

# **Endothelial function and vascular health of black South Africans: The significance of plasminogen activator inhibitor-1**

**A Jacobs**



**orcid.org/0000-0003-1122-8249**

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Promoter: Prof AE Schutte

Co-promoter: Prof M Pieters

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Student number: 13062778

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## PREFACE

This thesis, titled “Endothelial function and vascular health of black South Africans: The significance of plasminogen activator inhibitor-1”, is presented in article format in accordance with the guidelines of the North-West University and comprises six chapters, as outlined below. The research articles included in this thesis were submitted to or published in international peer-reviewed journals.

### *Chapter layout of this thesis:*

Chapter 1: Literature overview, motivation, aims, objectives and hypotheses

Chapter 2: Study design and methodology

Chapter 3: Research article 1 (submitted to *Microvascular Research*)

Chapter 4: Research article 2 (published in *Nutrition, Metabolism & Cardiovascular Diseases*)

Chapter 5: Research article 3 (published in *Journal of Hypertension*)

Chapter 6: Summary of main findings and conclusion

The *Vancouver* referencing style was used throughout the thesis, except for chapters 3–5 where the prescribed styles according to the author instructions of the above-mentioned journals were used.

In order to improve the legibility and flow of this thesis for examination purposes, I deviated from certain journals’ author instructions by inserting tables and figures in the text, by keeping the line spacing consistent throughout and by not numbering the sections and subsections in the research article chapters. Text throughout the thesis has also been justified.

## **AUTHOR CONTRIBUTIONS**

### **Mr A Jacobs**

Mr Jacobs, as the PhD candidate, was involved in the initial design and planning of this PhD study, as well as data collection and analysis in both the African-PREDICT and PURE-SA studies. He was involved in the design and planning of the research articles, as well as the interpretation of results. He was responsible for conducting the literature review, writing of the research articles and all sections of this thesis, and for the statistical analyses of research articles 1 and 2 (Chapters 3 and 4).

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Prof. Schutte, as promoter and Principal Investigator of the African-PREDICT study, supervised and provided intellectual and technical input throughout all stages of this PhD study. This includes being involved in the initial design and planning of this PhD study and all research articles, interpretation of results, critical evaluation of and contribution to all research articles and the final thesis.

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### **Prof. W Smith**

As a co-author of research article 1 (Chapter 3), Prof. Smith contributed to the planning of the article, interpretation of results, provided intellectual input regarding the retinal microvasculature and critically revised the article.

### **Dr C Ricci**

As a co-author of research article 3 (Chapter 5) and an expert biostatistician, Dr Ricci conducted the statistical analysis for this article, aided in interpretation of the results and provided intellectual input.

## Statement by the co-authors

The following is a statement from the co-authors verifying their individual roles in the study and giving their permission that the research articles may form part of this thesis:

*Hereby, I declare that I 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 PhD thesis of Mr Adriaan Jacobs.*



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Professor AE Schutte



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Professor M Pieters



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Doctor C Ricci



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Professor W Smith

## PUBLICATIONS RELATED TO PhD STUDY

Jacobs A, Schutte AE, Ricci C, Pieters M. Plasminogen activator inhibitor-1 activity and the 4G/5G polymorphism are prospectively associated with blood pressure and hypertension status. *Journal of Hypertension*. 2019 Dec;37(12):2361-2370; doi: 10.1097/HJH.0000000000002204.  
[https://journals.lww.com/jhypertension/Fulltext/2019/12000/Plasminogen\\_activator\\_inhibitor\\_1\\_activity\\_and\\_the.8.aspx](https://journals.lww.com/jhypertension/Fulltext/2019/12000/Plasminogen_activator_inhibitor_1_activity_and_the.8.aspx)

Jacobs A, Pieters M, Schutte AE. The association of PAI-1 with 24 h blood pressure in young healthy adults is influenced by smoking and alcohol use: The African-PREDICT study. *Nutrition, Metabolism and Cardiovascular Diseases*. 2020;30:2063-71; doi.org/10.1016/j.numecd.2020.07.003.  
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## **SUMMARY**

### **Background and motivation**

Plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor known for its classic role in fibrinolysis, has in recent years gained attention for its association with impaired vascular function and blood pressure. On a genetic level, the 4G/5G polymorphism in the promoter region of the PAI-1 (*SERPINE1*) gene, known to influence PAI-1 levels, has also been associated with blood pressure. It is speculated that microvascular dysfunction not only accompanies, but possibly also precedes, macrovascular dysfunction. Yet limited studies have investigated the association of microvascular function with PAI-1. The observed association of PAI-1 with blood pressure is partly attributed to PAI-1's alternative roles in endothelial dysfunction and vascular remodelling. Whether elevations in PAI-1 precede or result from elevated blood pressure is still an ongoing debate, and prospective studies that may shed light on the sequence of events are limited. The majority of previous studies in this field were performed in older individuals and in patients already presenting with hypertension, with very limited information on the potential role of PAI-1 in the early phases of cardiovascular disease development.

