Haemostatic markers and cardiovascular function in black and white South Africans: The SABPA study

L Lammertyn

20088310

Thesis submitted in fulfillment of the requirements for the degree *Philosophiae Doctor* in Cardiovascular Physiology at the Potchefstroom Campus of the North-West University

Promoter: Prof R Schutte

Co-promoters: Prof AE Schutte

Prof M Pieters

April 2015

ACKNOWLEDGEMENTS

I would like to thank the following people for their input and support over the course of this project

- To my promotors, thank you for your profession input, time, honesty, guidance and support throughout the duration of my studies.
- Carina Mels and Wayne Smith for your intellectual input and guidance;
- Me. Christien Terblanche and her team from Cum Laude Language Practitioners for the language editing;
- The SABPA participants for their willingness to participate and for allowing me to use the data;
- DAAD-NRF for providing me with a scholarship for the duration of my studies;
- Shani Botha and Chiné Pieterse for your support, encouragement and for providing distractions during the difficult times;
- Close friends and family, thank you for all your understanding and support;
- My parents and brother, thank you for your encouragement, love and support throughout my academic career;
- Lastly, to all the people who said no, because it's because of you that I did it myself.

TABLE OF CONTENTS

PREFACE

This thesis is presented in the article format and includes three peer-reviewed published or submitted articles. This format is approved, supported and defined by the North-West University guidelines for PhD-studies. The first chapter of this thesis consists of a detailed literature review and states the aims, objectives and hypotheses of the study. Chapters 2, 3 and 4 present the articles in the format in which it was originally submitted for publication in the respective journals. The promoter and co-promoters were included as the co-authors of each paper, as well as additional researchers from the Hypertension in Africa Research Team where applicable. The first author was responsible for the initiation and all parts of this thesis, including literature searches, data mining, statistical analyses, the interpretation of results, as well as the writing of the research papers. All co-authors gave their consent that the research articles may form part of the thesis.

The first article was published in *Thrombosis Research*, the second article was accepted for publication in *Clinical and Experimental Hypertension*, while the third and final article was submitted to *Journal of Hypertension.* All relevant references are provided at the end of each chapter according to the instructions for authors provided by the specific journal in which the papers were published or where they have been submitted for publication. The Vancouver reference style was used for the remaining chapters.

AUTHOR CONTRIBUTIONS

The contribution of each of the authors was:

L Lammertyn: Responsible for initial proposal of the study, along with all extensive literature searches, critical evaluation of study protocol and methodology, data collection during 2009, 2011 and 2012, statistical analyses, design and planning of research articles and thesis, interpretation of results and writing of all sections.

R Schutte: **Promoter:** Guidance, intellectual input, data collection and critical evaluation of statistical analyses and the final product.

AE Schutte: **Co-promoter:** Guidance, intellectual input, data collection and critical evaluation of statistical analyses and the final product.

M Pieters: **Co-Promoter:** Guidance, intellectual input, and critical evaluation of statistical analyses and the final product.

CMC Mels: **Co-author:** Intellectual and well-grounded input regarding oxidative stress in the paper accepted for publication in *Clinical and Experimental Hypertension (Chapter 3).*

W Smith: **Co-author:** Valued expert input and collection of data concerning the retinal vessel calibres in the submitted article to *Journal of Hypertension (Chapter 4)*.

STATEMENT BY THE AUTHORS

The following is a statement of the co-authors to verify their individual contributions and involvement in this study and to grant their permission that the relevant research articles may form part of this thesis:

I hereby declare that I have approved the aforementioned manuscripts and that my role in this study, as stated above, is representative of my actual contribution. I also give my consent that these manuscripts may be published as part of the Ph.D. thesis of Leandi Lammertyn.

Prof. R Schutte

Prof AE Schutte

Prof. M Pieters

Dr. CMC Mels

Dr. W Smith

SUMMARY

Motivation

In the black population of South Africa, cardiovascular disease (CVD) is rapidly increasing due to urbanisation. Stroke is usually accompanied by a prothrombotic haemostatic profile. Changing lifestyle factors that accompany the urbanisation process could have a negative impact on the haemostatic profile of black South Africans. Elevated levels of pro-coagulant factors, von Willebrand factor (vWF), fibrinogen and fibrin D-dimer have been reported in the black population, which could increase the black population's susceptibility to CVD. However, low levels of plasminogen activator inhibitor-1 (PAI-1) previously reported in the black population could contribute towards a pro-fibrinolytic state, which may counteract the hypercoagulant state. This may have a beneficial effect on the haemostatic profile of the black population. More investigation into the haemostatic profile of black South Africans is therefore needed to determine if an altered haemostatic profile exists in this group, and if so, to what extent these alterations may relate to cardiovascular dysfunction. This study included markers of both the coagulation (vWF, fibrinogen, fibrin D-dimer) and fibrinolytic (PAI-1, fibrin D-dimer and fibrinolytic potential) systems in an attempt to investigate the haemostatic profile of the black population of South Africa, and for comparison purposes that of the white population as well. The relationship of these markers' with selected markers of cardiovascular function was also examined to determine if they could possibly contribute to an increase in cardiovascular risk, especially in the black population.

Aims

The aims of this study were to first compare coagulation and fibrinolysis markers in the black and white populations of South Africa. Furthermore, to determine if associations exist between the selected components of the haemostatic system and markers of cardiovascular function, especially in the black population of South Africa, who tends to be at a higher cardiovascular risk due to altered metabolic and haemostatic profiles.

Methodology

The *Sympathetic activity and Ambulatory Blood Pressure in Africans* (SABPA) study was a prospective cohort study that consisted of 409 participants at baseline (2008-2009) that were equally distributed according to both ethnicity (200 black; 209 white) and gender (black, 101 men, 99 women; white, 101 men, 108 women). At follow-up (2011/2012) the cohort totalled 359 participants (170 black, 88 men and 82 women; 189 white, 93 men and 96 women). Data from baseline measurements were used for the first two manuscripts (chapters 2 and 3), while follow-up data was used for the third manuscript (chapter 4). vWF, fibrinogen, PAI-1, fibrin D-dimer, CLT, serum peroxides, glutathione, glutathione peroxidase and reductase activity were determined, and ambulatory blood pressure and the retinal vessel calibres were measured. The groups were stratified by ethnicity as specified by statistical interaction terms. T-tests and chi-square tests were used to compare means and proportions, respectively. Pearson and partial regression analyses were used to determine correlations between the components of the haemostatic system and cardiovascular function markers. This was followed by multiple linear regression analyses to investigate whether independent associations exist between the variables in both ethnic groups. P-values ≤0.050 were deemed significant.

Results and conclusion of each manuscript

The first manuscript (chapter 2) compares the haemostatic profiles of the black and white population to determine whether ambulatory blood pressure is related to components of the haemostatic system. The black participants displayed a prothrombotic profile with significantly higher vWF, fibrinogen, PAI-1, fibrin D-dimer and a longer CLT than their white counterparts. Furthermore, partial and multiple linear regression analyses showed a positive association of systolic and diastolic blood pressure with fibrin D-dimer in the black population, while a negative association existed between ambulatory blood pressure and CLT in the white population. These associations suggest that fibrin D-dimer may contribute, at least in part, to the high prevalence of hypertension in the black population.

The second manuscript (chapter 3) determined associations between markers of the haemostatic and oxidant-antioxidant systems in the black and white populations. In addition to the prothrombotic profile that exists in the black population, this group also had significantly higher serum peroxides (oxidative stress) and lower glutathione peroxidase activity (antioxidant) levels. Multiple linear regression analyses indicated positive associations between fibrinogen and serum peroxides in both populations. In the white population, an additional positive association was found between serum peroxide and CLT. In the black population, vWF and CLT were negatively associated with GPx activity. The results suggest that there are ethnic-specific relationships between the haemostatic and oxidant-antioxidant systems.

The third manuscript (chapter 4) investigated the relationships between the retinal vessel calibres and components of the haemostatic system in the black and white population. The investigation focussed specifically on arteriolar diameters in the lower median, since a narrow arteriolar diameter is known to be associated with elevated blood pressure. In both ethnic groups, a narrower arteriolar calibre was accompanied by narrower venular calibres. Independent positive associations were found between the central retinal vein equivalent (CRVE) and fibrinogen in the black population, as well as vWF and CLT in the white population. In addition, independent negative associations were found between the central retinal artery equivalent and CLT in the black population and with vWF in the white population. The results suggest that haemostatic alterations are linked to early vascular changes that may differ between ethnicities.

General conclusion

Ethnic-specific relationships between the components of the haemostatic system and measures of cardiovascular function are evident. The prothrombotic profile that is observed in the black population, together with the adverse associations of the haemostatic components with blood pressure, a compromised oxidant-antioxidant profile, and retinal vessel calibres may contribute, at least in part, to the high cardiovascular and cerebrovascular risk evident in this population group.

Key words: black, African, ethnicity, cardiovascular, von Willebrand factor, fibrinogen, plasminogen activator inhibitor-1, fibrin D-dimer, clot lysis time, blood pressure, oxidative stress, antioxidant capacity, retinal vessel calibres.

LIST OF TABLES

CHAPTER 4

CHAPTER 4: DATA SUPPLEMENT

LIST OF FIGURES

CHAPTER 1

CHAPTER 2: DATA SUPPLEMENT

ABBREVIATIONS

a	Activated
ABPM	Ambulatory blood pressure
act	Activity
ag	Antigen
ARIC	Arteriosclerosis Risk In Communities
AVR	Arteriolar-to-venular ratio
BMI	Body mass index
cAMP	cyclic Adenosine monophosphate
CLT	Clot lysis time
CRAE	Central retinal arteriolar equivalent
CRP	C-reactive protein
CRVE	Central retinal venular equivalent
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
EDTA	Ethylenediaminetetraacetic
ET-1	Endothelin-1
F	Factor
FpA	Fibrinopeptide A
FpB	Fibrinopeptide B
FVII	Factor VII
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSH	Oxidised glutathione / Glutathione disulphide
H_2O_2	Hydrogen peroxide
HbA1c	Glycosylated haemoglobin A1c
HDL-C	High density lipoprotein cholesterol
HIV	Human Immunodeficiency Virus

hs-CRP	high sensitivity C-reactive protein
ICAM-1	Intercellular adhesion molecule-1
IL-1	Interleukin-1
IL-6	Interleukin-6
LDL-C	Low density lipoprotein cholesterol
MESA	Multi-Ethnic Study of Atherosclerosis
NO	Nitric oxide
p	Probability
PAI-1	Plasminogen activator inhibitor-1
PC	Protein C
PGI	Prostacyclin
PP	Pulse pressure
ROS	Reactive oxygen species
SABPA	Sympathetic activity and Ambulatory Blood Pressure in Africans
SBP	Systolic blood pressure
$SO^{\cdot}$	Super oxide
SOD	Superoxide dismutase
TC	Total cholesterol
TC: HDL-C	Total cholesterol: high-density lipoprotein cholesterol ratio
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TNF-α	Tissue necrosis factor-α
t-PA	tissue Plasminogen activator
u-PA	urokinase Plasminogen activator
vWF	von Willebrand factor
α	Alpha
β	Beta
γ	Gamma
γ-GT / GGT	Gamma-glutamyltransferase

SD	Standard deviation
n / N	Number of participants
r	Regression coefficient
Tot R^2	Total relative predictive power of a model
Adj R^2	Adjusted relative predictive power of a model

MEASURING UNITS

%	percentage
µg/L	micrograms per litre
µM	micrometre
µmol/L	micromole per litre
cm	centimetres
g/L	grams per litre
kcal/day	kilocalories per day
kg	kilogram
kg/m^2	kilogram per square metre
mg/L	milligrams per litre
min	minutes
mm	millimetres
mmHg	millimetre of mercury
mmol/L	micromole per litre
MU	measuring units
N / n	number of participants
ng/ml	nanogram per millilitre
nm	nanometre
U/L	units per litre

CHAPTER 1

Background, literature overview, aims, objectives and hypotheses

1.1 Background

The World Health Organization estimates that approximately 60% of deaths worldwide are attributed to non-communicable diseases of which 30% are caused by cardiovascular disease (CVD) [1]. Specifically low and middle income countries have the highest rates of CVD mortality [1,2]. The incidence of non-communicable diseases is rapidly increasing in sub-Saharan Africa due to epidemiological transition, characterised by increasing urbanisation and associated changes in lifestyle factors [3,4]. The black population of South Africa has a high prevalence of hypertension [5,6], diabetes mellitus [7] and obesity [8], which increases their cardiovascular risk. Hypertension has been identified as a major contributor to stroke risk in sub-Saharan Africa [4]. In comparison with individuals from high income countries, people from African descent have a higher prevalence of stroke that also seems to occur at a younger age [9,10]. A hypercoagulable state has been related to stroke [11] and elevated levels of haemostatic factors such as von Willebrand factor (vWF) [12,13], fibrinogen [14,15], plasminogen activator inhibitor-1 (PAI-1) [16,17] and fibrin D-dimer [18,19] have been implicated as additional risk factors in CVD states.

Pieters *et al*. [20] reported that urbanisation and the resultant increase in non-communicable diseases in black South Africans could possibly have a negative effect on their haemostatic profile. Black individuals tend to have higher vWF, fibrinogen and fibrin D-dimer levels when compared to white individuals [21-23]. However, low levels of PAI-1 have also been reported in the black population from South Africa, which may on the other hand have beneficial effects on their haemostatic profile by counteracting the hypercoagulant state with increased fibrinolysis [24]. Therefore, it is still uncertain to what extent the haemostatic system contributes to cardiovascular dysfunction in black South Africans and further investigation is needed. Key markers of the haemostatic system were identified and analysed (vWF, fibrinogen, fibrin D-dimer and PAI-1 as well as fibrinolytic potential) in an attempt to characterise the haemostatic profile of our population. Moreover, the relationships with blood pressure, oxidative stress and microvascular function were also investigated to determine their possible involvement in CVD risk in both black and white South Africans. A brief literature overview of the haemostatic system and its relationship with cardiovascular function follows.

1.2 The haemostatic process

The haemostatic system (**Figure 1**) consists of several processes (primary haemostasis, secondary haemostasis or coagulation and fibrinolysis) that prevent excessive blood loss and regulate tissue repair at the site of vascular injury [25,26]. Any disruption in the balance of these processes can lead to bleeding disorders or excessive thrombus formation, the latter of which has been found to precede acute clinical manifestations of coronary, cerebrovascular and peripheral artery disease [27,28]. The exact mechanisms by which the haemostatic factors contribute to the development of arterial disease are difficult to determine because of the influence by various genetic (ethnicity and gender) and non-genetic factors (environment and lifestyle).

Primary and secondary haemostasis occurs concomitantly and generates an environment that prevents blood loss and aids in wound healing. When injury to the vessel wall occurs, collagen and tissue factor (TF) are exposed to the flowing blood at the site of injury. The exposed collagen triggers the initiation of primary haemostasis while tissue factor initiates secondary haemostasis [29]. During the primary phase, vWF is released from surrounding endothelial cells and platelets and rapidly binds to the exposed collagen [30]. Once bound, the vWF molecule undergoes a conformation change that exposes its platelet binding sites, especially under high shear conditions, thereby promoting platelet adhesion and aggregation at the site of injury [31]. An unstable platelet plug is rapidly formed and is quickly stabilised by fibrin during secondary haemostasis [32].

The primary objective of secondary haemostasis is thrombin generation, which mediates the conversion of soluble fibrinogen into insoluble fibrin that surrounds the platelet plug [33]. This forms a strong and stable fibrin network that protects the vessel wall from further damage and promotes wound healing [34]. Tissue factor (TF) is the primary initiator of secondary

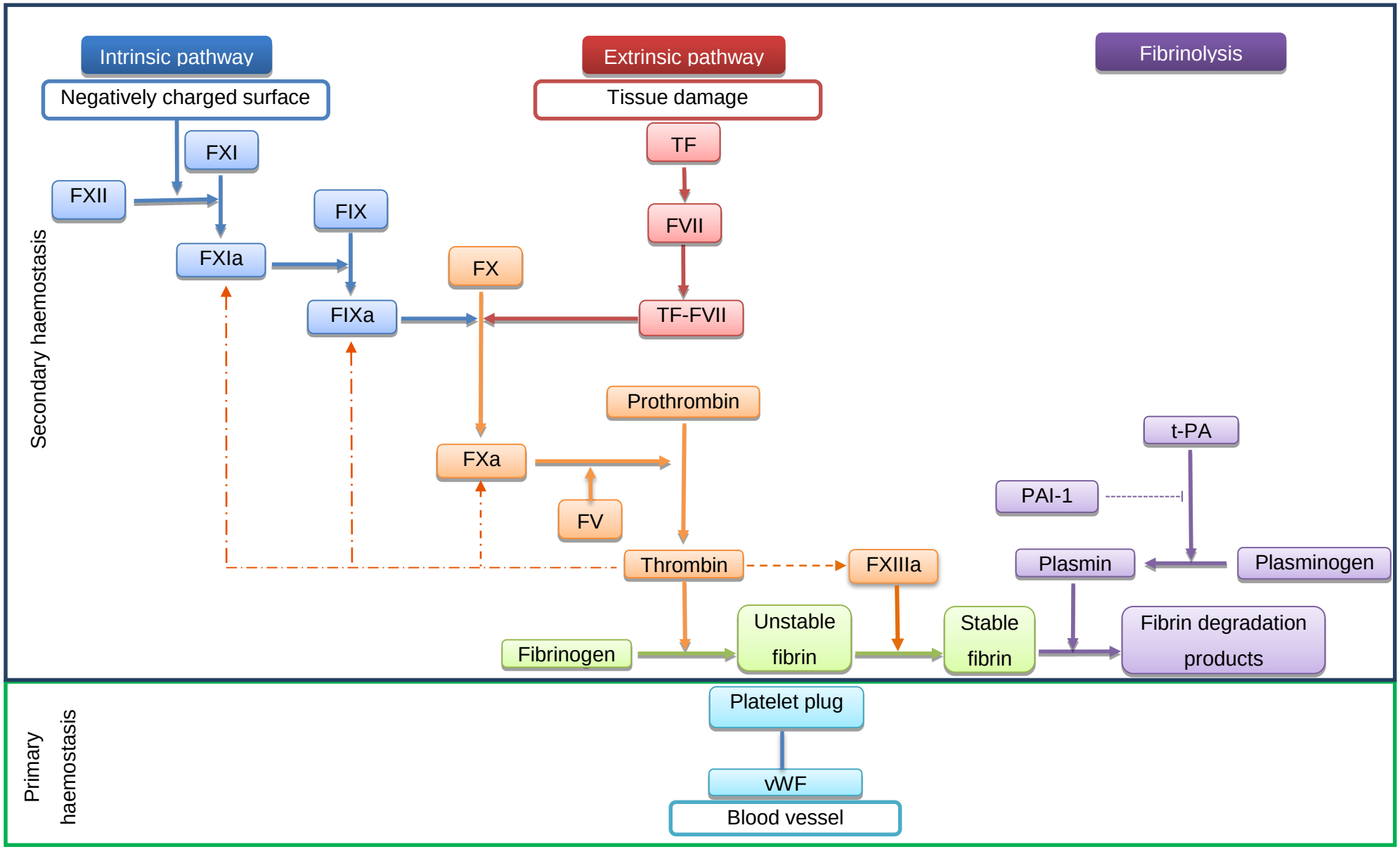

Figure 1: Schematic representation of coagulation and fibrinolysis. Abbreviations: a, activated; F, factor; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; t-PA, tissue plasminogen activator; vWF, von Willebrand factor [25].

haemostasis when damage to the vessel wall has occurred and is located on the plasma membranes of the subendothelial cells [35]. Circulating activated plasma protein factor VII (FVIIa) binds to TF to form the TF-FVIIa complex that activates Factor IX and Factor X. Activated Factor X binds with Factor V to form the prothrombinase complex that converts prothrombin to thrombin [36-38]. The amount of thrombin generated by the extrinsic pathway is however not adequate to produce large amounts of thrombin [39,40]. Nonetheless, the amount of thrombin produced is enough to trigger a positive feedback effect that activates Factor XI, Factor VIII, Factor V as well as the platelets, thereby rapidly amplifying the amount of thrombin produced [25,26]. The intrinsic pathway is activated when circulating Factor XII comes in contact with a negatively charged surface such as the exposed collagen fibres. Once Factor XII is activated, it triggers a cascade of enzymatic reactions that result in the activation of Factor XI, Factor IX and Factor X [41].

It is important to note that both pathways result in the activation of Factor X. This is followed by a set of enzymatic reactions that forms the common pathway [26]. First FIXa binds to FVIII to form the FIX-FVIII complex, also known as the tenase complex that results in the activation of Factor X. Activated Factor X leads to the formation of the prothrombinase complex that is responsible for the formation of thrombin. The formed thrombin then converts fibrinogen into fibrin monomers to form a fibrin clot [26,34,40]. At first, an unstable fibrin clot is formed that consists of fibrin monomers connected with noncovalent bonds. However, the fibrin clot is quickly stabilised by Factor XIII, which is activated by thrombin and calcium [42,43]. Activated Factor XIII results in the formation of covalent bonds between the fibrin monomers, which aid in the formation of cross-linkages between the fibrin fibres. This results in the formation of a stable and dense fibrin clot that seals and protects the site of injury, thereby allowing wound healing to occur [44].

Once wound healing has occurred, the fibrinolytic system is activated to limit further fibrin deposition by dissolving the fibrin clot and restoring normal blood flow to the area [25]. Fibrinolysis consists of enzymatic interactions between plasminogen activators and inhibitors, which result in the degradation of fibrin into fibrin degradation products such as fibrin D-dimer [45]. The fundamental action of the plasminogen activator system is the conversion of plasminogen to

plasmin by tissue-type plasminogen activator (t-PA) [46]. Plasmin degrades the fibrin clots by binding to the lysine binding sites on the surface of fibrin fibres, which lead to transverse cuts of the fibrin fibres. This results in the generation of soluble fibrin degradation products [25]. t-PA activity is regulated by plasminogen activator inhibitor-1 (PAI-1) to prevent the premature lysis of a fibrin clot [47].

1.2.1 von Willebrand factor (vWF)

vWF is a large multimeric plasma glycoprotein that is synthesised in both endothelial cells and megakaryocytes and stored in Weibel-Palade bodies and α-granules, respectively [48-50]. From here it is released in response to secretagogues such as thrombin, fibrin and histamine [30,49,51] (**Figure 2**)

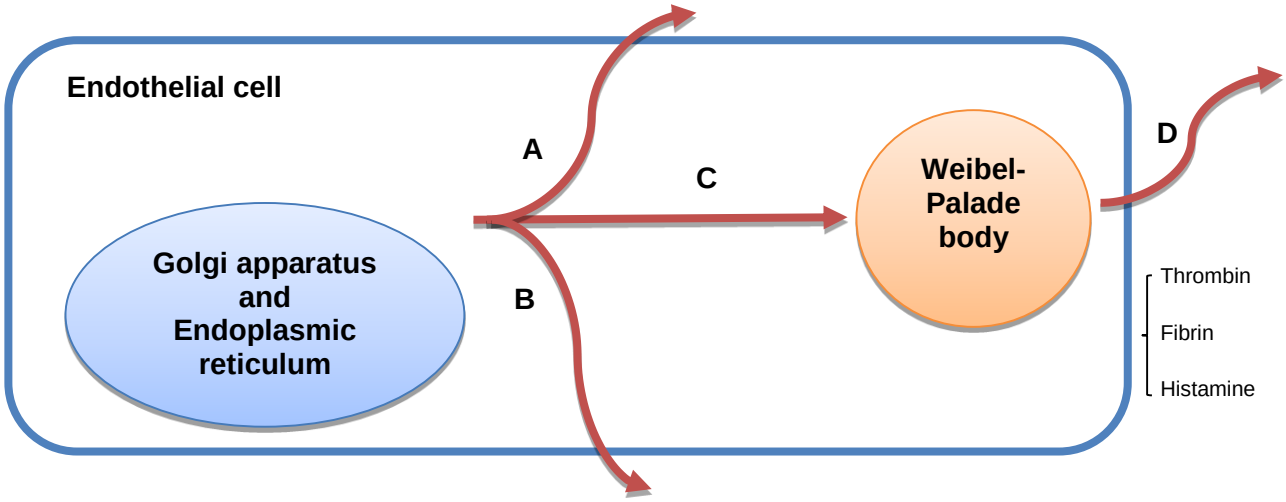

Figure 2: von Willebrand factor synthesis from an endothelial cell, modified from Lip GYH *et al*. [52]. A = constitutive secretion into plasma; B = constitutive secretion to the subendothelium; C = regulated pathway for storage in the Weibel-Palade body; D = Controlled exocytosis of Weibel-Palade body mediated by secretagogues.

Normal circulatory antigen levels range between 75-125% and it has a half-life of approximately 18 hours [53,54]. vWF antigen is released from the endothelial cells at a steady state, while the α-granules only release vWF at the site of injury upon platelet activation [55]. vWF is responsible for the formation of a platelet plug at the site of injury by mediating platelet aggregation and adhesion to exposed subendothelial tissues [31]. Furthermore, it also acts as a carrier molecule for FVIII, thereby protecting FVIII from premature degradation and clearance [56]. Circulating vWF

levels increase with endothelial cell disturbance and/or dysfunction, therefore vWF is considered to be an indicator of endothelial health [30,52].

vWF is synthesised as a large 2813 amino acid precursor (pre-pro-vWF) that consists of a 22 amino acid signal peptide, a 741 amino acid pro-peptide and 2050 amino acid mature vWF subunit [30,57]. vWF undergoes several complex post-translational modifications as it moves along the secretory pathway [55]. Firstly, the signal peptide is cleaved, followed by dimerisation of pro-vWF through inter-subunit C-terminal disulphide bond formation at the carboxyl terminal ends [58]. This is followed by the addition of 12 N-linked and 10 O-linked oligosaccharide side chains that undergo glycosylation by a series of glycosidases and glycosyltransferases in the endoplasmic reticulum [59]. Thereafter, multimerisation of the vWF dimers occur through another round of disulphide bond formation, which is followed by the cleavage of the pro-peptide [60] (**Figure 3**).

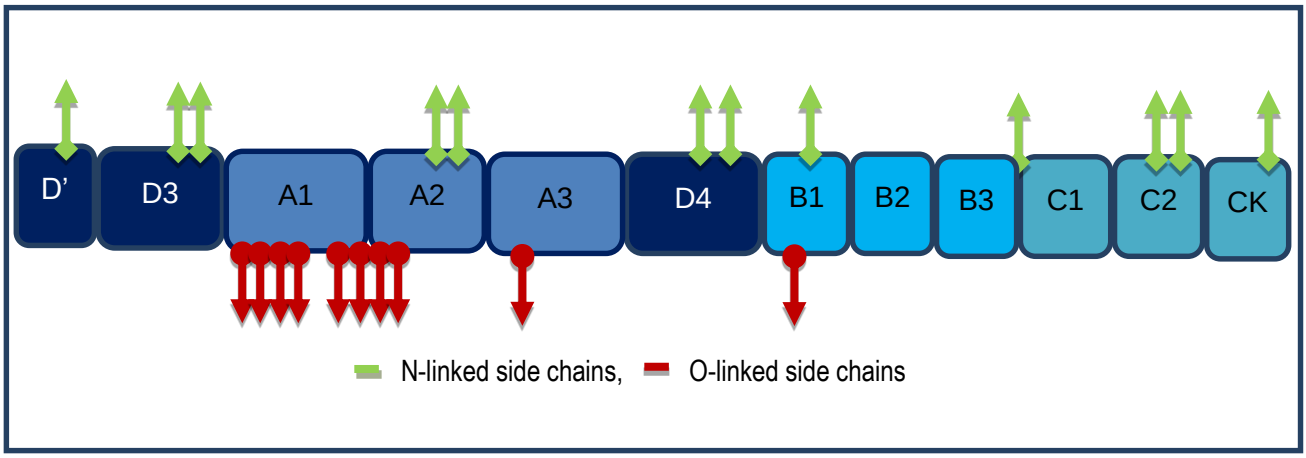

Figure 3: Positions of the 12 N-linked and 10 O-linked oligosaccharide side chains on a mature von Willebrand factor monomer as depicted by McGrath *et al*. [55].

The mature vWF molecule consists of several subunits and a series of oligomers that are either directly released into the plasma or stored in the Weibel-Palade bodies, from where it will be secreted in a regulated manner as high molecular weight multimers (HMWM) of mature vWF [30,57]. The HMWM of vWF can consist of a minimum of 2 or a maximum of 40 linked vWF subunits [57]. These HMWM of vWF has a high affinity for collagen and platelets binding and are therefore highly efficient in inducing platelet aggregation, especially during high shear conditions

[61,62]. **Figure 4** depicts the domains and their respective binding sites that each vWF subunit comprises of [31]. The accessibility of domain A1 and A3 are dependent on the dynamic forces of the blood exerted on the vWF molecule because of the unique coiled structure of vWF. This coiled structure of vWF promotes its ability to bind platelets to the subendothelium during conditions of high shear stress [31].

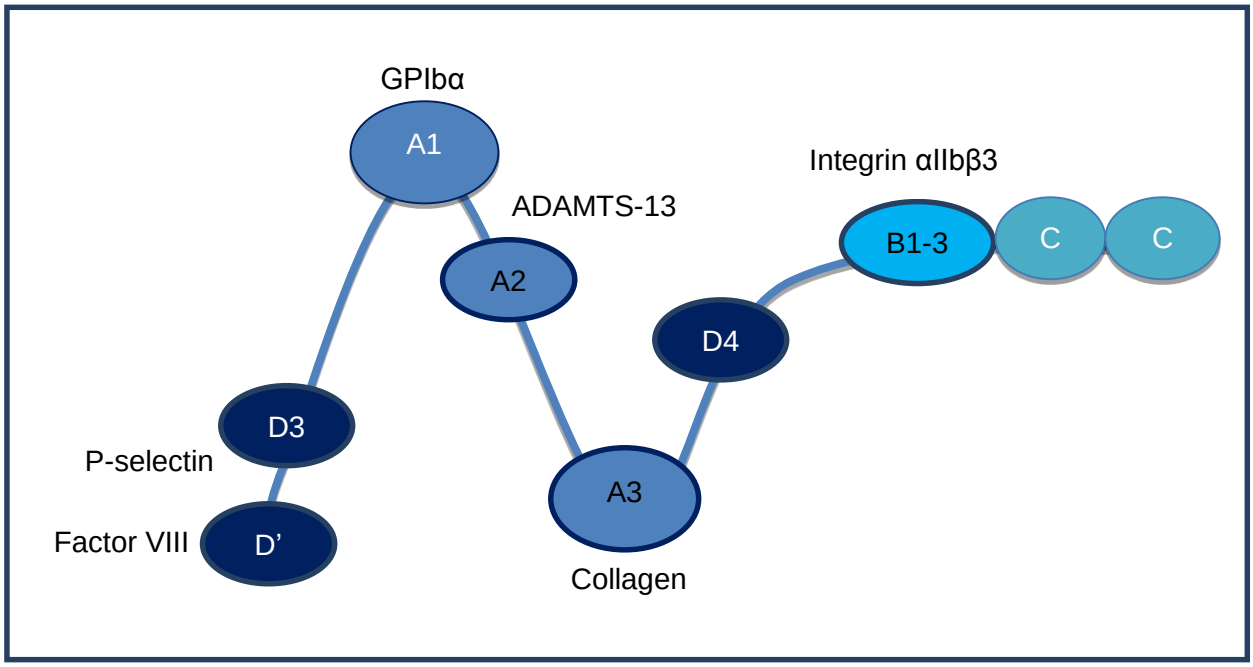

Figure 4: Schematic representation of a mature von Willebrand factor monomer with its domains and their respective binding and cleavage sites from Reininger *et al*. [31].

Once vWF is released at the site of injury, it is immediately anchored to the surface of the extracellular matrix by means of the P-selectin and collagen binding sites [63,64]. Fluid shear stress stretches the vWF molecule, thereby exposing the various domains of the vWF subunits and their binding sites. Platelets rapidly bind to the exposed GP1bα binding sites and promote platelet plug formation [65,66]. Therefore vWF is considered as the bridge between the exposed subendothelial layer and the platelets. Fluid shear stress does not only promote platelet plug formation but also mediates the proteolysis of vWF by exposing the A2 domain. vWF is cleaved by the metalloproteinase ADAMTS-13 (A Disintegrin And Metalloproteinase with ThromboSpondin type 1 motif 13) into smaller less active forms, thereby preventing overt thrombus formation during conditions of elevated shear stress [67-69]. The measurement of vWF may be of clinical value, since elevated circulatory vWF levels indicate endothelial disturbance or dysfunction [70]. Previous investigators found that vWF is an independent marker of

cardiovascular risk and is independently associated with coronary heart disease and stroke [71-74].

1.2.2 Fibrinogen

Fibrinogen is a soluble glycoprotein with a molecular weight of 340 KDa that is primarily synthesised by hepatocytes [75]. Normal plasma levels range between 1.5 to 4.0 g/L [53]. Once secreted, fibrinogen has a half-life of approximately 100 hours [76]. Fibrinogen is the precursor for fibrin that is responsible for stabilisation of the platelet plug and is seen as an essential component of the haemostatic system [77].

A fibrinogen molecule consists of two outer D domains and a central E domain that are connected to each other by two α helical coiled segments (**Figure 5**). The molecule is comprised of two sets of three polypetide chains namely, Aα, Bβ and γ [78-81]. The D domain consists of the Bβ and γ-C termini while the globular Aα-C termini is located close to the E domain. Each of the Aα chains contains an fibrinopeptide A (FpA) and fibrinopeptide B (FpB) sequence that is cleaved by thrombin upon activation. Cleavage of the FpA sequence exposes a polymerisation site termed E_A. The exposed E_A site combines with a complementary binding pocket D_a in the D domain of a neighbouring molecule to form double stranded twisting fibrin fibres [82-85]. The cleavage of FpB occurs at a slower rate than FpA cleavage. Once FpB is cleaved by thrombin, the polymerisation site E_B is exposed. This interacts with a complementary D_b site in the β chain segment of the D domain of another fibrinogen molecule. This interaction allows lateral binding of fibrin fibres that contributes to fiber thickness and tensile strength of a fibrin molecule [86-88].

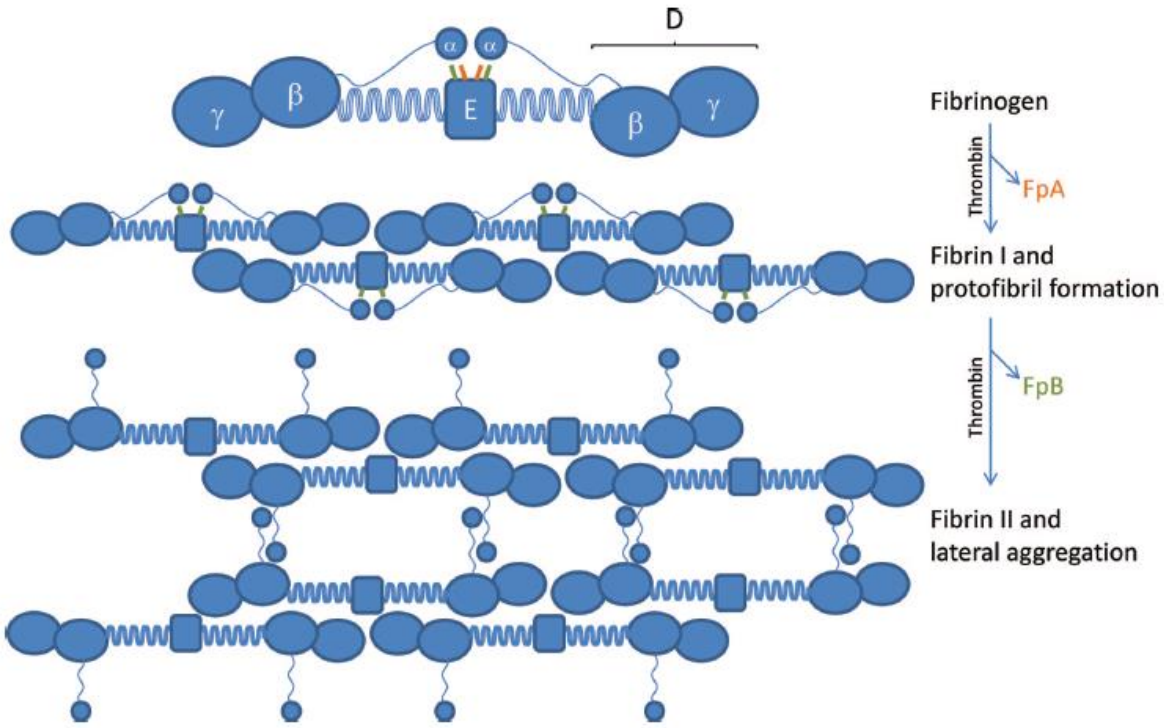

Figure 5: Schematic representation of a fibrinogen molecule that is cleaved by thrombin to form fibrin fibres as depicted by Undas *et al*. [89].

Fibrinogen is an independent risk marker of cardiovascular disease and may increase an individual's cardiovascular risk through increased fibrin formation and alteration of the fibrin network structure [14,90,91]. In addition, elevated fibrinogen can increase an individual's cardiovascular risk by increasing plasma viscosity and red blood cell aggregation [92,93] decreasing blood fluidity and enhancing platelet aggregation. Moreover, fibrinogen also acts as an acute phase reactant during inflammatory states [94,95], possibly contributing to endothelial dysfunction.

1.2.3 Fibrin D-dimer

Haemostasis is followed by fibrinolysis to prevent overt thrombus formation from occurring. During fibrinolysis the cross-linked fibrin clot is cleaved by plasmin into fibrin degradation products of which fibrin D-dimer is the best characterised [96,97] (**Figure 6**). Fibrin D-dimer is not only considered a marker of subsequent lysis but also of increased thrombus formation since it originates from cross-linked fibrin that is formed during thrombus formation [18,98]. Circulating fibrin D-dimer levels are easily measured and suitable for routine clinical and epidemiological

purposes [18]. The half-life of fibrin D-dimer is approximately 48 hours and normal reference values range between 0-500 µg/ml [18,53]. The clearance of fibrin D-dimer occurs via the urinary and the reticulo-endothelial system [18,98].

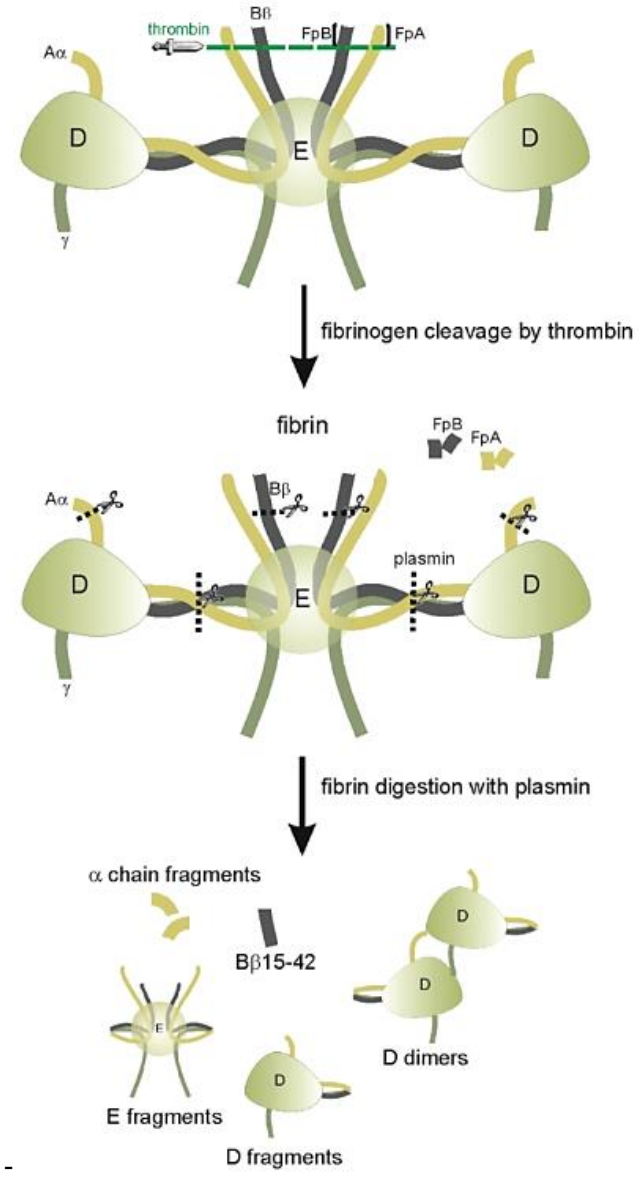

Figure 6: Fibrinolysis of a fibrin(ogen) molecule into fibrin fragments such as fibrin D-dimers, D and E fragments, Bβ15–42 and α chain fragments. The scissors mark plasmin cleavage sites, while the knife marks thrombin cleavage sites from Jennewein *et al*. [99].

