival (p<0.0001). In addition, higher ETP above the 90th percentile (p=0.034) and 97.5th percentile (p=0.00097) of the reference sample (**Figure 1D**), measured at 5 pM TF was associated with worse survival. No such associations were observed for ETP at 1 pM TF.

Considering the strong positive association between TGA and the use of oral contraceptives or hormone replacement therapy, individuals taking these medications were excluded from Cox regression analysis. In the first model, adjustments were made for age, sex, and vitamin K antagonist use, whereas in the second and third models, additional adjustments were made for CVRFs and CVD, respectively. As demonstrated by the second model in **Table 4A, B**, Cox regression analysis confirmed an increased mortality for individuals with a lag time at 1 pM TF above the 95th percentile of the reference [HR= 1.55, 95% confidence interval (95% CI): 1.14-2.11; p=0.0058] and with ETP at 5 pM TF above the 95th percentile of the reference (HR=1.53, 95% CI: 1.09-2.15; p=0.015), independently of the presence of CVRFs. After additional adjustment for CVD, lag time at 1 pM TF (HR=1.46, 95% CI: 1.07-2.00; p=0.018) and ETP at 5 pM TF (HR=1.50, 95% CI: 1.06-2.13; p=0.023) remained associated with mortality.

Figure 1A-F Survival over 10 years for markers of TGA above and below reference limits.

Figure 1A

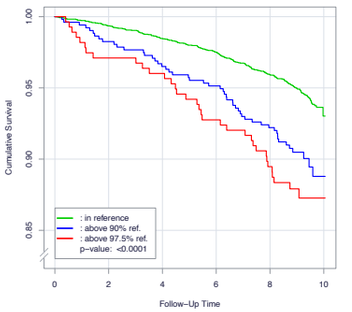

Figure 1B

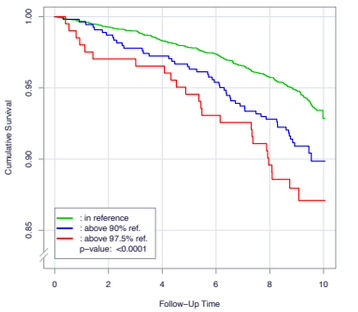

Figure 1C

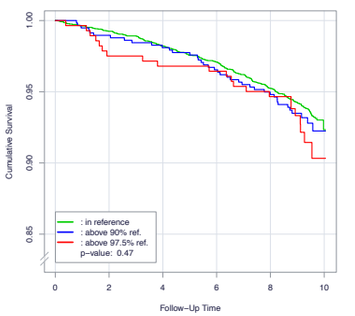

Figure 1D

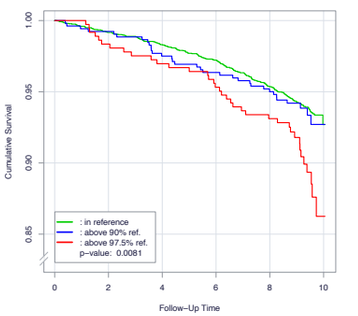

Figure 1E

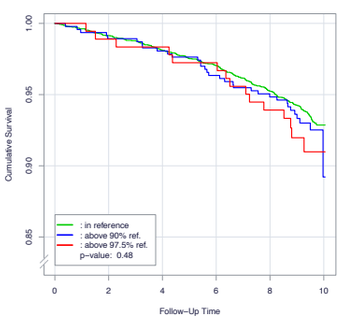

Figure 1F

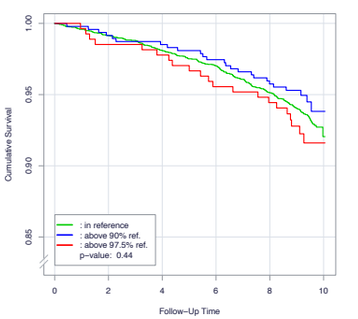

Figure legend: Kaplan-Meier survival curves of the overall study sample demonstrating the 10-year survival of individuals with the TGA parameters lag time (upper panels), endogenous thrombin potential (ETP) (middle panels), and peak height (lower panels) within the range of the reference group (green line), individuals above the 90th percentile of the reference group (blue line), and individuals above the 97.5th percentile of the reference group (red line), at 1 (upper panels) and 5 pM (lower panels) TF. For the lag times at both 1 and 5 pM TF, p<0.001 for the difference between the reference and the 90th percentile, as well as for the reference and the 97.5th percentile. For the ETP at 5 pM TF, p=0.034 for the difference between the reference and the 90th percentile and p=0.00097 for the difference between the reference and the 97.5th percentile.

Table 4A. Prognostic value of markers of thrombin generation measured at 1 pM TF for mortality

TGA parameter	First model*			Second model**			Third model***		
	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Lag time below 5% of reference	1.22	(0.58/2.60)	0.60	1.11	(0.52/2.37)	0.78	1.14	(0.53/2.42)	0.74
Lag time above 95% of reference	1.54	(1.14/2.08)	0.0055	1.55	(1.14/2.11)	0.0058	1.46	(1.07/2.00)	0.018
ETP below 5% of reference	1.08	(0.75/1.56)	0.66	1.11	(0.77/1.60)	0.58	1.05	(0.72/1.52)	0.80
ETP above 95% of reference	1.41	(0.98/2.03)	0.061	1.44	(0.99/2.07)	0.051	1.41	(0.97/2.04)	0.071
Peak height below 5% of reference	1.14	(0.78/1.67)	0.51	1.18	(0.81/1.74)	0.39	1.12	(0.76/1.65)	0.56
Peak height above 95% of reference	1.28	(0.82/2.01)	0.27	1.29	(0.83/2.03)	0.26	1.39	(0.89/2.18)	0.15

Cox regression model performed in the overall study sample with mortality as outcome and markers of thrombin generation as predictors. Models were *adjusted for age, sex and vitamin K antagonists. **adjusted for age, sex, vitamin K antagonists and CVRF's. ***adjusted for age, sex, vitamin K antagonists, CVRF's, and CVD.

Table 4B. Prognostic value of markers of thrombin generation measured at 5 pM TF for mortality

TGA parameter	First model*			Second model**			Third model***		
	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Lag time below 5% of reference	1.08	(0.40/2.90)	0.89	0.98	(0.36/2.63)	0.96	1.08	(0.40/2.92)	0.88
Lag time above 95% of reference	1.29	(0.92/1.79)	0.14	1.25	(0.89/1.75)	0.20	1.17	(0.83/1.65)	0.36
ETP below 5% of reference	1.35	(0.94/1.94)	0.11	1.36	(0.94/1.97)	0.10	1.26	(0.87/1.83)	0.23
ETP above 95% of reference	1.55	(1.11/2.17)	0.0099	1.53	(1.09/2.15)	0.015	1.50	(1.06/2.13)	0.023
Peak height below 5% of reference	1.07	(0.72/1.59)	0.76	1.09	(0.73/1.63)	0.66	1.02	(0.68/1.52)	0.92
Peak height above 95% of reference	1.18	(0.78/1.78)	0.44	1.21	(0.80/1.83)	0.37	1.23	(0.81/1.86)	0.34

Cox regression model, performed in the overall study sample, with mortality as outcome and markers of thrombin generation as predictors. Models were *adjusted for age, sex and vitamin K antagonists, **adjusted for age, sex, vitamin K antagonists and CVRFs, and ***adjusted for age, sex, vitamin K antagonists, CVRFs, and CVD.

Discussion

The formation of thrombin is one of the key processes underlying thrombotic diseases and its role in CVD due to atherosclerosis attracted new interest with recent data showing superior efficacy of a combined strategy of aspirin and low-dose direct oral anticoagulation in reducing atherothrombotic events.[17] Hence, limiting TGA by inhibiting F-Xa provides an interesting approach to lower cardiovascular risk. In this study we explore the clinical determinants of TGA measured in plasma, in a large population-derived study. Our data provide important insights into the effects of CVRFs in males and females. This study is the first to demonstrate the positive association of TGA parameters, ETP as a global measure of procoagulant and anticoagulant action in plasma and lag time, with total mortality, independent of age, sex and CVRFs.

The presented reference values may be generalized to other laboratories. However, the reference ranges should be used with caution as the (pre-) analytical conditions of the assay may influence the reference ranges and standardization between laboratories is needed, as well as confirmation of the observed data. The reference values of the TGA parameters as well as the mean and median values of the TGA parameters in the overall study sample showed sex-specific differences with females having shorter lag times and higher ETP and peak height, compared to males. The sex differences in TGA can be partly explained by the strong influence of female endogenous sex hormones on the coagulation cascade, as higher levels of fibrinogen and lower levels of protein S, antithrombin and protein C were observed in females, compared to males, irrespective of hormonal treatment.[18]

Following a sex-stratified, fully adjusted, large, multivariable model analysis we show that age, obesity and dyslipidemia are the most important clinical factors linked with higher TGA potential. Furthermore, this study demonstrates the effect of different groups of medication on TGA, with hormone-containing drugs being positively associated and anticoagulant and antiarrhythmic drugs being inversely associated with TGA potential.

Few studies have described the effect of age on TGA parameters.[10,11,19] Collectively, these studies suggest that TGA potential enhances with increasing age, indicated by shorter lag time and higher ETP and peak height. However, these studies had rather small sample sizes and included a homogeneous population of healthy volunteers. In the present analysis, age was positively associated with lag time in both males and females. In males, ETP and peak height increased with age, whereas in females the amount of TGA showed a rather negative trend with less strong associations compared to those in males.

Other positive determinants of lag time observed in this study were obesity and dyslipidemia, which may partly be explained by increased levels of TFPI,

a lipoprotein-associated coagulation inhibitor. It has been suggested that free TFPI is a major determinant of lag time.[10] Elevated TFPI levels have been reported in individuals with impaired glucose tolerance and type 2 diabetes mellitus[20] and it has been suggested that these TFPI levels were elevated due to related obesity.[21] Smid and colleagues found that a prolongation in lag time in patients with previous MI may be due to release of TFPI.[7] In addition to lag time, both ETP and peak height showed positive associations with obesity and dyslipidemia. Total body fat percentage and body mass index have been positively associated with lag time, ETP and peak height in females, independently of age, prior CVD, glucose metabolism and smoking status, though no associations were observed in males.[22] The present study demonstrates strong relations of obesity with a longer lag time and higher ETP and peak height in both males and females, independently of potential confounders. The association of ETP and peak height with obesity may be attributed to a low-grade inflammation observed in obese individuals.[23]

The results on associations between therapy and TGA parameters showed that use of VKA was positively associated with lag time and negatively associated with ETP and peak height, as expected from previous studies.[24-26] Aspirin showed no effect on TGA (*data not shown*), in line with recent findings from the COMPASS trial, in which treatment with a combination of aspirin and rivaroxaban, a direct F-Xa inhibitor, showed a superior effect on prevention of the manifestation of atherothrombosis in atherosclerotic disease, as compared to treatment with aspirin alone.[17]

Furthermore, intake of oral contraceptives or hormone replacement therapy was associated with a shorter lag time and higher ETP and peak height, in line with previous reports.[10,27-29] The influence of estrogen-containing medication on the TGA potential has been linked through increased levels of the coagulation factors II, VII, VIII, and X and fibrinogen, decreased levels of the natural anticoagulants, antithrombin and protein S, and acquired resistance to APC.[27,28]

Hitherto, only a limited number of studies have explored the association between TGA and mortality. The PROSPER study, including only elderly individuals, showed positive associations of vascular mortality with lag time and peak height and total mortality with lag time.[5] However, after adjustment for interleukin-6 and C-reactive protein levels, the associations were no longer significant, indicating that inflammation may be contributing to higher TGA in these individuals. In another smaller study, higher ETP and peak height (at 5 pM TF), independently of age, sex and CVRFs, were associated with increased risk of cardiovascular death in patients with acute coronary syndrome.[30] In the present large, adult, population-based study, we demonstrate a positive association between lag time at 1 pM TF and total mortality, which remained significant after adjusting for traditional CVRFs and CVD. Furthermore, this study highlights the relation between higher ETP (above the 95th percentile of the reference group) at 5 pM TF, as a global measure of both procoagulant and anticoagulant forces in the plasma, and increased risk of death, independently of CVRFs and CVD.

These findings indicate that both lag time and ETP are potential biomarkers for increased mortality risk, beyond the traditional CVRFs. As discussed for the previous published PROSPER study, the association between a prolonged lag time and total mortality is not only a surprising and counterintuitive observation, but also one that is difficult to explain. With a risk of being too speculative, potential mechanisms might include consumption of initiators of coagulation before the system overshoots to start actual thrombosis. In other words, a constant (weak) prothrombotic trigger activates the coagulation system which is subsequently down-regulated by the natural anticoagulants antithrombin and TFPI until, at a certain moment, the prothrombotic trigger increases and the system becomes overactivated and anticoagulants can no longer prevent thrombosis. In such a scenario, consumption of F-VII or F-XII, for example, could lead to a prolonged lag time in a sensitive *in vitro* assay. Another potential contributor to the prolonged lag time could be altered TFPI levels between subjects. However, assessing the TFPI levels in the presented cohort is part of another study and beyond the scope of the current study.

Limitations of our study are that we measured TGA in PPP after one-step centrifugation of whole blood (10 minutes at 2,000 x *g*) in contrast to recommendations (two-step centrifugation, 2000 x *g* for 5 minutes, 10,000 x *g* for 10 minutes). A previous small-scale analysis by Loeffen and colleagues[16] showed that in order to eliminate residual platelets and microparticles, which may contribute to variability in TGA results, double-centrifuged samples are preferable. We cannot, therefore, exclude that residual platelets and microparticles contributed to the observed associations between CVRFs and TGA parameters. Next, we had only cumulative mortality data available, so conclusions could not be made regarding associations between TGA variables and specific causes of mortality. However, the standardized clinical investigation of the cardiovascular profile, standardized laboratory measurements of the large GHS sample and availability of prospective mortality data are essential strengths of our study, which delivers important evidence on the TGA as a potential tool for improving risk stratification for CVD.

In conclusion, this is the first, large, population-based study demonstrating an important relation between TGA parameters, such as the time to minimum thrombin formed or the amount of thrombin formed, and total mortality. Further research is required on the underlying mechanism as well as to explore the potential role of the parameters as independent biomarkers for increased mortality risk. The observed association of TGA and traditional

CVRFs, particularly obesity, is an important finding in light of the growing "globesity'' issue worldwide.[31]

Funding

The GHS is funded through the government of Rhineland-Palatinate ("Stiftung RheinlandPfalz für Innovation", contract AZ 961–386261/733), the research programs "Wissen schafft Zukunft" and "Center for Translational Vascular Biology (CTVB)" of the Johannes Gutenberg- University of Mainz, and its contract with Boehringer Ingelheim and PHILIPS Medical Systems, including unrestricted grants for the GHS. This work was supported by the German Federal Ministry of Education and Research (BMBF 01EO1003) and the Center for Translational Vascular Biology (CTVB) of the University Medical Center Mainz (to P. S. Wild). H. ten Cate is a Fellow of the Gutenberg Research Foundation.

Acknowledgments

We are indebted to all study participants and all co-workers of the GHS, who were involved in the planning and conduct of this study.

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Appendix: Supplemental Material

Definition of classical cardiovascular risk factors

Diabetes mellitus and dyslipidemia were defined as individuals with a definite diagnosis of by a physician. Additional definition of diabetes was a blood glucose level of ≥126mg/dl in the baseline examination after an overnight fast of at least 8 hours or a blood glucose level of ≥200mg/dl in the baseline examination after a fasting period <8 hours. Dyslipidemia was additionally defined as a LDL/HDL-ratio of >3.5. Hypertension was diagnosed, if antihypertensive drugs are taken, or a mean systolic blood pressure of ≥140mmHg or a mean diastolic blood pressure of ≥90mmHg (in the 2nd and 3rd standardized measurement after 8 and 11 minutes of rest). Smoking was classified into non-smokers (never smokers and former smokers) and smokers (occasional smoker, i.e. <1 cigarette/day, and smoker, i.e. ≥1 cigarette/day). Obesity defined as a body-mass index ≥ 30 kg/m². Self-reported CAD, MI, HF, stroke, DVT, PE and PAD indicated personal history of CVD. A positive family history was defined as history of MI or stroke in a female first-degree relative ≤65 years or a male first-degree relative ≤60 years.