Numerous modifiable and non-modifiable factors are known to influence PAI-1 levels. These include health behaviours such as smoking, alcohol consumption and obesity, especially central obesity, and non-modifiable factors such as sex, genetics and Black ethnicity. The extent to which these determinants of PAI-1 influence the associations of PAI-1 with blood pressure and microvascular function is largely unknown.

Black South Africans are prone to hypertension development and are known to display unique cardiovascular and haemostatic profiles. The investigation of the associations of PAI-1 (as a potential early marker of vascular dysfunction) with retinal microvascular function and blood pressure across the life course of Black South Africans will aid in elucidating the relationship of PAI-1 with micro- and macrovascular function related to hypertension.

### **Aim**

The overarching aim of this study was to investigate the associations of blood pressure and microvascular function with PAI-1 in Black South Africans, and to investigate to what extent these associations are influenced by selected modifiable and non-modifiable factors.

## Methodology

Data from two studies were used: The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT), which included Black and White individuals aged 20–30 years, and the South African arm of the Prospective Urban and Rural Epidemiological (PURE) study, which included Black individuals older than 30 years of age.

In young, apparently healthy individuals we investigated the associations of plasma PAI-1 activity ( $\text{PAI-1}_{\text{act}}$ ) with retinal vasodilatory responses to flicker light provocation. We furthermore investigated the association of  $\text{PAI-1}_{\text{act}}$  with 24-hour blood pressure (24 h BP) and determined the influence of smoking and alcohol use on these associations. In the older cohort of Black individuals we investigated the associations of  $\text{PAI-1}_{\text{act}}$  and the 4G/5G polymorphism with brachial and central blood pressure measures, as well as with hypertension status, in both cross-sectional and prospective analysis (over a 10-year period).

## Results

In young individuals (aged 20–30 years), maximal retinal venular dilation was independently and inversely associated with  $\text{PAI-1}_{\text{act}}$  (Adj.  $R^2=0.11$ ;  $\beta=-0.15$ ;  $p=0.001$ ) in the total group ( $n=518$ ). In exploratory subgroup analysis, this association persisted in White women only (Adj.  $R^2=0.07$ ;  $\beta=-0.23$ ;  $p=0.005$ ) and was more robust in non-smokers and individuals of younger age, those with lower blood pressure, those with higher low-density lipoprotein cholesterol and also in individuals with higher levels of inflammation and greater central adiposity (all  $p<0.05$ ).

In the same young cohort ( $n=1156$ ), 24 h blood pressure measures (systolic and diastolic blood pressure [SBP and DBP] as well as pulse pressure [PP]) were positively associated with  $\text{PAI-1}_{\text{act}}$  in age-adjusted models in most groups (split for sex and ethnicity) ( $p<0.05$ ). Upon multivariate adjustment and stratification based on self-reported alcohol use and smoking, the 24 h BP– $\text{PAI-1}_{\text{act}}$  association persisted only in Black men who consumed alcohol (24 h SBP [ $B=4.22$ ,  $p<0.001$ ], DBP [ $B=2.04$ ,  $p=0.015$ ] and PP [ $B=2.18$ ,  $p=0.013$ ]) and smoked (24 h SBP [ $B=6.10$ ,  $p<0.001$ ] and PP [ $B=4.33$ ,  $p=0.001$ ]).

In the older PURE cohort of Black individuals,  $\text{PAI-1}_{\text{act}}$  associated positively with brachial and central blood pressure measures at most time points in cross-sectional analysis. In multivariate analysis,  $\text{PAI-1}_{\text{act}}$  independently and prospectively associated with brachial SBP ( $r=0.0815$ ) and PP ( $r=0.0832$ ) in the total group, and with central PP in women ( $r=0.1125$ ; all  $p<0.05$ ). Furthermore, both elevated  $\text{PAI-1}_{\text{act}}$  and the 4G/4G (vs the 5G/5G) genotype increased the odds of hypertension development in the total group (1.04 [1.01; 1.08] and

1.82 [1.07; 3.12], respectively). The presence of central obesity either decreased or nullified the associations of PAI-1<sub>act</sub> with BP and hypertension development.

## **Conclusion**

In young apparently healthy adults, PAI-1 was positively associated with 24 h blood pressure and impaired retinal microvascular function. In older individuals, PAI-1 was positively associated with brachial and central blood pressure in both cross-sectional and prospective analysis, and both elevated PAI-1 levels and the 4G/4G genotype increased the odds of developing hypertension. These associations differ in women and men and between ethnicities, and are influenced by modifiable factors such as central obesity, smoking and alcohol consumption. Our data provide evidence that PAI-1 is associated with microvascular function, blood pressure and hypertension development throughout the life course.