Elevated fibrin D-dimer levels are indicative of a procoagulant state that favours the development and progression of cardiovascular disorders [100]. Koening *et al*. [101] found that elevated fibrin D-dimer levels predict the risk of future coronary events independently of conventional risk factors in healthy individuals. These findings are reinforced by a meta-analysis of six studies that reported

an odds ratio of 1.79 (95% CI, 1.36 to 2.36; p<0.001) for coronary heart disease when fibrin D-dimer were stratified by tertiles [102]. Furthermore, elevated fibrin D-dimer is also associated with an increased risk of myocardial infarction, ischemic stroke and coronary death in high risk patients [101,103-105]. The elevated levels may occur due to increased fibrin formation, reduced renal clearance, increased prevalence of cardiovascular risk factors, occult disease and/or inflammation [23,106]. On the other hand, low levels of fibrin D-dimer occur when plasmin formation is inhibited by PAI-1, which is also associated with an increased cardiovascular risk [107]. There is a complex relationship between fibrin D-dimer and PAI-1. Elevated PAI-1 results in low levels of fibrin D-dimer, while fibrin D-dimer possesses conformational-dependent signalling epitopes that regulate PAI-1 expression, which may be exposed upon plasmin cleavage of cross-linked fibrin [107,108]. Therefore, the measurement of both fibrin D-dimer and PAI-1 are essential for the assessment of the fibrinolytic system.

1.2.4 Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is a single chained glycoprotein that is a member of the serine protease inhibitor (serpin) superfamily, which consists of 379 amino acids in length and has a molecular weight of about 48-52 KDa [46,109]. PAI-1 is primarily derived from the liver, but several other sources such as the vascular endothelium, megakaryocytes, smooth muscle cells, macrophages, the spleen and adipose tissue have also been identified [110-112]. Normal plasma values range between 4.0 - 43.0 ng/ml and a biological half-life of 8-10 min has been suggested [17,53,113].

Endothelial cells contribute to a large amount of circulating PAI-1 caused by hormonal, metabolic or inflammatory stimuli [16]. Therefore, elevated levels of PAI-1 have been suggested as an indicator of endothelial dysfunction [114]. Recently Brogen *et al.* [115] reported that large amounts of active PAI-1 are released from platelets during thrombus formation, which stabilises fibrin clots and prevents premature lysis of the fibrin clot that promotes wound healing [115]. PAI-1 is secreted into the bloodstream in its active form and exhibits a unique conformational flexibility that allows it to be in an active, latent or substrate form [116] (**Figure 7**). PAI-1 inhibits the activity of the plasma serpin enzymes t-PA and urokinase plasminogen activator (u-PA) by blocking their

active sites, thereby preventing the conversion of plasminogen into plasmin and ultimately the lysis of a fibrin clot [117].

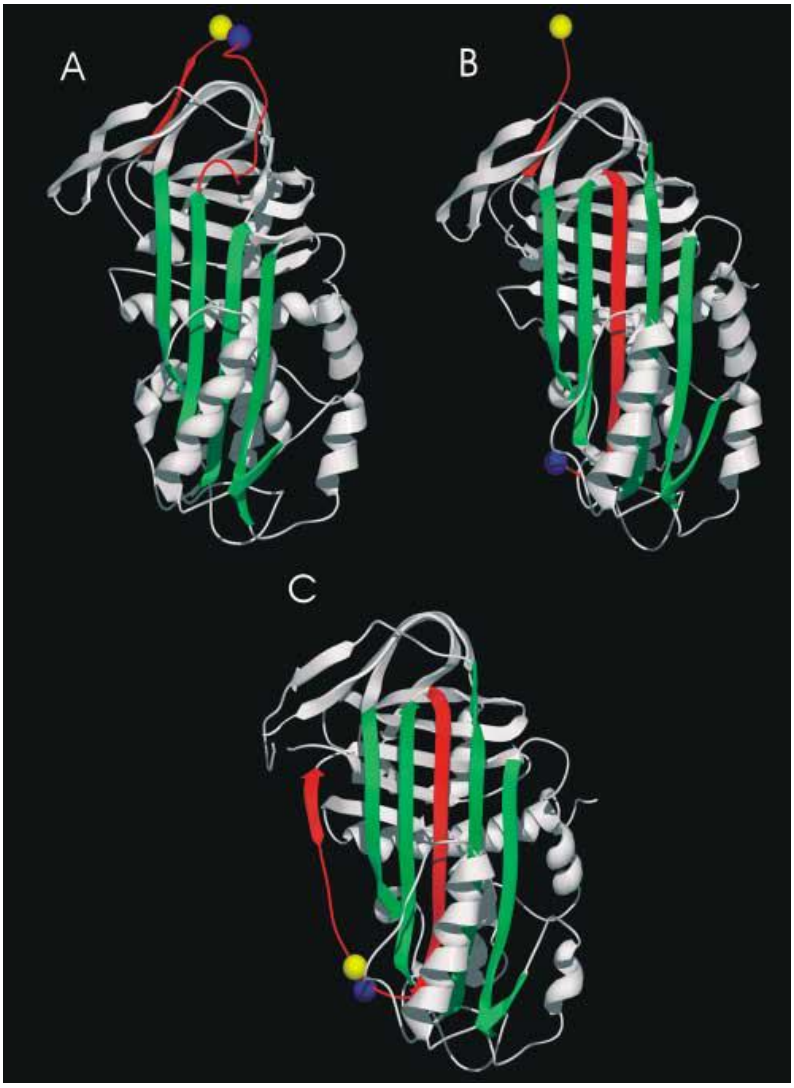

Figure 7: The structure of PAI-1 in the active (A), cleaved (B) and latent (C) from, Gils *et al*. [118]. The β-sheet is indicated in green, the reactive site loop in red and the reactive site residues Arg_{346}-Met_{347} are represented as blue and yellow spheres, respectively.

Structural information about the native inhibitory form of PAI-1 is elusive because of its inherent conformational instability and rapid conversion to a latent structure [109]. Generally the crystal structure of the serpin proteins consist of three β-sheets and nine α-helixes with an exposed reactive central loop [119]. The gene for PAI-1 is located on chromosome seven, and is composed of nine exons and eight introns [120]. The most important regulatory elements of the PAI-1 gene include a glucocorticoid response element that mediates aldosterone responsiveness [121,122],

a very low-density lipoprotein response site [123], and two Sp1 sites that mediate glucose and glucosamine responsiveness [124,125]. There are two well characterised functional domains for PAI-1. The first is encoded by exon 3 and 4, which has a binding site for vitronectin. This anchors the PAI-1 molecule to the extracellular matrix. The second domain is the reactive centre loop that is encoded by exon 8 [109]. The reactive centre loop undergoes conformational changes after PAI-1 binds to vitronectin and is primarily required for the inhibition of both t-PA and u-PA [118,126].

Overproduction of PAI-1 will suppress fibrinolysis, consequently leading to pathological fibrin deposition [127,128]. Plasma PAI-1 levels peak in the early morning, coinciding with the highest incidence of acute myocardial infarction and non-occlusive ischaemic coronary events [17]. Several studies have reported that the association between PAI-1 and cardiovascular events are not independent of components of the metabolic syndrome and suggests that elevated PAI-1 may be the link between the metabolic syndrome and cardiovascular risk [129,130].

1.2.5 Fibrinolytic potential

The contribution of individual components of the fibrinolytic system to the global fibrinolytic potential of an individual can be difficult to interpret, especially during disease states. As described earlier, the fibrinolytic system is influenced by several components that may increase or decrease fibrin clot breakdown [45]. The interpretation of an individual's fibrinolytic potential by means of a global fibrinolytic assay is therefore of value, since it provides information regarding both fibrin generation and lysis [131]. Several turbidimetric [132-135] and non-turbidimetric [136,137] global fibrinolytic assays are available to determine the fibrinolytic potential of an individual. However, these assays do not all include all the components of fibrin formation and degradation and can therefore not be considered a true representation of an individual's fibrinolytic potential. A plasma-based turbidimetric clot lysis assay recently developed by Lisman *et al*. [138] is considered to be a true reflection of fibrinolytic potential, since it is influenced by levels of proteins involved both in the coagulation, as well as the lysis pathways. In this assay clotting is initiated by TF, therefore clot formation is dependent on the endogenous concentrations of the different coagulation factors,

which are known to influence lysis rate. This assay expresses an individual's plasma fibrinolytic potential as clot lysis time (CLT), which is determined from a turbidity curve of fibrin polymerisation, in which clot formation was induced by adding TF and clot breakdown by t-PA [138] (**Figure 8**).

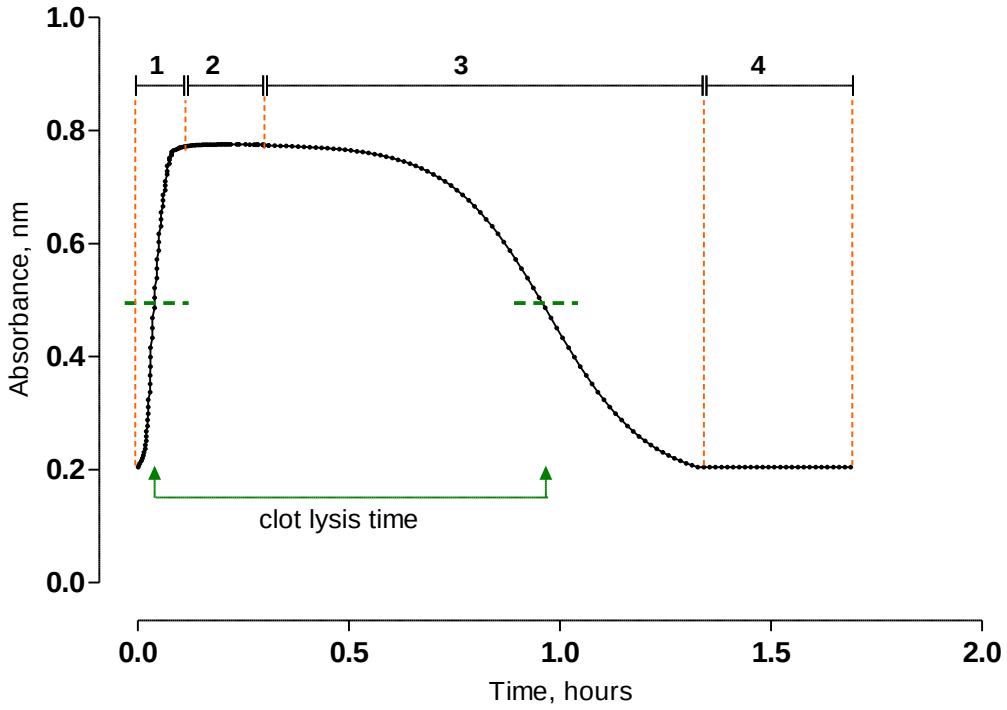

Figure 8: A clot lysis profile in normal plasma. The curve was generated by plotting absorbance (Y-axis) as a function of time (X-axis). Coagulation was initiated by the addition of tissue factor, phospholipids and calcium chloride, while fibrinolysis was initiated by the addition of tissue type plasminogen activator. The profile consists of 4 distinct parts: 1 – clot formation; 2 – latency; 3 – clot dissolution; 4 – latency.

Clot lysis time is defined as the time from the midpoint in the transition from the initial baseline to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot [138]. CLT can be affected by factors that influence the fibrin binding characteristics of t-PA, concentrations of fibrinolysis inhibitors and the structure of the fibrin clot [134]. The thickness of the fibrin fibres and permeability of a fibrin clot have a major impact on fibrinolysis. For example, fibrin networks composed of thin fibres with a decreased permeability lyse slower than clots formed with thicker fibres and increased permeability [139,140] (**Figure 9**). Previous investigators have reported that

a decreased fibrinolytic potential, as indicated by increased CLT, is indicative of hypofibrinolysis and has been associated with increased cardiovascular risk in patients with CVD [138,141].

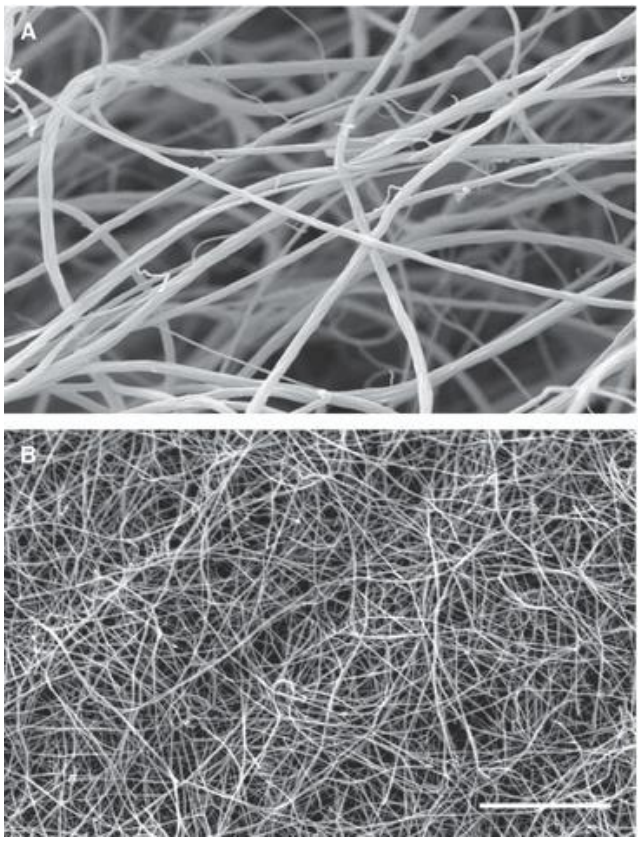

Figure 9: Scanning electron micrographs of fibrin clots. A-fibrin clot with thick fibres and increased permeability, B-fibrin clot with thin fibres and decreased permeability from Weisel *et al*. [142]. Magnification bar = 5 µm.

1.3 Determinants of the haemostatic markers

Several genetic and environmental factors that influence circulating levels of the haemostatic markers have been reported. Circulating levels of vWF [143,144], fibrinogen [145,146], PAI-1 [147,148] and fibrin D-dimer [149] are controlled to a substantial degree by genetic factors. However, environmental factors seem to be the primary determinants of circulating haemostatic factor levels. Table 1 lists the most conventional factors that influence the selected haemostatic components.

Table 1: Determinants of haemostatic markers.

	vWf	Fibrinogen	Fibrin D-dimer	PAI-1	Fibrinolytic potential
Age	↑ [21]	↑ [150, 151]	↑ [23, 152]	↑ [46, 153]	↑ [141]
Gender	↑ Women [21]	↑ Women [154, 155]	↑ Women [152, 156]	↑ Men [157, 158]	-
Ethnicity	↑ African [21, 159]	↑ African [22, 151]	↑ African [23, 160]	↓ African [160, 161]	-
Obesity	↑ [21, 162]	↑ [154, 163]	↑ [156]	↑ [164, 165]	↑ [141, 166]
Cholesterol	↑ [167]	↑ [91]	-	↑ [129, 168]	↑ [141, 166]
Glucose	↑ [169]	↑ [170]	-	↑ [171, 172]	↑ [131, 166]
Smoking	-	↑ [90, 173]	↑ [152]	↑ [174, 175]	-
Drinking	Moderate ↓ [21, 176]	Moderate ↓ [177]	-	Excessive ↑ [176, 178]	-
Regular physical activity	↓ [21, 179]	↓ [180, 181]	-	↓ [182, 183]	-
Contraceptives	↑ [184, 185]	↑ [186, 187]	↑ [156]	↓ [188, 189]	↑ [141]
Menopause	-	↑ [190]	-	↑ [190, 191]	-
Hormone replacement therapy	↓ [192]	-	-	↓ [193, 194]	-
Diurnal changes	↑ Midday [195]	↑ Afternoon [195, 196]	↑ Afternoon [195]	↑ Morning [197, 198]	-
Seasonal differences	↑ Winter [195]	↑Winter [199, 200]	-	↑ Winter [199]	-

1.4 Haemostasis and cardiovascular function

1.4.1 Haemostasis and blood pressure

The pathogenesis of hypertension is multifactorial [201,202] and numerous physiological alterations have been described in hypertensive individuals, including endothelial dysfunction [203], systemic inflammation [204,205], enhanced oxidative stress and a reduced fibrinolytic potential [141,206,207]. Clinical and laboratory evidence suggests that hypertension is related to a prothrombotic state that may be brought on by changes disrupting the balance between the coagulation and fibrinolytic pathways [208,209]. Elevated blood pressure contributes to all the components of Virchows Triad that consist of endothelial dysfunction and changes to both blood

constituents and flow, which results in the enhanced activation of the coagulation system, thereby promoting thrombogenesis [209,210]. This may explain why hypertensive complications such as myocardial infarction and stroke are thrombotic rather than haemorrhagic in nature. Furthermore hypertension is also related to conditions such as atrial fibrillation [211,212], heart failure [213] and left ventricular hypertrophy [214] that are accompanied by a prothrombotic state (**Figure 10**).

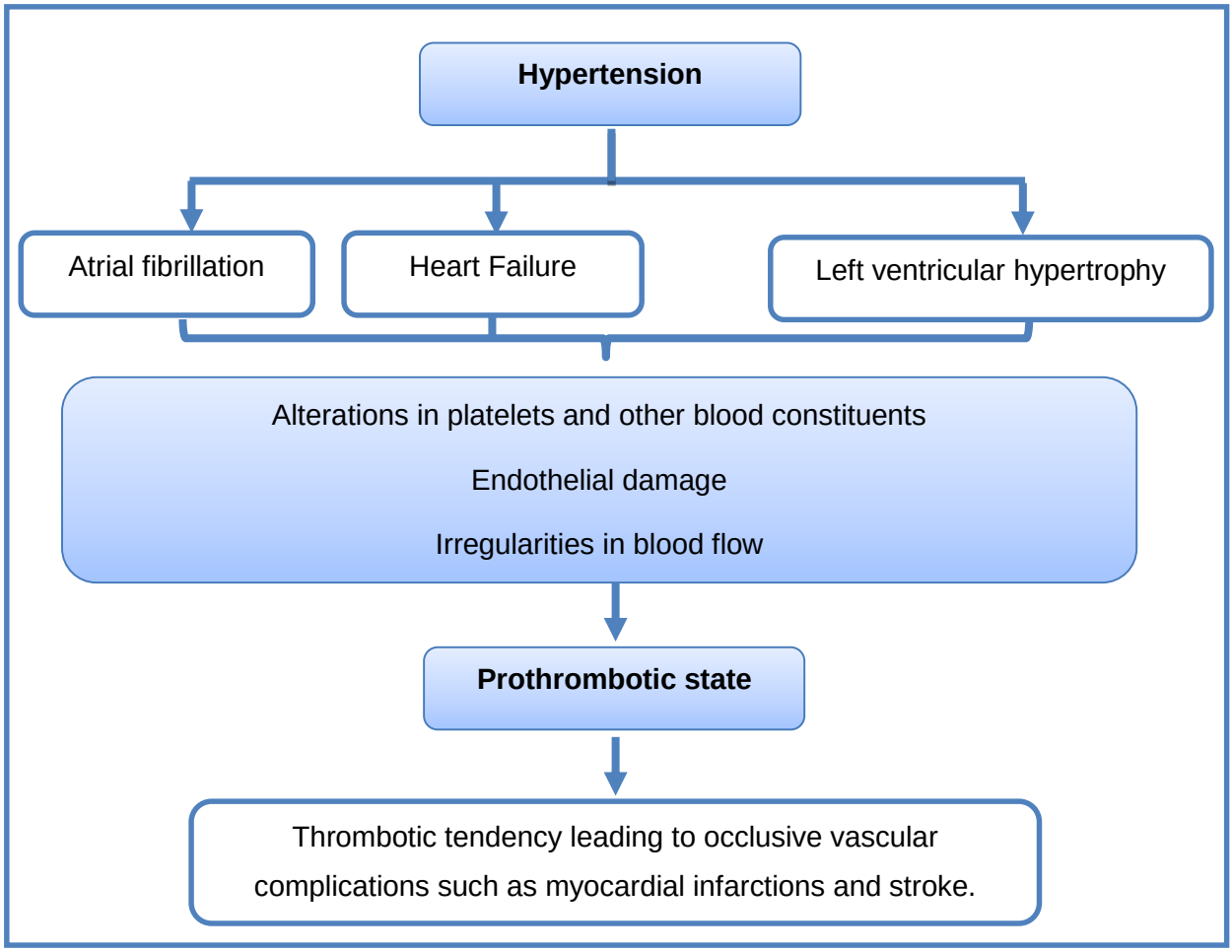

Figure 10: Sequence of events in hypertensive cardiovascular disease leading to the prothrombotic state. Adapted from Varughese *et al*. [210].

Associations of fibrinogen [92,215], vWF [216], PAI-1 [206,207], fibrin D-dimer [152,214] and CLT [141] with blood pressure have been reported. Fibrinogen overproduction clearly accompanies hypertension progression and may even precede its development [217]. The exact mechanism by which fibrinogen may promote hypertension progression and development is still not fully understood. However, its role as a determinant of plasma viscosity has received much attention, since plasma viscosity is normally elevated in patients with hypertension and blood pressure

correlates positively with fibrinogen [92,218]. High levels of vWF were reported in hypertensives, but it tends to normalise again after the successful treatment of hypertension [216]. Junker *et al*. [219] speculated that an increase in vWF expression only occurs in patients with sustained hypertension, since low vWF levels have been found in newly diagnosed hypertensive patients. It was suggested that the presence of endothelial dysfunction or damage, as represented by an increase in vWF, may be one mechanism by which patients with hypertension is at a greater risk for thrombogenesis [52].

PAI-1 seems elevated in hypertensive patients and may precede hypertension development [206,207,220]. However, generally elevated blood pressure levels seem to result in the increased secretion of PAI-1 through hypertension-induced shear stress and/or endothelial dysfunction [221]. Elevated PAI-1 levels may suggest that a reduced fibrinolytic potential exists in individuals with hypertension, although investigators determining the fibrinolytic potential with the euglobin fibrinolytic assay, were unable to find any significant association with blood pressure [219]. Meltzer *et al*. [141] who determined the fibrinolytic potential using the method described by Lisman *et al*. [138] did report associations between blood pressure and CLT, thereby indicating that elevated blood pressure may be accompanied by a decreased fibrinolytic potential. Independent associations between fibrin D-dimer and blood pressure have also been reported in hypertensive patients [152,222]. Furthermore, fibrin D-dimer levels were also higher in black hypertensives when compared to their white hypertensive counterparts, suggesting that fibrin turnover is higher in people from African descent [222]. This may be a possible explanation for the increased cardiovascular morbidity and mortality among the black population with hypertension. The detailed mechanisms that result in the increased activity of the haemostatic system seen in hypertensives, remain unclear and further investigation is needed.

1.4.2 Haemostasis and endothelial function

The endothelium is an active endocrine organ that regulates vascular tone, control blood fluidity and exert antiplatelet, anticoagulant and fibrinolytic properties [223,224]. Under normal conditions nitric oxide (NO), prostacyclin, and bradykinin are released by the vascular endothelium.

Prostacyclin and NO act synergistically to inhibit platelet aggregation, while bradykinin stimulates the production of t-PA that aids fibrinolysis [225]. Excessive stimulation of the endothelial cells by a variety of stimuli that include diabetes mellitus, smoking, hypertension and inflammation upsets the homeostasis of the vascular endothelium. This results in endothelial dysfunction that contributes to atherosclerosis development and ultimately an increase in cardiovascular events [223,224] (**Figure 11**). Endothelial dysfunction is characterised by the suppression of antiplatelet and anticoagulant activity and the expression of procoagulant activity [223] (**Figure 12**).

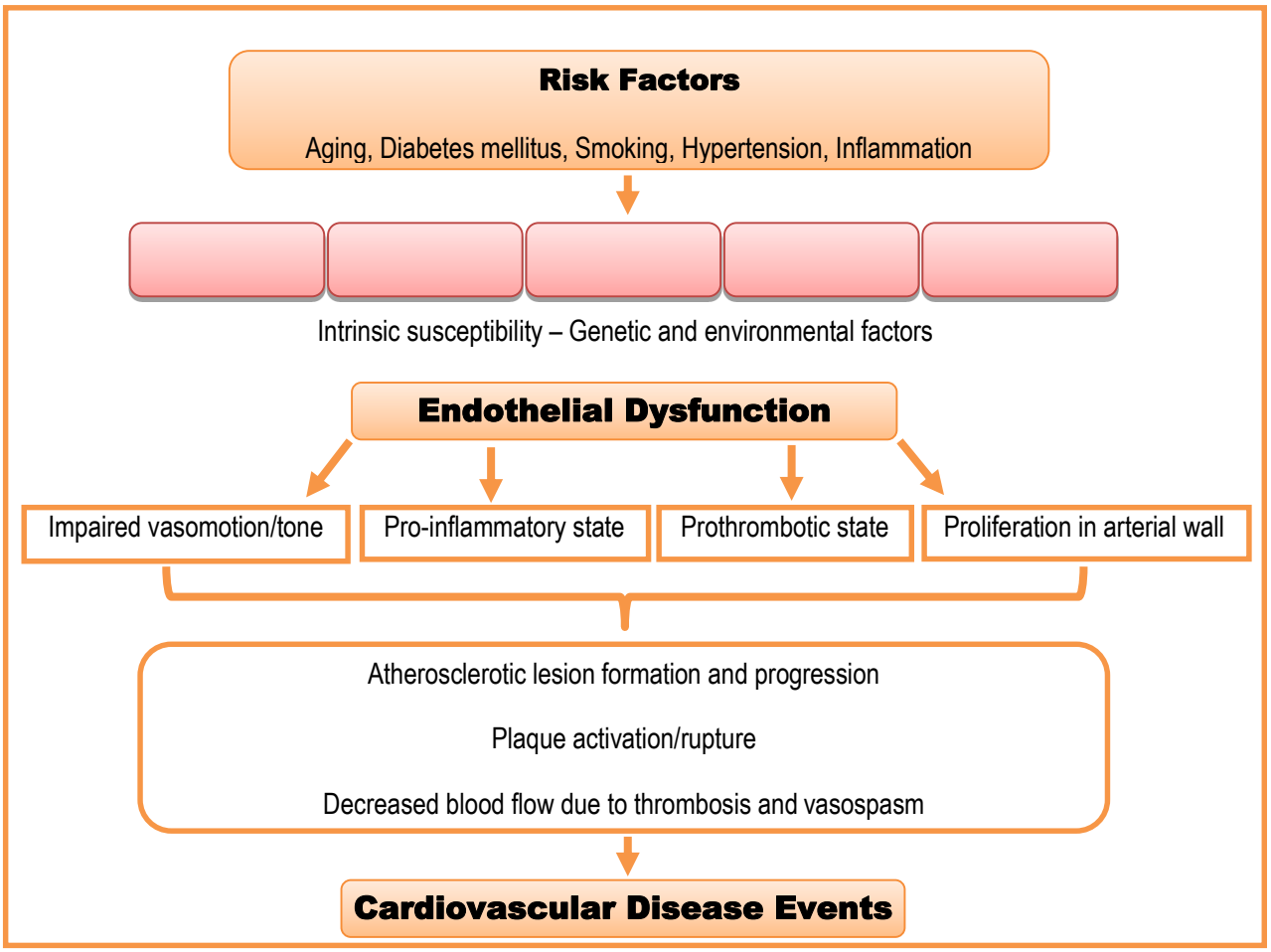

Figure 11: The role of endothelial dysfunction in the pathogenesis of cardiovascular disease events. Adapted from Widlandsky *et al*. [224].

Inflammatory mediators play a crucial role in endothelial dysfunction that disrupts the normal functioning of the vascular endothelium and impairs vascular tone regulation. Inflammation and haemostasis are linked processes that elicit a vicious cycle, since the activation of the one results in the activation of the other [226,227]. The main inflammatory mediators involved in inflammation-

induced coagulation activation are interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1). IL-6 and TNF-α are involved in the up-regulation of TF and increased synthesis of fibrinogen from the hepatocytes, while IL-1 is involved in the down regulation of the protein C pathway that forms part of the anticoagulant system [227]. Furthermore, fibrinogen and fibrin can also stimulate the expression of the inflammatory mediators by activating specific receptors on mononuclear or endothelial cells [228].

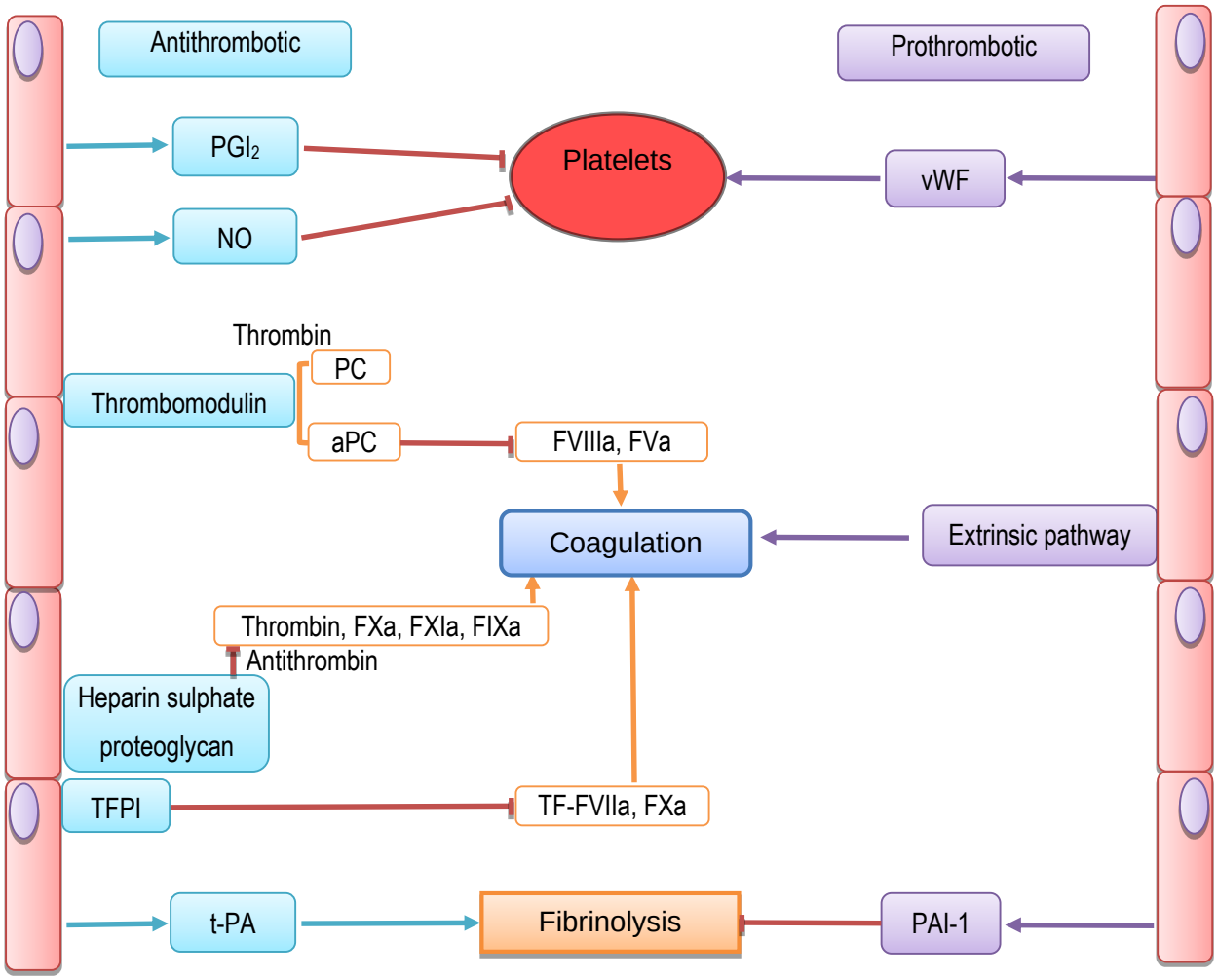

Figure 12: Endothelial cells produce antithrombotic and prothrombotic molecules. Adapted from Wu *et al*. [223]. Abbreviations: a, activated; F, factor; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1, PGI_2, prostacyclin; TFPI, tissue factor pathway inhibitor; t-PA, tissue plasminogen activator; PC, Protein C; vWF, von Willebrand factor.

The release of vWF and PAI-1 are promoted by several antagonists that result in increased platelet adhesion and aggregation and decreased fibrinolysis [30]. Studies on cultured endothelial cells have identified several pathways and antagonists that lead to the increased secretion of vWF from the Weibel-Palade bodies. Firstly, thrombin, histamine, superoxide anions and several

inflammatory markers mediate the acute release of vWF through an increase in intracellular cytosolic free calcium [229-231].

Secondly, epinephrine, adenosine, prostacyclin and vasopressin induce vWF release through the activation of V2 receptors that mediate signalling via cyclic adenosine monophosphate (cAMP) [232]. PAI-1 expression is also up-regulated by cytokines (TNF-α [233], IL-1 [234], tissue growth factor β [235]) and hormones (glucocorticoid [236], insulin [237], angiotensin II [238]). Hypoxic conditions and elevated shear stress can also induce the secretion of vWF and PAI-1 from endothelial cells. However their signal transductions are still unclear [239-241]. Furthermore, Hoekstra *et al*. [242] reported that PAI-1 release is not only increased as a response to proinflammatory cytokines but in turn, also stimulates the synthesis of cytokines.

IL-6 is the main stimulator for the release of both C-reactive protein (CRP) and fibrinogen from hepatic cells [243,244]. Although CRP and fibrinogen are strongly correlated with each other, fibrinogen is preferred for the determination of an individual's cardiovascular risk, since it is more specifically related to vascular disease states [245,246]. Fibrinogen influences the endothelium by binding to the intercellular adhesion molecule-1 receptor on the endothelial membrane. This stimulates the release of endothelin-1 (ET-1) from the Weibel-Palade bodies and increase endothelial permeability [217,245,247]. The increased permeability of the endothelial cells enhances fibrinogens deposition in the subendothelial matrix, where it is converted into fibrin and degraded into fibrin degradation products. Both fibrin and its degradation products induce monocyte chemotaxis and promote smooth muscle cell chemotaxis and proliferation [245,248]. Activation of the coagulation cascade by IL-6 and/or TNF-α also increases circulating fibrin D-dimer levels [249,250]. However, this link seems to be bi-directional, since elevated fibrin D-dimer also stimulates neutrophil and monocyte activation, which in turn secretes cytokines (such as IL-6 and TNF-α) [251-253]. A study by Yevdokimova *et al*. [107] also reported that elevated fibrin D-dimer may be involved in the down-regulation of NO production, which contributes to endothelial dysfunction and promotes oxidative stress development.

1.4.3 Haemostasis and oxidative stress

Under normal conditions oxidants are released at a steady state from the endothelium and play a role in gene expression, cell growth, vasodilatation and oxygen sensing in various cell types [254]. One of the most well characterised oxidants is superoxide, which is dismutated enzymatically to form hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) [254]. H_2O_2 is stable and moves easily through membranes with the aid of transporters that are responsible for the oxidation of key thiol residues on proteins and low molecular weight thiolating agents such as glutathione (GSH) [255]. H_2O_2 has an important signalling role in mitochondrial metabolism and the regulation of cellular processes [256]. It is converted to water by either catalase or glutathione peroxidase (GPx) [255]. The production of oxidants is carefully maintained by antioxidant enzymes (SOD, catalase and GPx) and oxidant scavengers (Vitamin E and glutathione), which reduce the bioavailability of oxidants and in doing so, protect the various cells against the potentially damaging accumulation of intra- and extracellular oxidants [256].

Oxidative stress occurs when oxidants are overproduced and/or when antioxidant enzyme levels are diminished. Both cases result in an altered equilibrium between oxidants and antioxidants in favour of oxidants [255,257]. Chronically induced oxidative stress contributes to endothelial dysfunction [258,259]. The most abundant non-protein thiol that defends against oxidative stress is the tripeptide GSH [260]. GSH exists in its thiol-reduced (GSH) or oxidised glutathione disulphide (GSSG) form and is resistant to intracellular degradation that is only metabolised extracellularly [260,261]. The cycling between GSH/GSSG removes the oxidants and protects the cells against oxidative injury [262,263]. For instance, when GPx reduces H_2O_2, two molecules of GSH binds with one molecule of H_2O_2, which leads to the formation of water and GSSG. GSSG is then converted back to its reduced state via the enzyme glutathione reductase [264]. Excessive oxidative stress conditions cause GSSG to increase. This in turn reduces the GSH/GSSG ratio [262]. GPx is the rate-limiting factor in this reaction and originates from a family of tetrameric enzymes, that contain the unique amino acid, selenocysteine, within their active sites. At present, four isoforms of GPx has been identified, i.e. cellular GPx (GPx-1), gastrointestinal GPx (GPx-2), extracellular GPx (GPx-3) and phospholipid hydroperoxide GPx (GPx-4) [255]. GPx-3 is

considered an important antioxidant enzyme in the extracellular compartment since it reduces H_2O_2, thereby protecting the endothelium against oxidative stress and maintaining the vasorelaxant and antithrombotic properties of the endothelium [265-268]. Overproduction of oxidants have been related to increased thrombus formation through the enhancement of thrombin formation and PAI-1 activity [269,270] (**Figure 13**).

An *in vitro* study by Matsushita *et al*. [271] showed that low levels of H_2O_2 inhibit thrombin-induced vWF secretion from endothelial cells. However, inverse relationships have been reported between diminished GPx activity and reduced GSH levels with vWF caused by increased oxidative stress in people from European descent [167,272,273]. This indicates that excessive oxidative damage may promote thrombus formation. Fibrinogen is also highly susceptible to oxidative modification by reactive oxygen species during tissue injury and inflammatory conditions [274]. Once the fibrinogen molecule is oxidised, it undergoes several posttranslational modifications that seem to alter its functional properties. This alters fibrinogen's interaction with platelets, endothelial, and other cells via cell membrane fibrinogen receptors and modifies the formation and architecture of the fibrin network [275-279].

There are conflicting results on whether oxidised fibrinogen is prothrombotic [276,281,282] or antithrombotic [277,279,283]. The oxidative modifications of fibrinogen is site-specific and vary depending on the method and oxidant being used [280] (**Table 2)**. It has been suggested that the site of oxidative modification of fibrinogen depends on the oxidant [275,280] and that the extent to which oxidised fibrinogen influences the haemostatic system depends on the intensity and duration of oxidation.

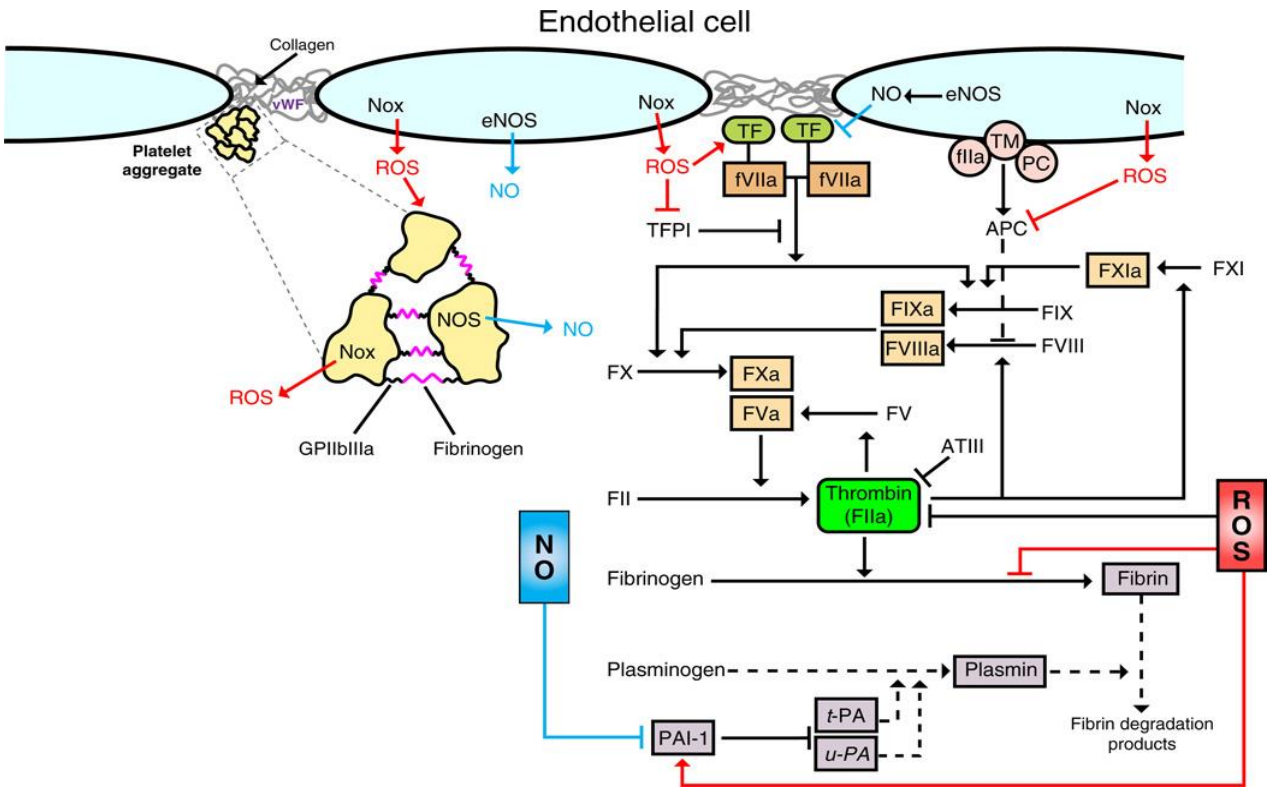

Figure 13: The role of reactive oxygen species and nitric oxide in coagulation and platelet aggregation from Kvietys *et al*. [270]. Abbreviations: a, activity; APC, activated protein C; ATIII, anti-thrombin III; C; eNOS, endothelial nitric oxide synthase; F, factor; NO, nitric oxide; Nox, nitrogen oxide; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species, TFPI, tissue factor pathway inhibitor; t-PA, tissue plasminogen activator; u-PA, urokinase plasminogen activator.