Categorization of medication

Medications were classified according to the ATC classification system. The following medication groups were selected for analysis: antithrombotic agents (excluding direct oral anticoagulants) (B01), sex hormones and modulators of the genital system (G03), cardiac therapy (C01), diuretics (C03), antigout preparations (M04) and immunosuppressants (L04). For the use of oral contraceptives and/or hormone replacement therapy, both ATC-code and self-reported information was used.

Table S1. Sample characteristics of the study sample (n=4843) and reference subsample (n=1210)

	Study sample		**Reference subsample**	
	Male	**Female**	**Male**	**Female**
Subjects (n)	51.0% (2471)	49.0% (2372)	44.7% (541)	55.3% (669)
Age, y (IQR)	56.0 (46.0/66.0)	55.0 (45.0/64.0)	47.0 (42.0/56.0)	48.0 (41.0/54.0)
BMI, kg/m² (IQR)	27.2 (24.9/30.0)	25.6 (22.9/29.5)	25.0 (23.3/26.7)	23.6 (21.6/25.9)
Traditional CVRFs (n)				
Diabetes	9.8% (242)	5.2% (123)	--	--
Obesity	25.4% (628)	22.8% (542)	--	--
Smoking	20.9% (515)	17.5% (415)	25.0% (135)	21.8% (146)
Hypertension	56.5% (1395)	46.1% (1092)	--	--
Systolic blood pressure, mmHg (SD)	135.6 (16.7)	129.7 (18.0)	123.5 (8.8)	117.8 (10.0)
Diastolic blood pressure, mmHg (SD)	83.2 (9.5)	84.5 (9.6)	79.2 (5.8)	76.9 (6.5)
Dyslipidemia	37.2% (917)	21.8% (517)	--	--
FH of MI/stroke	34.5% (853)	39.0% (924)	28.7% (155)	32.0% (214)
History of Diseases (n)				
MI	4.7% (116)	1.5% (35)	--	--
Stroke	2.3% (57)	1.5% (35)	--	--
AF	3.8% (93)	1.5% (35)	--	--
PAD	4.4% (107)	3.9% (93)	--	--
CAD	6.9% (167)	2.1% (50)	--	--
CHF	1.6% (39)	1.5% (36)	--	--
DVT	2.9% (70)	5.2% (123)	--	--
PE	0.2% (5)	0.3% (6)	--	--
History of cancer	7.9% (194)	10.0% (236)	4.6% (25)	7.6% (51)
Medication (n)				
Antithrombotic agents	15.9% (391)	9.4% (223)	--	--
Oral contraceptives	--	6.4% (151)	--	--
HRT	--	12.3% (292)	--	--

IQR, interquartile range.

Table S2B. Multivariable linear regression in the overall study sample for TGA parameters in females at 5 pM tissue factor (TF)

Variable	Females (N)	Log(lag time [min])			ETP [nM.min]			Peak height [nM]		
		beta	95% CI	p-value	beta	95% CI	p-value	beta	95% CI	p-value
Age [10y]		0.0307	(0.0240/0.0374)	<0.0001	1.08	(-11.5/13.7)	0.87	-9.93	(-12.9/-7.00)	<0.0001
Diabetes	123	-0.0176	(-0.0458/0.0105)	0.22	-36.6	(-89.7/16.5)	0.18	-2.24	(-14.6/10.1)	0.72
Obesity	542	0.0418	(0.0266/0.0570)	<0.0001	154	(125/182)	<0.0001	21.8	(15.1/28.4)	<0.0001
Smoking	415	0.0108	(-0.00516/0.0267)	0.19	-8.86	(-38.9/21.2)	0.56	-6.33	(-13.3/0.648)	0.076
Hypertension	1092	0.0127	(-0.000763/0.0261)	0.065	5.37	(-20.0/30.7)	0.68	-2.07	(-7.95/3.82)	0.49
Dyslipidemia	517	0.0337	(0.0186/0.0488)	<0.0001	46.1	(17.6/74.5)	0.0015	-3.46	(-10.1/3.14)	0.30
FH of MI/stroke	924	-0.00320	(-0.0156/0.00916)	0.61	2.98	(-20.3/26.3)	0.80	3.21	(-2.20/8.62)	0.25
History of MI	35	-0.0395	(-0.101/0.0223)	0.21	-66.7	(-183/49.9)	0.26	-0.837	(-27.9/26.2)	0.95
History of stroke	35	-0.0611	(-0.119/-0.00363)	0.037	72.4	(-35.9/181)	0.19	36.3	(11.1/61.4)	0.0047
History of CAD	50	0.0198	(-0.0244/0.0641)	0.38	-48.0	(-131/35.5)	0.26	-6.48	(-25.8/12.9)	0.51
History of AF	35	-0.0441	(-0.108/0.0193)	0.17	73.8	(-45.9/193)	0.23	15.5	(-12.3/43.2)	0.28
History of PAD	93	0.0274	(-0.00358/0.0583)	0.083	-16.0	(-74.3/42.4)	0.59	-6.69	(-20.2/6.85)	0.33
History of VTE	124	-0.0184	(-0.0476/0.0107)	0.22	26.7	(-28.3/81.7)	0.34	10.0	(-2.76/22.8)	0.12
History of CHF	36	-0.0799	(-0.141/-0.0188)	0.010	4.78	(-111/120)	0.94	9.44	(-17.3/36.2)	0.49
History of cancer	236	-0.000209	(-0.0208/0.0203)	0.98	-16.2	(-54.9/22.6)	0.41	0.497	(-8.49/9.49)	0.91
Hormone replacement therapy	223	-0.0343	(-0.0525/-0.0161)	0.00023	44.7	(10.3/79.0)	0.011	20.9	(12.9/28.9)	<0.0001
Oral contraceptives	151	-0.0964	(-0.122/-0.0708)	<0.0001	325	(276/373)	<0.0001	103	(92.2/115)	<0.0001
Vitamin K antagonists	292	0.543	(0.480/0.606)	<0.0001	-841	(-960/-721)	<0.0001	-160	(-188/-132)	<0.0001

The analysis was adjusted for age, vitamin K antagonists, oral contraceptives, HRT, CVRFs and CVD.

Table S2A. Multivariable linear regression in the overall study sample for TGA parameters in males at 5 pM tissue factor (TF)

Variable	Males (N)	Log(lag time [min])			ETP [nM.min]			Peak height [nM]		
		beta	95% CI	p-value	beta	95% CI	p-value	beta	95% CI	p-value
Age [10y]		0.00690	(0.000786/0.0130)	0.027	6.88	(-3.44/17.2)	0.19	3.51	(1.01/6.01)	0.0059
Diabetes	242	-0.0366	(-0.0582/-0.0150)	0.00090	-44.1	(-80.5/-7.63)	0.018	9.12	(0.304/17.9)	0.043
Obesity	628	0.0242	(0.0102/0.0382)	0.00073	53.4	(29.8/77.0)	<0.0001	3.05	(-2.67/8.76)	0.30
Smoking	515	0.0116	(-0.00283/0.0260)	0.12	30.8	(6.39/55.1)	0.013	-4.17	(-10.1/1.73)	0.17
Hypertension	1395	0.0119	(-0.000832/0.0246)	0.067	5.09	(-16.4/26.5)	0.64	-1.60	(-6.79/3.59)	0.55
Dyslipidemia	917	0.0310	(0.0190/0.0430)	<0.0001	66.1	(45.8/86.3)	<0.0001	1.58	(-3.32/6.48)	0.53
FH of MI/stroke	853	-0.00417	(-0.0163/0.00796)	0.50	16.2	(-4.22/36.7)	0.12	1.62	(-3.33/6.57)	0.52
History of MI	116	0.000491	(-0.0358/0.0359)	1.00	-45.9	(-106/14.6)	0.14	-11.1	(-25.8/3.53)	0.14
History of stroke	57	0.0270	(-0.0140/0.0680)	0.20	-47.9	(-117/21.3)	0.17	-10.5	(-27.2/6.27)	0.22
History of CAD	167	-0.0181	(-0.0471/0.0108)	0.22	14.6	(-34.2/63.4)	0.56	8.71	(-3.11/20.5)	0.15
History of AF	93	0.0167	(-0.0176/0.0509)	0.34	-0.102	(57.9/57.7)	1.00	-3.56	(-17.6/10.4)	0.62
History of PAD	107	0.0215	(-0.00948/0.0525)	0.17	-6.87	(-59.1/45.4)	0.80	-8.98	(-21.6/3.66)	0.16
History of VTE	71	-0.0346	(-0.0702/0.000981)	0.057	-21.0	(-81.1/39.0)	0.49	1.22	(-13.3/15.8)	0.87
History of CHF	39	-0.0432	(-0.0925/0.00619)	0.087	-17.1	(-100/66.1)	0.69	16.5	(-3.66/36.6)	0.11
History of cancer	194	0.00633	(-0.0156/0.0282)	0.57	31.1	(-5.86/68.0)	0.099	5.44	(-3.50/14.4)	0.23
Vitamin K antagonists	391	0.498	(0.454/0.543)	<0.0001	-860	(-934/-785)	<0.0001	-148	(-166/-130)	<0.0001

The analysis was adjusted for age, vitamin K antagonists, CVRFs and CVD.

Chapter 3: Biochemical Determinants of Thrombin Generation in a General Population with Arterial and Venous Disease Background

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Under revision at Thrombosis Journal

Abstract

Background: The current study aims to identify the biochemical determinants of plasma TGA in a large population-based study by comparing individuals with a history of arterial or venous thrombosis to cardiovascular healthy individuals.

Methods: This study comprised 502 individuals with a history of arterial disease, 195 with history of venous thrombosis and 1402 cardiovascular healthy individuals (reference group) from the population-based GHS. Calibrated Automated Thrombography was assessed and coagulation factors were measured by means of BCS XP Systems. To assess the biochemical determinants of TGA variables, a multiple linear regression analysis, adjusted for age, sex and antithrombotic therapy, was conducted.

Results: The lag time, the time to form the first thrombin, was mainly positively associated with the natural coagulant and anticoagulant factors in the reference group, i.e. higher factors result in a longer lag time. The same determinants were negative for individuals with a history of arterial or venous thrombosis, with a 10 times higher effect size. Endogenous thrombin potential, or area under the curve, was predominantly positively determined by F-II, F-VIII, F-X and F-IX in all groups. However, the effect sizes of the reported associations were 4 times higher for the arterial and venous disease groups in comparison to the reference group.

Conclusion: This large-scale analysis demonstrated a stronger effect of the coagulant and natural anticoagulant factors on the thrombin potential in individuals with a history of arterial or venous thrombosis as compared to healthy individuals, which implicates sustained alterations in the plasma coagulome in subjects with a history of thrombotic vascular disease, despite intake of antithrombotic therapy.

Introduction

TGA is established as an important research tool for exploring the plasma "coagulome" in relation to clinical risks for bleeding or thromboembolism. For a bleeding tendency, like hemophilia subjects lacking F-VIII or F-IX, reduced peak height and ETP of the TGA curve have been observed, supporting a state of hypocoagulability.[1–5] Correction of such factor deficiency normalized the TGA profile.[6] In thrombosis research, the reported findings on TGA are conflicting, e.g. whereas an increased thrombin potential is frequently reported in venous thrombosis, for subjects with arterial thrombosis data are quite inconsistent.[7–10] While some studies show positive associations of increased peak height and/or ETP to outcomes like ischemic stroke, other studies show reverse associations of increased lag time and/or lower peak height levels in patients that suffered a myocardial infarction or stroke.[11,12] The reasons for these discrepancies are not fully understood but might include variations in coagulation factor concentrations, release of TFPI from the endothelium as well as effects of specific medication. Solid evidence based on a comprehensive set of different data is still missing.[13]

VTE and arterial thrombotic diseases share several risk factors and several studies have shown that the risk of arterial thrombosis is increased in those that suffered a first VTE and vice versa.[14] Therefore, one would expect that also the plasma coagulome, assessed by the TGA, would reflect certain similarities between subjects with VTE or arterial thrombosis.[15] However, given the observed discrepant associations with TGA data, different profiles between venous and arterial thrombotic disease may also be present.

In order to address these issues, we carried out the present study to identify the biochemical determinants (coagulation factors and natural anticoagulants) of the TGA parameters in individuals with a history of either an arterial cardiovascular disease or venous thrombotic disease compared to cardiovascular healthy group within the population-based GHS.

Methods

Research design

The GHS is a prospective, observational, single center cohort study, designed for population-based health research, in the Rhine-Main region in Germany. With a total of 15,010 individuals between 35 and 74 years enrolled at the baseline examination, the GHS aims to assess the consequences of diseases and environmental factors in addition to the inherited predisposition on the development and progression of asymptomatic and symptomatic disease. The study has been conducted in accordance with the tenets of the revised Declaration of Helsinki. The study protocol was approved by the local ethics committee and by the local and federal data safety commissioners (Ref. No. 837.020.07 [5555]). Written informed consent was obtained from all participants for laboratory analyses, clinical examinations, sampling of biomaterial and use of data records for research purposes. During the baseline visit, every participant underwent a comprehensive, standardized 5-hour clinical examination program, as reported elsewhere.[16,17] The baseline visit comprised of a detailed computer-assisted interview covering cardiovascular risk factors, life style, socioeconomic status, and other areas. The prevalence of cardiovascular disease was determined by history taking. In addition to the clinical assessment, a large biobank has been established for future biochemical and genetic analyses. As part of the follow-up, a standardized computer-assisted telephone interview and an inventory of primary and secondary endpoints were done 2.5 years after baseline visit. In addition, participants undergo a quinquennial, extensive clinical examination in the research facility similar to the baseline visit. Primary endpoints of the study were MI and cardiovascular death. Secondary endpoints were cerebrovascular accident, diabetes mellitus, heart failure, atrial fibrillation or death caused by the previously named diseases. Details of the study protocol and the further purposes of the study are discussed elsewhere.[18]

Study sample

The overall study sample consisted of the first 5000 subjects enrolled into the GHS between April 2007 and October 2008. After excluding subjects without biomaterial available or without complete TGA assessment (one or several TGA parameters were missing), 4843 individuals were successfully included in the present analysis.

The reference group was defined as apparently cardiovascular healthy subjects without history of cardiovascular disease (MI, CHF, CAD, PAD, VTE, AF) or the presence of CVRFs (obesity, dyslipidemia, arterial hypertension, diabetes mellitus) and included 1402 individuals. Individuals with a self-reported history

of inherited coagulation abnormalities were excluded from the reference sample. The arterial disease group was defined as individuals with a history of MI, CAD, stroke or PAD and included 502 individuals. The venous disease group was defined as individuals with a history of DVT or PE and included 195 individuals. Individuals that did not meet the above mentioned criteria for the various groups were excluded from the current analysis. For a detailed definition of traditional CVRFs and categorization of medications, please see **Appendix: Supplemental Material of chapter 2.**

Blood sampling and laboratory assessment

Venous blood sampling was performed according to standard operating procedures and the blood was collected in trisodium citrate (0.109 M, 1:9 vol:vol) monovette plastic tubes, while the subject was in fasting state (i.e. overnight fast, if subject was examined before 12 p.m.. and 5 hour fast, if subject was examined after 12 p.m.). PPP was prepared by one-step centrifugation at 2,000 x *g* at room temperature for 10 minutes. After preparation the PPP was aliquoted and immediately stored at -80 °C in the biobank of the GHS study center.