## **Keywords**

Blood pressure; hypertension; microvascular function; plasminogen activator inhibitor-1; 4G/5G polymorphism

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GraphPad Prism version 5.03 (GraphPad Software Inc., CA, USA) was used for the graphical representations in Chapters 3 and 4.

## LIST OF ABBREVIATIONS

|                        |                                                                                                                |
|------------------------|----------------------------------------------------------------------------------------------------------------|
| <b>ABPM</b>            | Ambulatory blood pressure monitoring                                                                           |
| <b>African-PREDICT</b> | African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension |
| <b>AUTHeR</b>          | Africa Unit for Transdisciplinary Health Research                                                              |
| <b>AVR</b>             | Arteriolar-to-venular ratio                                                                                    |
| <b>bDBP</b>            | Brachial diastolic blood pressure                                                                              |
| <b>BMI</b>             | Body mass index                                                                                                |
| <b>BP</b>              | Blood pressure                                                                                                 |
| <b>bPP</b>             | Brachial pulse pressure                                                                                        |
| <b>bSBP</b>            | Brachial systolic blood pressure                                                                               |
| <b>cfPWV</b>           | Carotid-femoral pulse wave velocity                                                                            |
| <b>cIMT</b>            | Carotid intima-media thickness                                                                                 |
| <b>cPP</b>             | Central pulse pressure                                                                                         |
| <b>CRAE</b>            | Central retinal arteriolar equivalent                                                                          |
| <b>CRP</b>             | C-reactive protein                                                                                             |
| <b>CRVE</b>            | Central retinal venular equivalent                                                                             |
| <b>cSBP</b>            | Central systolic blood pressure                                                                                |
| <b>CEN</b>             | Centre of Excellence for Nutrition                                                                             |
| <b>CVD</b>             | Cardiovascular disease                                                                                         |
| <b>DALY</b>            | Disability-adjusted life-year                                                                                  |
| <b>DBP</b>             | Diastolic blood pressure                                                                                       |
| <b>DNA</b>             | Deoxyribonucleic acid                                                                                          |
| <b>ECM</b>             | Extracellular matrix                                                                                           |
| <b>EDTA</b>            | Ethylenediaminetetraacetic acid                                                                                |
| <b>ELISA</b>           | Enzyme-linked immunosorbent assay                                                                              |

|               |                                                             |
|---------------|-------------------------------------------------------------|
| <b>eNOS</b>   | Endothelial nitric oxide synthase                           |
| <b>ESC</b>    | European Society of Cardiology                              |
| <b>ESH</b>    | European Society of Hypertension                            |
| <b>FMD</b>    | Flow-mediated dilation                                      |
| <b>GBD</b>    | Global Burden of Diseases, Injuries, and Risk Factors Study |
| <b>GC-MS</b>  | Gas chromatography-mass spectrometry                        |
| <b>GGT</b>    | Gamma-glutamyl transferase                                  |
| <b>HART</b>   | Hypertension in Africa Research Team                        |
| <b>HbA1c</b>  | Glycated haemoglobin                                        |
| <b>HDL-C</b>  | High-density lipoprotein cholesterol                        |
| <b>HIV</b>    | Human immunodeficiency virus                                |
| <b>HREC</b>   | Health Research Ethics Committee                            |
| <b>HRP</b>    | Horseradish peroxidase                                      |
| <b>ICAM-1</b> | Intercellular adhesion molecule 1                           |
| <b>IL-6</b>   | Interleukin-6                                               |
| <b>LDL-C</b>  | Low-density lipoprotein cholesterol                         |
| <b>MAP</b>    | Mean arterial pressure                                      |
| <b>MCP-1</b>  | Monocyte chemoattractant protein-1                          |
| <b>MESA</b>   | Multi-Ethnic Study of Atherosclerosis                       |
| <b>NCD</b>    | Non-communicable disease                                    |
| <b>NO</b>     | Nitric oxide                                                |
| <b>NOS</b>    | Nitric oxide synthase                                       |
| <b>NRF</b>    | National Research Foundation                                |
| <b>NVC</b>    | Neurovascular coupling                                      |
| <b>NWU</b>    | North-West University                                       |
| <b>PA</b>     | Plasminogen activator                                       |