Azizova *et al*. [278] found that when fibrinogen is only oxidised by 10 percent, the extrinsic pathway is only moderately activated, while a 20 percent oxidation of fibrinogen supresses both the extrinsic and intrinsic pathways. Furthermore, Vadseth *et al*. [276], who investigated post translational modification of fibrinogen after exposure to myeloperoxidase and H_2O_2 or hypochlorite in patients with a history of CVD, reported that fibrin clots formed in an oxidative environment has a decreased permeability that tends to be more resilient towards lysis. Oxidised fibrinogen has also been shown to have a reduced capacity to stimulate t-PA expression, which is responsible for inducing lysis, thereby promoting thrombus formation [282]. Furthermore, PAI-1 expression is redox sensitive and tends to increase with oxidative stress and decrease with an increase in antioxidants [284-286].

Table 2: Site specific oxidative modifications of fibrinogen and effects on fibrin function and clot structure from Martinez *et al*. [280].

	***In vitro* photo-oxidised fibrinogen**	***In vitro* hypochlorite-oxidised fibrinogen**	***Ex vivo* plasma of smokers and non-smokers**
Type of modification	Oxidised histidine	Methionine sulfoxide	3-Nitrotyrosine
Site of modification	Bβ His^{16}; unknown Aα	Aα Met^{476} [a], Bβ Met^{367}, γ Met^{78}	Bβ Tyr^{292} [b], Bβ Tyr^{422}
Monomer association	Increased	NA	NA
Fibrin Polymerisation	Decreased	Decreased	Increased
Final turbidity	Decreased	Decreased	Increased
Fibrin clot lysis	NA	Slower	Slower
Viscoelastic properties	NA	Decreased stiffness and viscosity	Increased stiffness and viscosity
Fibrin clot structure	NA	Increased fiber density, decreased fiber diameter, decreased pore size.	Increased fiber clusters

[a] most abundant modification; [b] most frequent modification.

Inflammation has been identified as the main initiator that disrupts the balance between NO and superoxide within endothelial cells often resulting in increased oxidative stress [270]. The intricate relationship between oxidative stress, inflammation and endothelial dysfunction on the haemostatic system may not only be relevant for vascular thrombotic disease but also have major consequences in the pathogenesis of microvascular dysfunction [227].

1.4.4 Haemostasis and the microvasculature

Investigation of the structure and function of the microvasculature is important for the early detection of cardiovascular changes [287]. The retina has been identified as a convenient site to assess microvessels with a non-invasive technique that is reproducible [288,289]. The retinal blood vessels also share many anatomical and physiological similarities with the cerebral- and

coronary blood vessels [288,289]. Therefore, changes in the retinal blood vessels are likely to reflect similar changes in the cerebral- and coronary blood vessels. The retinal blood vessels are assessed from digital images from where the arteriolar-to-venular ratio (AVR) and central retinal artery (CRAE) and vein (CRVE) equivalents are determined by means of computer assisted programs [287,290]. Changes in the calibre size of the microvessels provide valuable information about the influence of systemic, environmental and genetic risk factors on early structural changes of the microvasculature [287]. For instance, arteriolar narrowing is associated with the presence and severity of hypertension [291-294], whereas venular widening is associated with inflammation [295,296], diabetes [297,298], dyslipidaemia [298,299] and stroke incidence [300,301]. Previous investigators from the United States of America reported a decreased AVR in black participants when compared to their white counterparts, which was caused by both a larger CRAE and CRVE [295,302].

Fibrinogen, vWF and PAI-1 were related to retinal microvascular changes in both the Artherosclerosis Risk In Communities study (ARIC) [295] and Multi-Ethnic Study of Atherosclerosis (MESA) [302]. Both studies reported a positive association between CRVE and fibrinogen and suggested that venular widening was brought about by inflammation with fibrinogen being an acute phase protein. Elevated levels of fibrinogen have also been shown to influence the microcirculation by increasing plasma viscosity, altering vascular reactivity and compromising the endothelial cell layer [217] (**Figure 14**). Plasma viscosity is among others determined by fibrinogen and overproduction leads to a decrease in blood fluidity and increase in shear stress [93,217]. Increased shear stress promotes thrombus formation through the activation of the surrounding endothelial cells and platelets, which in turn results in the increased expression of several adhesion molecules and integrins [303-307]. These effects increase blood flow resistance or, in severe cases, allow blood flow stagnation to occur in the micro vessels [308,309].

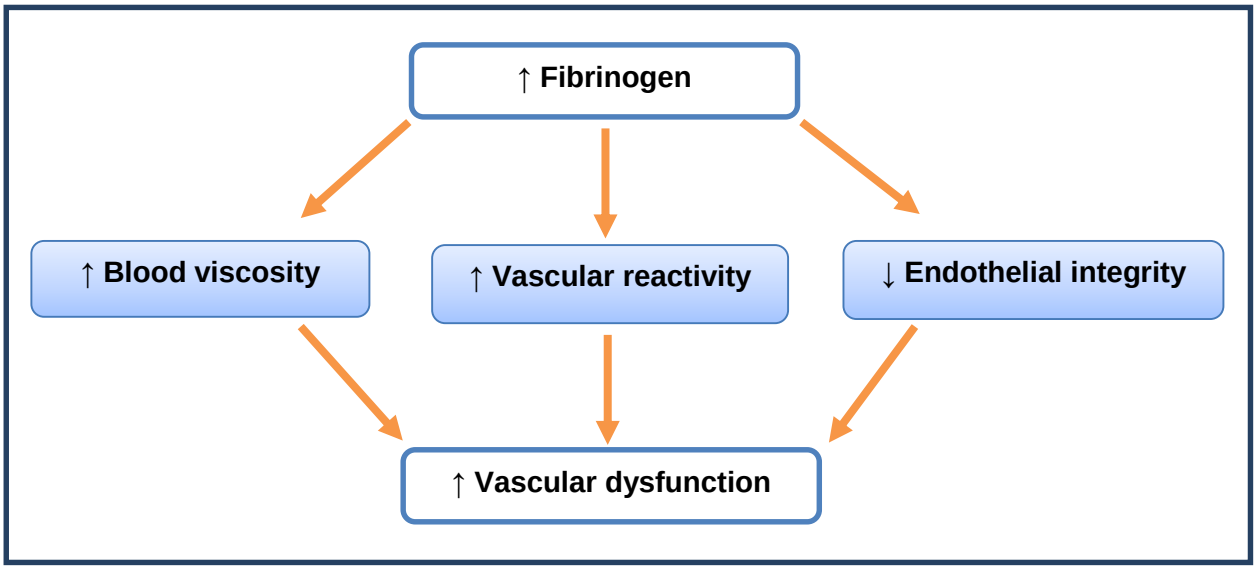

Figure 14: Schematic representation of fibrinogen induced vascular dysfunction. Adapted from Lominadze *et al*. [217].

Fibrinogen also has vasoactive effects [310,311] and has the ability to increase endothelial permeability [217,312,313] by binding to intracellular adhesion molecule-1 (ICAM-1) and the $\alpha_v\beta_{III}$ or $\alpha_v\beta_1$ integrins. A study by Sen *et al*. [247] showed that once fibrinogen is bound to ICAM-1, extracellular signal regulated kinase 1/2 is activated. This results in the release of ET-1 from the Weibel-Palade bodies, thereby promoting vasoconstriction [247]. The binding of fibrinogen to integrin $\alpha_v\beta_{III}$ or $\alpha_v\beta_I$ on the endothelial membrane has been found to induce opposing effects. Fibrinogens binding to integrin $\alpha_v\beta_{III}$ prompted vasodilation while $\alpha_v\beta_I$ binding prompted vasoconstriction [314,315]. Furthermore, the binding of fibrinogen to ICAM-1 and integrin $\alpha_v\beta_I$ also increases endothelial permeability by altering the vascular tight junction proteins and activation of the matrix metalloproteinases [312,313]. All these effects of fibrinogen contribute to endothelial dysfunction, which exacerbates microcirculatory complications during CVD.

Furthermore, the ARIC study also found that higher vWF was associated with an increased AVR that was prompted by arteriolar widening [295,296]. No association between vWF and any of the vessel calibres was found in the MESA study. However a weak positive association between PAI-1 and CRVE was reported [302]. The investigators of the MESA study suggested that both inflammation and endothelial dysfunction may play a role in retinal vessel calibre changes based on the associations of fibrinogen and PAI-1 with the vessel calibres. The exact mechanisms

involved in arteriolar narrowing and venular widening remain unknown. However, epidemiological studies have consistently shown that venular widening is related to systemic inflammation [296,302]. Arteriolar narrowing, which is largely influenced by blood pressure, and forms part of the initial stages of hypertensive retinopathy, seems to be preferentially related to endothelial dysfunction. Since, the retinal blood vessels have no adrenergic vasomotor nerve supply, vascular tone is mainly regulated by myogenic and endothelial cell changes [287].

1.5 Aims

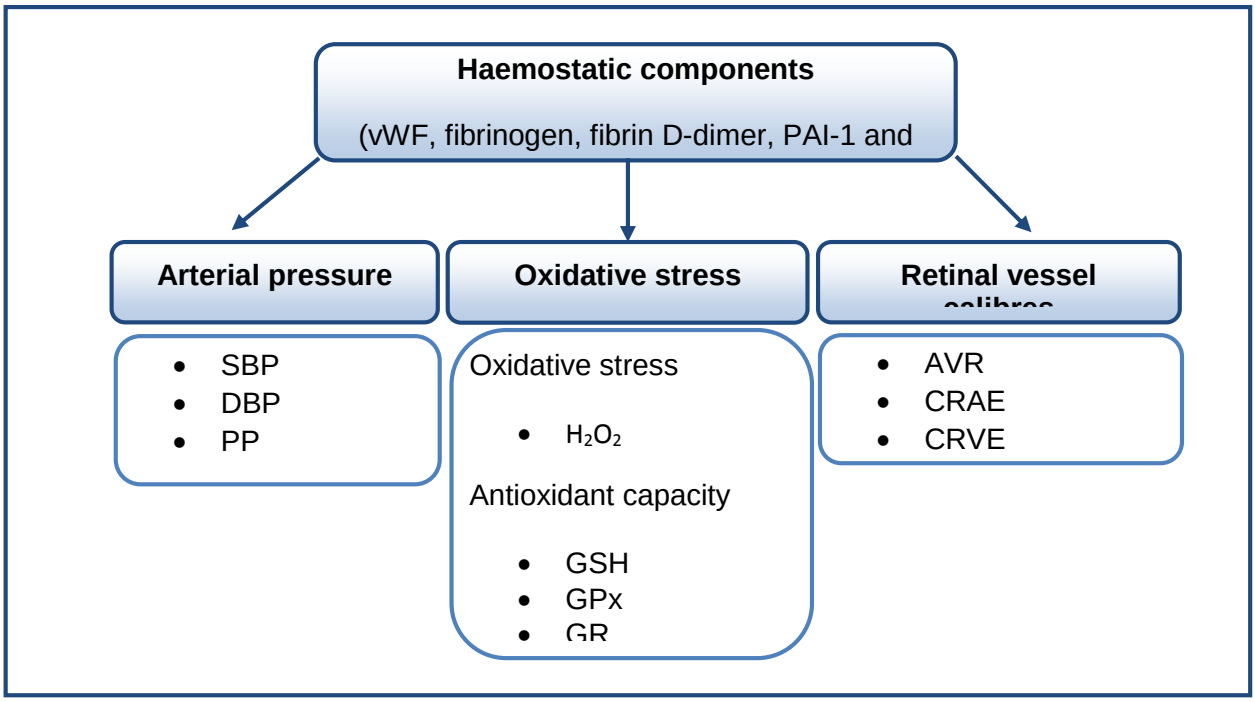

Figure 15: Components of the haemostatic system and cardiovascular function investigated. Abbreviations: vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; CLT, clot lysis time; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; H_2O_2, hydrogen peroxide; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; AVR, arteriolar-to-venular ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent.

The aims of this study are firstly, to compare markers of coagulation and fibrinolysis between a black and white population from South Africa; and secondly, to determine if associations exist between the selected components of the haemostatic system and markers of cardiovascular function in this bi-ethnic population (**Figure 15**). This is especially relevant for the black population

who tends to be at higher cardiovascular risk due to altered metabolic [5-8] and haemostatic profiles [21-23] compared to their white counterparts.

1.6 Objectives

- To investigate potential ethnic differences in selected markers of coagulation and fibrinolysis in black and white South Africans;
- To investigate ethnic-specific associations of selected coagulation and fibrinolysis markers with (1) ambulatory blood pressure, (2) markers of oxidative stress and antioxidant capacity, and (3) measures of microvascular structure.

1.7 Hypotheses

- Compared to the white population, the black population exhibits higher levels of vWF, fibrinogen and fibrin D-dimer, lower levels of PAI-1 and a shorter CLT;
- Ethnic differences exist in the relationships of the ambulatory blood pressures with the haemostatic components;
- Oxidative stress and antioxidant markers are associated with the haemostatic components in both ethnicities, with more adverse associations seen in the black population;
- The retinal vessel calibres are associated with the haemostatic components in the black population.

1.8 References

1. Mathers C, Fat DM, Boerma J. The global burden of disease: 2004 update: World Health Organization; 2008.

2. Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Measuring the global burden of disease and risk factors, 1990–2001. Global burden of disease and risk factors. 2006; 1:1-14.

3. BeLue R, Okoror TA, Iwelunmor J, Taylor KD, Degboe AN, Agyemang C, *et al*. An overview of cardiovascular risk factor burden in sub-Saharan African countries: a socio-cultural perspective. Global Health. 2009; 5 (1):10.

4. Dalal S, Beunza JJ, Volmink J, Adebamowo C, Bajunirwe F, Njelekela M, *et al*. Non-communicable diseases in sub-Saharan Africa: what we know now. Int J Epidemiol. 2011; 40 (4):885-901.

5. Opie LH, Seedat YK. Hypertension in sub-Saharan African populations. Circulation. 2005; 112 (23):3562-8.

6. Peltzer K, Phaswana-Mafuya N. Hypertension and associated factors in older adults in South Africa. Cardiovasc J Afr. 2013; 24 (3):67-71.

7. Peer N, Steyn K, Lombard C, Lambert EV, Vythilingum B, Levitt NS. Rising diabetes prevalence among urban-dwelling black South Africans. PloS One. 2012; 7 (9):e43336.

8. Tibazarwa K, Ntyintyane L, Sliwa K, Gerntholtz T, Carrington M, Wilkinson D, *et al*. A time bomb of cardiovascular risk factors in South Africa: results from the Heart of Soweto Study “Heart Awareness Days”. Int J Cardiol. 2009; 132 (2):233-9.

9. Connor MD, Walker R, Modi G, Warlow CP. Burden of stroke in black populations in sub-Saharan Africa. Lancet Neurol. 2007; 6 (3):269-78.

10. Mensah GA. Epidemiology of stroke and high blood pressure in Africa. Heart. 2008; 94 (6):697-705.

11. Matijevic N, Wu K. Hypercoagulable states and strokes. Curr Atheroscler Rep. 2006; 8 (4):324-9.

12. Lip GY, Blann AD. von Willebrand factor and its relevance to cardiovascular disorders. Brit Heart J. 1995; 74 (6):580-3.

13. Cooney M-T, Dudina AL, O'Callaghan P, Graham IM. von Willebrand factor in CHD and stroke: Relationships and therapeutic implications. Curr Treat Options Cardiovasc Med. 2007; 9 (3):180-90.

14. Ernst E, Resch KL. Fibrinogen as a cardiovascular risk factor: A meta-Analysis and review of the literature. Ann Intern Med. 1993; 118 (12):956-63.

15. Danesh J, Lewington S, Thompson SG, Lowe G, Collins R, Kostis J, *et al*. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. JAMA. 2005; 294 (14):1799-809.

16. Vaughan DE. PAI-1 and atherothrombosis. J Thromb Haemost. 2005; 3 (8):1879-83.

17. Huber K, Christ G, Wojta J, Gulba D. Plasminogen Activator Inhibitor Type-1 in Cardiovascular Disease: Status Report 2001. Thromb Res. 2001; 103, Supplement 1 (0):S7-19.

18. Lip GY, Lowe GD. Fibrin D-dimer: a useful clinical marker of thrombogenesis? Clin Sci. 1995; 89 (3):205-14.

19. Lowe G. Fibrin D-dimer and cardiovascular risk. Semin Vasc Med. 2005; 5: 387-98.

20. Pieters M, Vorster HH. Nutrition and hemostasis: a focus on urbanization in South Africa. Mol Nutr Food Res. 2008; 52 (1):164-72.

21. Conlan MG, Folsom AR, Finch A, Davis CE, Sorlie P, Marcucci G, *et al*. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. Thromb Haemost. 1993; 70 (3):380-5.

22. Folsom AR, Wu KK, Conlan MG, Finch A, Davis CE, Marcucci G, *et al*. Distributions of hemostatic variables in blacks and whites: population reference values from the Atherosclerosis Risk in Communities (ARIC) Study. Ethn Dis. 1992; 2 (1):35-46.

23. Pieper CF, Rao KMK, Currie MS, Harris TB, Cohen HJ. Age, functional status, and racial differences in plasma D-dimer levels in community-dwelling elderly persons. J Gerontol A-Biol. 2000; 55 (11):M649-57.

24. Greyling A, Pieters M, Hoekstra T, Oosthuizen W, Schutte AE. Differences in the association of PAI-1 activity with the metabolic syndrome between African and Caucasian women. Nutr Metab Cardiovasc Dis. 2007; 17 (7):499-507.

25. Norris LA. Blood coagulation. Best Pract Res Clin Obstet Gynaecol. 2003; 17 (3):369-83.

26. Green D. Coagulation cascade. Haemodial Int. 2006; 10 (2):S2-4.

27. Tzoulaki I, Murray GD, Price JF, Smith FB, Lee AJ, Rumley A, *et al*. Hemostatic factors, inflammatory markers, and progressive peripheral atherosclerosis the edinburgh artery study. Am J Epidemiol. 2006; 163 (4):334-41.

28. Kannel WB. Overview of hemostatic factors involved in atherosclerotic cardiovascular disease. Lipids. 2005; 40 (12):1215-20.

29. Furie B, Furie BC. Mechanisms of thrombus formation. New Engl J Med. 2008; 359 (9):938-49.

30. Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. J Thromb Haemost. 2006; 4 (6):1186-93.

31. Reininger AJ. Function of von Willebrand factor in haemostasis and thrombosis. Haemophilia. 2008; 14 Suppl 5:11-26.

32. Lisman T, Weeterings C, de Groot PG. Platelet aggregation: involvement of thrombin and fibrin(ogen). Front Biosci. 2005; 10:2504-17.

33. Crawley J, Zanardelli S, Chion C, Lane D. The central role of thrombin in hemostasis. J Thromb Haemost. 2007; 5 (1):95-101.

34. Mann KG, Brummel-Ziedins K, Orfeo T, Butenas S. Models of blood coagulation. Blood Cells Mol Dis. 2006; 36 (2):108-17.

35. Steffel J, Lüscher TF, Tanner FC. Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. Circulation. 2006; 113 (5):722-31.

36. Osterud B, Rapaport SI. Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. Proc Natl Acad Sci USA. 1977; 74 (12):5260-4.

37. Camerer E, Kolstø A-B, Prydz H. Cell biology of tissue factor, the principal initiator of blood coagulation. Thromb Res. 1996; 81 (1):1-41.

38. Morrissey JH. Tissue factor: an enzyme cofactor and a true receptor. Thromb Haemost. 2001; 86 (1):66-74.

39. Orfeo T, Brufatto N, Nesheim ME, Xu H, Butenas S, Mann KG. The factor V activation paradox. J Biol Chem. 2004; 279 (19):19580-91.

40. Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. Arterioscler Thromb Vasc Biol. 2002; 22 (9):1381-9.

41. Colman RW, Hirsh J, Marder VJ, Colman, Hirsh, Marder, *et al*. Hemostasis and thrombosis: basic principles and clinical practice: Lippincott Williams & Wilkins Philadelphia; 2006.

42. Hornyak TJ, Shafer JA. Interactions of factor XIII with fibrin as substrate and cofactor. Biochemistry. 1992; 31 (2):423-9.

43. Ariëns RA, Lai T-S, Weisel JW, Greenberg CS, Grant PJ. Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. Blood. 2002; 100 (3):743-54.

44. Clark RAF. Wound repair. Overview and general considerations. In: The molecular and cellular biology of wound repair. 2nd ed, edited by Clark RAF. New York, Plenum, 1996: 3-50.

45. Rijken D, Lijnen H. New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost. 2009; 7 (1):4-13.

46. Cesari M, Pahor M, Incalzi RA. Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. Cardiovasc Ther. 2010; 28 (5):e72-91.

47. Ajjan R, Ariëns R. Cardiovascular disease and heritability of the prothrombotic state. Blood Rev. 2009; 23 (2):67-78.

48. McGrath RT, McRae E, Smith OP, O'Donnell JS. Platelet von Willebrand factor--structure, function and biological importance. Brit J Haematol. 2010; 148 (6):834-43.

49. Mayadas TN, Wagner DD. von Willebrand-factor biosynthesis and processing. Ann NY Acad Sci. 1991; 614:153-66.

50. Tsai HM, Nagel RL, Hatcher VB, Seaton AC, Sussman, II. The high molecular-weight form of endothelial-cell vonWillebrand-factor is released by the regulated pathway. Brit J Haematol. 1991; 79 (2):239-45.

51. Wagner DD. Cell biology of von Willebrand factor. Annu Rev Cell Biol. 1990; 6:217-46.

52. Lip GYH, Blann A. von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? Cardiovasc Res. 1997; 34 (2):255-65.

53. Kratz A, Ferraro M, Sluss PM, Lewandrowski KB. Normal reference laboratory values. New Engl J Med. 2004; 351 (15):1548-63.

54. Over J, Sixma JJ, Bruine MH, Trieschnigg MC, Vlooswijk RA, Beeser-Visser NH, *et al*. Survival of 125 iodine-labeled Factor VIII in normals and patients with classic hemophilia. Observations on the heterogeneity of human Factor VIII. J Clin Invest. 1978; 62 (2):223-34.

55. McGrath RT, McRae E, Smith OP, O'Donnell JS. Platelet von Willebrand factor structure, function and biological importance. Brit J Haematol. 2010; 148 (6):834-43.

56. Vlot AJ, Koppelman SJ, Bouma BN, Sixma JJ. Factor VIII and von Willebrand factor. Thromb Haemost. 1998; 79 (3):456-65.

57. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem. 1998; 67:395-424.

58. Perutelli P, Molinari AC. von Willebrand factor, von Willebrand factor-cleaving protease, and shear stress. Cardiovasc Hematol Agents Med Chem. 2007; 5 (4):305-10.

59. Wagner DD, Marder VJ. Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. J Cell Biol. 1984; 99 (6):2123-30.

60. Vischer UM, Wagner DD. von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. Blood. 1994; 83 (12):3536-44.

61. Sporn LA, Marder VJ, Wagner DD. von Willebrand factor released from Weibel-Palade bodies binds more avidly to extracellular matrix than that secreted constitutively. Blood. 1987; 69 (5):1531-4.

62. Arya M, Anvari B, Romo GM, Cruz MA, Dong JF, McIntire LV, *et al*. Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. Blood. 2002; 99 (11):3971-7.

63. Padilla A, Moake JL, Bernardo A, Ball C, Wang Y, Arya M, *et al*. P-selectin anchors newly released ultralarge von Willebrand factor multimers to the endothelial cell surface. Blood. 2004; 103 (6):2150-6.

64. Michaux G, Pullen TJ, Haberichter SL, Cutler DF. P-selectin binds to the D'-D3 domains of von Willebrand factor in Weibel-Palade bodies. Blood. 2006; 107 (10):3922-4.

65. Ruggeri ZM. Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation. Best Pract Res Clin Haematol. 2001; 14 (2):257-79.

66. Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. J Clin Invest. 2000; 105 (6):699-701.

67. Lopez JA, Dong JF. Shear stress and the role of high molecular weight von Willebrand factor multimers in thrombus formation. Blood Coagul Fibrinolysis. 2005; 16 (1):S11-6.

68. Chauhan AK, Motto DG, Lamb CB, Bergmeier W, Dockal M, Plaimauer B, *et al*. Systemic antithrombotic effects of ADAMTS13. J Exp Med. 2006; 203 (3):767-76.

69. Furlan M. Von Willebrand factor: molecular size and functional activity. Ann Hematol. 1996; 72 (6):341-8.

70. Blann AD. Plasma yon Willebrand factor, thrombosis, and the endothelium: the first 30 years. Thromb Haemost. 2006; 95 (1):49-55.

71. Folsom AR, Wu KK, Rosamond WD, Sharrett AR, Chambless LE. Prospective study of hemostatic factors and incidence of coronary heart disease: The Atherosclerosis Risk in Communities (ARIC) Study. Circulation. 1997; 96 (4):1102-8.

72. Whincup PH, Danesh J, Walker M, Lennon L, Thomson A, Appleby P, *et al*. von Willebrand factor and coronary heart disease: prospective study and meta-analysis. Eur Heart J. 2002; 23 (22):1764-70.

73. Bongers TN, de Maat MP, van Goor ML, Bhagwanbali V, van Vliet HH, Gomez Garcia EB, *et al*. High von Willebrand factor levels increase the risk of first ischemic stroke: influence of ADAMTS13, inflammation, and genetic variability. Stroke. 2006; 37 (11):2672-7.

74. O'Callaghan PA, Fitzgerald A, Fogarty J, Gaffney P, Hanbidge M, Boran G, *et al*. New and old cardiovascular risk factors: C-reactive protein, homocysteine, cysteine and von Willebrand factor increase risk, especially in smokers. Eur J Cardiovasc Prev Rehabil. 2005; 12 (6):542-7.

75. Tennent GA, Brennan SO, Stangou AJ, O'Grady J, Hawkins PN, Pepys MB. Human plasma fibrinogen is synthesized in the liver. Blood. 2007; 109 (5):1971-4.

76. Haidaris P, Francis C, Sporn L, Arvan D, Collichio F, Marder V. Megakaryocyte and hepatocyte origins of human fibrinogen biosynthesis exhibit hepatocyte-specific expression of gamma chain-variant polypeptides. Blood. 1989; 74 (2): 743-50.

77. Wolberg AS, Campbell RA. Thrombin generation, fibrin clot formation and hemostasis. Transfus Apher Sci. 2008; 38 (1):15-23.

78. Henschen A, Lottspeich F, Kehl M, Southan C. Covalent structure of fibrinogen. Ann NY Acad Sci. 1983; 408 (1):28-43.

79. Blombäck B, Hessel B, Hogg D. Disulfide bridges in NH 2-terminal part of human fibrinogen. Thromb Res. 1976; 8 (5):639-58.

80. Huang S, Cao Z, Davie E. The role of amino-terminal disulfide bonds in the structure and assembly of human fibrinogen. Biochem Biophys Res Commun. 1993; 190 (2):488-95.

81. Zhang J-Z, Redman C. Identification of B beta chain domains involved in human fibrinogen assembly. J Biol Chem. 1992; 267 (30):21727-32.

82. Scheraga HA, Laskowski Jr M. The fibrinogen-fibrin conversion. Adv Protein Chem. 1957; 12:1-131.

83. Blombäck B, Hessel B, Hogg D, Therkildsen L. A two-step fibrinogen-fibrin transition in blood coagulation. Nature. 1978; 275 (5680):501-5.

84. Krakow W, Endres G, Siegbl B, Schekaga H. An electron microscopic investigation of the polymerization of bovine fibrin monomer. J Mol Biol. 1972; 71 (1):95-103.

85. Fowler W, Hantgan R, Hermans J, Erickson H. Structure of the fibrin protofibril. Proc Natl Acad Sci USA. 1981; 78 (8):4872-6.

86. Shainoff JR, Dardik BN. Fibrinopeptide B in fibrin assembly and metabolism: Physiologic significance in delayed release of the peptide. Ann NY Acad Sci. 1983; 408 (1):254-68.

87. Medved L, Litvinovich S, Ugarova T, Lukinova N, Kalikhevich V, Ardemasova Z. Localization of a fibrin polymerization site complementary to Gly-His-Arg sequence. FEBS letters. 1993; 320 (3):239-42.

88. Everse SJ, Spraggon G, Veerapandian L, Riley M, Doolittle RF. Crystal structure of fragment double-D from human fibrin with two different bound ligands. Biochemistry. 1998; 37 (24):8637-42.

89. Undas A, Ariens RA. Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases. Arterioscler Thromb Vasc Biol. 2011; 31 (12):e88-99.

90. Kannel WB, D'Agostino RB, Belanger AJ. Fibrinogen, cigarette smoking, and risk of cardiovascular disease: Insights from the Framingham Study. Am Heart J. 1987; 113 (4):1006-10.

91. Stec JJ, Silbershatz H, Tofler GH, Matheney TH, Sutherland P, Lipinska I, *et al*. Association of fibrinogen with cardiovascular risk factors and cardiovascular disease in the Framingham offspring population. Circulation. 2000; 102 (14):1634-8.

92. Letcher RL, Chien S, Pickering TG, Sealey JE, Laragh JH. Direct relationship between blood pressure and blood viscosity in normal and hypertensive subjects: role of fibrinogen and concentration. Am J Med. 1981; 70 (6):1195-202.

93. Lowe G, Lee A, Rumley A, Price J, Fowkes F. Blood viscosity and risk of cardiovascular events: the Edinburgh artery study. Brit J Haematol. 1997; 96 (1):168-73.

94. Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Springer Semin Immunopathol. 2012; 34 (1): 43-62.

95. Ernst E. Fibrinogen as a cardiovascular risk factor-interrelationship with infections and inflammation. Eur Heart J. 1993; 14:82-7.

96. Gaffney PJ, Brasher M. Subunit structure of the plasmin-induced degradation products of crosslinked fibrin. Biochim Biophys Acta. 1973; 295 (1):308-13.

97. Gaffney P. D-dimer. History of the discovery, characterisation and utility of this and other fibrin fragments. Fibrinolysis. 1993; 7:2-8.

98. Lowe GD, Rumley A. Use of fibrinogen and fibrin D-dimer in prediction of arterial thrombotic events. Thromb Haemost. 1999; 82 (2):667-72.

99. Jennewein C, Tran N, Paulus P, Ellinghaus P, Eble JA, Zacharowski K. Novel aspects of fibrin(ogen) fragments during inflammation. Mol Med. 2011; 17 (5-6):568.

100. Van Der Bom J, Bots M, Haverkate F, Meijer P, Hofman A, Kluft C, *et al*. Activation products of the haemostatic system in coronary, cerebrovascular and peripheral arterial disease. Thromb Haemost. 2001; 85 (2):234-9.

101. Koenig W, Rothenbacher D, Hoffmeister A, Griesshammer M, Brenner H. Plasma fibrin D-dimer levels and risk of stable coronary artery disease results of a large case-control study. Arterioscler Thromb Vasc Biol. 2001; 21 (10):1701-5.

102. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, *et al*. Fibrin D-dimer and coronary heart disease: prospective study and meta-analysis. Circulation. 2001; 103 (19):2323-7.

103. Morange P, Bickel C, Nicaud V, Schnabel R, Rupprecht H, Peetz D, *et al*. Haemostatic factors and the risk of cardiovascular death in patients with coronary artery disease the atherogene study. Arterioscler Thromb Vasc Biol. 2006; 26 (12):2793-9.

104. Barber M, Langhorne P, Rumley A, Lowe GD, Stott DJ. D-Dimer predicts early clinical progression in ischemic stroke confirmation using routine clinical assays. Stroke. 2006; 37 (4):1113-5.

105. Reich LM, Heiss G, Boland LL, Hirsch AT, Wu K, Folsom AR. Ankle-brachial index and hemostatic markers in the Atherosclerosis Risk In Communities (ARIC) study cohort. Vasc Med. 2007; 12 (4):267-73.

106. Hager K, Platt D. Fibrin degeneration product concentrations (D-dimers) in the course of ageing. Gerontology. 1995; 41 (3):159-65.

107. Yevdokimova NY, Veselovska LD, Gogolinska GK, Buchanevich OM, Kosyakova GV, Gritsenko PG, *et al*. Fibrin D-dimer impairs the accumulation and anticoagulant properties of heparan sulphate and stimulates secretion of plasminogen activator inhibitor-1 by rabbit coronary endothelial cells. Acta Biochim Pol. 2003; 50 (1):279-89.

108. Olman MA, Hagood JS, Simmons WL, Fuller GM, Vinson C, White KE. Fibrin fragment induction of plasminogen activator inhibitor transcription is mediated by activator protein-1 through a highly conserved element. Blood. 1999; 94 (6):2029-38.

109. Ghosh AK, Vaughan DE. PAI-1 in tissue fibrosis. J Cell Physiol. 2012; 227 (2):493-507.

110. Chomiki N, Henry M, Alessi MC, Anfosso F, Juhan-Vague I. Plasminogen activator inhibitor-1 expression in human liver and healthy or atherosclerotic vessel walls. Thromb Haemost. 1994; 72 (1):44-53.

111. Zorio E, Gilabert-Estelles J, Espana F, Ramon LA, Cosin R, Estelles A. Fibrinolysis: the key to new pathogenetic mechanisms. Curr Med Chem. 2008; 15 (9):923-9.

112. Loskutoff DJ, van Mourik JA, Erickson LA, Lawrence D. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. Proc Natl Acad Sci USA. 1983; 80 (10):2956-60.

113. Kluft C, Verheijen J, Jie A, Rijken D, Preston F, Sue-Ling H, *et al*. The postoperative fibrinolytic shutdown: a rapidly reverting acute phase pattern for the fast-acting inhibitor of tissue-type plasminogen activator after trauma. Scan J Clin Lab Invest. 1985; 45 (7):605.

114. Diamantopoulos EJ, Andreadis EA, Vassilopoulos CV, Theodorides TG, Giannakopoulos NS, Chatzis NA, *et al*. Increased plasma plasminogen activator inhibitor-1 levels: a possible marker of hypertensive target organ damage. Clin Exp Hypertens. 2003; 25 (1):1-9.

115. Brogren H, Karlsson L, Andersson M, Wang L, Erlinge D, Jern S. Platelets synthesize large amounts of active plasminogen activator inhibitor 1. Blood. 2004; 104 (13):3943-8.

116. Levin EG, Santell L. Conversion of the active to latent plasminogen activator inhibitor from human endothelial cells. Blood. 1987; 70 (4):1090-8.

117. Gorog DA. Prognostic value of plasma fibrinolysis activation markers in cardiovascular disease. J Am Coll Cardiol. 2010; 55 (24):2701-9.

118. Gils A, Declerck PJ. The structural basis for the pathophysiological relevance of PAI-I in cardiovascular diseases and the development of potential PAI-I inhibitors. Thromb Haemost. 2004; 91 (3):425-37.

119. Nar H, Bauer M, Stassen J-M, Lang D, Gils A, Declerck PJ. Plasminogen activator inhibitor 1. Structure of the native serpin, comparison to its other conformers and implications for serpin inactivation. J Mol Biol. 2000; 297 (3):683-95.

120. Strandberg L, Lawrence D, Ny T. The organization of the human-plasminogen-activator-inhibitor-1 gene. Eur J Biochem. 1988; 176 (3):609-16.

121. Brown NJ, Kim KS, Chen YQ, Blevins LS, Nadeau JH, Meranze SG, *et al*. Synergistic effect of adrenal steroids and angiotensin II on plasminogen activator inhibitor-1 production. J Clin Endocrinol Metab. 2000; 85 (1):336-44.

122. van Zonneveld AJ, Curriden SA, Loskutoff DJ. Type 1 plasminogen activator inhibitor gene: functional analysis and glucocorticoid regulation of its promoter. Proc Natl Acad Sci USA. 1988; 85 (15):5525-9.

123. Eriksson P, Nilsson L, Karpe F, Hamsten A. Very-low-density lipoprotein response element in the promoter region of the human plasminogen activator inhibitor-1 gene implicated in the impaired fibrinolysis of hypertriglyceridemia. Arterioscler Thromb Vasc Biol. 1998; 18 (1):20-6.

124. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, *et al*. Hyperglycemia induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. Proc Natl Acad Sci USA. 2000; 97 (22):12222-6.

125. Chen YQ, Su M, Walia RR, Hao Q, Covington JW, Vaughan DE. Sp1 sites mediate activation of the plasminogen activator inhibitor-1 promoter by glucose in vascular smooth muscle cells. J Biol Chem. 1998; 273 (14):8225-31.

126. Gorlatova NV, Cale JM, Elokdah H, Li D, Fan K, Warnock M, *et al*. Mechanism of inactivation of plasminogen activator inhibitor-1 by a small molecule inhibitor. J Biol Chem. 2007; 282 (12):9288-96.

127. Weisberg AD, Albornoz F, Griffin JP, Crandall DL, Elokdah H, Fogo AB, *et al*. Pharmacological inhibition and genetic deficiency of plasminogen activator inhibitor-1 attenuates angiotensin II/salt-induced aortic remodeling. Arterioscler Thromb Vasc Biol. 2005; 25 (2):365-71.

128. Aso Y. Plasminogen activator inhibitor (PAI)-1 in vascular inflammation and thrombosis. Front Biosci. 2007; 12:2957-66.

129. Juhan-Vague I, Pyke SD, Alessi MC, Jespersen J, Haverkate F, Thompson SG. Fibrinolytic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. ECAT study group. Circulation. 1996; 94 (9):2057-63.

130. Folsom AR, Aleksic N, Park E, Salomaa V, Juneja H, Wu KK. Prospective study of fibrinolytic factors and incident coronary heart disease: The Atherosclerosis Risk in Communities (ARIC) Study. Arterioscler Thromb Vasc Biol. 2001; 21 (4):611-7.

131. Guimarães AH, De Bruijne EL, Lisman T, Dippel DW, Deckers JW, Poldermans D, *et al*. Hypofibrinolysis is a risk factor for arterial thrombosis at young age. Brit J Haematol. 2009; 145 (1):115-20.

132. He S, Bremme K, Blombäck M. A laboratory method for determination of overall haemostatic potential in plasma. Method design and preliminary results. Thromb Res. 1999; 96 (2):145-56.

133. Undas A, Brożek J, Jankowski M, Siudak Z, Szczeklik A, Jakubowski H. Plasma homocysteine affects fibrin clot permeability and resistance to lysis in human subjects. Arterioscler Thromb Vasc Biol. 2006; 26 (6):1397-404.

134. Carter AM, Cymbalista CM, Spector TD, Grant PJ. Heritability of clot formation, morphology, and lysis: the EuroCLOT study. Arterioscler Thromb Vasc Biol. 2007; 27 (12):2783-9.

135. Goldenberg NA, Hathaway WE, Jacobson L, Manco-Johnson MJ. A new global assay of coagulation and fibrinolysis. Thromb Res. 2005; 116 (4):345-56.