The TGA was assessed in the Laboratory for Clinical Thrombosis and Hemostasis, Maastricht University, the Netherlands, by the CAT assay (Thrombinoscope BV, Maastricht, The Netherlands), according to the recommendations.[19,20] The TGA was triggered by PPP Reagent Low (Stago) in freshly thawed PPP. The CAT method employs a low affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC) to continuously monitor thrombin activity in clotting plasma. TGA measurements were calibrated against the fluorescence curve obtained in a sample from the same plasma (80 µL), supplemented with a fixed amount of thrombin–alfa 2-macroglobulin complex (20 µL of Thrombin Calibrator; Thrombinoscope BV, Maastricht, The Netherlands) and 20 µL of the fluorogenic substrate and calcium chloride mixture. TGA parameters were derived from the TGA curve and include lag time (time to minimum thrombin formed [min]), peak height (the maximum amount of thrombin formed [nM]) and ETP (or area under the curve [nM.min]).

Coagulation factors were measured by means of BCS XP Systems in the Biomolecular laboratory at the Department of Epidemiology, University Medical Center Mainz, Germany. The coagulation factors II, V, VII, VIII, IX, X, XI, XII were determined using the clotting-based coagulation methodology, protein C and antithrombin by the chromogenic assay and vWF and protein S by using immunological-based assay. Reference values by the WHO standard provided by Siemens were used.

Total TFPI activity was assessed in PPP by the Actichrome TFPI activity assay (American Diagnostica, Stamford, CT, USA) in the Laboratory for Clinical Thrombosis and Hemostasis, Maastricht University, the Netherlands.

Data management and statistical analysis

A central data management unit conducted quality control on all data in this study. Statistical analysis was performed with software program R, version 3.3.1 (http://www.R-project.org). Data on coagulation factors and inhibitors are presented as mean (standard deviation) in case of normal distribution.

Multiple linear regressions were used to assess the associations between biochemical variables and TGA parameters in the reference group as well as in the arterial and venous disease group. The analyses were adjusted for age, sex and additionally for hormones (oral contraceptives and hormone replacement therapy = G03) and anticoagulant agents (B01AA, B01AB, B01AE, B01AF, B01AX) as these may affect the thrombin potential. Due to a skewed distribution, lag time, as a dependent variable, was log-transformed prior to the analysis. Estimated beta regression coefficients, presented with corresponding 95% CI, were calculated as per standard deviation to compare the effects of different coagulation-related factors on TGA parameters. Due to its explorative nature, a p-value threshold was not defined. However, to account for multiple testing and to avoid a false positive finding, a Bonferroni corrected p-value (0.00036) was set for the results on the multiple linear regression analyses.

Results

Baseline characteristics of the study sample

Baseline characteristics of the reference group, arterial and venous disease groups are shown in **Table 1**. The majority of the individuals in the arterial subsample were males (63.3%), whereas there was a preponderance of females in the reference group (60.5%) and the venous disease group (63.6%). The mean age in the reference group was 49.3 years and the mean age of the study population in the arterial and venous disease groups was 63.8 years and 61.3 years, respectively. In both the arterial and venous disease groups, hypertension (arterial disease group: 72.5%, venous disease group: 59.0%) was the most prevalent traditional CVRFs, followed by family history of MI/stroke (arterial disease group: 43.6%, venous disease group: 43.6%). Of the cardiovascular diseases, CAD was the most prevalent with 46.5% of the study subjects in the arterial disease group. In the venous disease group, 99.0% of the individuals had a history of DVT and 5.7% of the individuals had a history of PE. Of the arterial vascular diseases, PAD was predominant with 26.6% of the study subjects in the venous disease group. Anticoagulant therapy was most common in the arterial disease group (61.0%), followed by the venous disease group (35.4%) and the reference group (1.8%). While individuals from the reference group were most often taking oral contraceptive therapy (12.5%), individuals in the arterial disease group were most often using hormonal replacement therapy (11.3%).

Levels of coagulation factors and inhibitors

Levels of coagulation factors and inhibitors in the reference group, arterial and venous disease group are shown in **Table 2**. Most notably, the lag time was significantly prolonged in individuals with a history of arterial vascular disease or venous thrombosis in comparison to the cardiovascular healthy individuals. In addition, the ETP from the arterial disease group was lower compared to the reference group. The activity level of F-II, F-X and antithrombin were lower in the arterial and venous disease groups compared to the reference group. Differently, activity levels of F-VIII and F-XI, vWF activity and fibrinogen concentration were higher in both arterial and venous disease groups compared to reference group. The individuals from the arterial disease group compared to the control subjects showed additionally higher activity levels of F-IX and Protein S and slightly lower activity of F-XII.

Table 1. Study sample characteristics

	Reference group n=1402	**Arterial Disease group n=502**	**Venous Disease group n=195**
Sex (females)	60.5% (848)	36.7% (184)	63.6% (124)
Age(years)	49.3±9.8	63.8±8.5	61.3±10.1
BMI (kg/m²)	24.2 (22.2/26.3)	28.8 (25.7/32.2)	28.6 (25.5/31.5)
Cardiovascular risk factors			
Diabetes	0% (0)	20.1% (101)	9.7% (19)
Obesity	0% (0)	41.2% (207)	36.9% (72)
Smoking	22.5% (316)	16.2% (81)	11.3% (22)
Arterial hypertension	0% (0)	72.5% (364)	59.0% (115)
Dyslipidemia	0% (0)	40.1% (201)	30.8% (60)
Family history of MI/Stroke	31.1% (436)	43.6% (219)	43.6% (85)
Comorbidities			
CAD	0% (0)	46.5% (217)	9.1% (17)
MI	0% (0)	30.4% (151)	5.2% (10)
Stroke	0% (0)	18.5% (92)	6.2% (12)
AF	0% (0)	9.2% (45)	6.2% (12)
PAD	0% (0)	40.2% (200)	26.6% (51)
CHF	0% (0)	6.2% (31)	6.7% (13)
DVT	0% (0)	14.3% (71)	99.0% (193)
PE	0% (0)	1.6% (8)	5.7% (11)
Therapy			
Anti-coagulant agents*	1.8% (25)	61.0% (305)	35.4% (69)
Oral contraceptive therapy	12.5% (174)	6.4% (32)	9.7% (19)
Hormonal replacement therapy	5.5% (77)	5.6% (28)	11.3% (22)

* ATC codes: B01AA (vitamin K antagonists), B01AB (heparin group), B01AE (direct thrombin inhibitors), B01AF (direct factor Xa inibitors), B01AX (other antithrombotic agents).

Table 2. Parameters of thrombin generation and levels of natural coagulation and anti-coagulant factors in the reference, arterial and venous subsample

Variable	Group				
	Reference group (n=1402) Mean±SD	**Arterial Disease group (n=502) Mean±SD**	**p-value arterial disease vs. reference**	**Venous Disease group (n=195) Mean±SD**	**p-value venous disease vs. reference**
Lag Time [min]	4.94±1.03	6.56±3.71	**<0.0001**	6.50±3.95	**<0.0001**
ETP [nM.min]	1105.0±237.4	1045.0±360.8	**0.00056**	1048.5±423.3	0.070
Peak Height [nM]	118.37±54.81	113.09±55.36	0.067	113.50±60.32	0.29
Factor II [%]	116.9±17.6	108.7±28.9	**<0.0001**	104.8±32.7	**<0.0001**
Factor V [%]	116.6±18.5	118.9±19.9	**0.024**	118.4±22.0	0.29
Factor VII [%]	111.1±21.6	108.3±31.2	0.056	107.2±34.8	0.12
Factor VIII [%]	115.3±33.1	136.3±40.2	**<0.0001**	140.1±46.5	**<0.0001**
Factor IX [%]	106.3±14.4	110.9±21.7	**<0.0001**	107.4±24.3	0.53
Factor X [%]	113.10±19.09	105.59±33.53	**<0.0001**	103.87±38.95	**0.0013**
Factor XI [%]	106.8±18.2	110.2±19.8	**0.00090**	112.6±19.4	**<0.0001**
Factor XII [%]	105.6±24.3	102.1±24.9	**0.0071**	105.5±24.7	0.98
vWF [%]	103.1±35.5	127.7±45.0	**<0.0001**	127.7±48.3	**<0.0001**
Protein C [%]	113.3±17.2	111.3±26.1	0.097	110.2±28.3	0.13
Protein S [%]	95.1±16.4	100.3±24.1	**<0.0001**	93.4±24.7	0.34
Antithrombin [%]	102.8±9.8	98.1±11.9	**<0.0001**	100.8±10.7	**0.014**
TFPI Activity [U/mL]	1.57±0.53	1.63±0.57	0.040	1.64±0.52	0.084
Fibrinogen [mg/dL]	331±66	395±95	**<0.0001**	391±95	**<0.0001**

Determinants of thrombin generation

The multivariate analysis of the determinants of the TGA in the reference group, arterial and venous disease group is presented in **Figure 1A-C**. Presented in **Appendix: Supplemental Material table 1A-C** are beta per standard deviation SD, meaning that one SD change of the predictor (coagulation factors) leads to beta change in dependent variable (TGA parameter). The lag time in the arterial and venous disease group was strongly and negatively associated with coagulation F-II, F-V and F-VII and with the natural anticoagulants protein S, antithrombin and TFPI activity. Fibrinogen was negatively associated with the lag time in the arterial disease group and positively associated in the venous disease group. Differently, F-XII was positively associated with the lag time in the arterial disease group and negatively associated in the venous disease group. In general, the effect size of the reported biochemical determinants was 10 times higher for the arterial and venous disease groups compared to the reference group (e.g. F-II, beta estimate median: arterial: -0.18 vs venous -0.23 vs reference 0.017). In addition, the direction of the associations for the reference group was positive for all reported variables, except for F-VII that was negatively associated.

The ETP was strongly positively determined by F-II, F-VIII, F-X and F-IX in the reference group as well as the arterial and venous disease groups. (**Figure 1A-C)** In addition, antithrombin was a negative determinant for the ETP in the reference group, though no association was observed for antithrombin in the arterial and venous disease groups. VWF was a negative determinant for the ETP in both the arterial and venous disease group, whereas vWF was positively associated with the ETP in the reference group. Moreover, the effect size of the reported biochemical determinants was nearly 4 times higher for the arterial and venous disease groups in comparison to the reference group (e.g. F-VIII, beta estimate median: arterial 184 vs venous 217 vs reference 55.6).

There was a positive association between F-VIII, F-IX, F-II, F-X and the peak height in the reference group, arterial and venous disease group. (**Figure 1A-C**) Protein S was a negative determinant of the peak height in the reference group, whereas it was positively associated with the peak height in the arterial and venous disease group. In addition, F-IX was a negative determinant for the peak height in the reference group, though no association was found in the arterial and venous disease group. In general, the effect sizes for the reported biochemical determinants of the peak height were similar in all groups, with the exception of F-VII that had lesser effect in the arterial disease group compared to the reference group and venous disease group (beta estimate: arterial: 10.4 vs venous 24.4 reference 17.6).

Figure 1A. Graphical depiction of the biochemical determinants of the lag time.

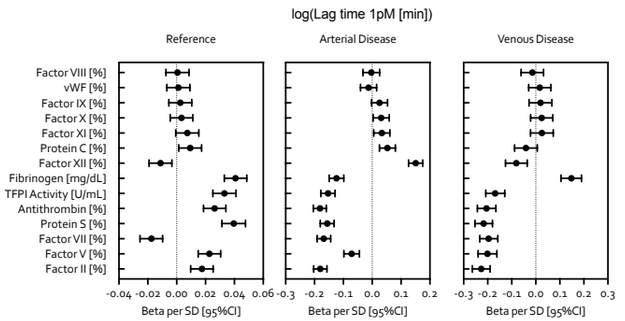

Figure 1B. Graphical depiction of the biochemical determinants of the ETP.

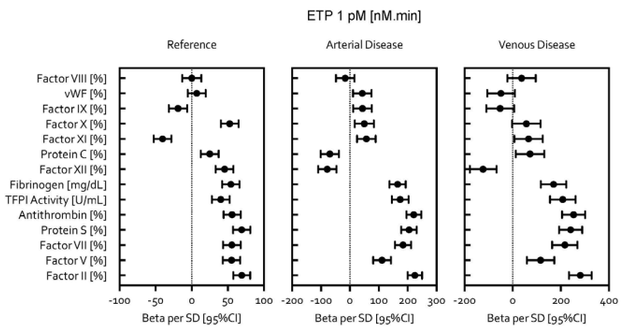

Figure 1C. Graphical depiction of the biochemical determinants of the peak height.

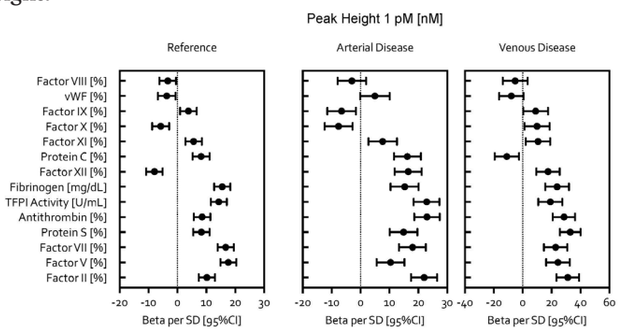

Figure legend: Graphical depiction of the biochemical determinants of the TGA parameters in the reference, arterial and venous disease group. Values are corresponding to supplemental table 1A-1C. The dots represent the beta per standard deviation with corresponding 95% confidence interval.

Discussion

This is the first large scale population-based study that has explored the biochemical determinants of the TGA parameters in individuals with a history of arterial vascular disease or venous thrombotic disease, as compared to cardiovascular healthy individuals. The main findings from our study show important distinct differences for the biochemical determinants between cardiovascular healthy individuals and those with a background of an arterial or venous disease. Whereas lag time was mainly negatively associated with the procoagulant and anticoagulant factors in the plasma, meaning higher factor levels result in a shorter lag time, the same associations were positive for the healthy individuals. Furthermore, the effect size for the biochemical parameters determining the lag time was about 10 times higher for the arterial and venous disease than for the reference group.

Dielis et al. previously investigated the coagulation factors as determinants of the TGA parameters at 1 pM TF (comparable to the applied PPP Reagent Low) and 13.6 pM TF in the absence or presence of thrombomodulin or in the absence or presence of activated protein C in a sample of healthy adults.[21] TFPI activity, protein S and fibrinogen were the strongest positive determinants of the lag time. Similarly, the results of the present study showed that TFPI activity, protein S and fibrinogen are strong positive determinants of the lag time in the control individuals. Fibrinogen was also positively associated with the lag time from the venous disease group. A possible explanation for the paradoxical association between fibrinogen and lag time may be the anticoagulant properties of fibrinogen by inhibiting the binding with thrombin directly as well as through accelerating the activation of plasminogen into plasmin by tissue plasminogen activator.[22] Interestingly, for the arterial disease individuals, higher fibrinogen concentration was associated with shorter lag time. These contrasting results raise the possibility of differential effects of fibrinogen on the initiation phase of the TG process in diseases affecting different vascular beds. F-VII was the unique coagulation factor that shared the same direction of association with the lag time for control subjects and disease individuals. F-VII is well known to play an important role in the initiation phase of the coagulation cascade by formation of the F-VIIa/TF complex that promotes the generation of the prothrombinase complex (F-Xa/F-Va) and ultimately leads to TG amplification.[23] Higher F-VII activity level and shorter lag time, shared by both control and disease individuals, confirms the role of F-VII in the ambient coagulation cascade reaction.