|                            |                                                                              |
|----------------------------|------------------------------------------------------------------------------|
| <b>PAI</b>                 | Plasminogen activator inhibitor                                              |
| <b>PAI-1</b>               | Plasminogen activator inhibitor-1                                            |
| <b>PAI-1<sub>act</sub></b> | Plasminogen activator inhibitor-1 activity                                   |
| <b>PAI-1<sub>ag</sub></b>  | Plasminogen activator inhibitor-1 antigen                                    |
| <b>PCR</b>                 | Polymerase chain reaction                                                    |
| <b>PHRI</b>                | Population Health Research Institute                                         |
| <b>PP</b>                  | Pulse pressure                                                               |
| <b>PURE study</b>          | Prospective Urban and Rural Epidemiological study                            |
| <b>PURE-SA study</b>       | South African arm of the Prospective Urban and Rural Epidemiological study   |
| <b>RVA</b>                 | Retinal vessel analyser                                                      |
| <b>SAMRC</b>               | South African Medical Research Council                                       |
| <b>SANPAD</b>              | South Africa - Netherlands Research Programme on Alternatives in Development |
| <b>SARCHI</b>              | South African Research Chairs Initiative                                     |
| <b>SBP</b>                 | Systolic blood pressure                                                      |
| <b>serpin</b>              | Serine protease inhibitor                                                    |
| <b>SES</b>                 | Socio-economic status                                                        |
| <b>SSA</b>                 | Sub-Saharan Africa                                                           |
| <b>TAFI</b>                | Thrombin activatable fibrinolysis inhibitor                                  |
| <b>TC</b>                  | Total cholesterol                                                            |
| <b>t-PA</b>                | Tissue-type plasminogen activator                                            |
| <b>TPR</b>                 | Total peripheral resistance                                                  |
| <b>u-PA</b>                | Urinary/urokinase-type plasminogen activator                                 |
| <b>VCAM-1</b>              | Vascular cell adhesion molecule 1                                            |
| <b>VLDL</b>                | Very low-density lipoprotein                                                 |
| <b>vWF</b>                 | von Willebrand factor                                                        |

**vWF<sub>ag</sub>** von Willebrand factor antigen

**WC** Waist circumference

**WHtR** Waist-to-height ratio

**24 h** 24-hour

# **CHAPTER 1**

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**Literature overview, motivation, aims,  
objectives and hypotheses**

## 1.1. Introduction

The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) identified high systolic blood pressure (SBP) as the leading risk factor for attributable deaths in 2019, accounting for an estimated 10.8 million deaths worldwide (1). Sub-Saharan Africa (SSA) is no exception, with the prevalence of cardiovascular disease (CVD) and its risk factors, including hypertension, being on the increase (2, 3). During 2017, 18.4% of deaths in South Africa were attributed to CVDs, making it the leading natural cause of mortality (4). Hypertension, a known cardiovascular risk factor, has an alarmingly high (and increasing) prevalence, especially among Black South Africans (5, 6).

At the vascular level, hypertension is associated with endothelial dysfunction (7) and vascular remodelling (8). Endothelial cells line the inner surface of the entire vascular system, i.e. from the large arteries to the small vessels of the microcirculation (9, 10). Endothelial dysfunction refers to normal endothelium-dependent functions (such as vasodilation) being compromised (11), whereas vascular remodelling entails structural alterations of the vascular wall (12, 13). Both of these have clinical significance and are considered early markers of hypertension development (7, 8, 13-17). Furthermore, it is known that several circulating biomarkers are associated with hypertension, endothelial dysfunction and vascular remodelling, with one of these biomarkers being plasminogen activator inhibitor-1 (PAI-1) (18-21).

PAI-1 is a serine protease inhibitor (serpin) that plays a classical role in fibrinolysis, where it regulates fibrin breakdown by inhibition of plasminogen activators (PAs) (22). Alternatively, circulating PAI-1 is also considered a marker of endothelial dysfunction since the vascular endothelium is one of the primary sites of PAI-1 production (23) and elevated levels may thus reflect endothelial stimulation or injury (19, 24, 25). PAI-1 has also been implicated in various cellular processes such as cellular migration (26-28), proliferation (27, 29, 30), apoptosis (31, 32) and adhesion (27, 33), and also affects the degradation of extracellular matrix (ECM) macromolecules (34, 35). In this regard, PAI-1 is thought to influence vascular structure by the process of vascular remodelling (18, 36, 37). Furthermore, positive associations between PAI-1 and blood pressure have consistently been reported (21, 38-43) and elevated PAI-1 has been found in patients with hypertension (44-49). Whether elevated PAI-1 precedes or results from elevated blood pressure is still under debate, since the former (21, 41, 42) as well as the latter (40, 43) have both been suggested.