136. Meade T, Ruddock V, Stirling Y, Chakrabarti R, Miller G. Fibrinolytic activity, clotting factors, and long-term incidence of ischaemic heart disease in the Northwick Park Heart Study. Lancet. 1993; 342 (8879):1076-9.

137. Rijken D, Hoegee de Nobel E, Jie A, Atsma D, Schalij MJ, Nieuwenhuizen W. Development of a new test for the global fibrinolytic capacity in whole blood. J Thromb Haemost. 2008; 6 (1):151-7.

138. Lisman T, de Groot PG, Meijers JC, Rosendaal FR. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. Blood. 2005; 105 (3):1102-5.

139. Collet J, Park D, Lesty C, Soria J, Soria C, Montalescot G, *et al*. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed dynamic and structural approaches by confocal microscopy. Arterioscler Thromb Vasc Biol. 2000; 20 (5):1354-61.

140. Collet JP, Lesty C, Montalescot G, Weisel JW. Dynamic changes of fibrin architecture during fibrin formation and intrinsic fibrinolysis of fibrin-rich clots. J Biol Chem. 2003; 278 (24):21331-5.

141. Meltzer ME, Doggen CJM, De Groot PG, Rosendaal FR, Lisman T. Reduced plasma fibrinolytic capacity as a potential risk factor for a first myocardial infarction in young men. Brit J Haematol. 2009; 145 (1):121-7.

142. Weisel JW. The mechanical properties of fibrin for basic scientists and clinicians. Biophys Chem. 2004; 112 (2-3):267-76.

143. de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. Lancet. 2001; 357 (9250):101-5.

144. Jenkins PV, O'Donnell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? Transfusion. 2006; 46 (10):1836-44.

145. Humphries SE, Luong L-A, Montgomery HE, Day IN, Mohamed-Ali V, Yudkin JS. Gene-environment interaction in the determination of levels of plasma fibrinogen. Thromb Haemost. 1999; 82:818-25.

146. Green FR. Fibrinogen polymorphisms and atherothrombotic disease. Ann NY Acad Sci. 2001; 936:549-59.

147. Eriksson P, Kallin B, van 't Hooft FM, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. Proc Natl Acad Sci USA. 1995; 92 (6):1851-5.

148. Dawson S, Hamsten A, Wiman B, Henney A, Humphries S. Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity. Arterioscler Thromb. 1991; 11 (1):183-90.

149. Lange L, Reiner A, Carty C, Jenny N, Cushman M, Lange E. Common genetic variants associated with plasma fibrin D-dimer concentration in older European-and African-American adults. J Thromb Haemost. 2008; 6 (4):654-9.

150. Fu A, Nair KS. Age effect on fibrinogen and albumin synthesis in humans; Am J Physiol. 1998; 275 (6): E1023-30.

151. Fibrinogen Collaboration Studies. Collaborative meta-analysis of prospective studies of plasma fibrinogen and cardiovascular disease. Eur J Cardiovasc Prev Rehabil. 2004; 11 (1):9-17.

152. Lee AJ, Fowkes GR, Lowe GD, Rumley A. Determinants of fibrin D-dimer in the Edinburgh Artery study. Arterioscler Thromb Vasc Biol. 1995; 15 (8):1094-7.

153. Gleerup G, Winther K. The effect of ageing on platelet function and fibrinolytic activity. Angiology. 1995; 46 (8):715-8.

154. Krobot K, Hense HW, Cremer P, Eberle E, Keil U. Determinants of plasma fibrinogen: relation to body weight, waist-to-hip ratio, smoking, alcohol, age, and sex. Results from the second MONICA Augsburg survey 1989-1990. Arterioscler Thromb Vasc Biol. 1992; 12 (7):780-8.

155. Ossei-Gerning N, Wilson IJ, Grant PJ. Sex differences in coagulation and fibrinolysis in subjects with coronary artery disease. Thromb Haemost. 1998; 79 (4):736-40.

156. Hughes R, Thomson K, Hopkins R, Weatherall M, Wiltshire C, Wilsher M, *et al*. Determinants of plasma D-dimer levels in a traveling population. J Thromb Haemost. 2005; 3 (11):2445-8.

157. Siegert G, Bergmann S, Jaross W. Relationship of plasminogen activator inhibitor activity and tissue-type plasminogen activator concentration with age, sex, risk factors for coronary heart disease and life style. Fibrinolysis. 1994; 8 (2):34-6.

158. Henry M, Tregouët DA, Alessi MC, Aillaud MF, Visvikis S, Siest G, *et al*. Metabolic determinants are much more important than genetic polymorphisms in determining the PAI-1 activity and antigen plasma concentrations: A family study with part of the Stanislas cohort. Arterioscl Thromb Vasc Biol. 1998; 18 (1):84-91.

159. Sukhu K, Poovalingam V, Mahomed R, Giangrande PL. Ethnic variation in von Willebrand factor levels can influence the diagnosis of von Willebrand disease. Clin Lab Haematol. 2003; 25 (4):247-9.

160. Lutsey PL, Cushman M, Steffen LM, Green D, Barr RG, Herrington D, *et al*. Plasma hemostatic factors and endothelial markers in four racial/ethnic groups: the MESA study. J Thromb Haemost. 2006; 4 (12):2629-35.

161. Festa A, D'Agostino R, Jr., Rich SS, Jenny NS, Tracy RP, Haffner SM. Promoter (4G/5G) plasminogen activator inhibitor-1 genotype and plasminogen activator inhibitor-1 levels in blacks, Hispanics, and non-Hispanic whites: the Insulin Resistance Atherosclerosis Study. Circulation. 2003; 107 (19):2422-7.

162. Blann AD, Bushell D, Davies A, Faragher EB, Miller JP, McCollum CN. von Willebrand factor, the endothelium and obesity. Int J Obes Relat Metab Disord. 1993; 17 (12):723-5.

163. Mertens I, Van Gaal L. Obesity, haemostasis and the fibrinolytic system. Obes Rev. 2002; 3 (2):85-101.

164. Sartori MT, Vettor R, De Pergola G, De Mitrio V, Saggiorato G, Della Mea P, *et al*. Role of the 4G/5G polymorphism of PaI-1 gene promoter on PaI-1 levels in obese patients: influence of fat distribution and insulin-resistance. Thromb Haemost. 2001; 86 (5):1161-9.

165. Mutch NJ, Wilson HM, Booth NA. Plasminogen activator inhibitor-1 and haemostasis in obesity. Proc Nutr Soc. 2001; 60 (3):341-7.

166. de Lange Z, Pieters M, Jerling JC, Kruger A, Rijken DC. Plasma clot lysis time and its association with cardiovascular risk factors in black Africans. PloS One. 2012; 7 (11):e48881.

167. Blann AD, Maxwell SR, Burrows G, Miller JP. Antioxidants, von Willebrand factor and endothelial cell injury in hypercholesterolaemia and vascular disease. Atherosclerosis. 1995; 116 (2):191-8.

168. Stiko-Rahm A, Wiman B, Hamsten A, Nilsson J. Secretion of plasminogen activator inhibitor-1 from cultured human umbilical vein endothelial cells is induced by very low density lipoprotein. Arteriosclerosis. 1990; 10 (6):1067-73.

169. Frankel DS, Meigs JB, Massaro JM, Wilson PW, O'Donnell CJ, D'Agostino RB, *et al*. Von Willebrand factor, type 2 diabetes mellitus, and risk of cardiovascular disease: the Framingham Offspring Study. Circulation. 2008; 118 (24):2533-9.

170. Kannel WB, D'Agostino RB, Wilson PW, Belanger AJ, Gagnon DR. Diabetes, fibrinogen, and risk of cardiovascular disease: the Framingham experience. Am Heart J. 1990; 120 (3):672-6.

171. Bastard JP, Pieroni L, Hainque B. Relationship between plasma plasminogen activator inhibitor 1 and insulin resistance. Diabetes Metab Res Rev. 2000; 16 (3):192-201.

172. Meigs JB, Mittleman MA, Nathan DM, Tofler GH, Singer DE, Murphy-Sheehy PM, *et al*. Hyperinsulinemia, hyperglycemia, and impaired hemostasis: the Framingham offspring study. JAMA. 2000; 283 (2):221-8.

173. Ernst E, Matria A, Schmölzl C, Magyarosy I. Dose effect relationship between smoking and blood rheology. Brit J Haematol. 1987; 65 (4):485-7.

174. Scarabin P-Y, Aillaud M-F, Amouyel P, Luc AEG, Ferrières J, Arveiler D, *et al*. Associations of fibrinogen, factor VII and PAI-1 with baseline findings among 10,500 male participants in a prospective study of myocardial infarction. Thromb Haemost. 1998; 80 (5):749-56.

175. Sasaki A, Kurisu A, Ohno M, Ikeda Y. Overweight/obesity, smoking, and heavy alcohol consumption are important determinants of plasma PAI-1 levels in healthy men. Am J Med Sci. 2001; 322 (1):19-23.

176. Mukamal KJ, Jadhav PP, D'Agostino RB, Massaro JM, Mittleman MA, Lipinska I, *et al*. Alcohol consumption and hemostatic factors: analysis of the Framingham offspring cohort. Circulation. 2001; 104 (12):1367-73.

177. Mennen LI, Balkau B, Vol S, Cacès E, Eschwège E, Group ftDS. Fibrinogen: A possible link between alcohol consumption and cardiovascular disease? Arterioscler Thromb Vasc Biol. 1999; 19 (4):887-92.

178. Djousse L, Pankow JS, Arnett DK, Zhang Y, Hong Y, Province MA, *et al*. Alcohol consumption and plasminogen activator inhibitor type 1: the National Heart, Lung, and Blood Institute Family Heart Study. Am Heart J. 2000; 139 (4):704-9.

179. Wannamethee SG, Lowe GDO, Whincup PH, Rumley A, Walker M, Lennon L. Physical activity and hemostatic and inflammatory variables in elderly men. Circulation. 2002; 105 (15):1785-90.

180. Bettega D, Zanettini R, Ferretti M. [Physical training exercise reduces the plasma levels of fibrinogen in subjects with mild hypertension]. Ann Ital Med Int. 1995; 10 (3):167-70.

181. Ernst E. Regular exercise reduces fibrinogen levels: a review of longitudinal studies. Brit J Sports Med. 1993; 27 (3):175-6.

182. DeSouza CA, Jones PP, Seals DR. Physical activity status and adverse age-related differences in coagulation and fibrinolytic factors in women. Arterioscler Thromb Vasc Biol. 1998; 18 (3):362-8.

183. Smith DT, Hoetzer GL, Greiner JJ, Stauffer BL, DeSouza CA. Effects of ageing and regular aerobic exercise on endothelial fibrinolytic capacity in humans. J Physiol. 2003; 546 (1):289-98.

184. Andersson HM, Siegerink B, Luken BM, Crawley JT, Algra A, Lane DA, *et al*. High VWF, low ADAMTS13, and oral contraceptives increase the risk of ischemic stroke and myocardial infarction in young women. Blood. 2012; 119 (6):1555-60.

185. Kluft C, Gevers Leuven JA, Helmerhorst FM, Krans HMJ. Pro-inflammatory effects of oestrogens during use of oral contraceptives and hormone replacement treatment. Vascul Pharmacol. 2002; 39 (3):149-54.

186. Task Force on Oral Contraceptives. A multicentre study of coagulation and haemostatic variables during oral contraception: variations with four formulations. WHO Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization, Geneva, Switzerland. Brit J Obstet Gynaecol. 1991; 98 (11):1117-28.

187. Balleisen L, Bailey J, Epping PH, Schulte H, van de Loo J. Epidemiological study on factor VII, factor VIII and fibrinogen in an industrial population: I. Baseline data on the relation to age, gender, body-weight, smoking, alcohol, pill-using, and menopause. Thromb Haemost. 1985; 54 (2):475-9.

188. Scarabin PY, Plu-Bureau G, Zitoun D, Bara L, Guize L, Samama MM. Changes in haemostatic variables induced by oral contraceptives containing 50 micrograms or 30 micrograms

oestrogen: absence of dose-dependent effect on PAI-1 activity. Thromb Haemost. 1995; 74 (3):928-32.

189. Scarabin PY, Vissac AM, Kirzin JM, Bourgeat P, Amiral J, Agher R, *et al*. Elevated plasma fibrinogen and increased fibrin turnover among healthy women who both smoke and use low-dose oral contraceptives--a preliminary report. Thromb Haemost. 1999; 82 (3):1112-6.

190. Meilahn EN, Cauley JA, Tracy RP, Macy EO, Gutai JP, Kuller LH. Association of sex hormones and adiposity with plasma levels of fibrinogen and PAI-1 in postmenopausal women. Am J Epidemiol. 1996; 143 (2):159-66.

191. Gebara OC, Mittleman MA, Sutherland P, Lipinska I, Matheney T, Xu P, *et al*. Association between increased estrogen status and increased fibrinolytic potential in the Framingham offspring study. Circulation. 1995; 91 (7):1952-8.

192. Lip GY, Blann AD, Jones AF, Beevers DG. Effects of hormone-replacement therapy on hemostatic factors, lipid factors, and endothelial function in women undergoing surgical menopause: implications for prevention of atherosclerosis. Am Heart J. 1997; 134 (4):764-71.

193. Shahar E, Folsom AR, Salomaa VV, Stinson VL, McGovern PG, Shimakawa T, *et al*. Relation of hormone-replacement therapy to measures of plasma fibrinolytic activity. Atherosclerosis Risk in Communities (ARIC) Study Investigators. Circulation. 1996; 93 (11):1970-5.

194. Cushman M, Meilahn EN, Psaty BM, Kuller LH, Dobs AS, Tracy RP. Hormone replacement therapy, inflammation, and hemostasis in elderly women. Arterioscler Thromb Vasc Biol. 1999; 19 (4):893-9.

195. Rudnicka AR, Rumley A, Lowe GDO, Strachan DP. Diurnal, seasonal, and blood-processing patterns in levels of circulating fibrinogen, fibrin D-dimer, C-reactive protein, tissue plasminogen activator, and von Willebrand factor in a 45-year-old population. Circulation. 2007; 115 (8):996-1003.

196. Bremner WF, Sothern RB, Kanabrocki EL, Ryan M, McCormick JB, Dawson S, *et al*. Relation between circadian patterns in levels of circulating lipoprotein(a), fibrinogen, platelets, and related lipid variables in men. Am Heart J. 2000; 139 (1):164-73.

197. Angleton P, Chandler WL, Schmer G. Diurnal variation of tissue-type plasminogen activator and its rapid inhibitor (PAI-1). Circulation. 1989; 79 (1):101-6.

198. Andreotti F, Davies GJ, Hackett DR, Khan MI, De Bart AC, Aber VR, *et al*. Major circadian fluctuations in fibrinolytic factors and possible relevance to time of onset of myocardial infarction, sudden cardiac death and stroke. Am J Cardiol. 1988; 62 (9):635-7.

199. Fröhlich M, Sund M, Russ S, Hoffmeister A, Fischer HG, Hombach V, *et al*. Seasonal variations of rheological and hemostatic parameters and acute-phase reactants in young, healthy subjects. Arterioscler Thromb Vasc Biol. 1997; 17 (11):2692-7.

200. van der Bom JG, de Maat MP, Bots ML, Hofman A, Kluft C, Grobbee DE. Seasonal variation in fibrinogen in the Rotterdam Study. Thromb Haemost. 1997; 78 (3):1059-62.

201. Oparil S, Zaman MA, Calhoun DA. Pathogenesis of hypertension. Ann Intern Med. 2003; 139 (9):761-76.

202. Beevers G, Lip GY, O'Brien E. The pathophysiology of hypertension. BMJ. 2001; 322 (7291):912-6.

203. Iiyama K, Nagano M, Yo Y, Nagano N, Kamide K, Higaki J, *et al*. Impaired endothelial function with essential hypertension assessed by ultrasonography. Am Heart J. 1996; 132 (4):779-82.

204. Rohde LE, Hennekens CH, Ridker PM. Survey of C-reactive protein and cardiovascular risk factors in apparently healthy men. Am J Cardiol. 1999; 84 (9):1018-22.

205. Bermudez EA, Rifai N, Buring J, Manson JE, Ridker PM. Interrelationships among circulating interleukin-6, C-reactive protein, and traditional cardiovascular risk factors in women. Arterioscler Thromb Vasc Biol. 2002; 22 (10):1668-73.

206. Poli KA, Tofler GH, Larson MG, Evans JC, Sutherland PA, Lipinska I, *et al*. Association of blood pressure with fibrinolytic potential in the Framingham offspring population. Circulation. 2000; 101 (3):264-9.

207. Wall U, Jern C, Bergbrant A, Jern S. Enhanced levels of tissue-type plasminogen activator in borderline hypertension. Hypertension. 1995; 26 (5):796-800.

208. Lee A. The role of rheological and haemostatic factors in hypertension. J Hum Hypertens. 1997; 11 (12):767-76.

209. Lip GY, Li-Saw-Hee FL. Does hypertension confer a hypercoagulable state? J Hypertens. 1998; 16 (7):913-6.

210. Varughese G, Lip GH. Is hypertension a prothrombotic state? Current Science Inc. 2005; 7 (3):168-73.

211. Marin F, Roldan V, Climent V, Ibanez A, Garcia A, Marco P, *et al*. Plasma von Willebrand factor, soluble thrombomodulin, and fibrin D-dimer concentrations in acute onset non-rheumatic atrial fibrillation. Heart. 2004; 90 (10):1162-6.

212. Inoue H, Nozawa T, Okumura K, Jong-Dae L, Shimizu A, Yano K. Prothrombotic activity is increased in patients with nonvalvular atrial fibrillation and risk factors for embolism. CHEST. 2004; 126 (3):687-92.

213. Chin B, Blann A, Gibbs C, Chung N, Conway D, Lip G. Prognostic value of interleukin-6, plasma viscosity, fibrinogen, von Willebrand factor, tissue factor and vascular endothelial growth factor levels in congestive heart failure. Eur J Clin Invest. 2003; 33 (11):941-8.

214. Lip GY, Blann AD, Jones AF, Lip PL, Beevers DG. Relation of endothelium, thrombogenesis, and hemorheology in systemic hypertension to ethnicity and left ventricular hypertrophy. Am J Cardiol. 1997; 80 (12):1566-71.

215. Landin K, Tengborn L, Smith U. Elevated fibrinogen and plasminogen activator inhibitor (PAI-1) in hypertension are related to metabolic risk factors for cardiovascular disease. J Intern Med. 1990; 227 (4):273-8.

216. Blann AD, Naqvi T, Waite M, McCollum CN. von Willebrand factor and endothelial damage in essential hypertension. J Hum Hypertens. 1993; 7 (2):107-11.

217. Lominadze D, Dean WL, Tyagi SC, Roberts AM. Mechanisms of fibrinogen-induced microvascular dysfunction during cardiovascular disease. Acta Physiol. 2010; 198 (1):1-13.

218. Koenig W, Sund M, Ernst E, Keil U, Rosenthal J, Hombach V. Association between plasma viscosity and blood pressure results from the MONICA-Project Augsburg. Am J Hypertens. 1991; 4 (6):529-36.

219. Junker R, Heinrich J, Schulte H, Erren M, Assmann G. Hemostasis in normotensive and hypertensive men: results of the PROCAM Study. J Hypertens. 1998; 16 (7):918-22.

220. Wang TJ, Gona P, Larson MG, Levy D, Benjamin EJ, Tofler GH, *et al*. Multiple biomarkers and the risk of incident hypertension. Hypertension. 2007; 49 (3):432-8.

221. Lip GY. Hypertension and the prothrombotic state. J Hum Hypertens. 2000; 14 (10-11):687-90.

222. Khaleghi M, Saleem U, McBane RD, Mosley Jr TH, Kullo IJ. African-American ethnicity is associated with higher plasma levels of D-dimer in adults with hypertension. J Thromb Haemost. 2009; 7 (1):34-40.

223. Wu M, KK, Thiagarajan M, P. Role of endothelium in thrombosis and hemostasis. Ann Rev Med. 1996; 47 (1):315-31.

224. Widlansky ME, Gokce N, Keaney JF, Vita JA. The clinical implications of endothelial dysfunction. J Am Coll Cardiol. 2003; 42 (7):1149-60.

225. Radomski MW, Palmer R, Moncada S. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Brit J Pharmacol. 1987; 92 (3):639-46.

226. Esmon CT. Inflammation and thrombosis. J Thromb Haemost. 2003; 1 (7):1343-8.

227. Levi M, van der Poll T. Two-Way Interactions Between inflammation and coagulation. Trends Cardiovasc Med. 2005; 15 (7):254-9.

228. Szaba FM, Smiley ST. Roles for thrombin and fibrin(ogen) in cytokine/chemokine production and macrophage adhesion *in vivo*. Blood. 2002; 99 (3):1053-9.

229. Birch KA, Pober JS, Zavoico GB, Means AR, Ewenstein BM. Calcium/calmodulin transduces thrombin-stimulated secretion: studies in intact and minimally permeabilized human umbilical vein endothelial cells. J Cell Biol. 1992; 118 (6):1501-10.

230. Lowenstein CJ, Morrell CN, Yamakuchi M. Regulation of Weibel–Palade body exocytosis. Trends Cardiovasc Med. 2005; 15 (8):302-8.

231. Hamilton K, Sims P. Changes in cytosolic Ca^{2+} associated with von Willebrand factor release in human endothelial cells exposed to histamine. Study of microcarrier cell monolayers using the fluorescent probe indo-1. J Clin Invest. 1987; 79 (2):600.

232. Kaufmann JE, Oksche A, Wollheim CB, Günther G, Rosenthal W, Vischer UM. Vasopressin-induced von Willebrand factor secretion from endothelial cells involves V2 receptors and cAMP. J Clin Invest. 2000; 106 (1):107-16.

233. Irigoyen J, Munoz-Canoves P, Montero L, Koziczak M, Nagamine Y. The plasminogen activator system: biology and regulation. Cell Mol Life Sci. 1999; 56 (1-2):104-32.

234. Emeis J, Kooistra T. Interleukin 1 and lipopolysaccharide induce an inhibitor of tissue-type plasminogen activator *in vivo* and in cultured endothelial cells. J Exp Med. 1986; 163 (5):1260-6.

235. Sawdey M, Podor T, Loskutoff D. Regulation of type 1 plasminogen activator inhibitor gene expression in cultured bovine aortic endothelial cells. Induction by transforming growth factor-beta, lipopolysaccharide, and tumor necrosis factor-alpha. J Biol Chem. 1989; 264 (18):10396-401.

236. Konkle BA, Schuster SJ, Kelly MD, Harjes K, Hassett DE, Bohrer M, *et al*. Plasminogen activator inhibitor-1 messenger RNA expression is induced in rat hepatocytes *in vivo* by dexamethasone. Blood. 1992; 79 (10):2636-42.

237. Samad F, Loskutoff D. Tissue distribution and regulation of plasminogen activator inhibitor-1 in obese mice. Mol Med. 1996; 2 (5):568-82.

238. Vaughan DE, Lazos SA, Tong K. Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. A potential link between the renin-angiotensin system and thrombosis. J Clin Invest. 1995; 95 (3):995-1001.

239. Pinsky DJ, Liao H, Lawson CA, Yan S-F, Chen J, Carmeliet P, *et al*. Coordinated induction of plasminogen activator inhibitor-1 (PAI-1) and inhibition of plasminogen activator gene expression by hypoxia promotes pulmonary vascular fibrin deposition. J Clin Invest. 1998; 102 (5):919-28.

240. Pinsky DJ, Naka Y, Liao H, Oz MC, Wagner DD, Mayadas TN, *et al*. Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. J Clin Invest. 1996; 97 (2):493-500.

241. Galbusera M, Zoja C, Donadelli R, Paris S, Morigi M, Benigni A, *et al*. Fluid shear stress modulates von Willebrand factor release from human vascular endothelium. Blood. 1997; 90 (4):1558-64.

242. Hoekstra T, Geleijnse JM, Schouten EG, Kluft C. Plasminogen activator inhibitor-type 1: its plasma determinants and relation with cardiovascular risk. Thromb Haemost. 2004; 91 (5):861-72.

243. Castell JV, Gómez-Lechón MJ, David M, Andus T, Geiger T, Trullenque R, *et al*. Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. FEBS letters. 1989; 242 (2):237-9.

244. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J. 1990; 265 (3):621-36.

245. Kamath S, Lip G. Fibrinogen: biochemistry, epidemiology and determinants. QJM. 2003; 96 (10):711-29.

246. Kaptoge S, White I, Thompson S, Wood A, Lewington S, Lowe G, *et al*. Associations of plasma fibrinogen levels with established cardiovascular disease risk factors, inflammatory markers, and other characteristics: individual participant meta-analysis of 154,211 adults in 31 prospective studies: the fibrinogen studies collaboration. Am J Epidemiol. 2007; 166 (8):867-79.

247. Sen U, Tyagi N, Patibandla PK, Dean WL, Tyagi SC, Roberts AM, *et al*. Fibrinogen-induced endothelin-1 production from endothelial cells. Am J Physiol Cell Physiol. 2009; 296 (4):C840-C7.

248. Koenig W. Fibrin(ogen) in cardiovascular disease: an update. Thromb Haemost. 2003; 89 (4):601-9.

249. Esmon C, Taylor F, Snow TR. Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C. Thromb Haemost. 1991; 66 (1):160-5.

250. Stouthard J, Levi M, Hack C, Veenhof C, Romijn H, Sauerwein H, *et al*. Interleukin-6 stimulates coagulation, not fibrinolysis, in humans. Thromb Haemost. 1996; 76 (5):738-42.

251. Ritchie DG, Levy BA, Adams MA, Fuller GM. Regulation of fibrinogen synthesis by plasmin-derived fragments of fibrinogen and fibrin: an indirect feedback pathway. Proc Natl Acad Sci USA. 1982; 79 (5):1530-4.

252. Robson S, Shephard E, Kirsch R. Fibrin degradation product D-dimer induces the synthesis and release of biologically active IL-1β, IL-6 and plasminogen activator inhibitors from monocytes *in vitro*. Br J Haematol. 1994; 86 (2):322-6.

253. Mandl J, Csala M, Lerant I, Banhegyi G, Biro J, Machovich R, *et al*. Enhancement of interleukin-6 Production by fibrinogen degradation product D in human peripheral monocytes and perfused murine liver. Scan J Immunol. 1995; 42 (1):175-8.

254. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: Animal and human studies. Circulation. 2003; 108 (17):2034-40.

255. Leopold JA, Loscalzo J. Oxidative enzymopathies and vascular disease. Arterioscler Thromb Vasc Biol. 2005; 25 (7):1332-40.

256. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007; 39 (1):44-84.

257. Sies H. Physiological society symposium: impaired endothelial and smooth muscle cell function in oxidative stress. Exp Physiol. 1997; 82:291-5.

258. Muller G, Goettsch C, Morawietz H. Oxidative stress and endothelial dysfunction. Hamostaseologie. 2007; 27 (1):5-12.

259. Loscalzo J. Oxidative stress in endothelial cell dysfunction and thrombosis. Pathophysiol Haemost Thromb. 2003; 32 (5-6):359-60.

260. Lu SC. Glutathione synthesis. Biochim Biophys Acta Gen Subjects. 2013; 1830 (5):3143-53.

261. Forman HJ, Zhang H, Rinna A. Glutathione: overview of its protective roles, measurement, and biosynthesis. Mol Aspects Med. 2009; 30 (1):1-12.

262. Lu SC. Regulation of glutathione synthesis. Mol Aspects Med. 2009; 30 (1-2):42-59.

263. Yang MS, Chan HW, Yu LC. Glutathione peroxidase and glutathione reductase activities are partially responsible for determining the susceptibility of cells to oxidative stress. Toxicology. 2006; 226 (2-3):126-30.

264. Jones DP, Carlson JL, Mody Jr VC, Cai J, Lynn MJ, Sternberg Jr P. Redox state of glutathione in human plasma. Free Radic Biol Med. 2000; 28 (4):625-35.

265. Loscalzo J. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. Circ Res. 2001; 88 (8):756-62.

266. Jin RC, Mahoney CE, Anderson L, Ottaviano F, Croce K, Leopold JA, *et al*. Glutathione peroxidase-3 deficiency promotes platelet-dependent thrombosis *In Vivo*. Circulation. 2011; 123 (18):1963-73.

267. Kenet G, Freedman J, Shenkman B, Regina E, Brok-Simoni F, Holzman F, *et al*. Plasma glutathione peroxidase deficiency and platelet insensitivity to nitric oxide in children with familial stroke. Arterioscler Thromb Vasc Biol. 1999; 19 (8):2017-23.

268. Takahashi K, Avissar N, Whitin J, Cohen H. Purification and characterization of human plasma glutathione peroxidase: a selenoglycoprotein distinct from the known cellular enzyme. Arch Biochem Biophys. 1987; 256 (2):677-86.

269. Ruef J, Peter K, Nordt TK, Runge MS, Kubler W, Bode C. Oxidative stress and atherosclerosis: its relationship to growth factors, thrombus formation and therapeutic approaches. Thromb Haemost. 1999; 82 Suppl 1:32-7.

270. Kvietys PR, Granger DN. Role of reactive oxygen and nitrogen species in the vascular responses to inflammation. Free Radic Biol Med. 2012; 52 (3):556-92.

271. Matsushita K, Morrell CN, Mason RJ, Yamakuchi M, Khanday FA, Irani K, *et al*. Hydrogen peroxide regulation of endothelial exocytosis by inhibition of N-ethylmaleimide sensitive factor. J Cell Biol. 2005; 170 (1):73-9.

272. Blankenberg S, Rupprecht HJ, Bickel C, Torzewski M, Hafner G, Tiret L, *et al*. Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. New Engl J Med. 2003; 349 (17):1605-13.

273. Ibrahim H, El-Meligi A, Abdel-Hamid M, Elhendy A. Relations between von Willebrand factor, markers of oxidative stress and microalbuminuria in patients with type 2 diabetes mellitus. Med Sci Monit. 2004; 10 (3):CR85-9.

274. Shacter E, Williams JA, Lim M, Levine RL. Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. Free Radic Biol Med. 1994; 17 (5):429-37.

275. Stikarova J, Kotlin R, Riedel T, Suttnar J, Pimkova K, Chrastinova L, *et al*. The effect of reagents mimicking oxidative stress on fibrinogen function. Sci World J. 2013; 2013:359-621.

276. Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, *et al*. Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. J Biol Chem. 2004; 279 (10):8820-6.

277. Shacter E, Williams JA, Levine RL. Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. Free Radic Biol Med. 1995; 18 (4):815-21.

278. Azizova OA, Aseichev AV, Piryazev AP, Roitman EV, Shcheglovitova ON. Effects of oxidized fibrinogen on the functions of blood cells, blood clotting, and rheology. Bull Exp Biol Med. 2007; 144 (3):397-407.

279. Tetik S, Kaya K, Demir M, Eksioglu-Demiralp E, Yardimci T. Oxidative modification of fibrinogen affects its binding activity to glycoprotein (GP) IIb/IIIa. Clin Appl Thromb Hemost. 2010; 16 (1):51-9.

280. Martinez M, Weisel JW, Ischiropoulos H. Functional impact of oxidative posttranslational modifications on fibrinogen and fibrin clots. Free Radic Biol Med. 2013; 65:411-8.

281. Paton LN, Mocatta TJ, Richards AM, Winterbourn CC. Increased thrombin-induced polymerization of fibrinogen associated with high protein carbonyl levels in plasma from patients post myocardial infarction. Free Radic Biol Med. 2010; 48 (2):223-9.

282. Upchurch Jr GR, Ramdev N, Walsh MT, Loscalzo J. Prothrombotic consequences of the oxidation of fibrinogen and their inhibition by aspirin. J Thromb Thrombolysis. 1998; 5 (1):9-14.

283. Tetik S, Kaya K, Yardimci T. Effect of oxidized fibrinogen on hemostatic system: *in vitro* study. Clin Appl Thromb Hemost. 2011; 17 (3):259-63.

284. Swiatkowska M, Szemraj J, Al-Nedawi KN, Pawlowska Z. Reactive oxygen species upregulate expression of PAI-1 in endothelial cells. Cell Mol Biol Lett. 2002; 7 (4):1065-71.

285. Dimova EY, Samoylenko A, Kietzmann T. Oxidative stress and hypoxia: implications for plasminogen activator inhibitor-1 expression. Antioxid Redox Signal. 2004; 6 (4):777-91.

286. Martina V, Bruno GA, Pannocchia A, Zumpano E, Tagliabue M, Trucco F, *et al*. PAI-1 reduction after treatment with glutathione in NIDDM. Fibrinolysis. 1996; 10 (0):63-5.

287. Sun C, Wang JJ, Mackey DA, Wong TY. Retinal vascular caliber: systemic, environmental, and genetic associations. Surv Ophthalmol. 2009; 54 (1):74-95.

288. Flammer J, Konieczka K, Bruno RM, Virdis A, Flammer AJ, Taddei S. The eye and the heart. Eur Heart J. 2013; 34 (17):1270-8.

289. Patton N, Aslam T, MacGillivray T, Pattie A, Deary IJ, Dhillon B. Retinal vascular image analysis as a potential screening tool for cerebrovascular disease: a rationale based on homology between cerebral and retinal microvasculatures. J Anat. 2005; 206 (4):319-48.

290. Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. Curr Eye Res. 2003; 27 (3):143-9.

291. Wang JJ, Rochtchina E, Liew G, Tan AG, Wong TY, Leeder SR, *et al*. The long-term relation among retinal arteriolar narrowing, blood pressure, and incident severe hypertension. Am J Epidemiol. 2008; 168 (1):80-8.

292. Kawasaki R, Cheung N, Wang JJ, Klein R, Klein BE, Cotch MF, *et al*. Retinal vessel diameters and risk of hypertension: the Multi Ethnic Study of Atherosclerosis. J Hypertens. 2009; 27 (12):2386-93.

293. Ikram MK, Witteman JC, Vingerling JR, Breteler MM, Hofman A, de Jong PT. Retinal vessel diameters and risk of hypertension: the Rotterdam Study. Hypertension. 2006; 47 (2):189-94.

294. Cheung CY-l, Ikram MK, Sabanayagam C, Wong TY. Retinal microvasculature as a model to study the manifestations of hypertension. Hypertension. 2012; 60 (5):1094-103.

295. Klein R, Sharrett AR, Klein BE, Chambless LE, Cooper LS, Hubbard LD, *et al*. Are retinal arteriolar abnormalities related to atherosclerosis?: The Atherosclerosis Risk in Communities study. Arterioscler Thromb Vasc Biol. 2000; 20 (6):1644-50.

296. Klein R, Klein BE, Knudtson MD, Wong TY, Tsai MY. Are inflammatory factors related to retinal vessel caliber?: The Beaver Dam Eye Study. Arch Ophthalmol. 2006; 124 (1):87-94.

297. Ikram MK, de Jong FJ, Vingerling JR, Witteman JC, Hofman A, Breteler MM, *et al*. Are retinal arteriolar or venular diameters associated with markers for cardiovascular disorders?: The Rotterdam study. Invest Ophthalmol Vis Sci. 2004; 45 (7):2129-34.

298. Wong TY, Duncan BB, Golden SH, Klein R, Couper DJ, Klein BE, *et al*. Associations between the metabolic syndrome and retinal microvascular signs: The Atherosclerosis Risk In Communities study. Invest Ophthalmol Vis Sci. 2004; 45 (9):2949-54.

299. Leung H, Wang JJ, Rochtchina E, Wong TY, Klein R, Mitchell P. Dyslipidaemia and microvascular disease in the retina. Eye. 2005; 19 (8):861-8.

300. McGeechan K, Liew G, Macaskill P, Irwig L, Klein R, Klein BE, *et al*. Prediction of incident stroke events based on retinal vessel caliber: a systematic review and individual-participant meta-analysis. Am J Epidemiol. 2009; 170 (11):1323-32.

301. Ikram M, De Jong F, Bos M, Vingerling J, Hofman A, Koudstaal P, *et al*. Retinal vessel diameters and risk of stroke: The Rotterdam study. Neurology. 2006; 66 (9):1339-43.

302. Wong TY, Islam FMA, Klein R, Klein BEK, Cotch MF, Castro C, *et al*. Retinal vascular caliber, cardiovascular risk factors, and Inflammation: The Multi-Ethnic Study of Atherosclerosis (MESA). Invest Ophthalmol Vis Sci. 2006; 47 (6):2341-50.

303. Shyy JY-J, Chien S. Role of integrins in endothelial mechanosensing of shear stress. Circ Res. 2002; 91 (9):769-75.

304. Davies PF, Zilberberg J, Helmke BP. Spatial microstimuli in endothelial mechanosignaling. Circ Res. 2003; 92 (4):359-70.

305. Ruggeri ZM. Mechanisms of shear-induced platelet adhesion and aggregation. Thromb Haemost. 1993; 70 (1):119-23.

306. Languino LR, Plescia J, Duperray A, Brian AA, Plow EF, Geltosky JE, *et al*. Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway. Cell. 1993; 73 (7):1423-34.

307. Spronk HM, Van Der Voort D, Ten Cate H. Blood coagulation and the risk of atherothrombosis: a complex relationship. Thromb J. 2004; 2 (1):12.

308. Vicaut E. Opposite effects of red blood cell aggregation on resistance to blood flow. J Cardiovasc Surg. 1995; 36 (4):361-8.

309. Chien S, Usami S, Dellenback RJ, Gregersen MI, Nanninga LB, Guest MM. Blood viscosity: influence of erythrocyte aggregation. Science. 1967; 157 (3790):829-31.

310. Hicks RC, Golledge J, Mir-Hasseine R, Powell JT. Vasoactive effects of fibrinogen on saphenous vein. Nature. 1996; 379 (6568):818-20.

311. Luscinskas FW, Lawler J. Integrins as dynamic regulators of vascular function. FASEB. 1994; 8 (12):929-38.

312. Tyagi N, Roberts AM, Dean WL, Tyagi SC, Lominadze D. Fibrinogen induces endothelial cell permeability. Mol Cell Biochem. 2008; 307 (1-2):13-22.

313. Patibandla PK, Tyagi N, Dean WL, Tyagi SC, Roberts AM, Lominadze D. Fibrinogen induces alterations of endothelial cell tight junction proteins. J Cell Physiol. 2009; 221 (1):195-203.

314. Mogford JE, Davis GE, Meininger GA. RGDN peptide interaction with endothelial alpha5beta1 integrin causes sustained endothelin-dependent vasoconstriction of rat skeletal muscle arterioles. J Clin Invest. 1997; 100 (6):1647-53.

315. Mogford JE, Davis GE, Platts SH, Meininger GA. Vascular smooth muscle αvβ3 integrin mediates arteriolar vasodilation in response to RGD peptides. Circ Res. 1996; 79 (4):821-6.

CHAPTER 2

D-dimer relates positively with increased blood pressure in black South Africans: The SABPA study

Published in *Thrombosis Research*

AUTHOR INSTRUCTIONS FOR THROMBOSIS RESEARCH

(http://www.elsevier.com/journals/thrombosis-research/0049-3848/guide-for-authors)

Thrombosis Research is an international journal with a goal of rapid dissemination of new information on thrombosis, haemostasis, and vascular biology to advance science and clinical care.

Essential title page information

Title. The title should be concise and informative. Avoid abbreviations where possible.

Author names and affiliations. State all authors' names, middle initial and last name. Present the authors' affiliation addresses below the names. Indicate all affiliations with a lower-case superscript number immediately after the author's name and in front of the appropriate address.

Corresponding author. Clearly indicate the corresponding author name and address.

Conflict of interest. Authors must state all potential conflicts of interest.

Word count should be provided on the title page. The word count should include all text, tables and legends except the abstract and reference list.

Abstract

A concise and factual abstract of no more than 250 words is required. The abstract should state the purpose of the research, the principal results and major conclusions. Structured abstracts consisting out of the following headings Introduction, Materials and Methods, Results and Conclusions are encouraged:

Keywords and Abbreviations

The abstract should be followed by a list of three to six keywords. Followed by a list of all abbreviations used. Only abbreviations firmly established in the field may be eligible.

Article structure

The article must be divided into clearly defined sections (Introduction, Material and methods, Results and Discussion). Each heading should appear on its own separate line.

Introduction.

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods.

Provide sufficient detail to allow the work to be reproduced. Authors should stipulate that informed consent was obtained when applicable to research on humans, with the comment that the study was approved by the institutional Ethics Committee on human research.

Results

Results should be clear and concise.

Discussion

Should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature.

Acknowledgements

Acknowledgement should be made to those who have made a substantial contribution to the study.

References

Text: Indicate references with Arabic numerals in square brackets e.g. [12] in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

Reference List: Should be numbered consecutively in the order they appear in the text.