Furthermore, Dielis and colleagues reported fibrinogen and F-XII as positive determinants for the ETP, which we confirmed in the present study.[21] As expected and as previously reported, antithrombin, a potent anticoagulant, was negatively associated with the ETP. In general, the present analysis

demonstrated that the direction of associations with coagulation factors and ETP were similar in the reference group, arterial and venous disease group.
The analysis of the levels of natural coagulation and anticoagulant factors showed that F-II, F-VIII, F-X and F-XI were significantly increased in the subjects with an arterial or venous disease background in comparison to the healthy individuals, which is in accordance with previous reports.[9,24–29] This finding illustrates a ''hypercoagulable'' state in these subjects and may explain the fourfold increased effect size of the associations with the reported coagulation factors and the ETP in arterial and venous disease groups compared to the reference group. This is further supported by evidence from previous TGA studies demonstrating its potential to expose hypercoagulability in plasma from patients with arterial and venous thrombosis.[30]

In contrast to the reference group, protein S, a natural anticoagulant, was a positive determinant for the peak height in individuals with a history of arterial or venous thrombotic disease. Our analysis confirms increased levels of coagulation factors in patients with an arterial or venous thrombotic disease background, which could potentially result in excessive activation of the activated protein C pathway to which protein S is a supporting cofactor. Therefore, as demonstrated by the analysis from the arterial disease group, levels of protein S may be elevated. However, the net effect of these pathological mechanism remains an increased thrombin generation which translates to the increased peak height. The effect sizes of the associations with the peak height were similar for healthy individuals and individuals with an arterial or venous thrombotic disease background.

Limitations to the study were: The TGA was measured in PPP after one-step centrifugation of whole blood (10 minutes at 2,000 x *g*), in contrast to standard recommendations (two-step centrifugation; 2,000 x *g* for 5 minutes, 10,000 x *g* for 10 minutes), which may affect the TGA results. The history of arterial or venous disease was self-reported by the participants. There was no data available for analysis on the time from the initial diagnosis of the arterial and/or venous event to study enrollment. Therefore, we were not able to investigate if different duration of disease has different impact on the coagulation and TGA profile. However, this study has important strengths, including the standardized clinical investigation of the participants' present cardiovascular profile and the comprehensive laboratory investigation of coagulation and anticoagulant factors.

In conclusion, this large-scale analysis of TGA biochemical determinants shows that the individual coagulation factors more strongly affect TGA parameters in individuals with a history of arterial or venous thrombosis as compared to cardiovascular healthy individuals. This illustrates the different effect size contribution of the coagulation factors to the hypercoagulable state of individuals at risk for a cardiovascular event and suggests that the coagulome might be tuned to a "hypersensitive" state increasing the risk for recurrence. Overall, the important finding of altered determinants of thrombin generation shows that in patients with a history of cardiovascular disease levels of coagulation factors should be taken into account. It also provides further rationale for the observed benefits of anticoagulant therapy in patients with cardiovascular disease at risk of atherothrombosis.

Funding

The Gutenberg Health Study is funded through the government of Rhineland-Palatinate ("Stiftung RheinlandPfalz für Innovation", contract AZ 961–386261/733), the research programs "Wissen schafft Zukunft" and "Center for Translational Vascular Biology (CTVB)" of the Johannes Gutenberg-University of Mainz, and its contract with Boehringer Ingelheim and PHILIPS Medical Systems, including unrestricted grants for the Gutenberg Health Study. H. ten Cate was a Fellow of the Gutenberg Research Foundation. H.M.H. Spronk and H. ten Cate received funding for research from Bayer and Pfizer, outside the work presented in this paper. There were no disclosures to report for the remaining authors.

Acknowledgments

We would like to express our gratitude to all participants and the staff who are involved in planning and conducting the GHS.

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Appendix: Supplemental Material

Supplemental table 1A-C. Biochemical determinants of the TG parameters in the reference subsample and arterial and venous subsample

1A. Lag time

Group:	Reference		Arterial disease		Venous disease	
log(Lag time 1pM [min])	Beta per SD (95% CI)	p value	Beta per SD (95% CI)	p value	Beta per SD (95% CI)	p value
Factor II [%]	0.0175(0.00972/0.0253)	<0.0001	-0.18 (-0.203/-0.157)	<0.0001	-0.227(-0.264/-0.190)	<0.0001
Factor V [%]	0.0227 (0.0149/0.0305)	<0.0001	-0.0712 (-0.0981/-0.0444)	<0.0001	-0.201 (-0.240/-0.162)	<0.0001
Factor VII [%]	-0.0175 (-0.0254/-0.00972)	<0.0001	-0.168 (-0.191/-0.144)	<0.0001	-0.196 (-0.233/-0.158)	<0.0001
Protein S [%]	0.0396 (0.0314/0.0477)	<0.0001	-0.156 (-0.180/-0.132)	<0.0001	-0.217 (-0.253/-0.180)	<0.0001
Antithrombin [%]	0.0263 (0.0186/0.0341)	<0.0001	-0.181 (-0.204/-0.159)	<0.0001	-0.205 (-0.243/-0.166)	<0.0001
TFPI Activity [U/mL]	0.0331 (0.0251/0.0411)	<0.0001	-0.153 (-0.178/-0.129)	<0.0001	-0.169 (-0.208/-0.129)	<0.0001
Fibrinogen [mg/dL]	0.0408 (0.0330/0.0486)	<0.0001	-0.124 (-0.149/-0.0986)	<0.0001	0.148 (0.105/0.190)	<0.0001
Factor XII [%]	-0.0112 (-0.0191/-0.00332)	0.0053	0.151 (0.126/0.175)	<0.0001	-0.081 (-0.126/-0.0356)	0.00047
Protein C [%]	0.0094 (0.00148/0.0173)	0.02	0.0527 (0.0250/0.0804)	0.00019	-0.041 (-0.0883/0.00620)	0.089
Factor XI [%]	0.00737 (-0.000621/0.0154)	0.071	0.0333 (0.00536/0.0612)	0.019	0.0254 (-0.0218/0.0726)	0.29
Factor X [%]	0.00335 (-0.00445/0.0112)	0.4	0.0312 (0.00340/0.0591)	0.028	0.0246 (-0.0214/0.0706)	0.29
Factor IX [%]	0.00253 (-0.00542/0.0105)	0.53	0.0255 (-0.00218/0.0531)	0.071	0.0195 (-0.0280/0.0670)	0.42
vWF [%]	0.00121 (-0.00681/0.00923)	0.77	-0.0127 (-0.0405/0.0152)	0.37	0.0166 (-0.0294/0.0627)	0.48
Factor VIII [%]	0.000547 (-0.00746/0.00855)	0.89	-0.00312 (-0.0320/0.0257)	0.83	-0.0144 (-0.0612/0.0324)	0.55

1B. ETP

Group:	Reference		Arterial disease		Venous disease	
ETP 1pM [nM.min]	Beta per SD (95% CI)	p value	Beta per SD (95% CI)	p value	Beta per SD (95% CI)	p value
Factor II [%]	69.3 (57.5/81.0)	<0.0001	225 (200/250)	<0.0001	281 (234/328)	<0.0001
Factor VII [%]	55.2 (43.2/67.2)	<0.0001	111 (80.8/142)	<0.0001	116 (59.2/173)	<0.0001
Factor VIII [%]	55.6 (43.4/67.8)	<0.0001	184 (156/212)	<0.0001	217 (164/269)	<0.0001
Factor IX [%]	69.2 (57.3/81.1)	<0.0001	205 (178/231)	<0.0001	241 (193/289)	<0.0001
Factor X [%]	55.9 (44.1/67.8)	<0.0001	221 (196/247)	<0.0001	253 (205/301)	<0.0001
Factor XI [%]	40.2 (27.9/52.6)	<0.0001	174 (145/203)	<0.0001	208 (155/261)	<0.0001
Factor XII [%]	54.2 (42.1/66.2)	<0.0001	165 (137/193)	<0.0001	170 (117/222)	<0.0001
vWF [%]	45.5 (33.1/57.8)	<0.0001	-78.6 (-110/-46.8)	<0.0001	-123 (-178/-67.1)	<0.0001
Protein C [%]	25 (12.6/37.3)	<0.0001	-69.5 (-101/-38.2)	<0.0001	71.9 (12.9/131)	0.017
Antithrombin [%]	-40.4 (-52.5/-28.3)	<0.0001	57 (24.8/89.2)	0.00052	66 (6.74/125)	0.029
Fibrinogen [mg/dL]	52.6 (40.3/65.0)	<0.0001	49.9 (16.8/83.0)	0.0031	56.6 (-2.58/116)	0.061
TFPI Activity [U/mL]	-19 (-31.7/ -6.32)	0.0033	43.4 (11.0/75.7)	0.0085	-52.2 (-110/5.90)	0.078
Factor V [%]	6.96 (-5.47/19.4)	0.27	42.8 (10.8/74.9)	0.0089	-48.7 (-106/9.06)	0.098
Protein S [%]	0.0931 (-13.1/13.3)	0.99	-16.2 (-48.3/15.9)	0.32	37.1 (-21.7/95.8)	0.22

1C. Peak height

Group:	Reference		Arterial disease		Venous disease	
Peak height 1pM [nM]	Beta per SD (95% CI)	p value	Beta per SD (95% CI)	p value	Beta (per SD) (95% CI)	p-value
Factor II [%]	10.2 (7.38/13.0)	<0.0001	22 (17.5/26.5)	<0.0001	31.2 (23.4/39.0)	<0.0001
Factor VII [%]	17.6 (14.9/20.4)	<0.0001	10.4 (5.60/15.2)	<0.0001	24.4 (16.2/32.6)	<0.0001
Factor VIII [%]	16.7 (13.9/19.5)	<0.0001	18 (13.3/22.6)	<0.0001	22.8 (14.7/30.9)	<0.0001
Factor IX [%]	8.28 (5.48/11.1)	<0.0001	14.9 (10.1/19.7)	<0.0001	32.9 (25.7/40.1)	<0.0001
Factor X [%]	8.53 (5.66/11.4)	<0.0001	23 (18.6/27.4)	<0.0001	28.6 (20.8/36.3)	<0.0001
vWF [%]	14.3 (11.6/17.1)	<0.0001	22.9 (18.4/27.3)	<0.0001	19.1 (10.8/27.4)	<0.0001
Protein C [%]	15.5 (12.7/18.3)	<0.0001	15.3 (10.4/20.1)	<0.0001	23.8 (15.6/32.0)	<0.0001
Protein S [%]	-7.93 (-10.8/-5.11)	<0.0001	16.5 (11.8/21.2)	<0.0001	17.5 (9.46/25.6)	<0.0001
TFPI Activity [U/mL]	8.18 (5.27/11.1)	<0.0001	16.2 (11.6/20.9)	<0.0001	-11 (-19.4/-2.55)	0.011
Factor V [%]	5.6 (2.77/8.44)	0.00011	7.66 (2.77/12.6)	0.0021	10.6 (2.08/19.0)	0.015
Factor XI [%]	-5.8 (-8.73/-2.87)	0.00011	-7.58 (-12.4/-2.75)	0.0021	9.92 (1.29/18.6)	0.024
Factor XII [%]	3.79 (0.916/6.67)	0.0098	-6.55 (-11.5/-1.60)	0.0094	8.98 (0.470/17.5)	0.039
Antithrombin [%]	-3.7 (-6.75/-0.652)	0.017	4.99(-0.101/10.1)	0.055	-7.86 (-16.3/0.545)	0.067
Fibrinogen [mg/dL]	-3.35 (-6.23/-0.476)	0.022	-2.99 (-7.91/1.94)	0.23	-5.19 (-13.8/3.42)	0.24

The multiple linear regression models were adjusted for age, sex and medication. * ATC codes: B01AA (vitamin K antagonists), B01AB (heparin group), B01AE (direct thrombin inhibitors), B01AF (direct factor Xa inhibitors), B01AX (other antithrombotic agents).

Part 2: Coagulation Proteins as Markers for Cardiovascular Disease and Mortality

Chapter 4: Relation between Tissue Factor Pathway Inhibitor Activity and Cardiovascular Risk Factors and Diseases in a Large Population Sample

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Thrombosis and Haemostasis, 2021

Abstract

Background

TFPI is a potent anticoagulant protein in the extrinsic coagulation pathway. In the present study, we aim to identify the cardiovascular determinants for total TFPI activity and its association with CVD and total mortality.

Methods

Total TFPI activity was assessed in a selection of the population-based GHS (n=5,000). Statistical analysis was performed to identify the determinants for total TFPI activity as well as the associations with CVD and mortality.

Results

Multivariable linear regression analysis identified smoking (β 0.095 [0.054-0.136]) as a positive determinant for total TFPI activity, while diabetes (β -0.072 [-0.134 to -0.009]), obesity (β -0.063 [-0.101 to -0.024]) and history of CAD were negatively associated with total TFPI activity, independent of age, sex and the remaining CVRFs. After adjustment for lipoprotein levels, the association between total TFPI activity levels and obesity and CAD was lost. The analysis additionally revealed a strong positive association between total TFPI activity levels and low-density lipoprotein (LDL) (β 0.221 [0.204-0.237]). The Cox regression models revealed that a higher total TFPI activity, above 97.5th percentile of the reference group, was associated with an increased mortality risk (HR = 2.58 [95%CI: 1.49-4.47]), independent of age, sex, and cardiovascular risk profile.

Conclusion

In the GHS population-based cohort the highest percentage of total TFPI correlated with an increased mortality risk. While elevated TFPI may reflect endothelial cell activation, the associations between total TFPI activity and obesity and CAD, points to additional mechanistic interactions.

Introduction

TFPI, a Kunitz-type serine protease inhibitor, is a potent anticoagulant regulator of the extrinsic coagulation pathway. TFPI acts by inhibiting F-Xa, after which TFPI•F-Xa subsequently inhibits the TF-F-VIIa complex, the initiator of the extrinsic pathway.[1] Inhibition of the TF-F-VIIa complex by TFPI greatly depends on its cofactor protein S, which enhances the interaction between TFPI and F-Xa 10-fold.[2]

TFPI mainly originates from vascular endothelial cells and the majority of plasma TFPI is C-terminally truncated and lipid-bound. Only 10-20% of plasma TFPI circulates as a free full-length form and has previously been recognized as the active, and biologically more important, anticoagulant in vitro.[3-5] Free TFPI antigen levels correlate strongly with endothelial cell markers, whereas total TFPI antigen levels correlate more strongly with traditional CVRFs.[6] Male sex, current smoking, diabetes mellitus and increased LDL levels have been proposed as positive determinants of total TFPI antigen levels, while treatment with hormone replacement therapy or oral contraceptives was strongly associated with lower total TFPI levels.[7,8]

Conflicting results have been reported on the relation of TFPI activity with arterial and venous thrombotic disease. Evidence from a case-control study showed that individuals with a TFPI activity below the 5th percentile had a 2-fold increased risk for developing DVT, independent of hormonal state, compared to individuals above the 5th percentile.[9] In a large observational study, a decreased TFPI function posed patients with an unprovoked first time DVT at 10-fold increased risk for recurrent VTE.[10] Interestingly, previous epidemiological and pathological studies show positive associations between TFPI activity and MI in young women.[11] Increased expression of TFPI was observed in arterial atherosclerotic lesions in similar plaque regions as TF, suggesting an active role of TFPI in regulating TF-dependent procoagulant activity and possibly other pro-atherogenic mechanisms.[12,13]

Given these apparent diverging risk associations for total TFPI activity and venous or arterial vascular disease, we embarked on a more extended analysis of this protein in relation to history of CVD, mortality, as well as CVRFs, in a large population based study.

Methods and materials

Research design

The GHS, a population-based, prospective, observational, single-center cohort study in the Rhine-Main region in western mid-Germany, was designed to improve the individual risk prediction of CVD. At baseline examination, the study included a total of 15,010 individuals. The samples were drawn randomly from the governmental local registry offices in the City of Mainz and the district of Mainz-Bingen and was stratified 1:1 for sex and residence (urban and rural) and in equal strata for decades of age. Individuals between 35 and 74 years of age were enrolled, and written informed consent was obtained from all participants for laboratory analyses, clinical examinations, sampling of biomaterial and use of data records for research purposes. The study was designed in accordance with the tenets of the revised Helsinki protocol, which together with the sampling design was approved by the local ethics committee and by the local and federal data safety commissioners. Details of the study protocol and further purposes of the study are discussed elsewhere.[14] As part of the study, every participant underwent a comprehensive, standardized 5-hour clinical examination program. Clinical examination and assessment of CVRFs were performed as published elsewhere.[15,16] In addition to the clinical assessment, a large biobank has been established for biochemical and genetic analyses.