Most studies that have investigated the link between PAI-1 and elevated blood pressure were of a cross-sectional nature (38, 40, 43, 46). There is, however, a paucity of data linking PAI-1 levels prospectively to the development and progression of vascular deterioration. As

an early marker of endothelial dysfunction (50, 51), elevated PAI-1 has been linked to subclinical conditions (52) and early cardiovascular risk (53-55). Furthermore, PAI-1 is associated with vascular senescence (56, 57), which may explain its consistent association with established hypertension (44-49), CVD and events (58-60) later in life. When focusing on the Black South African population, which is prone to the development of hypertension (61, 62) and is known to display unique vascular (63-69) and haemostatic profiles (70, 71), it would be of importance to evaluate, across the life course from young healthy individuals to the elderly, the relationship between PAI-1 and hypertension development. Therefore, this study aimed to elucidate the associations of blood pressure and microvascular function (as indicators of endothelial function and vascular health) with PAI-1 in Black South Africans across the life course. The literature currently available on PAI-1's association with blood pressure and its involvement in compromised vascular health, as reflected by endothelial dysfunction and structural vascular alterations, will be discussed in the following sections.

## 1.2. Plasminogen activator inhibitor-1 (PAI-1)

Plasminogen activator inhibitor-1 is a single-chain glycoprotein that belongs to the serine protease inhibitor family (72, 73). It is the main plasminogen activator (PA) inhibitor in blood, accounting for 60% of plasma's PA inhibitor activity (74). It is expressed and secreted by various cell types and tissues, including endothelial cells, platelets, megakaryocytes, hepatocytes, neutrophils, macrophages, vascular smooth muscle cells (23), and adipocytes (75). In the circulation, PAI-1 exists in either active or inactive (latent) forms, or it may be in complex with tissue-type PA (t-PA) (76, 77).

### 1.2.1. *The classic role of PAI-1 in fibrinolysis*

Traditionally, PAI-1 has been associated with the fibrinolytic process (**Figure 1**). Fibrinolysis begins with the conversion of the inactive pro-enzyme plasminogen to its active form, plasmin. This process is mediated by PAs (22) and is the key reaction of the plasminogen activator system (78). Plasmin is then responsible for the degradation of fibrin and hence also the dissolution of blood clots, thereby producing fibrin degradation products (22, 78). The activation of plasminogen to plasmin can be inhibited by direct inhibitors of PAs, known as plasminogen activator inhibitors (PAIs). Both PAs and PAIs coexist in blood and the fibrinolytic potential of blood is determined by the balance or imbalance of these activators and inhibitors. For this reason, these components are tightly regulated (22).

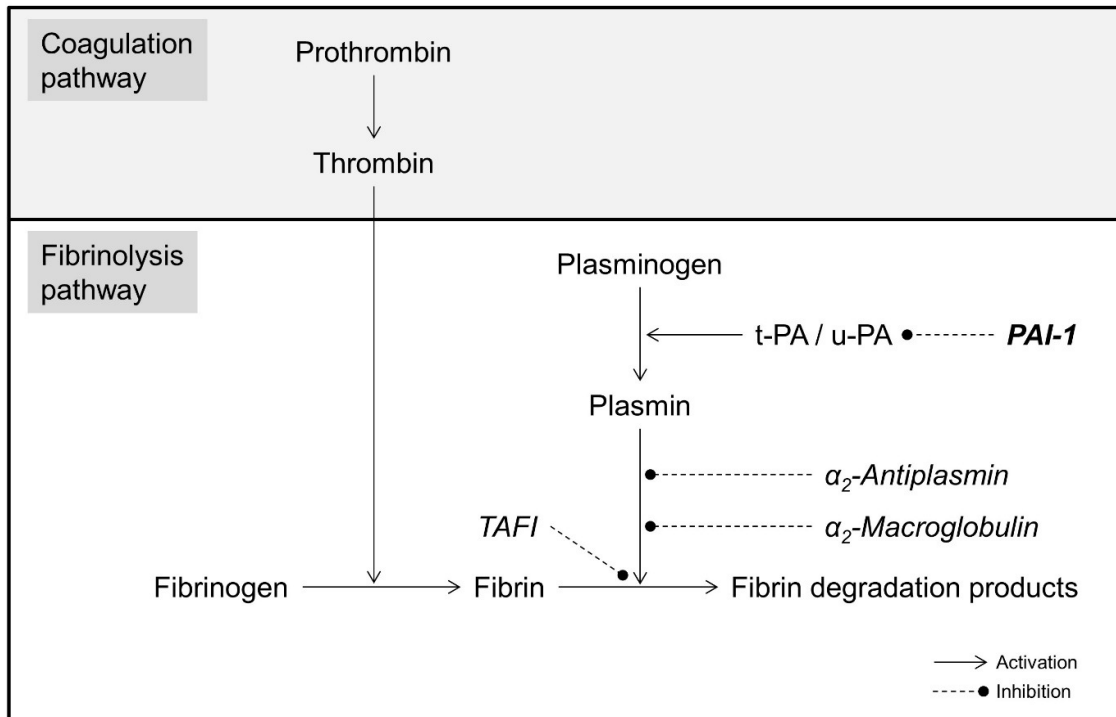


**Figure 1.** The classic role of PAI-1 in the fibrinolysis pathway as an inhibitor of plasminogen activators (t-PA and u-PA). Other inhibitors of fibrin degradation include thrombin activatable fibrinolysis inhibitor (TAFI),  $\alpha$ -antiplasmin and  $\alpha$ -macroglobulin.