Reference style template: Can be obtained for Endnote users at

http://endnote.com/downloads/style/thrombosis-research

Tables

Number tables consecutively in accordance with their appearance in the text. Each table should be submitted on their own page and contain a brief title. Place explanatory matter in a footnote below the table body.

Supplementary data

Electronic supplementary material that supports and enhances your scientific research is accepted. Supplementary files supplied are published online alongside the electronic version of your article. A List of the supplementary content with a concise and descriptive caption for each file should be submitted at the end of the manuscript.

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

D-dimer relates positively with increased blood pressure in black South Africans: The SABPA study

Leandi Lammertyn[1], Aletta E Schutte[1], Marlien Pieters[2] and Rudolph Schutte[1]

[1]Hypertension in Africa Research Team (HART); North-West University (Potchefstroom Campus); Potchefstroom; South Africa.

[2]Centre of Excellence for Nutrition (CEN); North-West University (Potchefstroom Campus); Potchefstroom; South Africa.

Correspondence:

Rudolph Schutte, PhD

Hypertension in Africa Research Team (HART),

Private Bag x6001,

North-West University (Potchefstroom Campus),

Potchefstroom,

2520

South Africa

Telephone: +27-18-299-2435

Facsimile: +27-18-299-1053

E-mail: rudolph.schutte@nwu.ac.za

Conflict of Interest: All authors declare no conflict of interest.

Word count: 3472

2.1 Abstract

Introduction: Hypertension is highly prevalent in black South Africans in which morbidity and mortality from stroke are on the increase. Elevated blood pressure and haemostatic markers can induce changes in blood rheology and endothelial function which could result in a procoagulant state that increases the risk for cerebrovascular disease. Information about the coagulation and fibrinolytic systems of people from African descent are limited. We therefore, investigated the haemostatic profile and its relationships with blood pressure in black South Africans.

Materials and methods: We measured ambulatory blood pressure and haemostatic markers of 201 black and 208 white school teachers. The haemostatic markers included measurements representing coagulation and fibrinolysis (von Willebrand factor, fibrinogen, plasminogen activator inhibitor-1, fibrin D-dimer and clot lysis time).

Results: Black participants displayed significantly higher blood pressure, von Willebrand factor, fibrinogen, plasminogen activator inhibitor-1 and D-dimer levels and a longer clot lysis times ($p \leq 0.001$). Single, partial and multiple regression analyses showed that systolic ($p \leq 0.011$) and diastolic blood pressure ($p = 0.010$) correlated positively with D-dimer in black participants, while systolic ($p \leq 0.001$) and daytime diastolic blood pressure ($p = 0.011$) correlated negatively with clot lysis time in white participants.

Conclusion: The black population had a more prothrombotic profile, with higher levels of coagulation markers and inhibited fibrinolysis, than the white study participants. The positive association between blood pressure and elevated D-dimer in the blacks may contribute to the high prevalence of hypertension and related increased cardiovascular and cerebrovascular risk in this group.

Key words: von Willebrand factor, fibrinogen, plasminogen activator inhibitor-1, fibrin D-dimer, clot lysis time, ethnicity.

2.2 Abbreviations

ABPM	Ambulatory blood pressure monitor
BMI	Body mass index
CLT	Clot lysis time
CRP	C-reactive protein
DBP	Diastolic blood pressure
EDTA	Ethylenediaminetetraacetic
HbA1c	Glycosylated haemoglobin A1c
HDL	High density lipoprotein cholesterol
HIV	Human immunodeficiency virus
LDL	Low density lipoprotein cholesterol
$PAI\text{-}1_{act}$	Plasminogen activator inhibitor-1 activity
$PAI\text{-}1_{ag}$	Plasminogen activator inhibitor-1 antigen
PP	Pulse pressure
SBP	Systolic blood pressure
TC	Total cholesterol
TC:HDL	Total cholesterol: high density lipoprotein cholesterol
t-PA	tissue Plasminogen activator
vWF_{ag}	von Willebrand factor antigen

2.3 Introduction

Abnormalities in the coagulation and fibrinolytic systems such as hypercoagulation and hypofibrinolysis are associated with cardiovascular [1,2] and cerebrovascular disease [3,4]. Morbidity and mortality from cardiovascular [5] and cerebrovascular [6] diseases are rapidly increasing in the black population of South Africa, a group with a high prevalence of hypertension [7,8], that contributes, at least in part, to the low life expectancy of 52.6 years [9].

Available evidence from European populations suggests that elevated blood pressure is associated with high circulating levels of several coagulatory [10,11] and fibrinolytic markers [12,13], inducing changes in blood constituents, flow and endothelial function [14-16]. These studies did not include people from African descent or distinguish between ethnicities. Information on the haemostatic system markers and their associations with cardiovascular function in black South Africans is limited. Current evidence suggest that black populations may have altered levels of haemostatic proteins [17-19] that could increase their cardiovascular and cerebrovascular risk.

We therefore investigated the blood pressure and haemostatic profiles of black and white South Africans and determined associations between ambulatory blood pressure and components of the haemostatic system in these groups.

2.4 Materials and methods

2.4.1 Study population

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study was a cross-sectional study involving school teachers (n=409) between the ages of 25 – 60 years from the North West Province, South Africa. Participants consisted of 200 black (101 men and 99 women) and 209 white (101 men and 108 women) participants. Exclusion criteria were: elevated ear temperature, dependence or abuse of psychotropic substances, regular blood donors, or individuals vaccinated in the previous three months. Informed consent was obtained prior to the commencement of measurements. Each participant completed a lifestyle questionnaire, including questions on smoking and alcohol habits as well as chronic medication use. The study complied

with all applicable international regulations and the Helsinki declaration for investigation of human participants. The Ethics Review Board of the North-West University (Potchefstroom Campus) approved the study.

2.4.2 Clinical Measurements

A 24-hour ambulatory blood pressure (ABPM) and electrocardiogram apparatus (Meditech CE120® Cardiotens, Budapest, Hungary) was attached to the participants at their workplace. The device was programmed to measure blood pressure at 30 minute intervals during the day (08:00 – 22:00) and every hour during the night (22:00 – 06:00). The electrocardiogram recorded measurements every 5 minutes for 20 seconds. The participants continued with their daily activities and were asked to record any abnormalities such as nausea, headache, physical activity and stress on their ambulatory diary cards. Each participant's energy expenditure during the day was calculated with a validated accelerometer device (Actical® accelerometers, Montréal, Québec). Participants reported to the Metabolic Research Unit of the North-West University at 16:30 where they were informed of the procedures of the following day. They received a standardised meal at 18:00, and final snacks and drinks at 20:30, and were requested to go to bed at around 22:00. At 06:00, the ABPM apparatus was removed, and anthropometric measurements and blood sampling performed. The 24-hour blood pressure and electrocardiogram data were downloaded onto a database using the CardioVisions 1.9.0 Personal Edition software (Meditech, Budapest, Hungary).

2.4.3 Anthropometric Measurements

All measurements were taken in triplicate with calibrated instruments. Stature was measured to the nearest 0.1 cm with a stadiometer (Invicta Stadiometer, IP 1465, London, UK), body mass to the nearest 0.1 kg (Precision Health Scale, A & D Company,Tokyo, Japan) and waist circumference to the nearest 0.1 cm with a unstretchable flexible 7 mm wide metal tape (Holtain, Crosswell, Wales) [20,21]. Body mass index (BMI) was calculated for each participant using the standard formula of weight / (height)^2.

2.4.4 Biochemical Measurements

A registered nurse collected blood samples with a sterile winged infusion set from the participants' antebrachial vein branches before 10:00. Serum samples were used for the analysis of C-reactive protein (CRP) and lipids. An ethylenediaminetetraacetic (EDTA) whole blood sample was used to analyse glycosylated haemoglobin A1c (HbA1c) and a sodium fluoride sample for fasting blood glucose determination. Citrated samples were used for the analysis of von Willebrand factor antigen (vWF_{ag}), fibrinogen, plasminogen activator inhibitor-1 antigen ($PAI\text{-}1_{ag}$), fibrin D-dimer and clot lysis time (CLT). The EDTA, sodium fluoride and citrate samples were centrifuged at 2000 x g for 15 minutes and stored at -80˚C until analysis. vWF_{ag} levels were determined with a sandwich enzyme-linked immunosorbent assay (ELISA). Polyclonal rabbit anti-vWF antibody and rabbit anti-vWF-horseradish peroxidase antibody (DAKO, Glostrup, Denmark) were used to perform the assay. The 6th International Standard for vWF/FVIII was used to create the standard curve against which the samples were measured. Plasma fibrinogen levels were determined with a viscosity-based clotting method using STAGO FIB kit (STAGO diagnostics, Asnières, France). The Liatest D-Dimer kit from STAGO was used to determine fibrin D-Dimer levels with an immuno-turbimetric method with a 540 nm detection limit. $PAI\text{-}1_{ag}$ levels were determined with the TriniLIZE $PAI\text{-}1_{ag}$ (Trinity Biotech, Bray, Ireland) kit using an ELISA. CLT was determined by studying the lysis of a tissue factor-induced clot by exogenous tissue-plasminogen activator (t-PA). Changes in turbidity during clot formation and lysis were monitored as described by Lisman *et al.* [22]. Tissue factor and t-PA concentrations were slightly modified to obtain comparable CLTs of about 60 minutes. The modified concentrations were 17 mmol/L CaCl, 60 ng/ml t-PA (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and 10 µmol/L phospholipids vesicles (Rossix, Mölndal, Sweden). Tissue factor was diluted 3000 times (Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany). CLT was defined as the time from the midpoint in the transition from the initial baseline to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot (supplementary Figure 1). The percentage of HbA1c was determined by means of the turbidimetric inhibition immunoassay using the Roche Integra 400 (Roche, Basil, Switzerland).

Fasting blood glucose, total cholesterol (TC), high density lipoprotein (HDL) cholesterol, high sensitivity CRP and gamma-glutamyltransferase were analysed with two sequential multiple analysers (Konelab 20i™, ThermoScientific, Vantaa, Finland; Unicel DXC 800, Beckman and Coulter, Munich, Germany) and low density lipoprotein (LDL) cholesterol was calculated by means of the Friedewald formula [LDL = TC - HDL - (0.45*triglycerides)]. Human immunodeficiency virus (HIV) status was determined directly after blood sampling with First Response (PMC Medical, India) rapid HIV tests according to the protocol of the National Department of Health of South Africa. The test was repeated with the Pareeshak (BHAT Bio-tech, India) card test to confirm the results.

2.4.5 Statistical Analyses

Statistica software v11.0 was used for database management and statistical analyses (Statsoft, Inc., 2011). Variables not normally distributed (Fibrin D-dimer, HbA1c, glucose, gamma-glutamyltransferase and energy expenditure) were logarithmically transformed. The central tendency and spread of these variables were represented by the geometric mean and the 5th and 95th percentile intervals. We tested for interaction with ethnicity for the association between systolic blood pressure and the haemostatic variables by using appropriate interaction terms in multiple regression analyses. Means and proportions were compared by a standard t-test and chi-square test, respectively. Analysis of covariance was used to determine mean values between groups. We used Pearson and partial correlations to determine associations between blood pressure and haemostatic markers, followed by multiple linear regression analyses. Significant covariates included in the multiple regression model were age, body mass index, gender, gamma-glutamyltransferase, physical activity, HbA1c, TC:HDL, triglycerides and C-reactive protein. A Bonferonni adjustment of the significance level was performed for the multiple linear regression analyses to protect against an inflated type 1 error, resulting in a more stringent p-value of ≤0.017. All p-values refer to a two-sided hypothesis.

2.5 Results

Table 1 lists the characteristics of the study population. Since significant interactions existed with ethnicity for the association between systolic blood pressure and the haemostatic variables (PAI-1_{ag}, p=0.002; fibrin D-dimer, p=0.006), our study population was divided into black and white groups. Although these groups were of similar age, the black participants were generally more obese (p<0.001) and had higher mean vWF_{ag} (p<0.001), fibrinogen (p<0.001), PAI-1_{ag} (p<0.001) and fibrin D-dimer (p<0.001) concentrations as well as a longer CLT (p<0.001) than the white participants. However the mean levels of vWF_{ag}, fibrinogen, PAI-1_{ag} and fibrin D-dimer were within their respective reference ranges [23]. 27.7% (fibrinogen), 17.6% (fibrin D-dimer), 20.2% (PAI-1_{ag}) and 31.9% (vWF_{ag}) black and 0.07% (Fibrinogen), 0.08% (fibrin D-dimer) and 19% (vWF_{ag}) white participants were not within the normal reference ranges. The black participants also had elevated ambulatory SBP (p<0.001), DBP (p<0.001) and PP (p=0.004) and 136 (68%) were hypertensive compared to the white participants of which 102 (49%) were hypertensive.

After adjusting for age, gender and BMI (Table 2), no significant correlations were found between any of the ambulatory blood pressures and vWF_{ag} (p≥0.11), fibrinogen (p≥0.15) or PAI-1_{ag} (p≥0.17). However in the black participants, positive correlations were found between all the ambulatory SBP (p≤0.034) as well as 24 hour and daytime DBP (p≤0.039) with fibrin D-dimer. In the white participants, we found negative correlations between all the ambulatory SBP (p≤0.041) measurements and pulse pressure (p=0.006) with CLT.

The aforementioned correlations were robust in multiple regression analyses (Table 3) which included additional known cardiovascular risk factors as covariates. An additional association was found between daytime DBP and CLT (p=0.011) in the white group. No associations existed between blood pressure, vWF_{ag} and fibrinogen in either ethnic group.

Table 1: Characteristics of the study population

	Black (n=200)	White (n=209)	p
Women, n (%)	99 (49.5)	108 (51.6)	0.66
Age, years	44.4 ± 8.1	45.0 ± 10.9	0.49
Body mass index, kg/m^2	30.1 ± 7.0	27.6 ± 5.9	<0.001
Waist circumference, cm (men)	93.6 ± 15.5	101.5 ± 14.4	<0.001
Waist circumference, cm (women)	93.6 ± 15.6	86.0 ± 13.3	<0.001
Haemostatic markers			
von Willebrand factor, %	92.8 ± 24.7	63.1 ± 15.0	<0.001
Fibrinogen, g/L	3.53 ± 0.89	3.07 ± 0.55	<0.001
Plasminogen activator inhibitor-1, ng/ml	35.4 ± 9.7	21.7 ± 6.7	<0.001
Fibrin D-dimer, µg/L	294 (80 – 1180)	209 (68 – 617)	<0.001
Clot lysis time, min	84.6 ± 18.8	75.3 ± 10.7	<0.001
Biochemical measurements			
Total cholesterol, mmol/L	4.60 ± 1.19	5.54 ± 1.28	<0.001
Total cholesterol: high density cholesterol	4.48 ± 2.05	4.99 ± 1.62	<0.001
High density lipoprotein cholesterol, mmol/L	1.12 ± 0.35	1.20 ± 0.41	0.039
Low density lipoprotein cholesterol, mmol/L	2.83 ± 0.98	3.80 ± 1.09	<0.001
Triglycerides, mmol/L	1.43 ± 1.27	1.20 ± 0.76	0.022
Glycosylated haemoglobin A1c, %	5.87 (5.10 – 7.40)	5.47 (5.00 – 6.10)	<0.001
Glucose, mmol/L	5.26 (4.04 – 7.06)	5.61 (4.70 – 6.81)	<0.001
C-reactive protein, mg/L	4.29 (1.76 – 31.05)	2.03 (1.01 – 8.99)	<0.001
Cardiovascular measurements			
24-hour Systolic blood pressure, mmHg	132.6 ± 15.2	123.7 ± 11.2	<0.001
24-hour Diastolic blood pressure, mmHg	83.1 ± 10.1	76.4 ± 7.5	<0.001
24-hour Pulse pressure, mmHg	49.5 ± 8.6	47.2 ± 6.9	0.004
Daytime systolic blood pressure, mmHg	138.1 ± 15.0	129.1 ± 11.2	<0.001
Daytime diastolic blood pressure, mmHg	88.8 ± 10.2	81.5 ± 8.5	<0.001
Nighttime systolic blood pressure, mmHg	123.2 ± 15.9	113.0 ± 12.1	<0.001
Nighttime diastolic blood pressure, mmHg	74.0 ± 11.6	66.3 ± 7.7	<0.001
Lifestyle			
Physical activity, kcal/day	2564 (1614 – 4022)	2884 (1905 – 4365)	<0.001
Gamma-glutamyltransferase, U/L	43.6 (20.0 – 154.7)	17.4 (7.0 – 74.0)	<0.001
Current smoking, n (%)	34 (17.0)	29 (13.9)	0.39
HIV infected, n (%)	19 (9.5)	0 (0.0)	<0.001
Intake of medications			
Anti-hypertensive medication, n (%)	43 (21.5)	18 (8.6)	<0.001
Anti-coagulant medication:			
Warfarin / Heparin, n (%)	2 (1.0)	0 (0.0)	0.15
Aspirin, n (%)	4 (2.0)	12 (5.7)	0.058

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval), or number of subjects (%).
Normal reference ranges: [23]
von Willebrand factor, %: 75 – 125.
Fibrinogen, g/L: 1.5 – 4.0.
Plasminogen activator inhibitor-1, ng/ml: 4.0 – 43.0.
Fibrin D-dimer, µg/L: 0 – 500.

Table 2: Adjusted correlations between ambulatory blood pressure and haemostatic markers.

	von Willebrand factor, %	Fibrinogen, g/L	Plasminogen activator inhibitor-1, ng/ml	Fibrin D-dimer, µg/L	Clot lysis time, min
Black (n=191)					
24-hour SBP, mmHg	r=0.065 p=0.38	r=-0.118 p=0.87	r=-0.044 p=0.54	**r=0.157 p=0.034**	**r= 0.126 p=0.094**
24-hour DBP, mmHg	r=0.003 p=0.97	r=-0.022 p=0.77	r=-0.065 p=0.37	**r=0.154 p=0.039**	r= 0.090 p=0.23
24-hour PP, mmHg	r=0.069 p=0.35	r=-0.020 p=0.79	r=-0.009 p=0.91	r=0.085 p=0.25	r= 0.083 p=0.27
Daytime SBP, mmHg	r=0.038 p=0.61	r=0.019 p=0.80	r=-0.016 p=0.82	**r=0.160 p=0.031**	**r= 0.132 p=0.079**
Daytime DBP, mmHg	r=-0.015 p=0.84	r=-0.015 p=0.84	r=-0.065 p=0.37	**r=0.154 p=0.039**	r= 0.071 p=0.34
Nighttime SBP, mmHg	r=0.117 p=0.11	r=-0.042 p=0.57	r=-0.101 p=0.17	**r=0.172 p=0.021**	r= -0.008 p=0.92
Nighttime DBP, mmHg	r=0.032 p=0.66	r=-0.065 p=0.38	r=-0.081 p=0.26	r=-0.117 p=0.12	r= 0.024 p=0.75
White (n=207)					
24-hour SBP, mmHg	r=-0.021 p=0.76	r=-0.049 p=0.48	r=0.011 p=0.86	r=0.039 p=0.58	**r=-0.163 p=0.024**
24-hour DBP, mmHg	r=-0.073 p=0.30	r=-0.017 p=0.81	r=-0.030 p=0.67	r=0.075 p=0.30	r=-0.030 p=0.68
24-hour PP, mmHg	r=0.029 p=0.68	r=-0.100 p=0.15	r=0.052 p=0.46	r=-0.017 p=0.81	**r=-0.198 p=0.006**
Daytime SBP, mmHg	r=-0.084 p=0.24	r=-0.074 p=0.29	r=0.053 p=0.45	r=0.004 p=0.96	**r=-0.154 p=0.032**
Daytime DBP, mmHg	r=-0.104 p=0.14	r=-0.024 p=0.73	r=0.020 p=0.78	r=0.050 p=0.49	r=-0.042 p=0.57
Nighttime SBP, mmHg	r=0.081 p=0.25	r=-0.003 p=0.96	r=-0.025 p=0.72	r=0.086 p=0.23	**r=-0.147 p=0.041**
Nighttime DBP, mmHg	r=0.053 p=0.46	r=0.023 p=0.75	r=-0.092 p=0.19	r=0.092 p=0.20	r=-0.034 p=0.64

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure.
Adjusted for age, gender and body mass index.

2.5.1 Sensitivity analyses

Since PAI-1 levels may influence CLT [24], we repeated the multiple regression analyses for CLT by additionally adding $PAI\text{-}1_{ag}$ to the models. After doing so, the results remained unchanged. The multiple regression analyses were also repeated to determine if antihypertensive and anticoagulation usage influenced the associations. By doing so, the results remained largely unchanged except that associations with DBP disappeared (supplementary Table 1).

Table 3: Multiple regression analyses of blood pressure with haemostatic markers.

	Black (n=191)								
	Plasminogen activator inhibitor-1, ng/ml			Fibrin D-Dimer, µg/L			Clot lysis time, min		
Dependent variable	**Total R^2**	**β (95% CI)**	**P**	**Total R^2**	**β (95% CI)**	**p**	**Total R^2**	**β (95% CI)**	**p**
24-hour SBP, mmHg		NS		0.309	0.180 (0.110 to 0.250)	0.011		NS	
24-hour DBP, mmHg		NS		0.334	0.178 (0.110 to 0.246)	0.010		NS	
24-hour PP, mmHg		NS			NS			NS	
Daytime SBP, mmHg		NS		0.310	0.180 (0.110 to 0.250)	0.010		NS	
Daytime DBP, mmHg		NS		0.319	0.179 (0.110 to 0.248)	0.010		NS	
Nighttime SBP, mmHg		NS		0.252	0.193 (0.121 to 0.265)	0.008		NS	
Nighttime DBP, mmHg		NS		0.265	0.149 (0.077 to 0.221)	0.040		NS	
	White (n=207)								
24-hour SBP, mmHg		NS			NS		0.492	-0.333 (-0.409 to -0.257)	<0.001
24-hour DBP, mmHg		NS			NS		0.407	-0.196 (-0.278 to -0.114)	0.018
24-hour PP, mmHg		NS			NS		0.269	-0.329 (-0.420 to -0.238)	0.004
Daytime SBP, mmHg		NS			NS		0.472	-0.353 (-0.431 to -0.275)	<0.001
Daytime DBP, mmHg		NS			NS		0.374	-0.216 (-0.301 to -0.131)	0.011
Nighttime SBP, mmHg		NS			NS		0.398	-0.257 (-0.340 to -0.174)	<0.001
Nighttime DBP, mmHg	0.304	-0.152 (0.013 to 0.291)	0.032		NS		0.303	-0.184 (-0.273 to -0.095)	0.040

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure.
Adjusted for age, body mass index, gender, gamma-glutamyltransferase, physical activity, glycosylated haemoglobin A1c, total cholesterol: high density lipoprotein ratio, triglycerides and C-reactive protein. P-values ≤0.017 were deemed significant.

2.6 Discussion

We found that black South Africans displayed significantly elevated blood pressure, and higher fibrinogen, vWF, PAI-1 and D-dimer as well as a longer CLT than the white participants. The ambulatory blood pressures of the black participants were positively associated with D-dimer independent of known cardiovascular risk factors, suggesting that an activated haemostatic system may contribute to blood pressure elevation. In the white participants, the lower PAI-1 and faster CLT, coupled with the negative associations between ambulatory SBP and CLT suggest a higher fibrinolytic potential compared to the black participants.

The higher fibrinogen and vWF in the black compared to white participants confirm previous findings [25-27] and together with the increased D-dimer levels, suggest that the black participants may have an increased thrombotic risk. Elevated PAI-1 influence the fibrinolytic system by inhibiting the lysis of a thrombus which generally results in a longer CLT [28]. The raised PAI-1_{ag} in the black participants contradicts previous studies which reported that blacks tend to have lower PAI-1 compared to whites [27,29]. It is important to note that these studies reported PAI-1 activity (PAI-1_{act}) instead of PAI-1_{ag} levels. However, other investigators reporting both PAI-1_{ag} and PAI-1_{act} levels found strong correlations between the two assays in Europeans [30,31].

D-dimer is a degradation product of cross-linked fibrin and serves as an indicator of both coagulation- and fibrinolysis activation [32]. Previous investigators reported that elevated D-dimer is associated with an increased risk of cardiovascular events [33]. It has been suggested that even moderately elevated levels of D-dimer may reflect increases in thrombin formation and fibrin turnover [34] and that D-dimer may exert an effect on the endothelial cells that promote the development of oxidative stress [35]. Augmented oxidative stress may impair vascular tone by decreasing nitric oxide bioavailability, impairing vasodilation and increasing blood pressure [36,37]. Therefore, the differences in mean levels and the positive associations observed between ambulatory blood pressure and D-dimer suggests that a prothrombotic state exist that may contribute to blood pressure elevation in the black participants. This may significantly increase

the cardiovascular risk of the black participants. Indeed several investigators [38-40] found that a procoagulatory state was associated with increased risk for coronary and cerebrovascular events.

In the white participants we found negative associations between ambulatory blood pressure and CLT's and a shorter CLT's were observed in individuals with higher blood pressure. This finding is in contrast with previous findings that reported a strong positive association between elevated blood pressure and CLT [41]. CLT is an indicator of fibrinolytic potential that is amongst others, influenced by the release of t-PA and PAI-1 from the surrounding endothelial cells [42,43]. t-PA is responsible for the activation of plasminogen into plasmin that results in the lysis of fibrin into fibrin degradation products and is released from the endothelial cells at a steady rate [44]. However, changes in blood constituents and flow as well as endothelial dysfunction may result in an increased release of t-PA and PAI-1 from the endothelial cells [15,45,46]. During conditions such as uncontrolled hypertension, t-PA release from the intracellular storage pools may be depleted and subsequently PAI-1 secretion may overtake t-PA production, impairing the fibrinolytic pathway that results in a longer CLT as well as the formation of a fibrin clot that is more resilient to lysis [47-49]. However, in our white participants of whom 51% were normotensive, the release of t-PA from the endothelial cells in response to changes in blood rheology and or endothelial damage might still be effective and therefore, the fibrin clot is rapidly lysed which results in a shorter CLT [43]. However, this is highly speculative and needs to be investigated further with special focus on the concentration of the exogenously added t-PA used in the CLT assay which may influence the results. Additional investigation into clot structure properties and other markers of the haemostatic system of this population group is therefore needed.

The present study should be interpreted within the context of its limitations and strengths. Although we attempted to determine a range of haemostatic markers representing the coagulation and fibrinolysis pathways, various markers were not measured such as prothrombin fragment 1 and 2, thrombin-activator fibrinolysis inhibitor, t-PA activity and PAI-1_{act}. Although the results were consistent after multiple adjustments, residual confounding cannot be excluded. Causality cannot be inferred, due to the cross-sectional nature of this study. Our subject group was recruited from

urban areas in the Potchefstroom district of the North West Province and cannot be seen as representative of the entire South African population. This was a well-designed study under controlled conditions in two ethnic and socio-economic homogeneous groups.

We conclude that in black South Africans, a less favourable cardiovascular, coagulation and fibrinolytic profile was observed when compared to white South Africans. The associations between blood pressure and D-dimer suggest that the prothrombotic state of this group may contribute, at least in part, to the high prevalence of hypertension and related cardiovascular and cerebrovascular risk in the black population.

2.7 Acknowledgements

The Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study would not have been possible without the voluntary collaboration of the participants and the Department of Education, North West province, South Africa. The authors gratefully acknowledge the technical assistance of Mrs. Tina Scholtz, Sr. Chrissie Lessing and Dr. Szabolcs Péter. Research included in the present study was partially funded by the National Research Foundation, DAAD-NRF, North-West University, North-West Department of Education, ROCHE Diagnostics, South Africa; and the Metabolic Syndrome Institute, France.

Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the DAAD-NRF.

2.8 Disclosure

The authors declare no competing commercial interest.

2.9 References

[1] Huber K, Christ G, Wojta J, Gulba D. Plasminogen activator inhibitor type-1 in cardiovascular disease: Status report 2001. Thromb Res. 2001;103:S7-S19.

[2] Folsom AR, Wu KK, Rosamond WD, Sharrett AR, Chambless LE. Prospective study of hemostatic factors and incidence of coronary heart disease: The Atherosclerosis Risk in Communities (ARIC) study. Circulation. 1997;96:1102-8.

[3] Smith FB, Lee AJ, Fowkes FGR, Price JF, Rumley A, Lowe GDO. Hemostatic factors as predictors of ischemic heart disease and stroke in the Edinburgh artery study. Arterioscler Thromb Vasc Biol. 1997;17:3321-5.

[4] Tohgi H, Kawashima M, Tamura K, Suzuki H. Coagulation-fibrinolysis abnormalities in acute and chronic phases of cerebral thrombosis and embolism. Stroke. 1990;21:1663-7.

[5] Sliwa K, Wilkinson D, Hansen C, Ntyintyane L, Tibazarwa K, Becker A, *et al*. Spectrum of heart disease and risk factors in a black urban population in South Africa (the Heart of Soweto Study): a cohort study. Lancet. 2008;371:915-22.

[6] Connor MD, Thorogood M, Casserly B, Dobson C, Warlow CP. Prevalence of stroke survivors in rural South Africa: results from the Southern Africa Stroke Prevention Initiative (SASPI) Agincourt field site. Stroke. 2004;35:627-32.

[7] Ogah OS, Rayner BL. Recent advances in hypertension in sub-Saharan Africa. Heart. 2013;19:1390-7.

[8] Kintscher U. The burden of hypertension. EuroIntervention. 2013;9:R12-R5.

[9] World Bank. World Development indicators 2013. Washington, DC: World Bank.

[10] Blann AD, Naqvi T, Waite M, McCollum CN. von Willebrand factor and endothelial damage in essential hypertension. J Hum Hypertens. 1993;7:107-11.

[11] Arikan E, Sen S. Endothelial damage and hemostatic markers in patients with uncomplicated mild-to-moderate hypertension and relationship with risk factors. Clin Appl Thromb Hemost. 2005;11:147-59.

[12] Poli KA, Tofler GH, Larson MG, Evans JC, Sutherland PA, Lipinska I, *et al*. Association of blood pressure with fibrinolytic potential in the Framingham offspring population. Circulation. 2000;101:264-9.

[13] Cigolini M, Targher G, Seidell JC, Tonoli M, Schiavon R, Agostino G, *et al*. Relationships of blood pressure to fibrinolysis: influence of anthropometry, metabolic profile and behavioural variables. J Hypertens. 1995;13:659-66.

[14] Fowkes FG, Lowe GD, Rumley A, Lennie SE, Smith FB, Donnan PT. The relationship between blood viscosity and blood pressure in a random sample of the population aged 55 to 74 years. Eur Heart J. 1993;14:597-601.

[15] Lip GY, Li-Saw-Hee FL. Does hypertension confer a hypercoagulable state? J Hypertens. 1998;16:913-6.

[16] Lominadze D, Dean WL, Tyagi SC, Roberts AM. Mechanisms of fibrinogen-induced microvascular dysfunction during cardiovascular disease. Acta Physiol. 2010;198:1-13.

[17] Ernst E, Resch KL. Fibrinogen as a cardiovascular risk factor a meta-analysis and review of the literature. Ann Intern Med. 1993;118:956-63.

[18] Conlan MG, Folsom AR, Finch A, Davis CE, Sorlie P, Marcucci G, *et al*. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. Thromb Haemost. 1993;70:380-5.

[19] Khaleghi M, Saleem U, McBane RD, Mosley Jr TH, Kullo IJ. African-American ethnicity is associated with higher plasma levels of D-dimer in adults with hypertension. J Thromb Haemost. 2009;7:34-40.

[20] Marfell-Jones M, Olds T, Stewart A, Carter L. International standards for anthropometric assessment: International Society for the Advancement of Kinanthropometry; 2006.

[21] Norton KI, Olds T, Commission AS. Anthropometrica: A textbook of body measurement for sports and health courses: University of New South Wales Press; 1996.

[22] Lisman T, de Groot PG, Meijers JC, Rosendaal FR. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. Blood. 2005;105:1102-5.

[23] Kratz A, Ferraro M, Sluss PM, Lewandrowski KB. Normal reference laboratory values. New Engl J Med. 2004;351:1548-63.

[24] Mutch NJ, Thomas L, Moore NR, Lisiak KM, Booth NA. TAFIa, PAI-1 and α2-antiplasmin: complementary roles in regulating lysis of thrombi and plasma clots. J Thromb Haemost. 2007;5:812-7.

[25] Miller CH, Haff E, Platt SJ, Rawlins P, Drews CD, Dilley AB, *et al*. Measurement of von Willebrand factor activity: relative effects of ABO blood type and race. J Thromb Haemost. 2003;1:2191-7.

[26] Folsom AR, Wu KK, Conlan MG, Finch A, Davis CE, Marcucci G, *et al*. Distributions of hemostatic variables in blacks and whites: population reference values from the Atherosclerosis Risk in Communities (ARIC) study. Ethn Dis. 1992;2:35-46.

[27] Lutsey PL, Cushman M, Steffen LM, Green D, Barr RG, Herrington D, *et al*. Plasma hemostatic factors and endothelial markers in four racial/ethnic groups: the MESA study. J Thromb Haemost. 2006;4:2629-35.

[28] Meltzer ME, Lisman T, de Groot PG, Meijers JCM, le Cessie S, Doggen CJM, *et al*. Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1. Blood. 2010;116:113-21.

[29] Greyling A, Pieters M, Hoekstra T, Oosthuizen W, Schutte AE. Differences in the association of PAI-1 activity with the metabolic syndrome between African and Caucasian women. Nutr Metab Cardiovasc Dis. 2007;17:499-507.

[30] Henry M, Tregouët DA, Alessi MC, Aillaud MF, Visvikis S, Siest G, *et al*. Metabolic determinants are much more important than genetic polymorphisms in determining the PAI-1 activity and antigen plasma concentrations: A family study with part of the Stanislas cohort. Arterioscl Thromb Vasc Biol. 1998;18:84-91.

[31] Alessi MC, Juhan-Vague I, Declerck PJ, Anfosso F, Gueunoun E, Collen D. Correlations between t-PA and PAI-1 antigen and activity and t-PA/PAI-1 complexes in plasma of control subjects and of patients with increased t-PA or PAI-1 levels. Thromb Res. 1990;60:509-16.

[32] Lip GY, Lowe GD. Fibrin D-dimer: a useful clinical marker of thrombogenesis? Clin Sci. 1995;89:205-14.

[33] Giansante C, Fiotti N, Cattin L, Da Col PG, Calabrese S. Fibrinogen, D-dimer and thrombin-antithrombin complexes in a random population sample: relationships with other cardiovascular risk factors. Thromb Haemost. 1994;71:581-6.

[34] Lowe GD, Rumley A. Use of fibrinogen and fibrin D-dimer in prediction of arterial thrombotic events. Thromb Haemost. 1999;82:667-72.

[35] Yevdokimova NY, Veselovska LD, Gogolinska GK, Buchanevich OM, Kosyakova GV, Gritsenko PG, *et al*. Fibrin D-dimer impairs the accumulation and anticoagulant properties of heparan sulphate and stimulates secretion of plasminogen activator inhibitor-1 by rabbit coronary endothelial cells. Acta Biochim Pol. 2003;50:279-89.

[36] Puddu P, Puddu GM, Cravero E, Rosati M, Muscari A. The molecular sources of reactive oxygen species in hypertension. Blood press. 2008;17:70-7.

[37] Touyz RM. Reactive oxygen species in vascular biology: role in arterial hypertension. Expert Rev Cardiovasc Ther. 2003;1:91-106.

[38] Smith A, Patterson C, Yarnell J, Rumley A, Ben-Shlomo Y, Lowe G. Which hemostatic markers add to the predictive value of conventional risk factors for coronary heart disease and ischemic stroke? The Caerphilly Study. Circulation. 2005;112:3080-7.

[39] Fibrinogen Collaboration Studies. Collaborative meta-analysis of prospective studies of plasma fibrinogen and cardiovascular disease. Eur J Cardiovasc Prev Rehabil. 2004;11:9-17.

[40] Wannamethee SG, Whincup PH, Lennon L, Rumley A, Lowe GD. Fibrin D-dimer, tissue-type plasminogen activator, von Willebrand factor, and risk of incident stroke in older men. Stroke. 2012;43:1206-11.

[41] Meltzer ME, Doggen CJM, De Groot PG, Rosendaal FR, Lisman T. Reduced plasma fibrinolytic capacity as a potential risk factor for a first myocardial infarction in young men. Brit J Haematol. 2009;145:121-7.

[42] Pearson JD. The control of production and release of haemostatic factors in the endothelial cell. Baillieres Clin Haematol. 1993;6:629-51.

[43] Emeis JJ. Regulation of the acute release of tissue-type plasminogen activator from the endothelium by coagulation activation products. Ann NY Acad Sci. 1992;667:249-58.

[44] Norris LA. Blood coagulation. Best Pract Res Clin Obstet Gynaecol. 2003;17:369-83.

[45] Sjogren LS, Doroudi R, Gan L, Jungersten L, Hrafnkelsdottir T, Jern S. Elevated intraluminal pressure inhibits vascular tissue plasminogen activator secretion and downregulates its gene expression. Hypertension. 2000;35:1002-8.

[46] Hrafnkelsdottir T, Wall U, Jern C, Jern S. Impaired capacity for endogenous fibrinolysis in essential hypertension. Lancet. 1998;352:1597-8.

[47] Collet JP, Lesty C, Montalescot G, Weisel JW. Dynamic changes of fibrin architecture during fibrin formation and intrinsic fibrinolysis of fibrin-rich clots. J Biol Chem. 2003;278:21331-5.

[48] Collet JP, Park D, Lesty C, Soria J, Soria C, Montalescot G, *et al*. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: dynamic and structural approaches by confocal microscopy. Arterioscler Thromb Vasc Biol. 2000;20:1354-61.

[49] Ridderstrale W, Ulfhammer E, Jern S, Hrafnkelsdottir T. Impaired capacity for stimulated fibrinolysis in primary hypertension is restored by antihypertensive therapy. Hypertension. 2006;47:686-91.

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Data supplement

D-dimer relates positively with increased blood pressure in black South Africans: The SABPA study

Leandi Lammertyn[1], Aletta E Schutte[1], Marlien Pieters[2] and Rudolph Schutte[1]

[1]Hypertension in Africa Research Team (HART); North-West University (Potchefstroom Campus); Potchefstroom; South Africa.

[2]Centre of Excellence for Nutrition (CEN); North-West University (Potchefstroom Campus); Potchefstroom; South Africa.

Table 1; Figure 1

Correspondence:

Rudolph Schutte, PhD

Hypertension in Africa Research Team (HART),

Private Bag x6001,

North-West University (Potchefstroom Campus),

Potchefstroom,

2520

South Africa

Telephone: +27-18-299-2435

Facsimile: +27-18-299-1053

E-mail: rudolph.schutte@nwu.ac.za

Conflict of Interest: All authors declare no conflict of interest.

Table S1: Multiple regression analyses of blood pressure with haemostatic markers.

	Black (n=153)								
	Plasminogen activator inhibitor-1, ng/ml			Fibrin D-Dimer, µg/L			Clot lysis time, min		
Dependent variable	**Total R^2**	**β (95% CI)**	**p**	**Total R^2**	**β (95% CI)**	**p**	**Total R^2**	**β (95% CI)**	**p**
24-hour SBP, mmHg		NS		0.305	0.217 (0.080 to 0.354)	0.002		NS	
24-hour DBP, mmHg		NS		0.326	0.208 (0.071 to 0.345)	0.003		NS	
24-hour PP, mmHg		NS			NS			NS	
Daytime SBP, mmHg		NS		0.308	0.213 (0.076 to 0.350)	0.003		NS	
Daytime DBP, mmHg		NS		0.307	0.204 (0.067 to 0.341)	0.004		NS	
Nighttime SBP, mmHg		NS		0.247	0.188 (0.043 to 0.333)	0.011		NS	
Nighttime DBP, mmHg		NS		0.248	0.181 (0.038 to 0.254)	0.015		NS	
	White (n=190)								
24-hour SBP, mmHg		NS			NS		0.496	-0.182 (-0.339 to -0.025)	0.024
24-hour DBP, mmHg		NS			NS			NS	
24-hour PP, mmHg		NS			NS		0.264	-0.223 (-0.413 to -0.033)	0.023
Daytime SBP, mmHg		NS			NS		0.464	-0.225 (-0.388 to -0.062)	0.007
Daytime DBP, mmHg		NS			NS		0.404	-0.154 (-0.241 to 0.017)	0.078
Nighttime SBP, mmHg		NS			NS		0.445	-0.341 (-0.506 to -0.176)	<0.001
Nighttime DBP, mmHg		NS			NS			NS	

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure.
Adjusted for age, body mass index, gender, gamma-glutamyltransferase, physical activity, glycosylated haemoglobin A1c, total cholesterol: high density lipoprotein ratio, triglycerides, C-reactive protein, antihypertension and anticoagulation medication. P-values of ≤0.017 were deemed significant.