Blood sampling and laboratory assessment

Venous blood sampling was performed according to standard operating procedures and the blood was collected in trisodium citrate (0.109 M, 1:9 vol:vol) monovette plastic tubes, while the subject was in fasting state (i.e. overnight fast, if subject was examined before 12 p.m. and 5 hour fast, if subject was examined after 12 p.m.). PPP was prepared by one-step centrifugation at 2,000 x *g* at room temperature for 10 minutes. After preparation the PPP was aliquoted and immediately stored at -80 °C. Total TFPI activity was assessed in PPP by the Actichrome TFPI activity assay (American Diagnostica, Stamford, CT, USA).

Study cohort and reference population

Total TFPI activity was measured in plasma of the first 5,000 participants of the GHS. After excluding individuals for which total TFPI activity levels could not be determined, 4779 individuals were included for statistical analysis.

The reference population was defined in order to carry out the cox-regression models, as explained further in the section on the statistical analysis. The reference population was defined as individuals without known history of CVD (MI, stroke, AF, CAD, stroke, PAD, CHF, VTE). Additionally, individuals suffering from diabetes mellitus, hypertension, dyslipidemia or a BMI higher than 45 kg/m^2 were excluded from the reference population. The definition of classical CVRFs is described in **Appendix: Supplemental Material of chapter 2**.

Categorization of medication

Medications were classified according to the ATC classification system. Lipid-modifying agents (C10) and sex hormones and modulators of the genital system (G03) were selected for analysis. For the use of oral contraceptives and/or hormone replacement therapy, both ATC-code and self-reported information was used.

Data management and statistical analysis

A central data management unit conducted quality control on all data in this study. Statistical analysis was performed with software program R, version 3.3.1 (www.R-project.com). The 10th and 90th percentiles of total TFPI activity were calculated for the sex-specific reference values and t-tests were used to determine significant sex-specific differences. Sex-specific nomograms were established depicting the age-related values of total TFPI activity of the lowest, as well as the highest percentiles of the reference group. Multiple linear regression models, adjusted for CVRFs, lipid profile, CVD, oral contraceptives, HRT and lipid-modifying agents were used to assess the clinical determinants of total TFPI activity. Lastly, Cox-regression models, adjusted for age, sex, CVRFs, lipid profile and CVD show the association between total TFPI activity and total mortality. The 2.5th and 97.5th percentile cut off in the Cox-regression models were based on the 2.5th and 97.5th percentile of the reference population. Because of the explorative character of the analysis, a significant threshold was not defined for p-values. A p-value of 0.05 was set as indication for statistical significance.

Results

Sample characteristics

The current GHS sample featured a well-balanced sex ratio, whereas a preponderance of females (60.9%) compared to males (39.1%) was observed in the reference population. The study cohort included 4,779 individuals and consisted of 2,426 males with a mean age of 56.0 years and 2,353 females with a mean age of 54.9 years. The reference population included a total of 1,480 individuals and comprised of 579 males with a mean age of 49.3 years and 901 females with a mean age of 48.7 years. Hypertension and family history of MI or stroke were the most prevalent traditional CVRFs in the study cohort. In the male population of the study cohort, a history of CAD was the most common CVD, while in the female population a history of VTE was more prevalent. A detailed overview of the sample characteristics of the study and reference population is given in **Table 1**.

Sex-specific total TFPI activity reference values and nomograms

Sex-specific percentiles of total TFPI activity were calculated in the reference population and were 0.9 U/mL for males in the 2.5th percentile and 0.8 U/mL for females. In the 97.5th percentile, total TFPI activity was 3.0 U/mL for males and 2.8 U/mL for females. Overall, as indicated by these data, females had lower total TFPI activity levels in both the 2.5th and the 97.5th percentiles compared to males. Nomograms, based on the reference population, relating total TFPI activity levels to age, were established and further subdivided by sex (**Fig. 1**). These sex-specific nomograms display age-related reference values of total TFPI activity in both sexes, where the age-related effect is stronger for the 2.5th, 50th and 97.5th percentile in females than in males.

Table 1. Sample characteristics of the study cohort (n=4779) and reference population (n=1480)

	Study cohort		**Reference population**	
	Male	**Female**	**Male**	**Female**
Subjects (n)	50.8% (2426)	49.2% (2353)	39.1% (579)	60.9% (901)
Age, y (SD)	56.0 (10.9)	54.9 (11.0)	49.3 (9.6)	48.7 (9.5)
BMI, kg/m² (SD)	27.7 (4.1)	26.7 (5.3)	25.7 (3.5)	24.7 (4.0)
Traditional CVRFs (n)				
Diabetes	9.8% (238)	5.3% (125)	--	--
Obesity	25.7% (624)	22.7% (535)	10.5% (61)	9.7% (87)
Smoking	21.0% (508)	17.5% (412)	26.1% (151)	21.8% (196)
Hypertension	56.6% (1372)	46.2% (1086)	--	--
Dyslipidemia	37.2% (900)	21.6% (508)	--	--
FH of MI/stroke	34.6% (839)	38.5% (905)	29.7% (172)	31.5% (284)
History of Diseases (n)				
MI	4.7% (113)	1.5% (35)	--	--
Stroke	2.4% (57)	1.5% (36)	--	--
AF	4.0% (95)	1.5% (36)	--	--
PAD	4.4% (105)	3.9% (91)	--	--
CAD	6.8% (163)	2.1% (50)	--	--
CHF	1.6% (38)	1.5% (36)	--	--
VTE	2.9% (69)	5.2% (122)	--	--
Medication (n)				
Antithrombotic agents	15.9% (384)	9.4% (221)	--	--
Oral contraceptives	--	6.5% (152)	--	10.0% (90)
HRT	--	12.3% (288)	--	8.9% (80)

Figure 1 Sex specific nomograms describing the association between age and TFPI activity

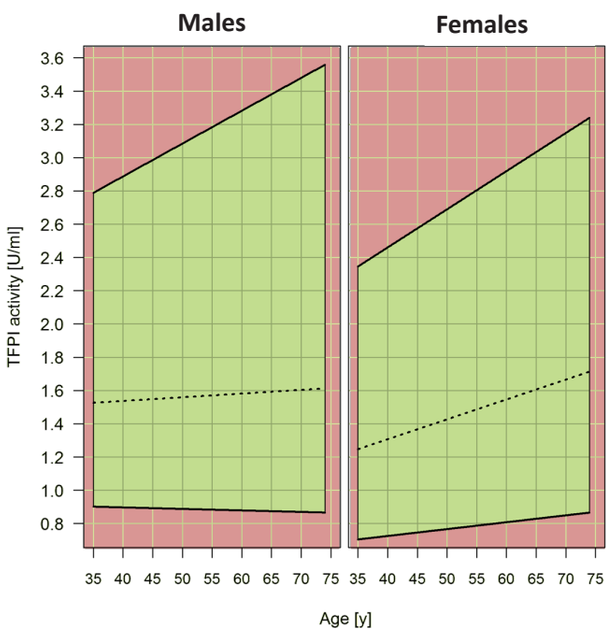

Figure legend: Sex-specific nomograms describing the association between age and TFPI activity. The dashed line represents the 50th percentile of the reference subsample. The upper line, bordering the upper red area and the green area, represents the 97.5th percentile of the reference subsample. The lower line, bordering the lower red area and the green area represents the 2.5th percentile of the reference subsample.

CVRFs, CVD and total TFPI activity levels

Multiple linear regression models were used to establish the clinical determinants of total TFPI activity levels and are demonstrated in **Table 2**. Age showed a positive association (β, 0.054 [0.039 to 0.0685], p<0.0001) and female sex showed a negative association with total TFPI activity levels (β, -0.067 [-0.099 to -0.035], p<0.0001), that remained after adjustment for potential confounders. The analysis demonstrated smoking (β, 0.0952 [0.0541 to 0.136], p<0.0001) as a positive determinant for total TFPI activity levels, whereas diabetes (β, -0.0716 [-0.134 to -0.00905], p=0.025) and obesity (β, -0.0627 [-0.101 to -0.024], p=0.0015) were negatively associated with total TFPI activity levels, in a model adjusted for age, sex and remaining CVRFs (**Table 2, Model 2.**). Further adjustments for history of CVD (Model 3) describes a negative relation between total TFPI activity levels and CAD (β, -0.160 [-0.256 to -0.0637], p=0.0011), independent of age, sex, and the remaining CVRFs and CVD. After adjustment for high-density lipoprotein levels (HDL), LDL and triglycerides, the association between total TFPI activity levels and obesity and CAD was lost (**Table 2, Model 4**).). Additionally, this adjustment revealed a strong positive association between total TFPI activity levels and LDL (β, 0.221 [0.204 to 0.237], p<0.0001). Oral contraceptives (β, -0.144 [-0.235 to -0.054], p=0.0018) and HRT (β, -0.0983 [-0.164 to -0.0324], p=0.0035) were both associated with lower levels of total TFPI activity in females (**Table 2**).

Total TFPI activity levels and total mortality

During the follow-up period until December 2017, with a median follow-up of 9.82 years (IQR: 9.42/10.3), a total of 333 deaths were reported. In a Cox regression model adjusted for age and sex (**Table 3**), a higher total TFPI activity, above 97.5th percentile of the reference group, was associated with an increased mortality risk (HR = 2.20 [95% CI: 1.26; 3.84], p=0.0056). Adjustments for CVRFs, CVD, oral contraceptives, HRT and LDL levels did not change the observed relation between total TFPI activity levels and mortality. However, the relation became stronger after additional adjustment for LDL levels (p=0.00074).

Table 2. Multivariable linear regression for TFPI activity

	Model 1		Model 2		Model 3		Model 4	
Variable	**Beta (95% CI)**	**p-value**	**Beta (95% CI)**	**p-value**	**Beta (95% CI)**	**p-value**	**Beta (95% CI)**	**p-value**
Age [10y]	0.054 (0.039/0.0685)	<0.0001	0.0642 (0.0481/0.0803)	<0.0001	0.0741 (0.0574/0.0908)	<0.0001	0.0419 (0.0259/0.0578)	<0.0001
Female sex	-0.067 (-0.099/-0.035)	<0.0001	-0.0664 (-0.0985/-0.0342)	<0.0001	-0.0772 (-0.11/-0.0443)	<0.0001	-0.0954 (-0.129/-0.0615)	<0.0001
Diabetes	-	-	-0.0716 (-0.134/-0.00905)	0.025	-0.0598 (-0.125/0.00575)	0.074	0.0686 (0.00608/0.131)	0.032
Obesity	-	-	-0.0627 (-0.101/-0.024)	0.0015	-0.0495 (-0.0893/-0.00978)	0.015	-0.0259 (-0.0647/0.0129)	0.19
Smoking	-	-	0.0952 (0.0541/0.136)	<0.0001	0.106 (0.0643/0.148)	<0.0001	0.110 (0.0707/0.149)	<0.0001
Hypertension	-	-	0.0135 (-0.022/0.0491)	0.46	0.0157 (-0.0202/0.0517)	0.39	0.00668 (-0.0272/0.0405)	0.70
FH of MI/stroke	-	-	-0.00245 (-0.0357/0.0308)	0.89	-0.00297 (-0.0368/0.0308)	0.86	-0.00952 (-0.0411/0.0221)	0.55
HDL (SD)	-	-	-	-	-	-	0.0121 (-0.00765/0.0318)	0.23
LDL (SD)	-	-	-	-	-	-	0.221 (0.204/0.237)	<0.0001
Triglycerides (SD)	-	-	-	-	-	-	-0.0188 (-0.0388/0.00121)	0.066
History of MI	-	-	-	-	-0.0871 (-0.205/0.0311)	0.15	-0.00869 (-0.120/0.103)	0.88
History of stroke	-	-	-	-	-0.114 (-0.239/0.0119)	0.076	-0.0673 (-0.185/0.0505)	0.26
History of CAD	-	-	-	-	-0.160 (-0.256/-0.0637)	0.0011	-0.0394 (-0.130/0.0513)	0.39
History of AF	-	-	-	-	0.0337 (-0.0698/0.137)	0.52	0.0697 (-0.0283/0.168)	0.16
History of PAD	-	-	-	-	0.0306 (-0.0556/0.117)	0.49	0.0302 (-0.0507/0.111)	0.46
History of VTE	-	-	-	-	-0.0412 (-0.128/0.0458)	0.35	-0.0389 (-0.120/0.0423)	0.35
History of CHF	-	-	-	-	-0.102 (-0.248/0.0441)	0.17	-0.0577 (-0.193/0.0780)	0.40
Use of oral contraceptives	-	-	-	-	-	-	-0.144 (-0.235/-0.054)	0.0018
Use of HRT	-	-	-	-	-	-	-0.0983 (-0.164/-0.0324)	0.0035
Use of lipid-modifying agents	-	-	-	-	-	-	0.00624 (-0.0453/0.0578)	0.81

Model 1 was adjusted for age and sex. Model 2 additionally adjusted for CVRFs except for dyslipidemia. Model 3 was additionally adjusted for CVD. Model 4 was additionally adjusted for oral contraceptives, HRT, lipid-modifying agents, HDL, LDL and triglyceride levels.

Table 3. Prognostic value of TFPI activity for mortality

TFPI activity above 97.5th percentile of the reference population			
	HR	**95% CI**	**p-value**
Model 1*	2.30	(1.34/3.94)	**0.0024**
Model 2†	2.28	(1.33/3.92)	**0.0028**
Model 3‡	2.32	(1.35/3.98)	**0.0024**
Model 4§	2.58	(1.49/4.47)	**0.00074**

*Adjusted for age and sex; †Adjusted for age, sex and CVRFs; ‡Adjusted for age, sex, CVRFs, CVD and oral contraceptives and HRT; §Adjusted for age, sex, CVRFs, CVD and oral contraceptives and HRT, and LDL levels.

Discussion

We report an increased mortality risk in individuals with higher total TFPI activity in a large epidemiological study of the general population. The Cox-regression models reveal that individuals with the highest total TFPI activity levels, above the 97.5th percentile of the reference population, are at 2.2 fold increased mortality risk, independent of CVRFs and CVD. Adjustment for LDL levels strengthened this risk association. The presented study demonstrated that diabetes, obesity and a history of CAD were, independently of age, sex and the remaining CVRFs and CVD, associated with lower total TFPI activity levels.

It has been reported that atherosclerotic plaques of dyslipidemic patients exhibit low levels of TFPI, as well as that individuals presenting with lower levels of TFPI are at increased risk for MI.[17,18] In contrast, subclinical atherosclerosis was associated with higher levels of TFPI, which may be precipitated by endothelial damage. Winckers et al. demonstrated higher levels of full length TFPI antigen in females with MI as well as that an increased TFPI activity posed females at a higher risk for MI.[11] These conflicting directions may partially be explained by a difference in study design, e.g. prospective versus retrospective study design. In an acute situation of MI or any arterial vascular disease, the high levels of TFPI may be a surrogate for endothelial damage, whereas in individuals with a history of CAD or CVRFs, lower levels of total TFPI activity may reflect chronic depletion due to sustained stimulation of coagulation.