The two main physiological PAs are tissue-type PA (t-PA) and urinary/urokinase-type PA (u-PA) (22, 78). Plasminogen activation by t-PA plays an important role in fibrin degradation in the blood (79), whereas u-PA facilitates activation of cell-bound plasminogen, leading to pericellular proteolysis (80). Thus, the fibrinolytic system and its components (including PAI-1) are not only involved in the dissolution of blood clots in the circulation, but also play a role within the vascular wall (22, 78). In this regard, PAI-1 has become known for its alternative role in influencing vascular function and structure, which is attributed mainly to its involvement in endothelial dysfunction (20) and vascular remodelling (18). These processes are some of the common denominators that may explain the positive association of PAI-1 with blood pressure, as will be discussed below.

### 1.2.2. Associations of PAI-1 with blood pressure

Impaired vascular structure and function, including endothelial dysfunction, are associated with elevated blood pressure or hypertension (7, 8). Numerous cross-sectional studies have reported positive associations between PAI-1 and blood pressure or hypertension (38-40, 43-49), whereas prospective studies on these associations are limited (21, 41, 42). Whether

elevated PAI-1 levels precede or result from elevated blood pressure is still under debate, since both scenarios have been suggested. It has been proposed that increased shear stress or endothelial dysfunction or damage caused by elevated blood pressure results in elevated PAI-1 levels (40, 43). On the other hand, associations of PAI-1 with blood pressure in prospective analysis (21, 41, 42) suggest a role for PAI-1 in the development of hypertension, including among Black South Africans. These prospective studies found that baseline PAI-1 was positively associated with blood pressure/hypertension incidence over mean/median follow-up periods of 3 to 5.3 years (21, 41, 42). Evidence from experimental studies in mice also support the notion that PAI-1 may play a causal role in hypertension development (56, 81). These studies showed that PAI-1 deficiency or inhibition attenuated the blood pressure increase induced by treatment with a nitric oxide synthase (NOS) inhibitor (56, 81). Explanations for PAI-1's possible contribution to the development of hypertension include its alternative roles in endothelial dysfunction (19, 20) and vascular remodelling (18) – both processes that are known to be associated with hypertension (7, 8) and which will be discussed below.

There is also evidence for a relationship between PAI-1 and blood pressure on a genetic level. The 4G/5G polymorphism in the PAI-1 (*SERPINE1*) gene promoter region that affects the expression of PAI-1 (82) has been associated with blood pressure (83, 84). A study by Martínez-Calatrava *et al.* (84) showed that individuals with the 4G/4G genotype were more likely to be hypertensive than individuals carrying the 5G allele, irrespective of plasma PAI-1 levels. Björck *et al.* (83) showed a gender-specific association of this polymorphism with central arterial blood pressure, where females carrying the 4G/4G genotype had higher central blood pressures in comparison with their 5G/5G genotype counterparts. Although the 4G/5G polymorphism's association with PAI-1 levels has been reported in a Black South African population (85) (see section 1.3), its association with blood pressure is yet to be determined.

### 1.2.3. PAI-1 and endothelial dysfunction

The endothelial monolayer that lines the inner surface of the blood vessels does not merely act as a barrier between the circulation and the sub-endothelial tissues. Instead, it is considered an important regulator of vascular homeostasis (10, 86). Normal endothelial function refers to its ability to regulate appropriate vascular tone, provide relative impermeability, and maintain an anti-coagulatory and anti-inflammatory surface by preventing adhesion and aggregation of platelets and leukocytes (86, 87). Many of these functions are achieved by the release of locally synthesised bioactive substances (86, 87).

Whenever the endothelium loses its ability to maintain this homeostatic balance, a state of endothelial dysfunction arises (11, 88). Hence, the characteristics of a dysfunctional endothelium include reduced vasodilation (due to decreased bioavailability of the vasodilator nitric oxide [NO]), and the development of a pro-coagulatory and pro-inflammatory surface (11, 88) (**Figure 2**).