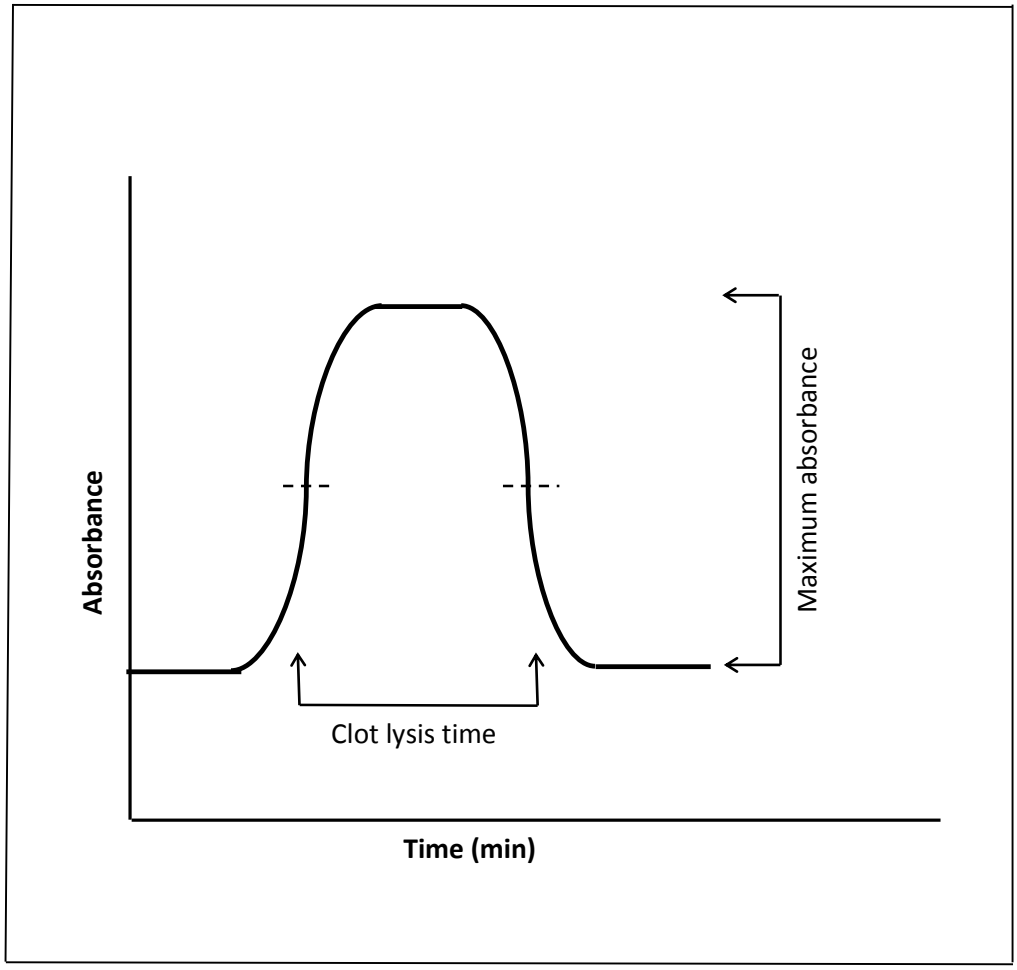

Figure S1: Variables calculated from Turbidity Curves. The clot lysis time measurements were obtained from turbidity curves of fibrin polymerisation triggered by the addition of tissue factor to plasma for clot formation and t-PA for subsequent lysis. Clot lysis time was calculated as the time from the midpoint (----) in the transition from the initial baseline to maximum absorbance to the midpoint (----) in the transition from maximum absorbance to the final baseline absorbance.

CHAPTER 3

Ethnic-specific relationships between haemostatic and oxidative stress markers in black and white South Africans: The SABPA study

Published in *Clinical and Experimental Hypertension*

AUTHOR INSTRUCTIONS FOR JOURNAL OF CLINICAL AND EXPERIMENTAL HYPERTENSION

(http://informahealthcare.com/page/ceh/Description#Instructions)

Clinical and Experimental Hypertension is a refereed journal in the English language for the rapid communication of new and significant data pertaining to human and animal hypertension of all types.

Essential title page information

Title. The title should be concise and informative.

Running head: A short brief title should be included of no more than 50 characters.

Authors. State all authors' names, middle initial(s) and last name.

Corresponding author. Clearly indicate the corresponding author name phone and fax numbers as well as their e-mail adress.

Conflict of interest. Authors must state all potential conflicts of interest.

Word count. A word count should be provided on the title page. The word count should include all text including the references but not tables and legends. The number of tables and supplementary files should also be indicated.

Abstract

A concise and factual abstract of no more than 250 words is required. The abstract should state the objectives of the study, basic methods, main results and principal conclusion.

Keywords and Abbreviations

The abstract should be followed by a list of three to ten keywords. Abbreviations should be divined on the first appearance in the text. Avoid the use of abbreviations in the title and abstract.

Acknowledgements

Acknowledgement should be made to those who have made a substantial contribution to the study. All financial sources should be acknowledged, including the names of private and public sector sponsors.

References

Text: Indicate references with Arabic numerals in parenthesis e.g. (12). Multiple references within one set of parentheses should be set off by comma, but no space.

Reference List: Should be numbered consecutively in the order they appear in the text.

Reference style for journal article should be as follow:

Flack JM, Gardin JM, Yunis C, Liu K. Static and pulsatile blood pressure correlates of left ventricular structure and function in black and white young adults: the CARDIA study. Am Heart J 1999; 138:856--864.

Tables

Number tables consecutively in accordance with their appearance in the text. Each table should be submitted on their own page and contain a brief title. Place explanatory matter in a footnote below the table body, all units should be included.

Supplementary data

Electronic supplementary material that supports and enhances your scientific research is accepted. Supplementary files supplied are published online alongside the electronic version of your article.

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Ethnic-specific relationships between haemostatic and oxidative stress markers in black and white South Africans: The SABPA study

Running head: Ethnicity, haemostasis and oxidative stress

Leandi Lammertyn[1], Catharina MC Mels[1], M Pieters[3], Aletta E Schutte[1,2] and Rudolph Schutte[1,2]

[1]Hypertension in Africa Research Team (HART); North-West University; Potchefstroom; South Africa; [2]MRC Research Unit for Hypertension and Cardiovascular Disease, North-West University, Potchefstroom, South Africa. [3]Centre of Excellence for Nutrition (CEN); North-West University; Potchefstroom; South Africa

Correspondence:

Rudolph Schutte, PhD

Hypertension in Africa Research Team (HART),

Private Bag x6001,

North-West University

Potchefstroom,

2520

South Africa

Telephone: +27-18-299-2435

Facsimile: +27-18-299-1053

E-mail: rudolph.schutte@nwu.ac.za

3.1 Abstract

Haemostatic- and oxidative stress markers are associated with increased cardiovascular risk. In the black population, evidence exists that both an imbalance in the haemostatic system and oxidative stress link with the development of hypertension. However, it is unclear whether these two risk components function independently or are related, specifically in the black population, who is known to have a high prevalence of stroke. We aimed to investigate associations between the haemostatic system and oxidative stress in black and white South Africans. We performed a cross-sectional study including 181 black (mean age, 44; 51.4% women) and 209 white (mean age, 45; 51.7% women) teachers. Several markers of the haemostatic- (von Willebrand factor, fibrinogen, plasminogen activator inhibitor-1, D-dimer and clot lysis time) and oxidant-antioxidant (serum peroxides, total glutathione, glutathione peroxidase- and glutathione reductase activities) systems were measured. Along with a worsened cardiovascular profile, the black group had higher haemostatic-, inflammation- and oxidative stress markers as well as decreased glutathione peroxidase activity. In multiple regression analyses, fibrinogen was positively associated with serum peroxides ($p<0.001$) in both ethnic groups. In the black population, we found negative associations of von Willebrand factor and clot lysis time with glutathione peroxidase activity ($p\leq0.008$), while a positive association existed between clot lysis time and serum peroxides ($p=0.011$) in the white population. We conclude that in the black population, decreased GPx activity accompanies an altered haemostatic profile, while in the white population associations may suggest that serum peroxides impair fibrin clot lysis.

Keywords: antioxidant capacity, hydrogen peroxide, glutathione peroxidase, fibrinogen, cardiovascular.

3.2 Introduction

Cardiovascular disease is increasing in South Africa, especially in the black population who has a high prevalence of hypertension and stroke (1-3). Cardiovascular disease development is associated with a thrombotic component initiated at least in part by underlying endothelial dysfunction (4). An imbalance between the oxidative/anti-oxidant system in favour of oxidative stress as reflected by elevated reactive oxygen species (ROS) (5-7) and/or a decreased antioxidant capacity (8,9) can influence the haemostatic balance either directly or indirectly towards a procoagulant state. Glutathione peroxidase (GPx) utilises glutathione (GSH) to metabolise hydrogen peroxides to water and in doing so, results in the formation of glutathione disulphide (GSSG). GSSG in turn is reduced back to GSH by glutathione reductase (GR) (10). GPx and GR are therefore crucial enzymes in the regulation of the glutathione/glutathione disulphide system and are important in the protection of the vascular wall (11,12). Both animal and human studies have shown that a compromised antioxidant capacity, as reflected by decreased GPx activity, predisposes an increased cardiovascular and thrombotic risk (8,13,14).

Ethnic differences in the functioning of the haemostatic system and oxidative stress seems to exist in blacks with higher thrombotic- (von Willebrand factor (vWF) (15), fibrinogen (16,17) and D-dimer (18), but lower plasminogen activator inhibitor-1 (PAI-1) (19)) and oxidative stress markers as compared to whites (20). This has been associated with the development and progression of cardiovascular disease (7,21-23). Evidence in the black population is limited, however associations of ROS (24) and D-dimer (25) with blood pressure have been reported. It is still unknown whether associations exist between components of the haemostatic system and oxidative stress in this population group and if these associations are comparable to what has been observed in whites (8,13,26).

We therefore investigated whether associations exist between components of the haemostatic system and oxidative stress, and if these associations differ between black and white populations in South Africa.

3.3 Materials and methods

3.3.1 Study population

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study was a cross-sectional study that was performed during 2008 and 2009, involving school teachers (n=409) between the ages of 25 – 60 years from the North West Province, South Africa. Participants consisted of 200 black (101 men and 99 women) and 209 white (101 men and 108 women) participants. Exclusion criteria were an elevated ear temperature, dependence or abuse of psychotropic substances, regular blood donors, or individuals vaccinated in the previous three months. For this sub-study, 19 participants were excluded due to human immunodeficiency virus infection. Written informed consent was obtained prior to the commencement of measurements. The study complied with all applicable international regulations and the Helsinki declaration for investigation of human participants. The Ethics Review Board of the North-West University (Potchefstroom Campus) approved the study. Each participant completed a lifestyle questionnaire, including questions about their cardiovascular health history, smoking and alcohol habits as well as chronic medication use.

3.3.2 Clinical measurements

A 24-hour ambulatory blood pressure (ABPM) and electrocardiogram apparatus (Meditech CE120® Cardiotens, Budapest, Hungary) was attached to the participants at their workplace. Each participant's energy expenditure during the day was calculated with a validated accelerometer device (Actical® accelerometers, Montréal, Québec). Participants reported to the Metabolic Research Unit of the North-West University at 16:30 where they were informed of the procedures of the following day. They received a standardised meal at 18:00, and final snacks and drinks at 20:30, and were requested to go to bed at around 22:00. At 06:00, the ABPM apparatus was removed, followed by anthropometric measurements and blood sampling. The 24-hour blood pressure and electrocardiogram data were downloaded onto a database using the CardioVisions 1.9.0 Personal Edition software (Meditech, Budapest, Hungary).

3.3.3 Anthropometric measurements

All measurements were taken in triplicate with calibrated instruments. Stature was measured to the nearest 0.1 cm with a stadiometer (Invicta Stadiometer, IP 1465, London, UK), body mass to the nearest 0.1 kg (Precision Health Scale, A & D Company,Tokyo, Japan) and waist circumference to the nearest 0.1 cm with a unstretchable flexible 7 mm wide metal tape (Holtain, Crosswell, Wales) (27,28). Body mass index (BMI) was calculated for each participant using the standard formula of weight/(height)^2.

3.3.4 Blood sampling

A registered nurse collected fasting blood samples with a sterile winged infusion set from the participants' antebrachial vein branches before 10:00 in the morning. The blood samples were centrifuged at 2000 x g for 15 minutes to obtain serum, sodium fluoride and citrate plasma samples. All samples were stored at -80˚C until analysis.

3.3.5 Biochemical analyses

High sensitivity C-reactive protein (hs-CRP), total cholesterol (TC), high density lipoprotein (HDL-C) cholesterol, low density lipoprotein (LDL-C), gamma-glutamyltransferase (γ-GT), glycosylated haemoglobin A1c (HbA1c) and fasting glucose were determined with methods described elsewhere [25]. A Quantikine high sensitivity enzyme-linked immunosorbent assay from R&D Systems (R&D Systems, Minneapolis, MN, USA) was used to determine Interleukin-6 (IL-6) from serum.

Citrated plasma samples were used for the analysis of von Willebrand factor antigen (vWF_{ag}), fibrinogen, plasminogen activator inhibitor-1 antigen ($PAI\text{-}1_{ag}$), fibrin D-dimer and clot lysis time (CLT). vWF_{ag} levels were determined with a sandwich enzyme-linked immunosorbent assay. Polyclonal rabbit anti-vWF antibody and rabbit anti-vWF-horseradish peroxidase antibody (DAKO, Glostrup, Denmark) were used to perform the assay. The 6th International Standard for vWF/FVIII was used to create the standard curve against which the samples were measured. Plasma fibrinogen levels were determined by means of a modified Clauss method using the FIB

kit from STAGO diagnostics (STAGO diagnostics, Asnières, France). The Liatest D-dimer kit from STAGO diagnostics (STAGO diagnostics, Asnières, France) was used to determine fibrin D-dimer levels with an immuno-turbimetric method with a 540 nm detection limit. PAI-1_{ag} levels were determined with the TriniLIZE PAI-1_{ag} (Trinity Biotech, Bray, Ireland) kit using an enzyme-linked immunosorbent assay. CLT was determined by studying the lysis of a tissue factor-induced clot by exogenous tissue-plasminogen activator. Changes in turbidity during clot formation and lysis were monitored as described by Lisman *et al*. (29). Tissue factor and tissue plasminogen activator concentrations were slightly modified to obtain comparable CLTs of about 60 minutes. The modified concentrations were 17 mmol/L calcium chloride, 60 ng/ml tissue plasminogen activator (Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and 10 µmol/L phospholipids vesicles (Rossix, Mölndal, Sweden). Tissue factor was diluted 3000 times (Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany). CLT was defined as the time from the midpoint in the transition from the initial baseline to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot.

The method described by Hayashi *et al*. (30) was used to determine one of the measurable ROS species, namely total peroxides that includes hydrogen peroxides and lipid peroxides, in serum samples. In this assay 1.0 mg/L hydrogen peroxide represents one unit of ROS. Total glutathione (GSH) were determined on ethylenediaminetetraacetic whole blood sample with the BIOXYTECH® GSH/GSSG-412^{TM} kit supplied by *Oxis*ResearchTM, a division of OXIS Health Products Inc., (Foster City, CA). Glutathione peroxidase (GPx) and glutathione reductase (GR) were measured with kits from Cayman Chemical Company (Cayman Chemical Company, Ann Arbor, MI, USA) on ethylenediaminetetraacetic plasma samples. ROS, GSH, GPx and GR were measured with the Bio-Tek Synergy HT microplate reader (Bio-Tek, Instruments., Inc., Highland Park, Winooski, USA).

3.3.6 Statistical analyses

Statistica software v12.0 was used for database management and statistical analyses (Statsoft, Inc., Tulsa, OK, 2012). Variables not normally distributed (fibrin D-dimer, hs-CRP, HbA1c, glucose, γ-GT, and energy expenditure) were logarithmically transformed. The central tendency and spread of these variables were represented by the geometric mean and the 5th and 95th percentile intervals. We tested for interaction with ethnicity by investigating associations between haemostatic markers and markers of oxidative stress with multiple regression analyses (supplementary Table S1). Results found served as motivation for ethnic split. Means and proportions were compared by an independent t-test and chi-square test, respectively. We used partial correlations to determine associations between oxidative and haemostatic markers, followed by forward stepwise multiple linear regression analyses. Covariates included in the models were age, body mass index, gender, γ-GT, current smoking, physical activity, HbA1c, TC:HDL-C, 24-hour systolic blood pressure and contraception usage. Sensitivity analyses were performed to test for the influence of inflammation by adding hs-CRP and IL-6 separately into the regression models. All p-values refer to a two-sided hypothesis.

3.4 Results

The general characteristics of the study population are listed in Table 1. Although the groups were of similar age, the black participants were generally more obese ($p<0.001$), had higher mean systolic and diastolic ($p<0.001$) blood pressures as well as an increased inflammatory state ($p<0.001$) compared to their white counterparts. The black participants also had a more detrimental haemostatic profile with higher vWF_{ag}, fibrinogen, $PAI\text{-}1_{ag}$, fibrin D-dimer levels and a longer CLT ($p<0.001$), as well as a mixed oxidant-antioxidant profile with higher serum peroxides ($p=0.012$) and lower GPx activity ($p<0.001$), while their total GSH levels ($p<0.001$) and GR activity ($p<0.001$) were higher.

Table 1: Characteristics of the study population

	Black (n=181)	White (n=209)	P
Women, n (%)	93 (51.4)	108 (51.7)	0.95
Age, years	44.4 ± 8.3	45.0 ± 10.9	0.52
Body mass index, kg/m^2	30.3 ± 7.1	27.6 ± 5.9	<0.001
Waist circumference, cm (men)	93.6 ± 15.8	101.5 ± 14.4	<0.001
Waist circumference, cm (women)	93.9 ± 15.8	85.0 ± 13.3	<0.001
Haemostatic markers			
von Willebrand factor, %	92.2 ± 24.7	63.1 ± 15.0	<0.001
Fibrinogen, g/L	3.58 ± 0.89	3.07 ± 0.55	<0.001
Plasminogen activator inhibitor-1, ng/ml	35.2 ± 9.6	21.6 ± 6.5	<0.001
Fibrin D-dimer, µg/L	295 (80 – 1175)	208 (68 – 610)	<0.001
Clot lysis time, min	84.2 ± 18.0	75.3 ± 10.7	<0.001
Oxidative stress markers			
Reactive oxygen species, mg/L	93.8 ± 24.6	87.8 ± 21.9	0.012
Glutathione, µM	904.0 ± 183.4	823.9 ± 134.0	<0.001
Glutathione peroxidase, nmol/min/ml	32.2 ± 12.3	36.0 ± 7.62	<0.001
Glutathione reductase, nmol/min/ml	7.60 ± 3.46	3.59 ± 2.08	<0.001
Biochemical measurements			
C-reactive protein, mg/L	4.48 (0.65 – 31.70)	2.03 (0.99 – 8.99)	<0.001
Interleukin-6, pg/ml	1.25 ± 0.69	0.98 ± 0.56	<0.001
Total cholesterol, mmol/L	4.65 ± 1.20	5.54 ± 1.28	<0.001
Total cholesterol: high density cholesterol	4.44 ± 2.10	4.99 ± 1.62	0.003
High density lipoprotein cholesterol, mmol/L	1.15 ± 0.35	1.20 ± 0.41	0.15
Low density lipoprotein cholesterol, mmol/L	2.87 ± 1.00	3.80 ± 1.09	<0.001
Triglycerides, mmol/L	1.41 ± 1.31	1.20 ± 0.76	0.042
Glycosylated hemoglobin A1c, %	5.92 (5.11 – 7.40)	5.47 (5.00 – 6.10)	<0.001
Glucose, mmol/L	5.27 (3.93 – 7.18)	5.61 (4.70 – 6.81)	<0.001
Gamma-glutamyltransferase, U/L	45.3 (20.0 – 154.5)	19.0 (7.0 – 74.0)	<0.001
Cardiovascular measurements			
24-hour Systolic blood pressure, mmHg	132.4 ± 15.4	123.5 ± 10.7	<0.001
24-hour Diastolic blood pressure, mmHg	82.8 ± 10.3	76.4 ± 7.5	<0.001
24-hour Pulse pressure, mmHg	49.6 ± 8.7	47.3 ± 7.0	0.003
Lifestyle			
Physical activity, kcal/day	2564 (1702 – 4018)	2910 (1914 – 4405)	<0.001
Current smoking, n (%)	29 (16.0)	29 (13.9)	0.57
Intake of medications			
Anti-hypertensive medication, n (%)	40 (22.1)	18 (8.6)	<0.001
Anti-coagulant medication, n (%)	2 (1.1)	0 (0)	0.13
Contraception, n (%)	17 (9.4)	7 (3.4)	0.013

Values are arithmetic mean ± SD, geometric mean (5^{th} to 95^{th} percentile interval), or number of subjects (%).
Normal reference ranges: (46)
von Willebrand factor, %: 75 – 125.
Fibrinogen, g/L: 1.5 – 4.0.
Plasminogen activator inhibitor-1, ng/ml: 4.0 – 43.0.
Fibrin D-dimer, µg/L: 0 – 500.

3.4.1 Associations of the haemostatic markers with peroxides and GPx activity.

In partial regression analyses (adjusted for age, gender and BMI; Table 2), a positive association was found between fibrinogen and serum peroxides (p<0.001) in both ethnic groups. In the black population, higher vWF_{ag} and longer CLT were associated with lower GPx activity (p<0.001 and p=0.030). No associations were found between the haemostatic components and GPx activity in the white population.

Table 2: Partial regression analyses between the haemostatic markers and markers of oxidative stress

	vWF, %	Fibrinogen, g/L	PAI-1, ng/ml	D-dimer, µg/L	CLT, min
			Black (n=175)		
ROS, mg/L	r=0.14; p=0.078	**r=0.27; p<0.001**	r=-0.06; p=0.45	r=0.11; p=0.16	r=0.04; p=0.61
GSH, µM	**r=-0.22; p=0.007**	r=-0.06; p=0.46	**r=-0.22; p=0.006**	r=-0.01; p=0.87	**r=-0.20; p=0.012**
GPx, nmol/min/ml	**r=-0.26; p<0.001**	r=0.02; p=0.86	r=-0.08; p=0.30	r=-0.04; p=0.60	**r=-0.18; p=0.030**
GR, nmol/min/ml	**r=0.17; p=0.030**	r=0.05; p=0.57	**r=0.19; p=0.014**	r=-0.04; p=0.65	**r=0.23; p=0.004**
			White (n=206)		
ROS, mg/L	r=0.08; p=0.27	**r=0.33; p<0.001**	r=0.02; p=0.77	r=0.08; p=0.30	r=0.12; p=0.097
GSH, µM	r=-0.04; p=0.63	r=0.04; p=0.54	r=0.00; p=0.98	r=-0.09; p=0.20	r=-0.08; p=0.28
GPx, nmol/min/ml	r=0.07; p=0.30	r=0.03; p=0.64	r=-0.02; p=0.81	r=0.04; p=0.54	r=0.02; p=0.84
GR, nmol/min/ml	**r=0.14; p=0.050**	r=0.03; p=0.65	r=0.07; p=0.35	**r=0.16; p=0.025**	r=0.07; p=0.34

Abbreviations: vWF, von Willebrand factor; PAI-1, Plasminogen activator inhibitor-1; CLT, Clot lysis time; hs-CRP, C-reactive protein; IL-6, Interleuken-6; ROS, Reactive oxygen species; GSH, Glutathione; GPx, Glutathione peroxidase; GR, Glutathione reductase.
Adjusted for age, gender and body mass index. Bold values indicate statistical significance p<0.05

After full adjustment (age, gender, body mass index, current smoking, γ-GT, physical activity, HbA1c, TC:HDL, 24-hour SBP and contraception usage; Table 3) in multiple regression analyses, the association between fibrinogen and serum peroxides (p<0.001) remained in both ethnic groups, as well as the negative associations of vWF_{ag} and CLT with GPx activity (p≤0.008) in the black population. We found additional associations between vWF_{ag} and serum peroxides (p=0.021) in the black population and CLT with serum peroxides (p=0.011) in the white population.

3.4.2 Associations of haemostatic markers with the glutathione/glutathione disulphide system.

In partial regression analyses (Table 2), vWF_{ag}, $PAI\text{-}1_{ag}$ and CLT were negatively associated with total GSH (p≤0.012) and positively associated with GR activity (p≤0.030) in the black population.

In the white population a positive association was found between vWF_{ag} and fibrin D-dimer, and GR activity ($p \leq 0.050$).

After full adjustments (Table 3), the negative associations of vWF_{ag} and PAI-1 with total GSH ($p \leq 0.002$) and the positive associations of vWF_{ag}, PAI-1_{ag} and CLT with GR activity ($p \leq 0.030$) remained in the black population, while only the association between vWF_{ag} and GR activity ($p=0.006$) remained in the white population.

3.4.3 Sensitivity analyses

Since oxidative stress is usually accompanied by a pro-inflammatory state, which may also influence haemostatic functioning, we additionally added hs-CRP (Table S2) and IL-6 (Table S3), separately to the models [31-33]. After adjusting for hs-CRP, both vWF_{ag} and fibrinogen were no longer significantly associated with serum peroxides, or vWF_{ag} with GR activity in the black population. After adjusting for IL-6, only vWF_{ag} was no longer associated with serum peroxides in the black population and GR activity in the white population. The positive associations of fibrinogen and CLT with ROS remained in the white population after adjusting for CRP and IL-6, respectively.

Table 3: Multiple regression analyses of haemostatic markers with oxidative stress markers

	Black (n=175)											
	von Willebrand factor, %			**Fibrinogen, g/L**			**Plasminogen activator inhibitor-1, ng/ml**			**Clot lysis time, min**		
Independent variables	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p
ROS, mg/L	0.12 0.08	0.197 (0.032 to 0.362)	0.021	0.36 0.34	0.277 (0.128 to 0.426)	<0.001		NS			NS	
GSH, µM	0.13 0.08	-0.240 (-0.389 to -0.091)	0.002		NS		0.12 0.08	-0.246 (-0.397 to -0.095)	<0.001	0.38 0.36	-0.162 (-0.287 to 0.037)	0.057
GPx, nmol/min/ml	0.16 0.13	-0.260 (-0.405 to -0.115)	<0.001		NS			NS		0.39 0.37	-0.177 (-0.302 to -0.052)	0.008
GR, nmol/min/ml	0.12 0.09	0.168 (0.017 to 0.319)	0.030		NS		0.12 0.08	0.215 (0.064 to 0.366)	0.006	0.39 0.37	0.176 (0.047 to 0.305)	0.014
	White (n=206)											
ROS, mg/L		NS		0.36 0.34	0.419 (0.301 to 0.537)	<0.001		NS		0.56 0.54	0.140 (0.034 to 0.246)	0.011
GSH, µM		NS			NS			NS			NS	
GPx, nmol/min/ml		NS			NS			NS			NS	
GR, nmol/min/ml	0.08 0.06	0.138 (0.003 to 0.273)	0.006		NS			NS			NS	

Abbreviations: ROS, reactive oxygen species; GSH, glutathione; GPx, glutathione peroxidase, GR, glutathione reductase. Adjusted for age, body mass index, gender, gamma-glutamyltransferase, current smoking, physical activity, glycosylated haemoglobin A1c, total cholesterol:high density lipoprotein ratio, 24-hour systolic blood pressure and contraception usage.

3.5 Discussion

We investigated ethnic differences with respect to the associations between several components of the haemostatic and oxidant-antioxidant systems. The black population with elevated ambulatory blood pressure had increased serum peroxides and decreased GPx activity that was associated with several haemostatic components, suggesting that oxidative stress may alter their haemostatic profile. In the white population, the association between CLT and serum peroxide suggests that oxidative stress may impair the lysis of a fibrin clot.

The link found between higher vWF and lower GPx activity in the black population confirms previous findings by Blann *et al*. (8), reporting lower GPx-1 activity and increased vWF levels in 48 white hypercholesterolaemic patients with vascular disease. Furthermore, Blankenberg *et al*. (13) reported increased cardiovascular risk with low levels of GPx activity in 636 white patients with suspected coronary artery disease. We also found an association between a longer CLT and decreased GPx activity. Decreased GPx activity may therefore predispose the vascular endothelium to excess oxidative damage and aid in the development and progression of atherosclerosis (8). Impairment of both ROS metabolism and nitric oxide activation may impede platelet inhibitory mechanisms leading to increased platelet aggregation and ultimately a prothrombotic state. The decreased GPx levels of the black population could therefore be an indication of a decreased antioxidant capacity, while the associations between vWF, CLT and GPx suggest that diminished GPx activity levels are associated with a prothrombotic profile.

Furthermore, vWF, PAI-1 and CLT were negatively associated with total GSH and positively with GR in the black population. Limited information is available on the relationship between the antioxidant markers and the components of the haemostatic markers. However, in agreement with our findings a previous study investigating relationships between vWF and reduced GSH in diabetic patients reported an inverse relationship between vWF and GSH (26). A study by Martina *et al*. (34) who evaluated the effect of GSH treatment on PAI-1 levels also reported an inverse relationship. Even though we used total GSH instead of reduced GSH it is possible that the total GSH levels of the black population could consist out of a decreased GSH/GSSG ratio. We could

argue that GR activity may be up-regulated in the black population in an attempt to restore the redox balance. However, this is highly speculative and needs to be confirmed. A previous study by Rybka *et al*. (35) who investigated the glutathione antioxidant defence system also reported that decreased GPx activity levels are accompanied by a concomitant increase in GR activity in patients with hypertension. When oxidative stress supersedes GPx availability, damage may occur to the surrounding cells which may at least in part, result in the release of several prothrombotic factors (36). The decreased GPx availability in the black population could alter their haemostatic profile and thereby their susceptibility to stroke. No associations were found between vWF and ROS, GSH or GPx in the white population, this could be due to the fact that their mean vWF levels were significantly lower than their black counterparts.

The positive association initially found between fibrinogen and ROS in both ethnic groups disappeared in the black population after adjusting for hs-CRP (supplementary Table S2), while the initial associations remained after adjusting for IL-6 (Supplementary Table S3). Although several *in vitro* studies exist regarding the relationship between fibrinogen and oxidative stress (37,38), epidemiological data regarding the relationship is lacking. Fibrinogen and hs-CRP are both markers of the acute phase response and their dependence on each other in the black population suggests that the association between fibrinogen and ROS could be mediated through the acute phase response (39). Overall, the black population had a worsened cardiovascular profile that may contribute to increased low grade inflammation or *vice versa*. The more pronounced inflammatory state in the black population could possibly explain the influence of hs-CRP on the fibrinogen and ROS relationship. Furthermore, fibrinogen is known to be highly susceptible to oxidative modifications which may affect changes in the formation and architecture of the fibrin network (38,40,41). However, controversy exists as to whether oxidised fibrinogen induces a prothrombotic (42,43) or antithrombotic (37,44) state. The positive association between CLT and ROS in the white population may serve as an indicator that oxidative stress influenced the architecture of the fibrin clot in such a manner that lysis occurs at a slower rate. Evidence from *in vitro* studies (42,45) suggests that oxidised fibrinogen may promote fibrin clot formation with increased fiber density, decreased fiber diameter and pore size that is lysed at a slower rate.

It is unclear why this association was not found in the black population who had higher levels of haemostatic-, oxidative stress and inflammatory markers as well as a worsened cardiovascular profile. Further studies are needed to gain insight into the haemostatic markers and oxidative stress interactions in different ethnicities.

The present study should be interpreted within the context of its limitations and strengths. Additional markers of oxidative stress such as superoxide dismutase and catalase could add valuable insight into the antioxidant capacity. Residual confounding cannot be excluded even though the results were consistent after multiple adjustments. Causality cannot be inferred, due to the cross-sectional nature of the study. Since our population group was only recruited from urban areas of the Potchefstroom district in the North West province of South Africa, our findings are not seen as representative of the entire South African population. Overall, this was a well-designed study under controlled conditions in two different ethnic groups, with a relatively similar socio-economic status.

We conclude that in the black population, decreased GPx activity accompanies an altered haemostatic profile, while in the white population associations may suggest that serum peroxides impair fibrin clot lysis.

3.6 Declaration of interest:

Except for funding received from Roche Diagnostics the authors declare no further conflicts of interest.

3.7 Acknowledgements

The Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study would not have been possible without the voluntary collaboration of the participants and the Department of Education, North West province, South Africa. Research included in the present study was partially funded by the National Research Foundation, North-West University, North-West Department of Education and ROCHE Diagnostics, South Africa; as well as the Metabolic Syndrome Institute, France. The funders had no role in study design, data collection and analysis,

decision to publish, or preparation of the manuscript. The authors gratefully acknowledge the technical assistance of Mrs. Tina Scholtz, Sr. Chrissie Lessing and Dr. Szabolcs Péter, as well as Prof Muriel Meiring for the analyses of vWF.

Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the DAAD-NRF.

3.8 References

(1) Sliwa K, Wilkinson D, Hansen C, Ntyintyane L, Tibazarwa K, Becker A, *et al*. Spectrum of heart disease and risk factors in a black urban population in South Africa (the Heart of Soweto Study): a cohort study. Lancet. 2008;371:915-22.

(2) Connor MD, Thorogood M, Casserly B, Dobson C, Warlow CP. Prevalence of stroke survivors in rural South Africa: results from the Southern Africa Stroke Prevention Initiative (SASPI) Agincourt field site. Stroke. 2004;35:627-32.

(3) Ogah OS, Rayner BL. Recent advances in hypertension in sub-Saharan Africa. Heart. 2013;99:1390-7.

(4) Carter AM, Cymbalista CM, Spector TD, Grant PJ. Heritability of clot formation, morphology, and lysis: the EuroCLOT study. Arterioscler Thromb Vasc Biol. 2007;27:2783-9.

(5) Ruef J, Peter K, Nordt TK, Runge MS, Kubler W, Bode C. Oxidative stress and atherosclerosis: its relationship to growth factors, thrombus formation and therapeutic approaches. Thromb Haemost. 1999;82:32-7.

(6) Sies H. Physiological society symposium: impaired endothelial and smooth muscle cell function in oxidative stress. Exp Physiol. 1997;82:291-5.

(7) Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol. 2005;25:29-38.

(8) Blann AD, Maxwell SR, Burrows G, Miller JP. Antioxidants, von Willebrand factor and endothelial cell injury in hypercholesterolaemia and vascular disease. Atherosclerosis. 1995;116:191-8.

(9) Kenet G, Freedman J, Shenkman B, Regina E, Brok-Simoni F, Holzman F, *et al*. Plasma glutathione peroxidase deficiency and platelet insensitivity to nitric oxide in children with familial stroke. Arterioscler Thromb Vasc Biol. 1999;19:2017-23.

(10) Lu SC. Regulation of glutathione synthesis. Mol Aspects Med. 2009;30:42-59.

(11) Leopold JA, Loscalzo J. Oxidative enzymopathies and vascular disease. Arterioscler Thromb Vasc Biol. 2005;25:1332-40.

(12) Yang MS, Chan HW, Yu LC. Glutathione peroxidase and glutathione reductase activities are partially responsible for determining the susceptibility of cells to oxidative stress. Toxicology. 2006;226:126-30.

(13) Blankenberg S, Rupprecht HJ, Bickel C, Torzewski M, Hafner G, Tiret L, *et al*. Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. New Engl J Med. 2003;349:1605-13.

(14) Jin RC, Mahoney CE, Anderson L, Ottaviano F, Croce K, Leopold JA, *et al*. Glutathione peroxidase-3 deficiency promotes platelet-dependent thrombosis *In Vivo*. Circulation. 2011;123:1963-73.

(15) Conlan MG, Folsom AR, Finch A, Davis CE, Sorlie P, Marcucci G, *et al*. Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) study. Thromb Haemost. 1993;70:380-5.

(16) Ernst E, Resch KL. Fibrinogen as a cardiovascular risk factor: A meta-analysis and review of the literature. Ann Intern Med. 1993;118:956-63.

(17) Folsom AR, Wu KK, Conlan MG, Finch A, Davis CE, Marcucci G, *et al*. Distributions of hemostatic variables in blacks and whites: population reference values from the Atherosclerosis Risk in Communities (ARIC) study. Ethn Dis. 1992;2:35-46.

(18) Lutsey PL, Cushman M, Steffen LM, Green D, Barr RG, Herrington D, *et al*. Plasma hemostatic factors and endothelial markers in four racial/ethnic groups: the MESA study. J Thromb Haemost. 2006;4:2629-35.

(19) Greyling A, Pieters M, Hoekstra T, Oosthuizen W, Schutte AE. Differences in the association of PAI-1 activity with the metabolic syndrome between African and Caucasian women. Nutr Metab Cardiovasc Dis. 2007;17:499-507.

(20) Lacy F, Kailasam MT, O'Connor DT, Schmid-Schonbein GW, Parmer RJ. Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity. Hypertension. 2000;36:878-84.

(21) Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. J Thromb Haemost. 2006;4:1186-93.

(22) Koenig W. Fibrin(ogen) in cardiovascular disease: an update. Thromb Haemost. 2003;89:601-9.

(23) Taverne YJHJ, Bogers AJJC, Duncker DJ, Merkus D. Reactive oxygen species and the cardiovascular system. Oxid Med Cell Longev. 2013;2013:15.

(24) Kruger R, Schutte R, Huisman H, Van Rooyen J, Malan N, Fourie C, *et al*. Associations between reactive oxygen species, blood pressure and arterial stiffness in black South Africans: the SABPA study. J Hum Hypertens. 2012;26:91-7.

(25) Lammertyn L, Schutte AE, Pieters M, Schutte R. D-dimer relates positively with increased blood pressure in black South Africans: The SABPA study. Thromb Res. 2014;133:1152-7.

(26) Ibrahim H, El-Meligi A, Abdel-Hamid M, Elhendy A. Relations between von Willebrand factor, markers of oxidative stress and microalbuminuria in patients with type 2 diabetes mellitus. Med Sci Monit. 2004;10:CR85-9.

(27) Marfell-Jones M, Olds T, Stewart A, Carter L. International standards for anthropometric assessment: International Society for the Advancement of Kinanthropometry; 2006.

(28) Norton KI, Olds T, Commission AS. Anthropometrica: A textbook of body measurement for sports and health courses: University of New South Wales Press; 1996.

(29) Lisman T, de Groot PG, Meijers JC, Rosendaal FR. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. Blood. 2005;105:1102-5.

(30) Hayashi I, Morishita Y, Imai K, Nakamura M, Nakachi K, Hayashi T. High-throughput spectrophotometric assay of reactive oxygen species in serum. Mutat Res. 2007;631:55-61.

(31) Harris MT, Davis WW, Le N-A, Eggleston B, Austin GE, Moussa M, *et al*. Free oxygen radicals in whole blood correlate strongly with high-sensitivity C-reactive protein. J Clin Lipidol. 2007;1:593-8.

(32) Gill R, Tsung A, Billiar T. Linking oxidative stress to inflammation: Toll-like receptors. Free Radic Biol Med. 2010;48:1121-32.

(33) Levi M, van der Poll T. Two-way interactions between inflammation and coagulation. Trends Cardiovasc Med. 2005;15:254-9.

(34) Martina V, Bruno GA, Pannocchia A, Zumpano E, Tagliabue M, Trucco F, *et al*. PAI-1 reduction after treatment with glutathione in NIDDM. Fibrinolysis. 1996;10:63-5.

(35) Rybka J, Kupczyk D, Kędziora-Kornatowska K, Motyl J, Czuczejko J, Szewczyk-Golec K, *et al*. Glutathione-related antioxidant defense system in elderly patients treated for hypertension. Cardiovasc Toxicol. 2011;11:1-9.

(36) Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004;134:489-92.

(37) Shacter E, Williams JA, Levine RL. Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. Free Radic Biol Med. 1995;18:815-21.

(38) Rosenfeld MA, Shegolihin AN, Bychkova AV, Leonova VB, Biryukova MI, Kostanova EA, *et al*. Oxidative modification of fibrinogen molecules. Dokl Biochem Biophys. 2013;452:255-8.

(39) Kaptoge S, White I, Thompson S, Wood A, Lewington S, Lowe G, *et al*. Associations of plasma fibrinogen levels with established cardiovascular disease risk factors, inflammatory markers, and other characteristics: individual participant meta-analysis of 154,211 adults in 31 prospective studies: the fibrinogen studies collaboration. Am J Epidemiol. 2007;166:867-79.