Interestingly, after additional adjusting for HDL, LDL and triglyceride levels, obesity and a history of CAD were no longer negatively associated with total TFPI activity levels, suggesting that this negative effect on total TFPI activity is mediated through lipoprotein particles. In addition, after additional adjustment for HDL and LDL, diabetes showed a positive association with total TFPI activity levels. Moreover, the linear regression model, adjusted for age, sex, CVRFs, CVD and lipid profile, displayed a strong positive link between total TFPI activity and LDL levels, in line with previous clinical data. However, the latter studies investigated free and total TFPI levels, while total TFPI activity was studied in the current report.[6,19] TFPI, in the lipid-bound form rather than the free variant, combines mainly with LDL, which may be important for its clearance.[20]

The positive association between smoking and total TFPI activity levels remained after additional adjustments for CVD and lipid profile. A similar association was previously observed in a large, population-based cohort. It was hypothesized that the higher levels of TFPI antigen as well as activity reflect endothelial dysfunction in smokers.[7]

The normograms as well as the linear regression models in the current analysis demonstrate lower levels of total TFPI activity in females compared to males. In addition, the current analysis demonstrates exogenous female sex hormones such as oral contraceptives and HRT to be negative determinants for the total TFPI activity, in accordance with the previous literature on TFPI levels and endogenous and exogenous female sex hormones. A recent study including healthy individuals revealed that females had profound lower levels of total TFPI compared to males. In addition, oral contraceptives were significantly associated with decreased total TFPI in plasma.[21] It was suggested in another study that TFPI levels in a patient population with previous VTE were down-regulated by oral contraceptive use and HRT.[22] A small-scale study, conducted in healthy female volunteers, shows a negative association between HRT and TFPI antigen levels, whereas a positive association between HRT and TFPI activity levels was observed.[23] It has been postulated that estrogen, endogenous as well as exogenous, regulates TFPI plasma concentration at the cellular level, by binding to the estrogen receptor on endothelial cells, thereby decreasing TFPI plasma levels.[24]

In accordance with numerous studies, age showed positive associations with total TFPI activity, suggesting that aging is linked with higher total TFPI activity, presumably mediated by increased endothelial damage with aging.[6,25] In addition to reported total and free TFPI antigen levels in the general population[7,18,25,26], the current study now adds reference values, clinical determinants of TFPI activity and the association with CVD to these analyses.

TFPI is mainly derived from and associated with vascular endothelial cells and to a lesser extend (5-10%) from platelets, suggesting that changes in TFPI plasma levels most likely reflect endothelial function. Therefore, it is important to understand the nature and direction of the association of TFPI with CVRFs and CVD. A key finding in this study is the association of TFPI activity with CVD mediated through LDL cholesterol. This, once again, highlights the role of lipids on the pathophysiological mechanism of CVD, which is of major concern with the current global obesity epidemic. In addition, independent from cholesterol subfractions, increased levels of total TFPI activity were associated with total mortality.

Funding

The GHS is funded through the government of Rhineland-Palatinate ("Stiftung RheinlandPfalz für Innovation", contract AZ 961–386261/733), the research programs "Wissenschafft Zukunft" and "Center for Translational Vascular Biology (CTVB)" of the Johannes Gutenberg-University of Mainz, and its contract with Boehringer Ingelheim and PHILIPS Medical Systems, including unrestricted grants for the GHS. This work was supported by the German Federal Ministry of Education and Research (BMBF 01EO1003) and the Center for Translational Vascular Biology (CTVB) of the University Medical Center Mainz (to P.S.W.). H. ten Cate was a Fellow of the Gutenberg Research Foundation.

Acknowledgments

We are grateful to all study participants and all co-workers who are involved in planning and conducting the GHS.

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Chapter 5: Lower Levels of vWF are Associated with Lower Risk of Cardiovascular Disease

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Under revision at Research and Practice in Thrombosis and Haemostasis

Abstract

Objective

The current study was undertaken to prospectively explore whether having low levels of vWF antigen and vWF activity reduce the risk for CVD and (cardiovascular) death.

Methods

VWF antigen and vWF activity were measured by enzyme-linked immunosorbent assay and an immunological-based assay, respectively, in a subsample of 4,857 individuals enrolled between April 2007 and October 2008 in the population-based GHS. VWF antigen and activity below the 20th percentile was set as a measure of ''low vWF''. Adjusted robust poisson regression models were used to analyse the relation between low vWF and the incidence of CVD. Consequent adjusted cox regression models as well as cumulative incidence plots were calculated to explore the relation between all-cause and cardiovascular mortality and low vWF.

Results

VWF activity levels <20th percentile (i.e. <76.2%) were associated with a decreased relative risk for CVD (95%CI: 0.37-0.95), independent of age and sex. After adjusting for levels of F-VIII, the association persisted (95%CI: 0.36-0.99). The cumulative incidence plots demonstrated that vWF antigen <20th percentile significantly correlated with decreased cardiovascular mortality. VWF antigen<20th percentile (i.e. <83%) was significantly associated with lower risk of all-cause mortality, independent of clinical factors (95%CI: 0.41-0.91).

Conclusion

The study demonstrated that having low vWF activity levels is associated with a lower risk for CVD, independent of age, sex, risk factors and F-VIII levels. Additionally, it reveals a decreased risk of cardiovascular and all-cause mortality in individuals with low levels of vWF antigen, shining new light on vWF as a potential target for novel therapies.

Introduction

VWF, a multimeric glycoprotein present in plasma, plays an important role in hemostasis. In vivo synthesis of vWF occurs in endothelial cells or megakaryocytes. Subsequently, depending on the site of the synthesis, vWF is either stored within endothelial cells in organelles known as Weibel-Palade bodies (WPBs) or within platelet α-granules. Following damage to the vascular wall, agonists (e.g. on endothelial cells: histamine, estrogen, fibrin; on platelets: ADP) will trigger the release of vWF from the WPBs and α-granules.[1] VWF mainly affects hemostasis by facilitating platelet adhesion and by stabilizing coagulation F-VIII thereby optimizing its function to enhance fibrin formation.[2] In addition, by recruiting platelets to the damaged vessel wall, via the interaction with the platelet glycoprotein 1b, vWF stimulates platelet adhesion and aggregation contributing to proinflammatory effects that may enhance atherosclerosis.[3,4]

In studies of the general population, vWF plasma levels show weak positive associations with CVD, whereas a more convincing association between vWF levels and CVD is observed in a high-risk population.[5,6] In addition, results from a recent study show that individuals with high vWF antigen levels have a higher risk of cardiovascular mortality than those with the lowest levels.[7] The relation between vWF activity and CVD has not yet been investigated. VWF levels are genetically determined by single-nucleotide polymorphisms (SNPs) in the vWF gene. To date, four SNPs have been identified to affect the vWF gene; rs7964777, rs7954855, rs7965413 and rs7966230. Numerous studies have demonstrated that the genetic variations in the vWF promoter region resulted in higher levels of vWF.[8,9] Higher levels of vWF have been repeatedly associated with cardiovascular disease, in particular arterial thrombosis, thus SNPs affecting the vWF may be directly linked to the incidence of arterial thrombosis.[10-12] In addition, novel genes that affect the vWF levels have been identified, such as ADAMTS13, TSP-1 and most recently soluble NSF attachment protein receptor (SNARE) genes. While more extensive research is required, the first results show an association between these genes, vWF and the risk of arterial thrombosis.[7,13,14]

A quantitative or qualitative deficiency of vWF, known as von Willebrand disease (VWD), poses an individual at greater risk of bleeding, most commonly manifested as epistaxis or menorrhagia.[15] Lower plasma levels of vWF have been associated with reduced development of aortic atherosclerosis in pigs.[16,17] Only few studies investigating the effect of VWD on CVD in humans have been performed, demonstrating a significant negative association between VWD and CVD.[18–20] VWD is an underdiagnosed condition, due to mild manifestation of symptoms, as well as a physiological wide range of vWF plasma levels in the population.[21] Therefore exploring the association between vWF antigen levels as a continuous variable, rather than linked to a diagnosis of VWD (dichotomous variable), and CVD in a general population may give different findings than observed in previous studies.[18,20] We hypothesized that the lower risk for CVD is not limited to VWD patients but also occurs in individuals at the lower end of the VWF activity and antigen range. Therefore, we carried out the present analysis within a subsample of the GHS to prospectively explore whether having low levels of vWF antigen and vWF activity reduces the risk for CVD and (cardiovascular) death.

Methods

Research design

The GHS is a population-based, prospective, observational, single center cohort study and included 15,010 individuals at baseline examination. The aim of the GHS is to improve individual cardiovascular risk stratification. The sample was drawn randomly from the governmental local registry offices in the City of Mainz and the district of Mainz-Bingen and was stratified 1:1 for sex and residence (urban and rural) and in equal strata for decades of age. Individuals between 35 and 74 years of age were enrolled, and written informed consent was obtained from all participants for laboratory analyses, clinical examinations, sampling of biomaterial and use of data records for research purposes. At the baseline visit, study participants underwent an extensive 5-hour standardized clinical examination program as further discussed elsewhere.[22,23] Next to this, a large biobank for further biochemical and genetic analyses was established. The study was designed in line with the tenets of the revised Helsinki protocol and the study protocol and sampling design were approved by the local ethics committee and by the local and federal data safety commissioners. Primary outcomes were incidence of MI and death related to CVD (MI, CAD, HF, AF, VTE, stroke, PAD). Secondary outcomes were the incidence of CAD, stroke, CHF, AF and PAD or all-cause mortality. Primary and secondary outcomes were assessed during the follow-up period through collection of the data on the participants from the University Medical Center in Mainz, as well as during the telephone interview after 2,5 years and the follow-up examination after 5 years. Afterwards the data on primary outcome were discussed by a research panel consisting of two physicians and one epidemiologist. Cardiovascular death was defined as mortality due to MI, CAD, stroke, PAD, HF, AF and VTE was assessed through the medical death certificate.

Follow-up

As part of the follow-up, a standardized computer-assisted telephone interview and an inventory of primary and secondary outcomes were done 2.5 years after baseline visit. In addition, participants undergo a quinquennial, extensive clinical examination in the research facility similar to the baseline visit.[22] During the follow-up period until June 2020 (median follow-up 10.1 years) 471 out of 5,000 participants were lost to follow-up. Reasons for loss to follow-up were death (126 individuals), discontinuation of participation by participant (420 individuals), relocation (59 individuals) and lack of success in contacting the participant (136).

Blood sampling and laboratory assessment

Venous blood sampling was performed according to standard operating procedures and the blood was collected in 3.2%(w/v) trisodium citrate (0.109 M, 1:9 vol:vol) monovette plastic tubes, while the subject was in fasting state (i.e. overnight fast, if subject was examined before 12 p.m. and 5 hour fast, if subject was examined after 12 p.m.). PPP was prepared by one-step centrifugation at 2,000 x *g* at room temperature for 10 minutes. After preparation the PPP was aliquoted and immediately stored at -80 °C. VWF antigen was measured by enzyme-linked immunosorbent assay (ELISA) in the Laboratory for Clinical Thrombosis and Hemostasis at Maastricht University Medical Center. VWF activity was measured by means of immunological-based assay at the departments of Clinical Epidemiology and Systems Medicine at University Medical Center Mainz. F-VIII (%) was measured by means of the Siemens BCS XP System, using the clotting-based coagulation methodology at the latter institution.

Study population

For this study, a subsample of the first 5,000 participants of the population-based GHS enrolled between April 2007 and October 2008, were included. Due to lack of material sent to Maastricht University Medical Center, resulting in a discrepancy between the total number of samples assessed at the two locations, 4,857 individuals remained for further analysis.

Definition of traditional cardiovascular risk factors

Diabetes mellitus and dyslipidemia were defined as individuals with a definite diagnosis of the respective disease by a physician. Additional definition of diabetes was a blood glucose level of ≥126mg/dl in the baseline examination after an overnight fast of at least 8 hours or a blood glucose level of ≥200mg/dl in the baseline examination after a fasting period <8 hours. Dyslipidemia was additionally defined as a LDL/HDL-ratio of >3.5. Hypertension was diagnosed, if antihypertensive drugs are taken, or a mean systolic blood pressure of ≥140mmHg or a mean diastolic blood pressure of ≥90mmHg (in the 2nd and 3rd standardized measurement after 8 and 11 minutes of rest). Smoking was classified into non-smokers (never smokers and former smokers) and smokers (occasional smoker, i.e. <1 cigarette/day, and smoker, i.e. ≥1 cigarette/day). Obesity defined as a body-mass index ≥ 30 kg/m^2. Self-reported CAD, MI, HF, stroke, DVT, PE and PAD indicated personal history of CVD. A positive family history was defined as history of myocardial infarction or stroke in a female first-degree relative ≤65 years or a male first-degree relative ≤60 years.

Data management and statistical analysis

A central data management unit conducted quality control on all data in this study. Statistical analysis was performed with software program R, version 3.3.1 (www.R-project.com). In order to study whether low levels of vWF affect the risk for cardiovascular disease and death, vWF antigen and activity below the 20th percentile were set as a measure of ''low vWF''. Robust poisson regression models, adjusted for age, sex, traditional CVRFs, and F-VIII were used to analyse the relation between low levels of vWF antigen and activity (i.e. below the 20th percentile) and the incidence of CVD.

Consequent cox regression models, each additionally adjusted for age, sex, CVRFs, CVD and F-VIII were calculated to explore the relation between all-cause and cardiovascular mortality and vWF antigen and activity below the 20th percentile. Cumulative incidence plots of cardiovascular mortality were calculated according to Gray's test, correcting for competing events. Due to the explorative nature of the current study, p-values were not set as a means of statistical significance.

Results

Sample characteristics

Details on the study population are reported in **Table 1A. and 1B**. A detailed overview of the socio-economic status of the study population can be found elsewhere.[24] Overall, there was a balanced sex ratio in the study population across all the individual percentiles of vWF antigen and vWF activity. As shown in Table 1A and 1B, both vWF antigen and activity increased with age (e.g. mean age was 60.4 years in the subgroup of vWF antigen >80%, as compared to a mean age of 51.1 years in the subgroup of vWF antigen <20). Arterial hypertension was the most prevalent CVRFs across all the percentiles of vWF antigen and activity, followed by dyslipidemia. The prevalence of all CVRFs, except for smoking, increased with higher vWF levels (**Table 1A and 1B**). Of the pre-existing comorbidities, CAD(4.6%) was predominant across all percentiles of vWF antigen and vWF activity, followed by PAD and VTE. Similarly, the incidence of CAD was highest across all the percentiles of vWF antigen and vWF activity, followed by PAD.

As shown in **Table 1A. and 1B.**, the group of vWF antigen and activity below the 20th percentile consisted of the highest number of subjects with blood group O (67.9% and 72.6%, respectively).

Distribution of vWF

In general, minimum and maximum measured values in the study population of vWF antigen were 28% and 369% and of vWF activity these were 22.8% and 300%, respectively, as demonstrated in **Table 2**. Furthermore, the median value of vWF antigen was 112% (Q1, 88%; Q3, 141%) and vWF activity was 107% (Q1, 81.6%; Q3 138%). The 20th percentile, which was the threshold set to be considered ''low vWF'', corresponded to vWF antigen levels of 83% and vWF activity levels 76.2% as shown in **Table 2**.

Low levels of vWF and incidence of cardiovascular disease

Results from the multiple robust poisson regression analyses for vWF antigen and vWF activity are presented in **Table 3**. VWF activity level below the 20th percentile (i.e. below 76.2%) was associated with a decreased relative risk for CVD (RR: 0.57, 95%CI: 0.35-0.90), independent of age and sex. After adjusting for traditional CVRFs, the association persisted (RR:0.59, 95%CI: 0.37-0.95). After additionally adjusting for levels of F-VIII, the association remained (RR: 0.60, 95%CI: 0.36-0.99). On the contrary, there was no association between vWF antigen and the incidence of CVD, independent of age, sex, CVRFs, and levels of F-VIII (**Table 3.**).