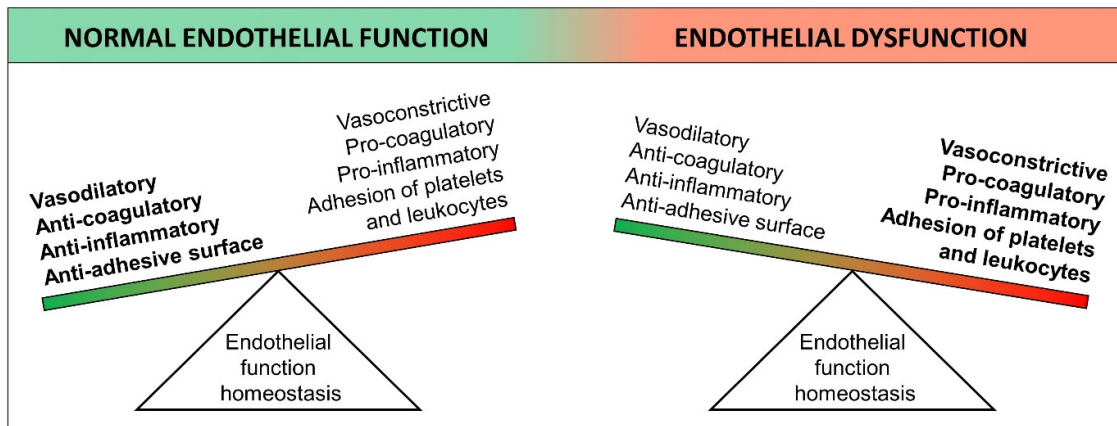


**Figure 2.** Homeostatic balance of normal endothelial function which, when disturbed, leads to endothelial dysfunction (adapted from Premer *et al.* (89) and Esper *et al.* (11)).

Although flow-mediated dilation (FMD) is considered the gold standard for the assessment of endothelial function (90), alternative methods are also used. Retinal microvascular function in response to flicker light provocation, for instance, is considered to reflect microvascular endothelial function (91), since NO has been shown to play a role in flicker-induced retinal vasodilation (92). Furthermore, measurement of circulating markers related to endothelial activation, endothelial dysfunction, and the accompanying haemostatic and inflammatory pathways is widely used to assess endothelial function. These biomarkers include PAI-1, von Willebrand factor (vWF), cellular adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and monocyte chemoattractant protein-1 (MCP-1) (20, 93).

The reason that PAI-1 is considered a marker of endothelial dysfunction is that the endothelium is one of the primary sources of PAI-1 production (23). Elevated PAI-1 may thus reflect endothelial stimulation or injury (19, 24, 25). An inverse association between PAI-1 levels and NO has been demonstrated (94, 95). Furthermore, PAI-1 has recently been shown to directly inhibit endothelial nitric oxide synthase (eNOS) enzyme activity (96), thereby reducing NO synthesis. As expected, reduced FMD (indicative of endothelial dysfunction) has been associated with elevated PAI-1 (97-99). Although literature on direct

correlations between PAI-1 and other circulating biomarkers of endothelial dysfunction is scarce (19, 100), concomitant increases in PAI-1 and these biomarkers under pathological conditions have been reported (19, 101, 102) and numerous studies have made use of circulating PAI-1 as a biomarker of endothelial dysfunction (19, 103-106).

There is a close interplay between endothelial function, haemostasis and inflammation, with endothelial dysfunction promoting pro-coagulatory and pro-inflammatory states (107, 108). Furthermore, PAI-1 is considered an acute-phase protein as its expression increases during inflammation (24). Hence, in addition to PAI-1's role in endothelial dysfunction, it is inevitably also closely linked to inflammation (20).

#### 1.2.4. *PAI-1 and vascular remodelling*

Vascular remodelling is a collective term describing structural changes that alter the vascular lumen size and/or the wall thickness (12, 13, 109), which in turn affect vascular function. These structural alterations are believed to contribute considerably to sustained increased vascular resistance associated with hypertension (8, 109). Indeed, essential hypertension has been associated with mainly inward eutrophic remodelling (110, 111) (decreased vascular lumen diameter whilst the amount of vascular wall material remains unaltered (12, 109)), whereas some forms of secondary hypertension have been associated with inward hypertrophic remodelling (112-114) (decreased vascular lumen diameter accompanied by an increase in vascular wall material (12, 109)). In mildly hypertensive patients, vascular remodelling of small arteries is already seen in the early stages of target organ damage (115). Furthermore, remodelling of small resistance arteries of the subcutaneous microcirculation, as reflected by greater media-to-lumen ratio, has been shown to predict cardiovascular events in a study population at high cardiovascular risk (116). In the retinal microvasculature, hypertension has been associated with narrower arteriolar and wider venular calibres (117, 118).