(40) Shacter E, Williams JA, Lim M, Levine RL. Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. Free Radic Biol Med. 1994;17:429-37.

(41) Azizova OA, Aseichev AV, Piryazev AP, Roitman EV, Shcheglovitova ON. Effects of oxidized fibrinogen on the functions of blood cells, blood clotting, and rheology. Bull Exp Biol Med. 2007;144:397-407.

(42) Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, *et al*. Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. J Biol Chem. 2004;279:8820-6.

(43) Upchurch GR, Ramdev N, Walsh MT, Loscalzo J. Prothrombotic consequences of the oxidation of fibrinogen and their Inhibition by aspirin. J Thromb Thrombolysis. 1998;5:9-14.

(44) Tetik S, Kaya K, Yardimci T. Effect of oxidized fibrinogen on hemostatic system: *in vitro* study. Clin Appl Thromb Hemost. 2011;17:259-63.

(45) Martinez M, Weisel JW, Ischiropoulos H. Functional impact of oxidative posttranslational modifications on fibrinogen and fibrin clots. Free Radic Biol Med. 2013;65:411-8.

(46) Kratz A, Ferraro M, Sluss PM, Lewandrowski KB. Normal reference laboratory values. New Engl J Med. 2004;351:1548-63.

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Data supplement

Ethnic-specific relationships between haemostatic and oxidative stress markers in black and white South Africans: The SABPA study

Running head: Ethnicity, haemostasis and oxidative stress

Leandi Lammertyn[1], Catharina MC Mels[1], M Pieters[3], Aletta E Schutte[1,2] and Rudolph Schutte[1,2]

[1]Hypertension in Africa Research Team (HART); North-West University; Potchefstroom; South Africa; [2]MRC Research Unit for Hypertension and Cardiovascular Disease, North-West University, Potchefstroom, South Africa. [3]Centre of Excellence for Nutrition (CEN); North-West University; Potchefstroom; South Africa

Tables 3

Correspondence:

Rudolph Schutte, PhD

Hypertension in Africa Research Team (HART),

Private Bag x6001,

North-West University

Potchefstroom,

2520

South Africa

Telephone: +27-18-299-2435

Facsimile: +27-18-299-1053

E-mail: rudolph.schutte@nwu.ac.za

Table S1: Interaction terms with ethnicity

	Total group (n=390)											
	ROS, mg/L			GSH, µM			GPx, nmol/min/ml			GR, nmol/min/ml		
	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p
vWF*Ethnicity	0.38 / 0.37	0.485 (0.136 to 0.834)	0.007	0.37 / 0.37	-0.471 (-0.932 to -0.010)	0.045	0.39 / 0.39	-0.693 (-1.005 to -0.381)	<0.001		NS	
Fibrinogen*Ethnicity		NS		0.13 / 0.12	-0.575 (-1.114 to -0.036)	0.037		NS			NS	
PAI-1*Ethnicity		NS			NS			NS			NS	
D-dimer*Ethnicity	0.11 / 0.10	0.419 (0.029 to 0.867)	0.037		NS			NS		0.08 / 0.07	-0.459 (-0.773 to -0.145)	0.004
CLT*Ethnicity		NS			NS		0.12 / 0.11	-0.523 (-0.905 to -0.141)	0.008		NS	

Abbreviations: ROS, reactive oxygen species; GSH, glutathione; GPx, Glutathione peroxidase, GR, glutathione reductase; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; CLT, clot lysis time.

Table S2: Multiple regression analyses of haemostatic markers with oxidative stress markers, after additional adjustment for high sensitivity C-reactive protein.

	Black (n=175)											
	von Willebrand factor, %			**Fibrinogen, g/L**			**Plasminogen activator inhibitor-1, ng/ml**			**Clot lysis time, min**		
Independent variables	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p
ROS, mg/L		NS			NS			NS			NS	
GSH, µM	0.16 / 0.13	-0.195 (-0.344 to -0.046)	0.012		NS		0.13 / 0.09	-0.273 (-0.426 to -0.120)	<0.001	0.41 / 0.37	-0.156 (-0.289 to -0.023)	0.022
GPx, nmol/min/ml	0.13 / 0.12	-0.242 (-0.387 to -0.097)	<0.001		NS			NS		0.42 / 0.38	-0.186 (-0.311 to -0.061)	0.005
GR, nmol/min/ml		NS			NS		0.13 / 0.09	0.241 (0.088 to 0.394)	0.002	0.41 / 0.38	0.198 (0.069 to 0.327)	0.003
	White (n=206)											
ROS, mg/L		NS		0.43 / 0.40	0.203 (0.050 to 0.356)	0.010		NS		0.56 / 0.54	0.140 (0.032 to 0.246)	0.011
GSH, µM		NS			NS			NS			NS	
GPx, nmol/min/ml		NS			NS			NS			NS	
GR, nmol/min/ml	0.11 / 0.08	0.141 (0.008 to 0.274)	0.041		NS			NS			NS	

Abbreviations: ROS, reactive oxygen species; GSH, glutathione; GPx, Glutathione peroxidase, GR, glutathione reductase. Adjusted for age, body mass index, gender, gamma glutamyl transferase, current smoking, physical activity, glycosylated haemoglobin A1c, total cholesterol: high density lipoprotein ratio, high sensitivity C-reactive protein, 24-hour systolic blood pressure and contraception usage.

Table S3: Multiple regression analyses of haemostatic markers with oxidative stress markers, after additional adjustment for interleukin-6.

	Black (n=175)											
	von Willebrand factor, %			**Fibrinogen, g/L**			**Plasminogen activator inhibitor-1, ng/ml**			**Clot lysis time, min**		
Independent variables	Tot R^2 / Adj R^2	β (95% CI)	P	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p
ROS, mg/L		NS		0.40 0.38	0.248 (0.101 to 0.395)	<0.001		NS			NS	
GSH, µM	0.16 0.14	-0.214 (-0.363 to -0.065)	0.005		NS		0.12 0.08	-0.246 (-0.401 to -0.091)	0.002	0.43 0.40	-0.305 (-0.438 to -0.172)	<0.001
GPx, nmol/min/ml	0.19 0.16	-0.244 (-0.389 to -0.099)	0.001		NS			NS		0.44 0.41	-0.189 (-0.312 to -0.066)	0.003
GR, nmol/min/ml	0.16 0.13	0.170 (0.023 to 0.317)	0.025		NS		0.13 0.06	0.237 (0.076 to 0.398)	0.005	0.43 0.40	0.165 (0.038 to 0.292)	0.013
	White (n=206)											
ROS, mg/L		NS		0.39 0.37	0.351 (0.208 to 0.494)	<0.001		NS		0.57 0.55	0.147 (0.037 to 0.257)	0.010
GSH, µM		NS			NS			NS			NS	
GPx, nmol/min/ml		NS			NS			NS			NS	
GR, nmol/min/ml		NS			NS			NS			NS	

Abbreviations: ROS, reactive oxygen species; GSH, glutathione; GPx, Glutathione peroxidase, GR, glutathione reductase. Adjusted for age, body mass index, gender, gamma glutamyl transferase, current smoking, physical activity, glycosylated haemoglobin A1c, total cholesterol: high density lipoprotein ratio, Interleukin-6, 24-hour systolic blood pressure and contraception usage.

CHAPTER 4

Retinal vessel calibres and haemostasis in black and white South Africans: The SABPA study

Published in *Journal of Hypertension*

AUTHOR INSTRUCTIONS FOR JOURNAL OF HYPERTENSION

(http://edmgr.ovid.com/jh/accounts/ifauth.htm)

The ***Journal of Hypertension*** publishes papers reporting original, clinical and experimental research of high standard that contributes to the advancement of knowledge in the field of hypertension.

Essential title page information

Title. The title should be concise and informative of no more than 20 words.

Running head: A short brief title should be included of no more than 40 characters.

Author names and affiliations. State all authors' names, middle initial(s) and last name. Present the authors' affiliation addresses below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address.

Corresponding author. Clearly indicate the corresponding author name and address.

Conflict of interest. Authors must state all potential conflicts of interest.

Word count. A word count should be provided on the title page. The word count should include all text including the references but not tables and legends. The number of tables and supplementary files should also be indicated.

Abstract

A concise and factual abstract of no more than 250 words is required. The abstract should state the objectives of the study, basic methods, main results and principal conclusion.

Keywords and Abbreviations

The abstract should be followed by a list of three to ten keywords. A list of abbreviations should follow. Abbreviations should also be defined on the first appearance in the text. Only standard abbreviations are allowed and avoid the use of abbreviations in the title and abstract.

Article structure

The article must be divided into clearly defined sections (Introduction, Methods, Results and Discussion). Each heading should appear on its own separate line.

Introduction.

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Methods.

Provide sufficient detail to allow the work to be reproduced. Authors should stipulate that the research was conducted within the guidelines of the declaration of Helsinki.

Results

Results should be clear and concise.

Discussion

Should explore the significance of the results of the work, not repeat them.

Acknowledgements

Acknowledgement should be made to those who have made a substantial contribution to the study.

References

Text: Indicate references with arabic numerals in square brackets e.g. [12] in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

Reference List: Should be numbered consecutively in the order they appear in the text.

Reference style template: Can be obtained for endnote users at http://endnote.com/downloads/style/journal-hypertension

Tables

Number tables consecutively in accordance with their appearance in the text. Each table should be submitted on their own page and contain a brief title. Place explanatory matter in a footnote below the table body.

Supplementary data

Electronic supplementary material that supports and enhances your scientific research is accepted. Supplementary files supplied are published online alongside the electronic version of your article. A List of the supplementary content with a concise and descriptive caption for each file should be submitted at the end of the manuscript.

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Word count: 4587

Tables: 4

Supplementary files: 1 (4 Tables)

Retinal vessel calibres and haemostasis in black and white South Africans: The SABPA study

Running head: Retinal vessel calibres and haemostasis

Leandi Lammertyn[a], Aletta E Schutte[ab], Wayne Smith[a], Marlien Pieters[c] and Rudolph Schutte[ab]

[a]*Hypertension in Africa Research Team (HART), North-West University, Potchefstroom, South Africa;*

[b]*MRC Research Unit for Hypertension and Cardiovascular Disease, North-West University, Potchefstroom, South Africa.*

[c]*Centre of Excellence for Nutrition (CEN), North-West University, Potchefstroom, South Africa.*

Correspondence:

Rudolph Schutte, PhD

Hypertension in Africa Research Team (HART),

Private Bag x6001,

North-West University,

Potchefstroom,

2520

South Africa

Telephone: +27-18-299-2435

E-mail: rudolph.schutte@nwu.ac.za

The present study was partially funded by the National Research Foundation, Medical Research Council, ROCHE Diagnostics, North-West University, North-West Department of Education, South Africa; as well as the Metabolic Syndrome Institute, France.

Conflict of Interest: All authors declare no conflict of interest.

4.1 Abstract

Objectives: Retinal arteriolar narrowing associates with hypertension development and indicates increased cardiovascular risk. Evidence on whether the retinal vessel calibres are related to the haemostatic system is limited, especially in the black hypertension-prone population with a high stroke incidence. We therefore investigated the relationships between haemostatic markers and retinal vessel calibres.

Methods: We performed a cross-sectional study involving 170 black (mean age, 58 years; 44% women) and 189 white (mean age, 49 years; 52% women) teachers, and determined ambulatory blood pressure, haemostatic factors (fibrinogen, von Willebrand factor, D-dimer, plasminogen activator inhibitor-1 and clot lysis time) and retinal vessel calibres (central retinal artery and vein equivalent). The black and white groups were stratified by median split of the retinal arteriolar calibre.

Results: Both ethnic groups with a smaller arteriolar calibre had higher systolic blood pressure and narrower venular calibres. In the black population the central retinal vein equivalent was positively (β=0.293; p=0.024) associated with fibrinogen, whereas in the white population the central retinal artery equivalent (β=-0.256; p=0.016) was negatively, and central retinal vein equivalent (β=0.234; p=0.021) positively associated with von Willebrand factor. Furthermore, clot lysis time was negatively associated with the central retinal artery equivalent (β=-0.390; p=0.014) in the black group and positively associated with the central retinal vein equivalent (β=0.275; p=0.008) in the white group.

Conclusion: Relationships between markers of haemostasis and the retinal vessel calibres exist and vary between ethnicities. Haemostatic alterations are linked to early retinal microvascular changes, and future studies should investigate whether it translates into elevated stroke risk.

Keywords: Arteriolar narrowing, von Willebrand factor, fibrinogen, hypertension, ethnicity

4.2 Abbreviations

ABPM	Ambulatory blood pressure monitoring
ARIC	Atherosclerosis Risk In Communities study
AVR	Arteriolar-to-venular ratio
CLT	Clot lysis time
CRAE	Central retinal artery equivalent
CRP	C-reactive protein
CRVE	Central retinal vein equivalent
CVD	Cardiovascular disease
HbA1c	Glycosylated haemoglobin A1c
HDL-C	High density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
MESA	Multi-Ethnic study of Atherosclerosis
PAI-1	Plasminogen activator inhibitor-1
SABPA	Sympathetic activity and Ambulatory Blood Pressure in Africans
TC	Total cholesterol
vWF	von Willebrand factor
γ-GT	Gamma-glutamyltransferase

4.3 Introduction

The burden of cardiovascular disease (CVD) is increasing worldwide with South Africa being no exception [1]. A recent report indicated that South Africa has a high prevalence of hypertension, especially among the black population, who also has a high stroke incidence [2,3]. The black population not only seems to have a worse cardiovascular profile than their white counterparts, but also a prothrombotic profile [4]. Investigation of the microvasculature for the early detection and prevention of vascular disease has gained particular interest in the fight against CVD [5]. The retina is a convenient site to study microvascular changes such as arteriolar narrowing and venular widening with reproducible non-invasive techniques [6].

The retinal vasculature shares many anatomical and physiological features with the cerebral and coronary vasculature which are exposed to similar intrinsic and environmental influences [5,7]. Retinal vessel calibre sizes convey different information about systemic diseases, with arteriolar narrowing being associated with elevated blood pressure [8-10], whereas venular widening relates more to inflammation and incidence of stroke [11,12]. The mechanisms contributing to retinal calibre changes are still unclear but is thought to be a reflection of cumulative vascular damage from conditions such as inflammation and endothelial dysfunction [11,13-16]. Haemostatic factors are key players in many of these processes and previous investigators have shown there is a relationship between markers of haemostasis and microvascular dysfunction [17-19].

Of the known retinal microvascular changes, evidence shows that arteriolar narrowing precedes the development of hypertension and is considered an early feature of mild hypertensive retinopathy [8,20]. Recently, we found elevated blood pressure to be associated with increased fibrin D-dimer in a black population [4]. However, evidence on whether the retinal vessel calibres are related to the haemostatic system is scant [11,21] and absent in the hypertension-prone black population of South Africa. We therefore focused on individuals with smaller retinal arteriolar calibres and investigated the relationships between components of the haemostatic system and retinal vessel calibres in black and white South Africans.

4.4 Materials and methods

4.4.1 Study population

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study was a population based cohort involving 409 school teachers between the ages of 25 – 60 years from the North West Province, South Africa at inception in 2008-2009. At that time, the study participants consisted of 200 black (49.5% women) and 209 white (51.6% women) participants. After 3 years, 359 (87.8%) participants returned for examinations during the months of February to May 2011-2012, of which 170 black (48.8% women) and 189 white (50.8% women) were used in the present analysis. The study complied with the Helsinki declaration for investigation of human participants [22]. The Health Research Ethics Committee of the North-West University (Potchefstroom Campus) approved the study. Written informed consent was obtained prior to the commencement of measurements.

4.4.2 Clinical measurements

A 24-hour ambulatory blood pressure (ABPM) and electrocardiogram apparatus (Meditech CE120® Cardiotens, Budapest, Hungary) was attached to the participants at their workplace. Each participant's energy expenditure for the week (7 days) was calculated with an Actiheart® (CamNtech Limited, Cambridgeshire, United Kingdom). All participants commenced with a 24-hour standardised diet. Participants reported to the North-West University at 15:00 where they were informed of the proceedings for the rest of the day and underwent retinal vessel imaging. The participants were then transported to a Metabolic Unit where they received dinner at 18:00 and final snacks and drinks at 20:30. They were requested to go to bed at around 22:00. At 07:30, the ABPM apparatus was removed, followed by anthropometric measurements and blood sampling. The 24-hour blood pressure and electrocardiogram data were downloaded onto a database using the CardioVisions 1.9.0 Personal Edition software (Meditech, Budapest, Hungary). A lifestyle questionnaire, including questions on smoking and alcohol habits, as well as chronic medication use, was completed by each participant.

4.4.3 Retinal photography and vessel calibre measurements

In 2011-2012 the participants had retinal photographs taken and only 302 (84.1%) had gradable retinal vessel measurements. The participants were in a non-fasting state. However, no food, drinks, exercise or smoking were permitted one hour prior to the assessment. The retinal photographs were predominantly taken of the right eye. Before the commencement of the retinal photographs, the participants' risk for acute anterior chamber angle glaucoma was determined with a small light source by a trained registered nurse. Thereafter, a drop of 1% tropicamide and 0.01% bensalconiumchloride (m/v) (Alcon) was administered into the eye to achieve mydriatic conditions. The retinal images were taken at 50º angle with a Zeiss Fundus Camera FF-450 Plus (Imedos, Jena, Germany) after pupil dilation was obtained.

Colour and monochrome fundus images centred on the optical disk were captured using Visualis 2.81 software (Imedos Systems, Jena, Germany). The retinal vessel calibres were determined from the monochrome image using VesselMap2 software (Imedos Systems, Jena, Germany) in a measuring zone of 0.5-1.0 optic disk diameters away from the optical disk margin. All first order vessels passing through this zone were manually selected and their borders automatically delineated. The colour image was used to aid with the correct identification of arterioles and venules. The calibres were measured in measuring units (MU), where one MU is equivalent to 1 µM in the normal Gullstrand eye. The 6 largest arterioles and venules were automatically selected by the software, and the central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) were calculated using the Knudson formula [23]. Thereafter the arteriolar-to-venular (AVR) ratio was calculated from the CRAE and CRVE values. All images were graded by the same two researchers working in unison.

4.4.4 Anthropometric measurements

All measurements were taken in triplicate with calibrated instruments. Stature was measured to the nearest 0.1 cm with a stadiometer (Invicta Stadiometer, IP 1465, London, UK), body mass to the nearest 0.1 kg (Precision Health Scale, A & D Company, Tokyo, Japan) and waist circumference to the nearest 0.1 cm with a unstretchable flexible 7 mm wide metal tape (Holtain,

Crosswell, Wales) [24,25]. Body mass index (BMI) was calculated for each participant using the standard formula of weight/(height)^2.

4.4.5 Blood sampling

A registered nurse collected fasting blood samples with a sterile winged infusion set from the participants' antebrachial vein branches before 10:00. The blood samples were centrifuged at 2000 x g for 15 minutes to obtain serum, sodium fluoride and citrate plasma samples. All samples were stored at -80˚C until analysis.

4.4.6 Biochemical analyses

High sensitivity C-reactive protein (CRP), total cholesterol (TC), high density lipoprotein (HDL-C) cholesterol, low density lipoprotein (LDL-C), gamma-glutamyltransferase (γ-GT), glycosylated haemoglobin A1c (HbA1c) and fasting glucose were determined with standard methods described elsewhere [4].

Citrated plasma samples were used for the analysis of von Willebrand factor antigen (vWF_{ag}), fibrinogen, plasminogen activator inhibitor-1 antigen ($PAI\text{-}1_{ag}$), fibrin D-dimer and clot lysis time (CLT). vWF_{ag} levels were determined with a sandwich enzyme-linked immunosorbent assay. Polyclonal rabbit anti-vWF antibody and rabbit anti-vWF-horseradish peroxidase antibody (DAKO, Glostrup, Denmark) were used to perform the assay. The 6th International Standard for vWF/FVIII was used to create the standard curve against which the samples were measured. Plasma fibrinogen levels were determined by means of a modified Clauss method using the FIB kit from STAGO diagnostics (STAGO diagnostics, Asnières, France). The Liatest D-dimer kit from STAGO diagnostics (STAGO diagnostics, Asnières, France) was used to determine fibrin D-dimer levels with an immuno-turbimetric method with a 540 nm detection limit. $PAI\text{-}1_{ag}$ levels were determined with the TriniLIZE $PAI\text{-}1_{ag}$ kit (Trinity Biotech, Bray, Ireland) using an enzyme-linked immunosorbent assay. CLT was determined by studying the lysis of a tissue factor-induced clot by exogenous tissue-plasminogen activator. Changes in turbidity during clot formation and lysis were monitored as described by Lisman *et al*. [26]. Tissue factor and tissue plasminogen activator

concentrations were slightly modified to obtain comparable CLTs of about 60 minutes. The modified concentrations were 17 mmol/L calcium chloride, 60 ng/ml tissue plasminogen activator (Actilyse, Boehringer Ingelheim, Ingelheim, Germany), and 10 µmol/L phospholipid vesicles (Rossix, Mölndal, Sweden). Tissue factor was diluted 3000 times (Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany). CLT was defined as the time from the midpoint in the transition from the initial baseline to maximum turbidity, which is representative of clot formation, to the midpoint in the transition from maximum turbidity to the final baseline turbidity, which represents the lysis of the clot.

4.4.7 Statistical analyses

Statistica software v12.0 was used for database management and statistical analyses (Statsoft, Inc., 2012). Variables not normally distributed (fibrin D-dimer, CRP, HbA1c, glucose, γ-GT, and energy expenditure) were logarithmically transformed. The central tendency and spread of these variables were represented by the geometric mean and the 5th and 95th percentile intervals. We tested for interaction with ethnicity when investigating associations between haemostatic markers and markers of retinal vessel calibres with multiple regression analyses. Results found served as motivation for the ethnic split (supplementary Table S1). A median split of CRAE was also performed based on our aims and on interactions found between the vascular calibres and haemostatic factors (supplementary Table S2), namely to determine if associations between retinal vessel calibres and haemostasis exist in a group with decreased arteriolar calibres that is considered to be at an increased risk for CVD. Means and proportions were compared by independent t-test and chi-square test, respectively. We used single regression analyses to determine associations between microvascular measures and markers of the haemostatic system. Forward stepwise multiple linear regression analyses were performed to determine independent associations between microvasculature measurements and markers of the haemostatic system. Covariates included in the models were age, body mass index, gender, γ-GT, current smoking, physical activity, HbA1c, TC:HDL-C, and 24-hour systolic blood pressure. CRAE was additionally adjusted for CRVE and *vice versa* [27]. All p-values refer to a two-sided hypothesis.

4.5 Results

Significant interactions were found between CRAE median split and ethnicity for the associations between the retinal vessel calibres and the haemostatic markers (Supplementary Table S1 and S2). Our study population was therefore stratified by median split for CRAE and thereafter by ethnicity, which allowed us to investigate the associations of retinal vascular calibres with the haemostatic variables in the high CVD risk group with arterial narrowing. Table 1 lists the general characteristics of the total black and white study population. The black population had higher mean systolic and diastolic blood pressures ($p<0.001$), fibrinogen ($p<0.001$) and PAI-1_{ag} ($p=0.001$) levels, as well as a longer mean CLT ($p=0.003$) than their white counterparts. With respect to their retinal vessel calibre profile, blacks tended to have smaller CRAE ($p=0.071$), with a larger CRVE ($p<0.001$), and consequently a lower AVR ($p<0.001$).

Table 1: Characteristics of the total study population

	Black (n=132)	White (n=170)	p
Women, n (%)	58 (43.9)	88 (51.8)	0.18
Age, years	47.5 ± 7.5	49.2 ± 9.8	0.046
Body mass index, kg/m^2	30.2± 6.5	28.8 ± 6.3	0.058
Waist circumference, cm (men)	98.3 ± 14.8	105.4 ± 13.0	0.001
Waist circumference, cm (women)	96.4 ± 16.7	87.0 ± 14.4	<0.001
Haemostatic markers			
von Willebrand factor, %	88.0 ± 19.4	90.4 ± 21.8	0.34
Fibrinogen, g/L	3.57 ± 0.72	2.81 ± 0.54	<0.001
Plasminogen activator inhibitor-1, ng/ml	41.7 ± 11.7	21.5 ± 9.2	<0.001
Fibrin D-dimer, µg/L	302 (219 – 692)	282 (218 – 550)	0.12
Clot lysis time, min	79.6 ± 8.8	76.4 ± 8.7	0.003
Biochemical measurements			
Total cholesterol, mmol/L	4.57 ± 1.00	4.33 ± 1.04	0.048
Total cholesterol: high density cholesterol	4.96 ± 1.71	4.45 ± 1.48	0.005
High density lipoprotein cholesterol, mmol/L	1.00 ± 0.34	1.06 ± 0.37	0.17
Low density lipoprotein cholesterol, mmol/L	2.95 ± 0.87	2.73 ± 0.90	0.033
Triglycerides, mmol/L	1.37 ± 0.83	1.22 ± 0.92	0.13
Glycosylated hemoglobin A1c, %	5.89 (5.25 – 7.08)	5.50 (5.13 – 6.03)	<0.001
Glucose, mmol/L	5.25 (4.37 – 7.08)	4.27 (2.95 – 6.03)	<0.001
C-reactive protein, mg/L	3.24 (0.43 – 13.18)	1.10 (0.16 – 6.45)	<0.001
Haematocrit, %	41.3 ± 4.23	42.4 ± 4.34	0.034
Microvascular measurements			
Arteriolar-to-venular ratio	0.60 ± 0.06	0.64 ± 0.04	<0.001
Central retinal artery equivalent, MU	148.6 ± 12.0	151.0 ± 10.7	0.071
Central retinal vein equivalent, MU	247.4 ± 18.6	236.5 ± 17.7	<0.001
Macrovasular measurements			
24-hour Systolic blood pressure, mmHg	134.2 ± 16.5	123.4 ± 12.0	<0.001
24-hour Diastolic blood pressure, mmHg	83.7 ± 10.8	75.8 ± 8.4	<0.001
Lifestyle			
Physical activity, kcal/day	3162 (1950 – 5754)	3162 (2042 – 5623)	0.91
Gamma-glutamyltransferase, U/L	35.5 (11.7 – 107.2)	17.8 (6.76 – 45.7)	<0.001
Current smoking, n (%)	21 (15.9)	22 (12.9)	0.46
HIV-infected, n (%)	18 (13.9)	1 (0.6)	<0.001
Intake of medications			
Anti-hypertensive medication, n (%)	40 (30.3)	33 (19.4)	0.028
Anti-coagulant medication, n (%)	22 (19.9)	22 (16.7)	0.36
Anti-inflammatory medication, n (%)	24 (18.2)	21 (12.4)	0.16
Contraception, n women (%)	11 (19.0)	5 (5.7)	0.038

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval), or number of subjects (%).

When stratifying the black and white groups by median split of the CRAE (Table 2), systolic blood pressure (p≤0.020) and AVR (p<0.001) were significantly higher, and CRVE (p<0.001) lower in the smaller CRAE subdivision for both ethnic populations. We found no differences in haemostatic markers between CRAE median split groups, except for fibrin D-dimer being significantly higher (p=0.035) in the smaller CRAE group in blacks only.

Table 2: Characteristics of the study population stratified by central retinal artery equivalent

	Black (N=132)			White (N=170)		
	Smaller CRAE <150.6 (N=71)	**Larger CRAE ≥150.6 (N=61)**	**p**	**Smaller CRAE <150.6 (N=80)**	**Larger CRAE ≥150.6 (N=90)**	**p**
Haemostatic markers						
vWF, %	89.2 ± 18.9	87.2 ± 20.2	0.57	90.8 ± 21.9	90.0 ± 21.7	0.81
Fibrinogen, g/L	3.55 ± 0.74	3.65 ± 0.69	0.40	2.84 ± 0.52	2.79 ± 0.56	0.54
PAI-1, ng/ml	41.0 ± 12.5	42.9 ± 11.1	0.36	22.1 ± 9.6	20.8 ± 1.15	0.34
Fibrin D-dimer, µg/L	324 (219 –759)	275 (218 – 617)	0.035	288 (219 – 550)	282 (219 – 676)	0.85
Clot lysis time, min	78.8 ± 8.7	80.2 ± 9.0	0.39	77.1 ± 8.5	75.7 ± 8.8	0.32
Microvascular measurements						
AVR	0.58 ± 0.05	0.64 ± 0.05	<0.001	0.63 ± 0.04	0.65 ± 0.04	<0.001
CRAE, MU	139.5 ± 7.6	159.2 ± 6.1	<0.001	142.0 ± 6.2	158.9 ± 6.9	<0.001
CRVE, MU	243.8 ± 18.8	251.6 ± 17.6	<0.001	227.0 ± 16.7	245.0 ± 13.9	<0.001
Macrovascular measurements						
24-hour SBP, mmHg	138.9 ± 17.6	128.5 ± 13.5	<0.001	125.8 ± 12.6	121.5 ± 11.3	0.020
24-hour DBP, mmHg	87.3 ± 10.5	79.2 ± 9.6	<0.001	77.1 ± 8.7	74.9 ± 8.0	0.088

Values are arithmetic mean ± SD, geometric mean (5^{th} to 95^{th} percentile interval), or number of subjects (%).
Abbreviations: vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; AVR, arteriolar-to-venular ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; SBP, systolic blood pressure; DBP, diastolic blood pressure.

4.5.1 Unadjusted analyses

In the smaller CRAE subdivision (Table 3), AVR correlated negatively (black p=0.020; white p=0.043) and CRVE positively (black p=0.017; white p=0.024) with fibrinogen in both ethnic groups. In the white group, a negative AVR and positive CRVE correlation were found with both vWF_{ag} (AVR p<0.001; CRVE p=0.031) and CLT (AVR p=0.007; CRVE p=0.002). We also performed similar analyses in the larger CRAE subdivision (Supplementary Table S3), and found that AVR correlated positively with fibrin D-dimer (p=0.029) in the black population, while CRVE correlated negatively with fibrin D-dimer (black p=0.017; white p=0.027) in both ethnic groups. An additional negative association was found between AVR and CLT (p=0.011) in the white population.

4.5.2 Adjusted analyses

Table 3: Single regression analyses of retinal vessel measurements with markers of the haemostatic system in participants with CRAE < 150.6 MU.

	Von Willebrand factor, %	Fibrinogen, g/L	Plasminogen activator inhibitor-1, ng/ml	Fibrin D-dimer, µg/L	Clot lysis time, min
			Black (N=69)		
AVR	r=0.13 p=0.31	**r=-0.28 p=0.020**	r=-0.02 p=0.86	r=0.02 p=0.86	r=-0.16 p=0.21
CRAE, MU	r=0.02 p=0.87	r=-0.09 p=0.49	r=-0.001 p=0.99	r=0.07 p=0.59	r=-0.12 p=0.35
CRVE, MU	r=-0.14 p=0.27	**r=0.29 p=0.017**	r=0.01 p=0.91	r=0.02 p=0.89	r=0.10 p=0.44
			White (N=78)		
AVR	**r=-0.37 p=0.001**	**r=-0.23 p=0.043**	r=-0.01 p=0.91	r=0.10 p=0.41	**r=-0.32 p=0.007**
CRAE, MU	r=-0.12 p=0.30	r=0.10 p=0.38	r=0.02 p=0.85	r=-0.01 p=0.93	r=0.01 p=0.27
CRVE, MU	**r=0.25 p=0.031**	**r=0.26 p=0.024**	r=0.01 p=0.92	r=-0.11 p=0.35	**r=0.35 p=0.002**

Abbreviations: AVR, arteriolar: venular ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent. Bold values denotes statistical significance, p<0.05.

When focussing again on participants in the smaller CRAE grouping, we confirmed after full adjustments for known covariates (age, gender, body mass index, smoking, γ-GT, physical activity, HbA1c, TC:HDL, systolic blood pressure; Table 4) the associations of AVR and CRVE with fibrinogen (AVR p=0.021; CRVE p=0.024) in the black population and vWF_{ag} (AVR p=0.015; CRVE=0.021) in the white population. The association between CRVE and CLT (p=0.008) in the white population was also confirmed. Additional negative associations were found between CRAE and CLT (p=0.014) in the black, as well as CRAE and vWF_{ag} (p=0.016) in the white population with a smaller CRAE. In the higher CRAE subdivision (Supplementary Table S4), former associations were confirmed after full adjustments and additional positive associations were found in the white population between AVR and fibrin D-dimer (p=0.034) as well as CRVE and CLT (p=0.038). No associations were found between the retinal vascular calibres and PAI-1 in either of the ethnic groups or CRAE subdivisions.

Table 4: Multiple regression analyses of the retinal vessel measurements with markers of the haemostatic system in the smaller central retinal artery equivalent.

Independent variables	**von Willebrand factor, %**			**Fibrinogen, g/L**			**Fibrin D-dimer, µg/L**			**Clot lysis time, min**		
Dependent variables	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p
Black (N=69)												
AVR		NS		0.23 0.17	-0.297 (-0.540 to -0.054)	0.021		NS			NS	
CRAE, MU		NS			NS			NS		0.37 0.28	-0.390 (-0.692 to -0.088)	0.014
CRVE, MU		NS		0.15 0.10	0.293 (0.044 to 0.542)	0.024		NS			NS	
White (N=78)												
AVR	0.21 0.18	-0.281 (-0.501 to -0.061)	0.015		NS			NS			NS	
CRAE, MU	0.40 0.34	- 0.256 (-0.458 to -0.054)	0.016		NS			NS			NS	
CRVE, MU	0.41 0.37	0.234 (0.040 to 0.428)	0.021		NS			NS		0.38 0.35	0.275 (0.079 to 0.471)	0.008

Abbreviations: AVR, arteriolar-to-venular ratio; CRAE, central retinal artery equivalent, CRVE, Central retinal vein equivalent. Adjusted for age, body mass index, gender, gamma-glutamyltransferase, current smoking, physical activity, glycosylated haemoglobin A1c, total cholesterol:high density lipoprotein ratio, 24-hour systolic blood pressure. CRAE was additionally adjusted for CRVE and *vice versa*.

4.5.3 Sensitivity analyses

The multiple regression analyses were repeated to determine if the inclusion of additional covariates (HIV status, antihypertensive, anticoagulation, anti-inflammatory medication or contraceptive usage, each entered separately) would influence the results, which was not the case.

4.6 Discussion

Retinal arteriolar narrowing relates to a higher risk for hypertension, cardiovascular disease development and mortality [28-30]. When focussing on individuals with smaller arteriolar calibres, we found that they had decreased AVR, narrower CRVE and higher blood pressures. Also, in these smaller CRAE groups, we found independent associations between specific haemostasis markers and retinal vessel calibres that differed distinctly between the black and white participants. These early vascular changes, as reflected by retinal vessel calibres, suggest an increased cardiovascular risk, especially in the black population, who is known to have an adverse cardiovascular and procoagulant profile when compared to whites [4,31].

A smaller arteriolar diameter, accompanied by a decreased AVR and narrower CRVE in both ethnicities confirms the observation of Liew *et al*. [27], who reported that individuals with narrower arterioles were also more likely to have narrower venules. In the black population the negative AVR and positive CRVE associations with fibrinogen suggest that fibrinogen, an acute phase protein, may play a role in venular widening. Previous studies that investigated older multi-ethnic populations from the United States also reported associations of AVR and CRVE with fibrinogen [11,21]. The Atherosclerosis Risk In Communities (ARIC) study [21] was the first to report a negative association between AVR and fibrinogen. Initially this association was thought to be associated with arteriolar narrowing. However, after further analyses of the vessel calibres, fibrinogen was associated with venular widening [11]. The Multi-Ethnic study of Atherosclerosis (MESA) [11], which investigated associations between biomarkers of inflammation and retinal vessel calibres, also reported a positive association between fibrinogen and CRVE. Both the ARIC and MESA study investigators suggested that fibrinogen may affect venular widening through its

role as an acute phase reactant. Although the mechanism is unclear, our result is consistent with other cohort studies that reported associations between inflammatory markers and venular widening [16,32]. It is unclear why we only found the relationship between CRVE and fibrinogen in the black population within the smaller CRAE subdivision. We may speculate that the ethnic differences may be explained by the significantly higher fibrinogen levels reported in the black population when compared to the white population [4]. Furthermore, the lack of a relationship between fibrinogen and CRVE in the larger CRAE group (supplementary Table S4) may indicate that fibrinogen only plays a role in the initial stages of venular widening. However, further investigation is needed.

Von Willebrand factor is a well-established measure of endothelial dysfunction [17,33]. We found a smaller CRAE and larger CRVE to be independently associated with vWF only in our white population within the smaller CRAE subdivision. This finding is in contrast to the ARIC study [21] which reported a positive association between AVR and vWF. This was linked to a wider arteriolar calibre after further analyses in a combined black and white population [16]. However, our negative association between CRAE and vWF supports the notion that arteriolar narrowing is accompanied by endothelial damage [14,34], which supports other findings on increased cardiovascular risk with arteriolar narrowing [9,13,35]. Notably we were unable to report any relationships between vWF and the retinal vessel calibres in the groups with a larger arteriolar calibre (supplementary Table S4). This warrants further investigation into the association between vWF and the retinal vessel calibres. It is also not clear why no associations between vWF and the retinal vessel calibres were found in the black population who had similar vWF levels as their white counterparts and a more adverse cardiovascular profile. In a previous study [36] we investigated associations between measures of arterial function and vWF in black and white South African women, not finding any associations between measures of arterial function and vWF in the black population. However, significant associations were apparent in white women with a more favourable cardiovascular profile [36]. Therefore, vWF may not be a reliable marker of cardiovascular alterations in the black South African population.

Our study is the first to report associations between the retinal vessel calibres and CLT. CLT is an indication of the plasma fibrinolytic potential of an individual, with a decreased fibrinolytic potential being associated with increased thrombotic risk [26]. The negative association with CRAE in the black population and positive association with CRVE in the white population suggest that impaired fibrinolysis may play a role in generalised arteriolar narrowing in the black population and venular widening in the white population. Furthermore, in the white population with a larger CRAE (supplementary Table S4), AVR was positively and CRVE negatively associated with fibrin D-dimer, while AVR was negatively and CRVE positively associated with CLT. This may suggest that an impaired fibrinolytic potential associates with venular widening, irrespective of retinal arteriolar calibre size. The ethnic differences in the associations of fibrinogen, vWF and CLT with the retinal vessel calibres could possibly be explained by genetic differences and/or varying systemic and environmental risk factors that were not controlled for in this study.

The present study should be interpreted within the context of its limitations and strengths. This was the first study to investigate associations between vascular calibres and the haemostatic system between two ethnic groups with similar socio-economic status in South Africa. Due to the cross-sectional nature of the study, causality cannot be inferred. Residual confounding cannot be excluded, even though the results were consistent after multiple adjustments. Our population group was only recruited from the urban areas of the Potchefstroom district in the North West Province of South Africa, therefore our findings cannot be seen as representative of the entire South African population.

We conclude that in individuals with a smaller arteriolar calibre, relationships between markers of haemostasis and the retinal vessel calibres exist that vary between ethnicities. Haemostatic alterations are linked to early retinal microvascular changes, and future studies should investigate whether it translates into elevated stroke risk.

4.7 Acknowledgements

The SABPA study would not have been possible without the voluntary collaboration of the participants and the Department of Education, North West province, South Africa. The authors

gratefully acknowledge the technical assistance of Mrs. Tina Scholtz, Sr. Chrissie Lessing and Dr. Szabolcs Péter, as well as Prof. Muriel Meiring for the analyses of vWF. Research included in the present study was partially funded by the National Research Foundation, Medical Research Council, North-West University, North-West Department of Education and ROCHE Diagnostics, South Africa; as well as the Metabolic Syndrome Institute, France. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and therefore, the NRF do not accept any liability in regard thereof.

4.8 References

1. Yusuf S, Reddy S, Ôunpuu S, Anand S. Global burden of cardiovascular diseases: Part II: variations in cardiovascular disease by specific ethnic groups and geographic regions and prevention strategies. Circulation. 2001; 104 (23):2855-64.

2. Lloyd-Sherlock P, Beard J, Minicuci N, Ebrahim S, Chatterji S. Hypertension among older adults in low- and middle-income countries: prevalence, awareness and control. Int J Epidemiol. 2014; 43 (1):116-28.

3. Mayosi BM, Flisher AJ, Lalloo UG, Sitas F, Tollman SM, Bradshaw D. The burden of non-communicable diseases in South Africa. Lancet. 2009; 374 (9693):934-47.

4. Lammertyn L, Schutte AE, Pieters M, Schutte R. D-dimer relates positively with increased blood pressure in black South Africans: The SABPA study. Thromb Res. 2014; 133 (6):1152-7.