Table 1A. Sample characteristics according to percentiles of vWF antigen

Variable	<20% N=942	20-80% N=2974	>80% N=941
Sex (Women)	50.1% (472)	49.1% (1460)	47.0% (442)
Age [y]	51.1±10.3	55.4±10.8	60.4±9.9
BMI [kg/m²]	25.7 (23.4/28.3)	26.5 (23.9/29.8)	27.7 (24.7/31.5)
Blood group (0-type)	67.9% (549)	37.0% (914)	15.6% (122)
Prevalence of CVRFs			
Diabetes	5.6% (53)	9.3% (275)	16.8% (158)
Hypertension	41.3% (389)	51.3% (1526)	61.7% (580)
Dyslipidemia	38.2% (360)	44.7% (1327)	52.5% (494)
Obesity	16.3% (154)	23.9% (712)	33.2% (312)
Smoking	21.3% (200)	19.4% (574)	17.0% (160)
FH of MI/Stroke	22.3% (210)	23.6% (702)	24.2% (228)
Comorbidities			
MI	1.6% (15)	2.7% (79)	6.1% (57)
CAD	2.6% (24)	4.1% (119)	8.2% (76)
PAD	2.5% (23)	3.8% (112)	6.9% (64)
HF	0.5% (5)	1.2% (37)	3.5% (33)
VTE	2.9% (27)	3.3% (99)	7.3% (68)
Stroke	1.1% (10)	1.8% (52)	3.2% (30)
AF	1.8% (17)	2.3% (67)	5.0% (47)
Incidence of comorbidities			
MI	0.6% (5)	0.8% (19)	1.0% (7)
CAD	1.1% (9)	1.4% (35)	3.4% (23)
PAD	1.0% (8)	1.6% (40)	2.1% (14)
HF	0.4% (3)	1.4% (35)	1.5% (11)
Stroke	0.5% (4)	0.9% (23)	1.3% (9)
AF	0.6% (5)	1.6% (40)	3.0% (21)

Table 1B. Sample characteristics according to percentiles of vWF activity

Variable	<20% N=991	20-80% N=2985	>80% N=994
Sex (Women)	47.9% (475)	48.5% (1449)	51.8% (515)
Age [y]	51.2±10.2	55.4±10.8	59.9±10.3
BMI [kg/m²]	25.6 (23.3/28.5)	26.5 (24.0/29.7)	27.4 (24.5/31.3)
Blood-type (0-type)	72.6% (618)	36.2% (900)	13.2% (108)
Prevalence of CVRFs			
Diabetes	6.0% (59)	9.8% (292)	14.2% (141)
Hypertension	43.3% (429)	51.2% (1528)	59.8% (594)
Dyslipidemia	39.3% (389)	44.9% (1340)	50.6% (502)
Obesity	17.7% (175)	23.6% (705)	32.0% (318)
Smoking	20.9% (207)	19.7% (586)	16.0% (159)
Family history of MI/Stroke	21.8% (216)	23.0% (687)	25.7% (255)
Pre-existing comorbidities			
MI	1.6% (16)	2.7% (80)	5.9% (58)
CAD	2.6% (26)	4.1% (121)	8.0% (78)
PAD	2.3% (23)	3.7% (111)	6.8% (67)
HF	0.5% (5)	1.3% (40)	3.2% (32)
VTE	3.2% (32)	3.4% (101)	6.8% (67)
Stroke	1.0% (10)	1.9% (56)	2.8% (28)
AF	2.0% (20)	2.5% (75)	4.1% (41)
Incidence of comorbidities			
MI	0.2% (2)	1.1% (27)	0.4% (3)
CAD	0.9% (8)	2.0% (49)	1.7% (12)
PAD	0.8% (7)	1.6% (39)	2.2% (16)
HF	0.6% (5)	1.3% (33)	1.6% (12)
Stroke	0.5% (4)	1.0% (24)	1.0% (8)
AF	0.7% (6)	1.6% (40)	3.1% (23)

Table 2. Distribution of vWF antigen and activity

Variable	Minimum	Mean (SD)	Median (Q1/Q3)	5th percentile	20th percentile	95th percentile	Maximum
vWF anigen [%]	28	122 (2.6)	112 (88/141)	61.2	83	236	369
vWF activity [%]	22.8	112 (9.5)	107 (81.6/138)	56.4	76.2	179	300

Q1, quartile 1; Q3, quartile 3.

Table 3. Multiple robust poisson regression analysis for the correlation between the incidence of CVD and vWF antigen and activity <20th percentile

Variable	Model 1*		Model 2†		Model 3‡	
	RR (95% CI)	p-value	RR (95% CI)	p-value	RR (95% CI)	p-value
vWF antigen <20th percentile	0.72 (0.46-1.11)	0.14	0.77 (0.49-1.19)	0.24	0.79 (0.50-1.26)	0.33
Sex (Women)	0.65 (0.49-0.86)	**0.003**	0.72 (0.54-0.97)	**0.03**	0.72 (0.54-0.96)	**0.029**
Age [5y]	1.47 (1.37-1.58)	**<0.0001**	1.48 (1.36-1.60)	**<0.0001**	1.47 (1.36-1.60)	**<0.0001**
Diabetes	-	-	1.12 (0.77-1.63)	0.55	1.11 (0.76-1.62)	0.57
Hypertension	-	-	1.28 (0.91-1.80)	0.15	1.27 (0.90-1.80)	0.16
Dyslipidemia	-	-	1.38 (1.03-1.85)	**0.028**	1.38 (1.03-1.84)	**0.029**
Obesity	-	-	1.36 (1.01-1.84)	**0.043**	1.36 (1.01-1.84)	**0.043**
Smoking	-	-	1.91 (1.359-2.70)	**0.0002**	1.93 (1.36-2.73)	**0.0002**
FH of MI/Stroke	-	-	0.98 (0.70-1.37)	0.91	0.98 (0.70-1.37)	0.92
Levels of F-VIII	-	-	-	-	1.00(0.99-1.00)	0.67
	Model 1*		**Model 2†**		**Model 3‡**	
Variable	**RR (95% CI)**	**p-value**	**RR (95% CI)**	**p-value**	**RR (95% CI)**	**p-value**
vWF activity<20th percentile	0.57 (0.35-0.90)	**0.018**	0.59 (0.37-0.95)	**0.029**	0.60 (0.36-0.99)	**0.046**
Sex (Women)	0.66 (0.50-0.88)	**0.004**	0.74 (0.55-0.98)	**0.036**	0.73 (0.55-0.98)	**0.036**
Age [5y]	1.47 (1.37-1.58)	**<0.0001**	1.47 (1.35-1.59)	**<0.0001**	1.47 (1.35-1.59)	**<0.0001**
Diabetes	-	-	1.11 (0.77-1.61)	0.56	1.11 (0.77-1.61)	0.57
Hypertension	-	-	1.31 (0.93-1.84)	0.12	1.31 (0.93-1.84)	0.12
Dyslipidemia	-	-	1.38 (1.03-1.84)	**0.028**	1.38 (1.03-1.84)	**0.028**
Obesity	-	-	1.40 (1.04-1.88)	**0.026**	1.40 (1.04-1.88)	0.026
Smoking	-	-	1.98 (1.41-2.76)	**<0.0001**	1.98 (1.41-2.78)	**<0.0001**
FH of MI/Stroke	-	-	0.96 (0.68-1.33)	0.81	0.96 (0.69-1.34)	0.82
Levels of F-VIII	-	-	-	-	1.00 (0.99-1.00)	0.88

*Model 1 is adjusted for age and sex †Model 2 is additionally adjusted for the CVRFs ‡Model 3 is additionally adjusted for F-VIII; RR, relative risk.

Cumulative incidence plots

As presented in **Figure 1A-B**, vWF antigen below the 20th percentile was significantly correlated with decreased death as a result of cardiovascular events (p=0.00011 for trend). Furthermore, the vWF activity below and within the 40th percentile was associated with a reduced mortality as a result of cardiovascular events (p=0.0027 for trend).

VWF and mortality

The analysis of the low levels of vWF antigen and vWF activity (i.e. below the 20th percentile) and risk of all-cause and cardiovascular mortality is shown in **Table 4**. During a follow-up period until June 2020 (median follow-up of 10.1 years), a total of 351 deaths were registered of which 81 deaths were registered as a result of cardiovascular events. VWF antigen below the 20th percentile (i.e. vWF antigen levels below 83%) was significantly associated with lower risk of all-cause mortality (HR: 0.60, 95%CI: 0.41-0.88), adjusted for age and sex. After adjusting for the traditional CVRFs and a history of CVD, the association remained significant (HR: 0.61, 95%CI: 0.41-0.91). However, after further adjusting for F-VIII, the association was lost (HR: 0.81, 95%CI: 0.53-1.23). Furthermore, vWF activity below the 20th percentile (i.e. vWF activity levels below 76.2%) was associated with a reduced risk for all-cause mortality (HR: 0.69, 95%CI: 0.49-0.99) in a model corrected for age, sex and traditional CVRFs. However, after additionally adjusting for pre-existing CVD and F-VIII, the association did not persist (HR: 0.99, 95%CI: 0.67-1.47).

In addition, subjects with vWF antigen levels below the 20th percentile showed a tendency for diminished risk of cardiac causes-related death (HR: 0.40, 95%CI: 0.14-1.10). After additionally adjusting for F-VIII levels, the association was lost (HR: 0.38 , 95%CI: 0.12-1.23).

Table 4. Cox regression models to determine the correlation between vWF antigen and activity levels <25th percentile and overall and cardiovascular mortality

	Model 1*		Model 2†		Model 3‡		Model 4‡‡	
All-cause mortality	**HR (95%CI)**	**p-value**	**HR (95%CI)**	**p-value**	**HR (95%CI)**	**p-value**	**HR (95%CI)**	**p-value**
vWF antigen <20th percentile	0.60 (0.41-0.88)	**0.009**	0.62 (0.42-0.92)	**0.018**	0.61 (0.41-0.91)	**0.016**	0.81 (0.53-1.23)	0.33
vWF activity <20th percentile	0.69 (0.49-0.99)	**0.047**	0.72 (0.51-1.03)	0.077	0.73 (0.50-1.05)	0.09	0.99 (0.67-1.47)	1
Cardiovascular mortality	**HR (95%CI)**	**p-value**	**HR (95%CI)**	**p-value**	**HR (95%CI)**	**p-value**	**HR (95%CI)**	**p-value**
vWF antigen <20th percentile	0.40 (0.14-1.10)	0.078	0.41 (0.15-1.13)	0.086	0.33 (0.10-1.058)	0.062	0.38 (0.12-1.23)	0.11
vWF activity <20th percentile	0.82 (0.39-1.71)	0.6	0.82 (0.39-1.71)	0.6	0.77 (0.35-1.70)	0.53	0.91 (0.41-2.04)	0.83

*Model 1 is adjusted for age and sex; †Model 2 is additionally adjusted for CVRFs; ‡Model 3 is additionally adjusted for CVD ‡‡Model 4 is additionally adjusted for levels of F-VIII.

Figure 1A and 1B Cumulative incidence plots for vWF antigen and activity percentiles and cardiovascular mortality

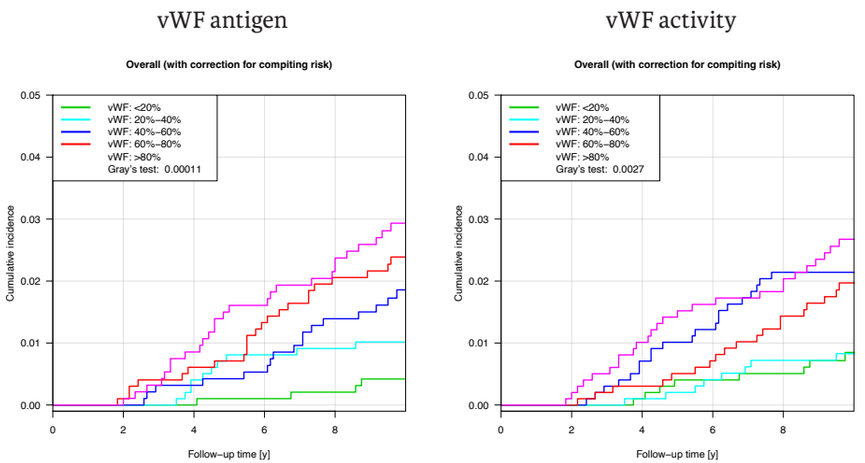

Figure legends:

Figure 1A. Cumulative incidence plots demonstrating the cumulative incidence of cardiovascular mortality during a follow-up period of 10 years, in individuals with vWF antigen levels below the 20th percentile (green line), between 20-40th percentile (turquoise line), between 40-60th percentile (blue line), between 60-80th percentile (red line), and above the 80th percentile (purple line). **Figure 1A**, p-value of 0.00011 for the difference between the percentiles. The plots are corrected for competing risks (death by non-cardiovascular causes).

Figure 1B. Cumulative incidence plots demonstrating the cumulative incidence of cardiovascular mortality during a follow-up period of 10 years, in individuals with vWF activity levels below the 20th percentile (green line), between 20-40th percentile (turquoise line), between 40-60th percentile (blue line), between 60-80th percentile (red line), and above the 80th percentile (purple line). **Figure 1B**, p-value of 0.0027 for the difference between the percentiles. The plots are corrected for competing risks (death by non-cardiovascular causes).

Discussion

One of the main findings of this study was that vWF activity below 76% was associated with a 40% decreased risk for CVD, independent of age, sex, CVRFs and previous CVD. Moreover, the current study shows that having vWF antigen levels below 83% correlated with a 40% decreased risk of all-cause mortality, which was lost after adjusting for F-VIII. In addition to this, the cumulative incidence plots show that vWF antigen below 83% and vWF activity below 76.2% result in significantly fewer deaths related to CVD.

To the best of our knowledge, this is the first study that demonstrated a lower risk of incident CVD in individuals with low levels of vWF activity and increased survival when having low levels of vWF antigen. Previously, Seaman and colleagues conducted a study to compare the incidence of CVD in a vWF deficient and vWF non deficient group. However, the definition of vWF deficiency was limited to vWF antigen (<0.5 IU/dL).[19]

VWF affects hemostasis by mediating platelet adhesion to the damaged vascular wall on the one hand, as well as by binding to F-VIII, thereby limiting its degradation on the other hand.[25] Hemostasis also plays a pivotal role in the pathogenesis of atherosclerosis, which eventually manifests as CVD.[26] Hence, border low vWF antigen and activity could reduce platelet adhesion and activation, reducing not only procoagulant effects of platelets but potentially also their inflammatory role. Platelets interact with inflammatory cells, shed extracellular vesicles and release various chemokines, all processes that are important in atherogenesis. It is therefore likely that reduced levels of WF may translate into reduced platelet binding and activation and subsequent reduced thrombo-inflammation and diminished build-up of atherosclerotic plaque. Although conceptually appealing, literature reports conflicting observations regarding this issue. Whereas Bilora and colleagues reported a significantly lower prevalence of carotid plaque in VWD patients in the late 90's, Sramek and co-workers could not find differences in prevalence of atherosclerotic plaque in type 3 VWD patients as compared to the reference sample.[27,28] However, these reports are from small-scale studies that were investigating the prevalence of atherosclerotic plaque rather than occurrence of CVD events. Besides, the studies included VWD type 3 patients, meaning patients have a full quantitative deficiency of vWF.

The link between blood group ABO and quantity and quality of vWF along with the relation with CVD has long been recognized.[29,30] Emerging recent data suggest that there is an enhanced clearance of vWF in subjects with blood group O, rather than a decreased synthesis.[31] It has been demonstrated that blood group O have 25% lower plasma levels of vWF compared to non-O blood group. Furthermore, in individuals with blood group O, vWF demonstrated enhanced susceptibility to ADAMTS13 proteolysis. Preliminary findings also suggested that the interaction of vWF in blood group O with platelets may also be reduced.[31]

In addition to the relation with vWF quantitative and qualitative properties, in the same study population we have previously shown that individuals with blood group O have lower F-VIII activity. Adjusted for sex and age, ABO blood group accounted for 18.3% of F-VIII activity variation.[32] Therefore, ABO blood group was omitted as confounder in the analysis. Furthermore, when the analysis was adjusted for levels of F-VIII, the association persisted, illustrating the effect of vWF on the incidence of CVD, despite the levels of F-VIII. Folsom et al. previously demonstrated that the incidence of CVD was affected by both vWF and F-VIII, independently.[33] However, based on the results it could not be determined whether the effect was mediated through one another or whether vWF and F-VIII are intertwined. The present analysis may suggest that the observed association is predominantly is determined by levels of vWF rather than F-VIII.

Holm and colleagues previously reported a decreased CVD-related mortality in hospitalized VWD patients. However, the analysis was conducted within a patient population, rather than assessing the individual vWF antigen and vWF activity test outcomes.[34] Our findings demonstrate that CVD-related and all-cause mortality is decreased in individuals with border-low vWF antigen levels, in addition to VWD patients. As in accordance with previous studies, this analysis demonstrated increasing levels of vWF with age.[35,36] The mechanism underlying the relation between age and vWF levels may be manifold, as described by a recent, extensive review by Alavi and colleagues. A pivotal underlying process is the age-related vascular dysfunction inflammation resulting from chronic endothelial stress. Indeed, vWF is a known marker of endothelial dysfunction. Moreover, there may be increased constitutive secretion of vWF through factors known to affect vWF levels like thrombin, histamine, vasopressin. It has also been postulated that vWF increases with age due to a decrease of ADAMTS13, resulting in the persistent circulation of ultra-large vWF multimers.[37]

The major strength of this large scale study is that it comprises cardiovascular healthy participants and participants with a medical history and therefore gives a correct overview of vWF in the general population. A limitation of the study was that there may have been unknown confounders that were not adjusted for, as they were not measured in the GHS.

To summarize, this study reveals associations between low levels of vWF antigen and activity and a diminished risk of (CVD-related) mortality. Importantly, these individuals were not identified as VWD patients. Thus, there was no bleeding tendency in these individuals and results showed primarily beneficial effects of low vWF levels. Overall, this may shine new light on vWF as a potential target for novel therapeutic prevention of CVD, however its implementation is questionable. 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.

Funding

The GHS is funded through the government of Rhineland-Palatinate (“Stiftung RheinlandPfalz für Innovation”, contract AZ 961–386261/733), the research programs “Wissenschafft Zukunft” and “Center for Translational Vascular Biology” of the Johannes Gutenberg-University of Mainz, and its contract with Boehringer Ingelheim and PHILIPS Medical Systems, including unrestricted grants for the Gutenberg Health Study. This work was supported by the German Federal Ministry of Education and Research (BMBF 01EO1003) and the Center for Translational Vascular Biology of the University Medical Center Mainz (to P.S. Wild). H. ten Cate was a Fellow of the Gutenberg Research Foundation. H.M.H. Spronk and H. ten Cate received funding for research from Bayer and Pfizer, outside the work presented in this paper.

Acknowledgments

We are grateful to all study participants and all co-workers who are involved in planning and conducting the GHS.

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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?

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Chapter 7: Impact paragraph

Despite global efforts, CVD is still the leading cause of disease and disability worldwide. Even though efforts to control traditional CVRFs have been effective, the rising numbers are concerning, predominantly in the developing countries.[1] By 2030 the total global cost of CVD is set to rise from approximately €713 billion (2010) to a staggering €863 billion.[2,3]

More than ever, there is medical and economical need for a different approach in managing prevention of CVD and a search for novel biomarkers, as a step towards personalized medicine. The TGA, a widely-used tool in thrombosis and hemostasis research, offers an estimate of the clotting potency of a given plasma sample. In a research setting, TGA is an established tool that is able to detect bleeding tendency (hypocoagulability) and venous thrombosis (hypercoagulability), while associations with arterial thrombosis are conflicting. The clinical validation of the TGA in relation to arterial and/or venous thrombosis remains to be addressed. Therefore, we researched TGA in the context of age, sex, traditional CVRFs, CVD and overall mortality (**chapter 2**) and investigated the biochemical determinants of the TGA (**chapter 3**). The analysis in **chapter 2** demonstrates that lag time (time needed for the thrombin to form) and ETP (overall amount of thrombin formed over time), two parameters of TGA, are associated with increased mortality. Due to the high prevalence of CVD in the general population, we may presume that the majority of the deaths are related to CVD. It illustrates that parameters of TGA might be valuable as a prognostic factor in the individual CVD risk assessment and deciding whether a patient needs additional anticoagulant or anti-inflammatory therapy.

At this point in time, TGA remains a relatively costly and labor-intensive test. However, its potential to develop into a bedside lab testing to address the overall coagulation ‘‘status’’ of a patient is feasible. The consequences of early detection of hypercoagulability to guide preventive management, in particular in high risk situations (post-operative, during pregnancy, COVID-19, cancer patients) could have impact on optimizing thrombosis prophylaxis, which would consequently improve the quality of life of patients.

A topical example: we learned from the COVID-19 epidemic that DVT or PE occurs in 21% of the hospitalized COVID-19 patients and 31% of the COVID-19 patients admitted to the intensive care unit, in spite of prophylactic doses of low-molecular-weight heparin (LMWH).[4] At present, we are not able to determine which COVID-19 patients are at risk for developing VTE under prophylactic LMWH. At the same time, over-prescribing or administering too high doses of LMWH to COVID-19 patients would increase bleeding risk, impacting quality of life, morbidity and mortality.[5] TGA could be used to detect patients at high risk, and could therefore be helpful to optimize individualized prophylactic LMWH treatment.

Chapter 4 and **chapter 5** describe the associations of TFPI and vWF on CVD(-related death). Inexpensive biomarkers for assessing the cardiovascular

risk are of increasing interest, as they may contribute to risk stratification, ultimately aiming for more cost effective disease management.[6] Timely observation of endothelial cell dysfunction through TFPI measurement may be a starting point for more expeditious use of endothelial cell protective medication, like statins. For the latter, further research is required to answer the question whether statins could reduce TFPI levels and endothelial dysfunction, regardless of baseline LDL levels.

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Chapter 8: Appendix

Curriculum Vitae

Pauline van Paridon was born on the 11th of September 1994 in Deventer, the Netherlands. After graduating from the Gymnasium in Groenlo and spending a year living and working in Denmark, she enrolled in the International Bachelor in Medicine at Maastricht University. From her first year onwards Pauline worked as a student assistant in the research department of Biochemistry and developed a special interest in research. After finishing her bachelor degree, Pauline embarked on a full-time research project on the thrombin generation assay and cardiovascular disease in the Gutenberg Health Study, which is a collaboration between the department of Biochemistry/Internal Medicine at Maastricht University and the Center of Thrombosis and Hemostasis at the University Medical Center Mainz (under supervision of Prof. Dr. Hugo ten Cate and Dr. Henri Spronk). Her work was financially supported by the Dutch Heart Foundation.

To this end, she spent 10 months at the University Medical Center Mainz, under supervision of Dr. Panova-Noeva and Prof. Dr. Philipp Wild. She presented her research work at national and international conferences (the Netherlands, Germany, France and the USA), for which she won several prizes and grants (the Society of Thrombosis and Hemostasis Research travel grant as well as the Arteriosclerosis, Thrombosis and Vascular Biology travel award for Young Investigators). From 2018 onwards Pauline worked as a medical intern in several hospitals in the Netherlands and abroad (Germany and India), while working part time on her research project. After obtaining her medical degree in May 2020, Pauline worked as a physician in the Netherlands, Greece and Suriname, while simultaneously completing her PhD dissertation. Next to an interest in (cardiovascular) research, Pauline is passionate about improving global healthcare. Pauline is starting her M.D. specialization in Global Health and Tropical Medicine (Arts Internationale Gezondheidszorg en Tropengeneeskunde) in July 2022 in the pediatrics department of Rijnstate hospital in Arnhem.

Publications

1. **van Paridon PCS**, Panova-Noeva M, van Oerle R, Schulz A, Prochaska JH, Arnold N, Schmidtmann I, Beutel M, Pfeiffer N, Münzel T, Lackner KJ, Hackeng TM, Ten Cate H, Wild PS, Spronk HMH. Relation between Tissue Factor Pathway Inhibitor Activity and Cardiovascular Risk Factors and Diseases in a Large Population Sample. Thromb Haemost. 2021 Feb;121(2):174-181.

2. **van Paridon PCS**, Panova-Noeva M, van Oerle R, Schultz A, Hermanns IM, Prochaska JH, Arnold N, Binder H, Schmidtmann I, Beutel ME, Pfeiffer N, Münzel T, Lackner KJ, Ten Cate H, Wild PS, Spronk HMH. Thrombin generation in cardiovascular disease and mortality - results from the Gutenberg Health Study. Haematologica. 2020 Sep 1;105(9):2327-2334.

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5. **van Paridon PCS**, Panova-Noeva M, van Oerle R, Schultz A, Hermanns IM, Prochaska JH, Arnold N, Schmidtmann I, Beutel ME, Pfeiffer N, Münzel T, Lackner KJ, Ten Cate H, Wild PS, Spronk HMH. Lower levels of vWF and a diminished risk of cardiovascular disease and mortality are linked through F-VIII. *Under revision at Research and Practice in Thrombosis and Haemostasis.*

Oral and poster presentations

d'Alessandro E, Becker C, Bergmeier W, Bode C, Bourne JH, Brown H, Buller HR, Ten Cate-Hoek AJ, Ten Cate V, van Cauteren YJM, Cheung YFH, Cleuren A, Coenen D, Crijns HJGM, de Simone I, Dolleman SC, Klein CE, Fernandez DI, Granneman L, van T Hof A, Henke P, Henskens YMC, Huang J, Jennings LK, Jooss N, Karel M, van den Kerkhof D, Klok FA, Kremers B, Lämmle B, Leader A, Lundstrom A, Mackman N, Mannucci PM, Maqsood Z, van der Meijden PEJ, van Moorsel M, Moran LA, Morser J, van Mourik M, Navarro S, Neagoe RAI, Olie RH, **van Paridon P**, Posma J, Provenzale I, Reitsma PH, Scaf B, Schurgers L, Seelig J, Siegbahn A, Siegerink B, Soehnlein O, Soriano EM, Sowa MA, Spronk HMH, Storey RF, Tantiwong C, Veninga A, Wang X, Watson SP, Weitz J, Zeerleder SS, Ten Cate H; Scientific Reviewer Committee. Thrombo-Inflammation in Cardiovascular Disease: An Expert Consensus Document from the Third Maastricht Consensus Conference on Thrombosis. Maastricht Consensus Conference on Thrombosis (MCCT), Maastricht, the Netherlands, 2019 (participation).

van Paridon PCS, Panova-Noeva M, van Oerle R, Schulz A, Prochaska JH, Arnold N, Schmidtmann I, Beutel M, Pfeiffer N, Münzel T, Lackner KJ, Hackeng TM, Ten Cate H, Wild PS, Spronk HMH. Relation between Tissue Factor Pathway Inhibitor Activity and Cardiovascular Risk Factors and Diseases in a Large Population Sample. American Society of Hematology (ASH), San Diego, USA, 2018 (poster presentation).

van Paridon PCS, Panova-Noeva M, van Oerle R, Schultz A, Hermanns IM, Prochaska JH, Arnold N, Binder H, Schmidtmann I, Beutel ME, Pfeiffer N, Münzel T, Lackner KJ, Ten Cate H, Wild PS, Spronk HMH. Thrombin generation in cardiovascular disease and mortality - results from the Gutenberg Health Study. Congress on Thrombosis and Haemostasis (ECTH), Marseille, France, 2018 (oral presentation).

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van Paridon PCS, Panova-Noeva M, van Oerle R, Schultz A, Hermanns IM, Prochaska JH, Arnold N, Binder H, Schmidtmann I, Beutel ME, Pfeiffer N, Münzel T, Lackner KJ, Ten Cate H, Wild PS, Spronk HMH. Thrombin generation in cardiovascular disease and mortality - results from the Gutenberg Health Study. Arteriosclerosis, Thrombosis, and Vascular Biology/Peripheral Arterial Disease

(ATVB/PVD) congress of the American Heart Association, San Fransisco, USA, 2018 (poster presentation).

van Paridon PCS, Panova-Noeva M, van Oerle R, Schultz A, Hermanns IM, Prochaska JH, Arnold N, Binder H, Schmidtmann I, Beutel ME, Pfeiffer N, Münzel T, Lackner KJ, Ten Cate H, Wild PS, Spronk HMH. Thrombin generation in cardiovascular disease and mortality - results from the Gutenberg Health Study. Society for Thrombosis and Haemostasis Research (GTH), Vienna, Austria, 2018 (oral presentation).

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Grants and awards

Travel Grant by the Dutch Heart Foundation, 2018.

Arteriosclerosis, Thrombosis, and Vascular Biology/Peripheral Arterial Disease (ATVB/PVD) Travel Award for Young Investigators, 2018.

Society for Thrombosis and Haemostasis Research (GTH) Travel Grant, 2018.

Dekker Student Grant by the Dutch Heart Foundation (DHF grant number: 2017SB015), 2017.

Acknowledgments

After years of hard work, combining my PhD research project with my medical studies and eventually clinical work, I am proud to have successfully delivered this thesis. However, it would not have been possible without the input and commitment of everyone that I have met and collaborated with along the way. Therefore, I would like to use this section to express my gratitude.

First of all I wish to thank my supervisor Hugo ten Cate. I want to thank you for all the opportunities that you have given me, the fruitful discussions and valuable feedback. I have learned tremendously from you as a scholar, and admire the way you combine your position in the scientific world with your clinical work.

To my supervisor Philipp Wild, to whom I owe the opportunity to perform my research in Mainz: I am grateful for the pleasant time I got to spend with you and your colleagues, and all the guidance you have provided.

To my co-supervisor, Henri Spronk; you were my tutor in the very first course of my bachelor's, and have since become my mentor. When I - as a first-year medicine student - expressed my interest in research, you immediately took me under your wing and offered me a position as a student assistant. I am eternally thankful for you allowing me to start my own research project. After years of working together, I can finally say that I finished my PhD. Thanks to you, I will pursue my career with a strong interest in medical research. I could not have done this without you and I am thankful to have had you as my co-supervisor!

To my co-supervisor in Mainz, Marina Panova-Noeva: Dear Marina, you made me grow as a doctor, researcher and person. I admire your critical attitude and impressive work, but also enjoy our mutual interest in Global Health. You made me feel part of the team in Mainz and were always willing to make time to explain or discuss the papers and thesis, even when you were not working at the Center of Thrombosis and Hemostasis in Mainz anymore. I hope that we will be able to collaborate again in the near future and I wish you all the best!

I am grateful for the colleagues at the department of Biochemistry/Internal Medicine at Maastricht University that I've met over the years; from the department's secretary, to the PhD students, post-docs, lab technicians and many more. In particular I'd like to thank René van Oerle; Dear René, When I was a first-year medical student, you explained the fundamentals of thrombin generation to me in great detail. That discussion laid the foundation for my eventual research. Without you and your team of lab technicians and interns, I would never have had the results for this thesis. I'd also like to thank Tilman Hackeng for his input, thoughts and discussions on the TFPI chapter.

I am grateful to all participants of the GHS: lab technicians, study nurses, statisticians, and study coordinators; the work presented in this thesis would not have been possible without you. In particular, I would like to thank Andreas Schulz for his work on statistical analyses, his patience in teaching me and his valuable input. I would also like to thank the GHS committee in Mainz; Prof. Lackner, Prof. Beutel, Prof. Münzel and Prof. Pfeiffer for their major contribution to the GHS and the papers.
To my office buddies Bianca and Vincent at Center for Thrombosis and Hemostasis in Mainz: I really enjoyed my time in Mainz, in office, but also outside the office like at the Christmas market and the summer party. I hope we will see each other again in the future!

A special word of appreciation for my dearest friends: Hanna, Jasmijn, Josephine, Louisa and Marijn. Without your support, producing this thesis would have been many times more difficult. Special thanks to my paranymphs, Hanna and Louisa; I am so honored to have you standing next to me on this exciting day. I truly appreciate all the help leading up to this day. I would also like to thank Wieke for her incredible work designing the cover and layout of the thesis.

To my dearest parents, Petra and Emile: I could not have been more lucky to have you as my parents. I want to thank you for all the support that you have given me; you supported me with fruitful discussions about my research project, visited me during my clinical work abroad and even worked by my side as colleagues in Greece!
To my brothers Pieter and Jurriaan; thank you for your continued support on this journey. I know that even though we joke about each other as siblings, you are proud of me as well as I am of you!

And last but not least: I want to thank Bas, my boyfriend, for his unconditional support and understanding. The course of my research meant we could not always be together in the same country. You are always there when I need you; whether it's making a temporary office space in Berlin during the COVID-19 pandemic, visiting me on the other side of the world, or lifting my spirits when needed.