5. Flammer J, Konieczka K, Bruno RM, Virdis A, Flammer AJ, Taddei S. The eye and the heart. Eur Heart J. 2013; 34 (17):1270-8.

6. Liew G, Wang JJ, Mitchell P, Wong TY. Retinal Vascular Imaging: A New Tool in Microvascular Disease Research. Circ Cardiovasc Imaging. 2008; 1 (2):156-61.

7. Patton N, Aslam T, MacGillivray T, Pattie A, Deary IJ, Dhillon B. Retinal vascular image analysis as a potential screening tool for cerebrovascular disease: a rationale based on homology between cerebral and retinal microvasculatures. J Anat. 2005; 206 (4):319-48.

8. Cheung CY-l, Ikram MK, Sabanayagam C, Wong TY. Retinal microvasculature as a model to study the manifestations of hypertension. Hypertension. 2012; 60 (5):1094-103.

9. Wang JJ, Rochtchina E, Liew G, Tan AG, Wong TY, Leeder SR, *et al*. The long-term relation among retinal arteriolar narrowing, blood pressure, and incident severe hypertension. Am J Epidemiol. 2008; 168 (1):80-8.

10. Leung H, Wang JJ, Rochtchina E, Tan AG, Wong TY, Klein R, *et al.* Relationships between age, blood pressure, and retinal vessel diameters in an older population. Invest Ophthalmol Vis Sci. 2003; 44 (7):2900-4.

11. Wong TY, Islam FMA, Klein R, Klein BEK, Cotch MF, Castro C, *et al.* Retinal vascular caliber, cardiovascular risk factors, and inflammation: The Multi-Ethnic Study of Atherosclerosis (MESA). Invest Ophthalmol Vis Sci. 2006; 47 (6):2341-50.

12. McGeechan K, Liew G, Macaskill P, Irwig L, Klein R, Klein BE, *et al.* Prediction of incident stroke events based on retinal vessel caliber: a systematic review and individual-participant meta-analysis. Am J Epidemiol. 2009; 170 (11):1323-32.

13. Sun C, Wang JJ, Mackey DA, Wong TY. Retinal vascular caliber: systemic, environmental, and genetic associations. Surv Ophthalmol. 2009; 54 (1):74-95.

14. Delles C, Michelson G, Harazny J, Oehmer S, Hilgers KF, Schmieder RE. Impaired endothelial function of the retinal vasculature in hypertensive patients. Stroke. 2004; 35 (6):1289-93.

15. Haefliger IO, Meyer P, Flammer J, Lüscher TF. The vascular endothelium as a regulator of the ocular circulation: a new concept in ophthalmology? Surv Ophthalmol. 1994; 39 (2):123-32.

16. Klein R, Klein BE, Knudtson MD, Wong TY, Tsai MY. Are inflammatory factors related to retinal vessel caliber?: The Beaver Dam Eye study. Arch Ophthalmol. 2006; 124 (1):87-94.

17. Lip GYH, Blann A. von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? Cardiovasc Res. 1997; 34 (2):255-65.

18. Lominadze D, Dean WL, Tyagi SC, Roberts AM. Mechanisms of fibrinogen-induced microvascular dysfunction during cardiovascular disease. Acta Physiol. 2010; 198 (1):1-13.

19. Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Semin Immunopathol.2012; 34(1): 43-62.

20. Ikram MK, Witteman JC, Vingerling JR, Breteler MM, Hofman A, de Jong PT. Retinal vessel diameters and risk of hypertension: The Rotterdam study. Hypertension. 2006; 47 (2):189-94.

21. Klein R, Sharrett AR, Klein BE, Chambless LE, Cooper LS, Hubbard LD, *et al.* Are retinal arteriolar abnormalities related to atherosclerosis?: The Atherosclerosis Risk In Communities study. Arterioscler Thromb Vasc Biol. 2000; 20 (6):1644-50.

22. Association WM. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013; 310 (20):2191.

23. Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. Curr Eye Res. 2003; 27 (3):143-9.

24. Marfell-Jones M, Olds T, Stewart A, Carter L. International standards for anthropometric assessment: International Society for the Advancement of Kinanthropometry; 2006.

25. Norton KI, Olds T, Commission AS. Anthropometrica: A textbook of body measurement for sports and health courses: University of New South Wales Press; 1996.

26. Lisman T, de Groot PG, Meijers JC, Rosendaal FR. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. Blood. 2005; 105 (3):1102-5.

27. Liew G, Wong TY, Mitchell P, Wang JJ. Are narrower or wider retinal venules associated with incident hypertension? Hypertension. 2006; 48 (2):e10.

28. Wong TY, Klein R, Klein BE, Tielsch JM, Hubbard L, Nieto FJ. Retinal microvascular abnormalities and their relationship with hypertension, cardiovascular disease, and mortality. Surv Ophthalmol. 2001; 46 (1):59-80.

29. Kawasaki R, Cheung N, Wang JJ, Klein R, Klein BE, Cotch MF, *et al.* Retinal vessel diameters and risk of hypertension: the Multi-Ethnic Study of Atherosclerosis. J Hypertens. 2009; 27 (12):2386.

30. Wang JJ, Liew G, Klein R, Rochtchina E, Knudtson MD, Klein BE, *et al.* Retinal vessel diameter and cardiovascular mortality: pooled data analysis from two older populations. Eur Heart J. 2007; 28 (16):1984-92.

31. Pieters M, Vorster HH. Nutrition and hemostasis: a focus on urbanization in South Africa. Mol Nutr Food Res. 2008; 52 (1):164-72.

32. Ikram MK, de Jong FJ, Vingerling JR, Witteman JC, Hofman A, Breteler MM, *et al*. Are retinal arteriolar or venular diameters associated with markers for cardiovascular disorders? The Rotterdam study. Invest Ophthalmol Vis Sci. 2004; 45 (7):2129-34.

33. Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. J Thromb Haemost. 2006; 4 (6):1186-93.

34. Kawagishi T, Matsuyoshi M, Emoto M, Taniwaki H, Kanda H, Okuno Y, *et al.* Impaired endothelium-dependent vascular responses of retinal and intrarenal arteries in patients with type 2 diabetes. Arterioscler Thromb Vasc Biol. 1999; 19 (10):2509-16.

35. Smith W, Wang JJ, Wong TY, Rochtchina E, Klein R, Leeder SR, *et al*. Retinal arteriolar narrowing is associated with 5-year incident severe hypertension the Blue Mountains Eye study. Hypertension. 2004; 44 (4):442-7.

36. Schutte R, Schutte AE, Van Rooyen JM, Huisman HW, Palmer IM, Fourie CM, *et al*. Von willebrand factor as marker of vascular function in south african women: the POWIRS Study. Am J Hypertens. 2008; 21 (12):1298-303.

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Data supplement

Retinal vessel calibres and haemostasis in black and white South Africans: The SABPA study

Running head: Retinal vessel calibres and haemostasis

Leandi Lammertyn[a], Aletta E Schutte[ab], Wayne Smith[a], Marlien Pieters[c] and Rudolph Schutte[ab]

[a]*Hypertension in Africa Research Team (HART), North-West University, Potchefstroom, South Africa;*

[b]*MRC Research Unit for Hypertension and Cardiovascular Disease, North-West University, Potchefstroom, South Africa.*

[c]*Centre of Excellence for Nutrition (CEN), North-West University, Potchefstroom, South Africa.*

Tables 4

Correspondence:

Rudolph Schutte, PhD

Hypertension in Africa Research Team (HART),

Private Bag x6001,

North-West University,

Potchefstroom,

2520

South Africa

Telephone: +27-18-299-2435

E-mail: rudolph.schutte@nwu.ac.za

Conflict of Interest: All authors declare no conflict of interest.

Table S1: Interaction terms with ethnicity

	Total group (n= 302)								
	AVR			CRAE, MU			CRVE, MU		
	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p
vWF*Ethnicity		NS			NS			NS	
Fibrinogen*Ethnicity		NS			NS			NS	
PAI-1*Ethnicity		NS			NS			NS	
D-dimer*Ethnicity	0.30 0.30	-6.73 (-8.26 to -5.20)	<0.001	0.09 0.08	-4.10 (-5.86 to -2.34)	<0.001		NS	
CLT*Ethnicity	0.18 0.17	-1.59 (-2.59 to -0.59)	0.002		NS		0.14 0.13	1.69 (0.67 to 2.71)	0.001

Abbreviations: AVR, arteriolar-to-venular ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; CLT, clot lysis time.

Table S2: Interaction terms with central retinal artery equivalent median split

	Total group (n= 302)								
	AVR			CRAE, MU			CRVE, MU		
	Tot R^2 / Adj R^2	β (95% CI)	P	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p
vWF*Median					-				
Fibrinogen*Median	0.26 / 0.25	-0.55 (-1.00 to -0.10)	0.014		-		0.22 / 0.21	0.76 (0.31 to 1.21)	0.001
PAI-1*Median	0.23 / 0.22	-0.36 (-0.61 to -0.11)	0.006		-		0.17 / 0.16	0.35 (0.10 to 0.60)	0.009
D-dimer*Median	0.18 / 0.17	2.47 (0.80 to 4.14)	0.004		-		0.26 / 0.25	-5.65 (-7.24 to -4.06)	<0.001
CLT*Median					-		0.20 / 0.19	-1.73 (-2.69 to -0.77)	<0.001

Abbreviations: AVR, arteriolar-to-venular ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; CLT, clot lysis time.

Table S3: Single regression analyses of retinal vessel measurements with markers of the haemostatic system in the larger central retinal artery equivalent.

	von Willebrand factor, %	Fibrinogen, g/L	Plasminogen activator inhibitor-1, ng/ml	Fibrin D-dimer, µg/L	Clot lysis time, min
			Black (N=59)		
AVR	r=-0.16 p=0.22	r=-0.05 p=0.71	r=0.08 p=0.56	**r=0.30 p=0.029**	r=-0.21 p=0.12
CRAE, MU	r=0.09 p=0.51	r=0.03 p=0.80	r=0.01 p=0.96	r=-0.01 p=0.94	r=0.02 p=0.91
CRVE, MU	r=0.22 p=0.11	r=0.04 p=0.79	r=-0.08 p=0.54	**r=-0.32 p=0.017**	r=0.21 p=0.12
			White (N=86)		
AVR	r=0.10 p=0.37	r=-0.02 p=0.85	r=0.04 p=0.72	r=0.14 p=0.20	**r=-0.28 p=0.011**
CRAE, MU	r=0.13 p=0.23	r=0.07 p=0.51	r=0.08 p=0.47	r=-0.12 p=0.27	r=-0.13 p=0.250
CRVE, MU	r=0.01 p=0.92	r=0.09 p=0.43	r=0.02 p=0.88	**r=-0.24 p=0.027**	r=0.20 p=0.070

Abbreviations: AVR, arteriolar-to-venular ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent.

Table S4: Multiple regression analyses of the retinal vessels measurements with markers of the haemostatic system in the larger central retinal artery equivalent.

Independent variables	**von Willebrand factor, %**			**Fibrinogen, g/L**			**Fibrin D-dimer, µg/L**			**Clot lysis time, min**		
Dependent variables	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p	Tot R^2 / Adj R^2	β (95% CI)	p
Black (N=59)												
AVR		NS			NS		0.24 0.20	0.268 (0.003 to 0.533)	0.053		NS	
CRAE, MU		NS			NS			NS			NS	
CRVE, MU		NS			NS		0.30 0.25	-0.245 (-0.490 to 0.0001)	0.056		NS	
White (N=86)												
AVR		NS			NS		0.27 0.21	0.217 (0.019 to 0.415)	0.034	0.26 0.21	-0.249 (-0.477 to -0.028)	0.031
CRAE, MU		NS			NS			NS			NS	
CRVE, MU		NS			NS		0.35 0.27	-0.265 (-0.457 to -0.073)	0.008	0.32 0.24	0.243 (0.018 to 0.468)	0.038

Abbreviations: AVR, arteriolar-to-venular ratio; CRAE, central retinal artery equivalent, CRVE, Central retinal vein equivalent. Adjusted for age, body mass index, gender, gamma glutamyl transferase, current smoking, physical activity, glycosylated haemoglobin A1c, total cholesterol:high density lipoprotein ratio, 24-hour systolic blood pressure. CRAE was additionally adjusted for CRVE and *vice versa*.

CHAPTER 5

General findings and conclusion

5.1 Introduction

This chapter summarises all the main findings of the three research articles. Each article's results are interpreted, compared to the relevant literature and discussed. Thereafter conclusions are drawn and recommendations made to future researchers investigating the relationship between haemostasis and cardiovascular function.

5.2 Summary of main findings and comparison with the literature

The relevant findings of each article were:

5.2.1 D-dimer relates positively with increased blood pressure in black and white South Africans (Chapter 2)

In this article I compared the haemostatic and blood pressure profiles of the black and white South African population, and investigated ethnic-specific associations between the selected haemostatic components and ambulatory blood pressure measures. The hypotheses put forward in chapter 1 stated firstly that compared to the white population, the black population exhibits higher levels of vWF, fibrinogen and fibrin D-dimer, lower levels of PAI-1 and a shorter CLT. The second hypothesis is that ethnic differences exist in the relationships of ambulatory blood pressure with the haemostatic components.

I found that the black population had higher levels of vWF, fibrinogen, PAI-1, fibrin D-dimer and a longer CLT than their white counterparts. Although the haemostatic markers were higher in the black population, they were still within their normal reference ranges [1]. The higher vWF, fibrinogen and fibrin D-dimer in the black population confirms previous findings [2-4]. However, the higher PAI-1 reported in this group is in contrast with the literature that reports lower PAI-1 in black compared to white populations [5,6]. To our knowledge, this is the first study to investigate ethnic differences in CLT. The longer CLT in the black population adds to the literature and could possibly be a result of the higher PAI-1 levels observed in this group. Therefore, the first hypothesis is only partially accepted due to PAI-1 being higher and CLT longer in the black population.

Moreover, positive associations independent of known cardiovascular risk factors existed between all the ambulatory blood pressure measures and fibrin D-dimer in the black population, which confirms the observation of Khaleghi *et al*. [7] in black hypertensive individuals. In the white population, independent inverse associations were reported between all the ambulatory blood pressure measures and CLT. These associations are in contrast with Meltzer *et al*. [8] who reported a positive association between blood pressure and CLT. Therefore, the second hypothesis is accepted since ethnic-specific relationships were found between the haemostatic components and ambulatory blood pressure measures.

5.2.2 Ethnic-specific relationships between haemostatic and oxidative stress markers in black and white South Africans (Chapter 3)

I investigated associations of oxidative stress (serum peroxides) and antioxidant markers (GSH, GPx and GR activity) with the components of the haemostatic system in black and white South Africans to determine if ethnic differences exist. We hypothesised (chapter 1) that the oxidative stress and antioxidant markers are associated with the haemostatic components in both ethnicities, with more adverse associations seen in the black population.

I found that the black population had a mixed oxidant-antioxidant profile with significantly higher serum peroxides, GSH and GR activity and lower GPx activity levels. Low levels of GPx activity is an indicator of a decreased antioxidant capacity [9]. When oxidative stress supersedes the antioxidant capacity, damage may occur to the endothelial cells, which results in the release of several prothrombotic factors [10]. In the black population, vWF and GPx activity correlated negatively, which confirms the findings of Blann *et al*. [9] that elevated vWF is accompanied by a decreased antioxidant capacity. A negative association between CLT and GPx activity also existed in the black population and adds to the existing literature by showing that decreased GPx activity is accompanied by an impaired fibrinolytic potential. Furthermore, in the black population the negative associations of vWF and PAI-1 with total GSH also confirm previous findings [11,12]. In addition, the positive associations of vWF and CLT with GR activity and negative association

with GPx activity in the black population, confirms the observation of Rybka *et al*. [13] that decreased GPx activity is accompanied by an increase in GR activity.

Serum peroxides were positively associated with fibrinogen in both ethnic groups in multiple regression analyses. Several *in vivo* studies show fibrinogen to be highly susceptible to oxidative stress [14,15]. However epidemiological data regarding this association is scant. Our study adds to the existing literature by confirming the relationship between fibrinogen and oxidative stress in both ethnic groups. In addition, the positive associations of serum peroxides with fibrinogen and CLT in the white population confirm the observation of Vadseth *et al*. [16] that a fibrin clot formed in an oxidised environment is more resilient to lysis. It is unclear why there was no association between serum peroxides and CLT in the black population who had higher levels of serum peroxides and a longer CLT than their white counterparts. Although the oxidative stress and antioxidant markers were more adversely related to the haemostatic components in the black population, the hypothesis is only partially accepted, since no independent associations were found between the haemostatic components and GSH and GPx activity that are markers of antioxidant capacity in the white population.

5.2.3 Retinal vessel calibres and haemostasis in black and white South Africans (Chapter 4)

Individuals with narrower arteriolar calibres tend to be at an increased CVD risk [17,18]. Therefore, I investigated associations between retinal vessel calibres and the haemostatic markers in individuals with a smaller CRAE diameter. A median split of CRAE was performed to identify indiviuals with narrow arterioles based on our aims and interactions found between the vascular calibres and haemostatic factors. The black population of the SABPA study has a worsened cardiovascular profile when compared to their white counterparts [19], and early vascular changes have already been observed in the black group by other investigators [20]. Therefore, the hypothesis was (chapter 1) that the retinal vessel calibres are associated with the haemostatic components in the black population.

In both ethnic groups, individuals with a smaller CRAE diameter also had narrower venular calibres and lower AVR when compared to individuals with a larger CRAE diameter. This result confirms a previous observation that individuals with narrower arterioles also tend to have narrower venules [21]. After performing forward stepwise multiple regression analyses, AVR was negatively and CRVE positively associated with fibrinogen in the black population. These results confirm findings from the ARIC study that associated fibrinogen with a decreased AVR [17], which was later reported to be due to venular widening after further analyses of the vessel calibres were performed [22]. Furthermore, in the white population, inverse associations existed between AVR, CRAE and vWF. This is in contrast with a previous study by Klein *et al*. [17] who reported a positive association between AVR and vWF, which was related to a wider arteriolar calibre [23]. Our study is the first to report associations between the retinal vessel calibres and CLT and indicates that narrower arterioles in the black population and wider venules in the white population in individuals with a smaller CRAE relates to a decreased fibrinolytic rate. The hypothesis is therefore rejected, since significant associations between the components of the haemostatic system and retinal vessel calibres were found in both ethnicities.

The results of the three articles both confirm and contradict existing knowledge, and add valuable insight to the relationship between components of the haemostatic system and cardiovascular function in black and white South Africans.

5.3 Chance and confounding

When reviewing the results obtained in the above-mentioned research the possibility of chance findings should be taken into account. Partial and forward stepwise regression analyses indicate that one out of twenty significant correlations might be due to chance. Confounders such as age, gender, body mass index, HbA1c, γ-GT, TC:HDL-C, physical activity and current smoking, in the partial and multiple regression analyses, could have influenced the results by causing over- or underestimation of the associations between the different haemostatic markers and cardiovascular function investigated in this study. Therefore, the number of covariates that could influence the relationship was kept to a minimum and only one covariate for every ten participants

was allowed. Furthermore, additional potential confounders, such as dietary intake, psychosocial stress, medication use and genetic characteristics were not taken into consideration and may have affected the outcome of this research.

5.4 Strengths and limitations

It is crucial to reflect on some of the strengths and limitations of our study.

5.4.1 Strengths

- The population group was equally distributed along ethnicity, gender and socio-economic status. This allowed us to compare ethnic as well as gender groups with the same socio-economic standing.
- The participants were fairly young (mean age of 45 years), allowing us to investigate relationships between the components of the haemostatic system and cardiovascular function in a general population from the working class.
- The measurements were conducted under controlled conditions in a Metabolic Research Unit to assure environmental stability such as room temperature, activity levels and dietary intake.
- A strict protocol was followed by trained research personnel to ensure the quality of all the measurements taken.
- Measurements were only conducted during the months of February and May to control for seasonal changes.
- An ambulatory blood pressure monitor and accredited physical activity accelerator device were worn by the participants at their place of work to get a representation of their blood pressure and physical activity profiles during a normal working day.
- Participants received standardised meals the day before the commencement of the measurements in an attempt to for control short-term dietary intake that could have influenced the results.
- Blood was drawn before 10:00 in the morning to control for diurnal changes.

- Blood samples were immediately sent to an onsite laboratory for processing, and stored at -80ºC to assure sample stability.

5.4.2 Limitations

- The study consisted of a relatively small sample size (n=409). However, adequate statistical power was obtained.
- South Africa is a country rich in culture and diversity, thus our population which was only recruited from the Potchefstroom district in the North West Province, cannot be seen as a representation of the entire South African population.
- Cause and effect cannot be determined, since the data was analysed cross-sectionally for all the articles. Although prospective data was available for the third article, the data was still examined cross-sectionally, since minimal change was observed with the haemostatic components and cardiovascular measurements during the short (three year) follow-up period. The microvascular data was also not available for baseline.
- The participant's blood group were not determined for this study. Therefore, we could not determine whether the variations in mean vWF levels between the ethnic groups were affected by different blood type profiles.
- The antigen levels of vWF and PAI-1 were measured instead of their activity levels. Furthermore, not all the components of the haemostatic and oxidant-antioxidant systems could be determined due to limited samples and funding.

5.5 Discussion of main findings

It is known that the black population has a high cardiovascular mortality rate, especially from stroke [24], and tends to suffer from heart failure rather than coronary heart disease [25]. The increased mortality rates from CVD in the black population are accompanied by a high prevalence of hypertension and diabetes mellitus [26]. Moreover, changes in the haemostatic system also play a primary role in CVD states [27-29] and elevated levels of vWF [30], fibrinogen [3], PAI-1 [31] and fibrin D-dimer [32] as well as a longer CLT [8] relates to CVD. Individuals from African

descent tend to have higher levels of vWF [2], fibrinogen [3] and D-dimer [33] that may contribute to their increased cardiovascular risk. However, lower PAI-1 activity levels have been reported in Africans when compared to their European counterparts [6]. This may counteract the effect of the elevated coagulation markers. To my knowledge, only a limited number of studies exist that compared the coagulation and fibrinolytic profiles with cardiovascular function in black and white South Africans. Therefore, I attempted to determine circulating levels of several components of both the coagulation and fibrinolytic systems to investigate the haemostatic profiles of both the black and white population. I also investigated the relationship of the haemostatic markers with blood pressure measures, oxidant-antioxidant markers and the retinal vessel calibres in both ethnic groups (Chapter 1, Figure 15). This was done to determine whether adverse relationships exist between the haemostatic markers and markers of cardiovascular function, which may increase the population's susceptibility towards CVD.

In the black population, the higher coagulation levels and inhibited fibrinolysis, when compared to their white counterparts, indicate an increased thrombotic risk. Notably, the prothrombotic profile in the black population was also accompanied by higher ambulatory blood pressure. Available evidence from European countries suggest that elevated blood pressure contributes to all the components of Virchow's triad [34,35] and is accompanied by elevated circulating haemostatic markers [36-39]. Therefore, the link found between D-dimer and blood pressure in the black population suggests that D-dimer, a marker of increased thrombin and/or fibrin turnover, may contribute to hypertension in this population or *vice versa*. Elevated D-dimer is suggested to exert an adverse effect on the surrounding endothelial cells, consequently promoting vasoconstriction of the blood vessels, thereby contributing to increased blood pressure [40]. However, in the white population, shorter CLT's were observed in individuals with higher blood pressure, which is in contrast with the existing literature [8] and warrants further investigation.

Endothelial dysfunction, initiated by increased inflammation and oxidative stress, results in the suppression of anticoagulant activity and the promotion of procoagulant activity [41,42]. After investigation of the oxidant-antioxidant system on haemostatic markers, it became evident that

increased oxidative stress (reflected by increased serum peroxides), together with a diminished antioxidant capacity (reflected by decreased GPx activity), accompany a prothrombotic profile in the black population. In the white population, oxidative stress seems to be related to an increase in circulating fibrinogen, as well as impaired fibrin clot lysis. The impaired clot lysis was probably due to the formation of a more resilient fibrin clot formed in the presence of an oxidative environment and in combination with increased fibrinogen concentrations [16].

The investigation of the retinal vessel calibres offered me the opportunity to investigate the microvasculature and its relationship with the haemostatic markers, with an approach that have not yet been implemented in our population. Our findings provided valuable information regarding the susceptibility of the microvascular system for systemic diseases in individuals who are known to be at increased CVD risk due to arteriolar narrowing. In individuals who have an increased susceptibility for hypertension development, such as the black population of South Africa [25,43], arterial narrowing may be of particular importance. Arteriolar narrowing was shown to precede the development of hypertension and is also considered an early feature of mild hypertensive retinopathy [44,45]. It was evident that in individuals with a smaller CRAE diameter, fibrinogen was related to venular widening in the black population, possibly through its role as an acute phase reactant. Previous studies investigating the influence of several inflammatory markers, including fibrinogen on the retinal vessel calibres, observed that inflammation is linked to venular widening [22,23]. In the white population, vWF was related to both arteriolar narrowing and venular widening, which indicates that endothelial dysfunction may contribute to early retinal vascular changes. These early vascular changes in both the black and white population may also result in the formation of fibrin clots that lyse at a slower rate, since associations were observed between CLT and arteriolar narrowing in the black and venular widening in the white population.

Although our study population was relatively small and the results cannot be generalised to the whole population of South Africa, valuable insight regarding the haemostatic profile of both black and white South Africans were obtained. The black population has a prothrombotic profile that is related to a compromised oxidant-antioxidant system, retinal vessel calibre changes and

increased blood pressure. However, in the white population adverse associations were also present with several haemostatic markers associating with oxidative stress and the retinal vessel calibres.

5.6 Recommendations for future studies

The following recommendations are made to future researchers investigating the haemostatic system and cardiovascular function.

- Additional components of the haemostatic system, such as thrombin generation, prothrombin fragment 1 and 2, t-PA activity, plasmin-anti-plasmin complex and thrombin-activatable fibrinolysis inhibitor should be determined to get a clearer indication on whether increased thrombotic risk is due to increased thrombus formation or decreased fibrinolysis.
- Further investigation into the fibrinolytic process as well as the fibrin clot properties are needed, particularly in the white population in whom associations were found between CLT and blood pressure, oxidative stress and retinal venular widening.
- Additional components of the oxidant-antioxidant system are needed to establish whether an impaired oxidant-antioxidant system exist in the black population and whether it is related to an impaired haemostatic profile, which may increase their susceptibility to CVD. Furthermore, investigation into the role of the inflammatory system and oxidative stress on the release of fibrinogen as well as the influence that oxidative stress may have on fibrinogen functionality is needed to determine to what extent oxidative stress may alter fibrinogen's properties.
- Emphasis must be placed on the investigation of the relationship between the haemostatic system and early microvascular changes, to identify early structural and functional changes that are accompanied by changes in the haemostatic system before the prominent onset of vascular disease.

- Larger prospective cohort studies that monitor the relationship between the haemostatic markers and cardiovascular function in young disease-free individuals are needed to determine which markers are early indicators of cardiovascular risk. Experimental studies are also needed to determine cause and effect of an impaired coagulation or fibrinolytic system on the cardiovascular system. By doing so, recommendations can be made for preventive and/or therapeutic interventions.

5.7 Conclusion

Evidence from this thesis confirm that the black population displays elevated ambulatory blood pressures, accompanied by a compromised oxidant-antioxidant system (increased serum peroxides and decreased GPx activity) and early vascular changes (decreased AVR, narrower CRAE and wider CRVE). The findings from this thesis further indicate that the black population has higher circulating haemostatic markers (vWF, fibrinogen, fibrin D-dimer, PAI-1) that is accompanied by a decreased fibrinolytic potential, which points towards a prothrombotic profile in the black South Africans. Moreover, the haemostatic markers were independently related to elevated blood pressures, a compromised oxidant-antioxidant system and retinal vessel calibres, suggesting that the prothrombotic state of the black population may contribute, at least in part, to the high cardiovascular and cerebrovascular risk observed in South Africa.

5.8 References

1. Kratz A, Ferraro M, Sluss PM, Lewandrowski KB. Normal reference laboratory values. New Engl J Med. 2004; 351 (15):1548-63.

2. Sukhu K, Poovalingam V, Mahomed R, Giangrande PL. Ethnic variation in von Willebrand factor levels can influence the diagnosis of von Willebrand disease. Clin Lab Haematol. 2003; 25 (4):247-9.

3. Fibrinogen Collaboration Studies. Collaborative meta-analysis of prospective studies of plasma fibrinogen and cardiovascular disease. Eur J Cardiovasc Prev Rehabil. 2004; 11 (1):9-17.

4. Lutsey PL, Cushman M, Steffen LM, Green D, Barr RG, Herrington D, *et al*. Plasma hemostatic factors and endothelial markers in four racial/ethnic groups: the MESA study. J Thromb Haemost. 2006; 4 (12):2629-35.

5. Festa A, D'Agostino R, Jr., Rich SS, Jenny NS, Tracy RP, Haffner SM. Promoter (4G/5G) plasminogen activator inhibitor-1 genotype and plasminogen activator inhibitor-1 levels in blacks, Hispanics, and non-Hispanic whites: The Insulin Resistance Atherosclerosis study. Circulation. 2003; 107 (19):2422-7.

6. Greyling A, Pieters M, Hoekstra T, Oosthuizen W, Schutte AE. Differences in the association of PAI-1 activity with the metabolic syndrome between African and Caucasian women. Nutr Metab Cardiovasc Dis. 2007; 17 (7):499-507.

7. Khaleghi M, Saleem U, McBane RD, Mosley Jr TH, Kullo IJ. African-American ethnicity is associated with higher plasma levels of D-dimer in adults with hypertension. J Thromb Haemost. 2009; 7 (1):34-40.

8. Meltzer ME, Doggen CJM, De Groot PG, Rosendaal FR, Lisman T. Reduced plasma fibrinolytic capacity as a potential risk factor for a first myocardial infarction in young men. Brit J Haematol. 2009; 145 (1):121-7.

9. Blann AD, Maxwell SR, Burrows G, Miller JP. Antioxidants, von Willebrand factor and endothelial cell injury in hypercholesterolaemia and vascular disease. Atherosclerosis. 1995; 116 (2):191-8.

10. Kvietys PR, Granger DN. Role of reactive oxygen and nitrogen species in the vascular responses to inflammation. Free Radic Biol Med. 2012; 52 (3):556-92.

11. Ibrahim H, El-Meligi A, Abdel-Hamid M, Elhendy A. Relations between von Willebrand factor, markers of oxidative stress and microalbuminuria in patients with type 2 diabetes mellitus. Med Sci Monit. 2004; 10 (3):CR85-9.

12. Martina V, Bruno GA, Pannocchia A, Zumpano E, Tagliabue M, Trucco F, *et al*. PAI-1 reduction after treatment with glutathione in NIDDM. Fibrinolysis. 1996; 10, 2 (0):63-5.

13. Rybka J, Kupczyk D, Kędziora-Kornatowska K, Motyl J, Czuczejko J, Szewczyk-Golec K, *et al*. Glutathione-related antioxidant defense system in elderly patients treated for hypertension. Cardiovasc Toxicol. 2011; 11 (1):1-9.

14. Shacter E, Williams JA, Levine RL. Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. Free Radic Biol Med. 1995; 18 (4):815-21.

15. Rosenfeld MA, Shegolihin AN, Bychkova AV, Leonova VB, Biryukova MI, Kostanova EA, *et al*. Oxidative modification of fibrinogen molecules. Dokl Biochem Biophys. 2013; 452 (1):255-8.

16. Vadseth C, Souza JM, Thomson L, Seagraves A, Nagaswami C, Scheiner T, *et al*. Pro-thrombotic State Induced by Post-translational modification of fibrinogen by reactive nitrogen species. J Biol Chem. 2004; 279 (10):8820-6.

17. Klein R, Sharrett AR, Klein BE, Chambless LE, Cooper LS, Hubbard LD, *et al*. Are retinal arteriolar abnormalities related to atherosclerosis?: The Atherosclerosis Risk In Communities study. Arterioscler Thromb Vasc Biol. 2000; 20 (6):1644-50.

18. Wang JJ, Rochtchina E, Liew G, Tan AG, Wong TY, Leeder SR, *et al*. The long-term relation among retinal arteriolar narrowing, blood pressure, and incident severe hypertension. Am J Epidemiol. 2008; 168 (1):80-8.

19. Lammertyn L, Schutte R, Schutte AE, Huisman HW, van Rooyen JM, Malan NT, *et al*. Associations of cholesterol and glucose with cardiovascular dysfunction in black Africans: the SABPA study. Clin Exp Hypertens. 2011; 33 (3):159-66.

20. Schutte R, Schutte AE, Huisman HW, van Rooyen JM, Malan NT, Péter S, *et al*. Blood glutathione and subclinical atherosclerosis in African men: the SABPA study. Am J Hypertens. 2009; 22 (11):1154-9.

21. Liew G, Wong TY, Mitchell P, Wang JJ. Are narrower or wider retinal venules associated with incident hypertension? Hypertension. 2006; 48 (2):e10.

22. Wong TY, Islam FMA, Klein R, Klein BEK, Cotch MF, Castro C, *et al*. Retinal vascular caliber, cardiovascular risk factors, and inflammation: The Multi-Ethnic Study of Atherosclerosis (MESA). Invest Ophthalmol Vis Sci. 2006; 47 (6):2341-50.

23. Klein R, Klein BE, Knudtson MD, Wong TY, Tsai MY. Are inflammatory factors related to retinal vessel caliber?: The Beaver Dam Eye study. Arch Ophthalmol. 2006; 124 (1):87-94.

24. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, *et al*. AHA statistical update. Circulation. 2013; 129:e28-292.

25. Sliwa K, Wilkinson D, Hansen C, Ntyintyane L, Tibazarwa K, Becker A, *et al*. Spectrum of heart disease and risk factors in a black urban population in South Africa (the Heart of Soweto Study): a cohort study. Lancet. 2008; 371 (9616):915-22.

26. Kurian AK, Cardarelli KM. Racial and ethnic differences in cardiovascular disease risk factors: a systematic review. Ethn Dis. 2007; (17):143-52.

27. Smith FB, Lee AJ, Fowkes FGR, Price JF, Rumley A, Lowe GDO. Hemostatic factors as predictors of ischemic heart disease and stroke in the Edinburgh artery study. Arterioscler Thromb Vasc Biol. 1997; 17 (11):3321-5.

28. Folsom AR, Rosamond WD, Shahar E, Cooper LS, Aleksic N, Nieto FJ, *et al*. Prospective study of markers of hemostatic function with risk of ischemic stroke. The Atherosclerosis Risk In Communities (ARIC) study. Circulation. 1999; 100 (7):736-42.

29. Smith A, Patterson C, Yarnell J, Rumley A, Ben-Shlomo Y, Lowe G. Which hemostatic markers add to the predictive value of conventional risk factors for coronary heart disease and ischemic stroke? The Caerphilly Study. Circulation. 2005; 112 (20):3080-7.

30. Cooney M-T, Dudina AL, O'Callaghan P, Graham IM. von Willebrand factor in CHD and stroke: Relationships and therapeutic implications. Curr Treat Options Cardiovasc Med. 2007; 9 (3):180-90.

31. Vaughan DE. PAI-1 and atherothrombosis. J Thromb Haemost. 2005; 3 (8):1879-83.

32. Wannamethee SG, Whincup PH, Lennon L, Rumley A, Lowe GD. Fibrin D-dimer, tissue-type plasminogen activator, von Willebrand factor, and risk of incident stroke in older men. Stroke. 2012; 43 (5):1206-11.

33. Hughes R, Thomson K, Hopkins R, Weatherall M, Wiltshire C, Wilsher M, *et al*. Determinants of plasma D-dimer levels in a traveling population. J Thromb Haemost. 2005; 3 (11):2445-8.

34. Lip GY, Li-Saw-Hee FL. Does hypertension confer a hypercoagulable state? J Hypertens. 1998; 16 (7):913-6.

35. Varughese G, Lip GH. Is hypertension a prothrombotic state? Curr Science. 2005; 7 (3):168-73.

36. Blann AD, Naqvi T, Waite M, McCollum CN. von Willebrand factor and endothelial damage in essential hypertension. J Hum Hypertens. 1993; 7 (2):107-11.

37. Arikan E, Sen S. Endothelial damage and hemostatic markers in patients with uncomplicated mild-to-moderate hypertension and relationship with risk factors. Clin Appl Thromb Hemost. 2005; 11 (2):147-59.

38. Poli KA, Tofler GH, Larson MG, Evans JC, Sutherland PA, Lipinska I, *et al*. Association of blood pressure with fibrinolytic potential in the Framingham offspring population. Circulation. 2000; 101 (3):264-9.

39. Cigolini M, Targher G, Seidell JC, Tonoli M, Schiavon R, Agostino G, *et al*. Relationships of blood pressure to fibrinolysis: influence of anthropometry, metabolic profile and behavioural variables. J Hypertens. 1995; 13 (6):659-66.

40. Yevdokimova NY, Veselovska LD, Gogolinska GK, Buchanevich OM, Kosyakova GV, Gritsenko PG, *et al*. Fibrin D-dimer impairs the accumulation and anticoagulant properties of

heparan sulphate and stimulates secretion of plasminogen activator inhibitor-1 by rabbit coronary endothelial cells. Acta Biochim Pol. 2003; 50 (1):279-89.

41. Wu M, KK, Thiagarajan M, P. Role of endothelium in thrombosis and hemostasis. Ann Rev Med. 1996; 47 (1):315-31.

42. Widlansky ME, Gokce N, Keaney JF, Vita JA. The clinical implications of endothelial dysfunction. J Am Coll Cardiol. 2003; 42 (7):1149-60.

43. Lloyd-Sherlock P, Beard J, Minicuci N, Ebrahim S, Chatterji S. Hypertension among older adults in low- and middle-income countries: prevalence, awareness and control. Int J Epidemiol. 2014; 43 (1):116-28.

44. Ikram MK, Witteman JC, Vingerling JR, Breteler MM, Hofman A, de Jong PT. Retinal vessel diameters and risk of hypertension: the Rotterdam study. Hypertension. 2006; 47 (2):189-94.

45. Cheung CY-l, Ikram MK, Sabanayagam C, Wong TY. Retinal microvasculature as a model to study the manifestations of hypertension. Hypertension. 2012; 60 (5):1094-103.

Annexure A

Turn it in originality reports

Turnitin Originality Reports

Chapter 1

TI (333228f1-215f-42c0-9c54-f0f1589fe06e)

- Processed on 27-Apr-2015 20:26 SAST
- ID: 534328483
- Word Count: 7090

Similarity Index: **7%**
Similarity by Source
Internet Sources: 2%
Publications: 9%
Student Papers: 1%

Chapter 2

TI (333228f1-215f-42c0-9c54-f0f1589fe06e)

- Processed on 27-Apr-2015 20:26 SAST
- ID: 534328521
- Word Count: 3769

Similarity Index: **26%**
Similarity by Source
Internet Sources: 13%
Publications: 27%
Student Papers: 12%

Chapter 3

TI (333228f1-215f-42c0-9c54-f0f1589fe06e)

- Processed on 27-Apr-2015 20:30 SAST
- ID: 534330731
- Word Count: 3984

Similarity Index: **21%**
Similarity by Source
Internet Sources: 8%
Publications: 20%
Student Papers: 8%

Chapter 4

TI (333228f1-215f-42c0-9c54-f0f1589fe06e)

- Processed on 27-Apr-2015 20:41 SAST
- ID: 534335457
- Word Count: 4511

Similarity Index: **14%**

Similarity by Source

Internet Sources: 8%

Publications: 15%

Student Papers: 11%

Chapter 5

TI (333228f1-215f-42c0-9c54-f0f1589fe06e)

- Processed on 27-Apr-2015 20:40 SAST
- ID: 534335489
- Word Count: 3013

Similarity Index: **1%**

Similarity by Source

Internet Sources: 0%

Publications: 1%

Student Papers: 0%

Annexure B

Declaration of language editing

cumlaude
languagepractitioners

Director: CME Terblanche - BA (Pol Sc), BA Hons (Eng), MA (Eng), TEFL

22 Strydom Street
Baillie Park, 2531

Tel 082 821 3083
cumlaudelanguage@gmail.com

23 April 2015

DECLARATION OF LANGUAGE EDITING

I, Christina Maria Etrecia Terblanche, hereby declare that I edited the thesis entitled:

Haemostatic markers and cardiovascular function in black and white South Africans: The SABPA study

for L. Lammertyn for the purposes of submission as a postgraduate study. No changes were permanently affected and were left to the discretion of the student.

Regards,

CME Terblanche

Cum Laude Language Practitioners (CC)

SATI accr nr: 1001066

PEG registered