Alterations in vascular structure entail changes in the amounts and arrangement of cells and non-cellular components in the vascular wall, as well as changes to the extracellular matrix (119). To achieve these changes, several cellular and extracellular processes are involved (8, 109). PAI-1 is increasingly implicated in processes related to vascular remodelling, such as cell proliferation (27, 29, 30), apoptosis (31, 32), cell migration (26-28), signalling (29, 120, 121), and cell adhesion (27, 33). PAI-1's involvement in these processes, as well as in ECM degradation (34, 35), has led to it also being implicated in the process of fibrosis (122-124).

PAI-1's involvement in the processes of remodelling is rather complex and the exact mechanisms explaining PAI's contribution to vasculopathy are still unclear (125). To complicate this even further, studies have yielded conflicting results as to whether PAI-1 promotes or inhibits the processes leading to vascular remodelling, which led to the concept of the "PAI-1 paradox" (18, 126, 127). Neointima formation after vascular injury in murine models, for instance, was found to be either promoted (128), inhibited (129, 130), or unaffected (131) by the activity of PAI-1. Explanations that have been proposed for the inconsistencies in PAI-1-related data include the type of model used (132), differences in clinical and experimental conditions, and time points after vascular injury (78).

#### *1.2.5. Associations of PAI-1 with measures of microvascular structure and function*

Microvascular structure and function can be assessed in different microvascular beds. The gold standard method (albeit invasive) for assessing microvascular structure is wire or pressure micromyography, but non-invasive methods of assessing e.g. the retinal microvasculature using fundoscopy are also available (133). Measurement of microvascular function generally entails the assessment of vasodilatory responses to induced hyperaemia (134-136).

The retinal, coronary and cerebral microvasculature beds have many structural and physiological features in common and are often subjected to similar intrinsic and environmental conditions (137, 138). Evaluation of the calibres and functionality of the retinal microcirculation is thus a convenient and non-invasive method of assessing the systemic microcirculation (135, 139). Measurement of retinal vessel calibres provides information on the central retinal arteriolar and venular equivalents (CRAE and CRVE, respectively), whereas retinal vessel changes in response to functional tests, such as flicker light provocation (mediated by neurovascular coupling [NVC] (135)), provide information on the microvasculature's functional capacity (135, 137, 139). Smaller retinal arteriolar calibre has been linked to higher blood pressure or hypertension (140-142), whilst larger venular calibre has generally been associated with markers of inflammation and endothelial dysfunction (142-147). Reduced retinal arteriolar and venular dilation to flicker light has been demonstrated in obesity (148) and patients with chronic heart failure (149), hypercholesterolaemia (150), diabetes (151) and diabetic retinopathy (151, 152). Additionally, impaired retinal venular dilation has recently been associated with serum leptin levels (153) and has been shown to predict all-cause mortality in end-stage renal disease patients (154).

Data on the associations of PAI-1 with microvascular structure and function are limited. Two studies which were conducted in coronary (136) and cutaneous (134) microvascular beds have reported associations between PAI-1 and impaired microvascular function. Furthermore, there are limited data available on PAI-1's associations with retinal vascular calibres. In the Multi-Ethnic Study of Atherosclerosis (MESA) conducted on individuals (mean age 62 years) free from clinical CVD residing in six U.S. communities, a weak positive association between PAI-1 and CRVE was found (142). Brazionis *et al.* (155) reported PAI-1 to be positively associated with CRAE and arteriolar-to-venular ratio (AVR) and negatively with CRVE in individuals with type 2 diabetes (mean age 64 years). A third study, conducted in Black and White teachers (mean ages 48 and 49 years, respectively), found no associations of PAI-1 with retinal vessel calibres (156).

Taken together, PAI-1's involvement in endothelial dysfunction and vascular remodelling (as reflected by its associations with impaired vascular structure and function) provides mechanistic insight into PAI-1's consistent associations with elevated blood pressure and hypertension.

### **1.3. Factors that influence PAI-1 levels**

Several modifiable and non-modifiable factors that may influence PAI-1 levels have been identified. These include health behaviours such as smoking, alcohol consumption and central obesity, and non-modifiable factors such as ethnicity, sex, age, and the PAI-1 4G/5G polymorphism. Hence, these factors may also influence the associations of PAI-1 with measures of vascular structure and function.

#### *Smoking and alcohol consumption*

Health behaviours such as smoking (157-160) and alcohol consumption (161-163) have shown positive correlations with PAI-1 levels. Smokers have higher PAI-1 than non-smokers (157, 158, 160), and short-term smoking cessation of habitual smokers has been shown to decrease PAI-1 levels (159). Likewise, alcohol consumption has been shown to increase PAI-1 (161, 162), with the extent of the increase associated with the level of alcohol consumed (163).

#### *Central obesity and triglycerides*

PAI-1 has consistently shown positive associations with measures of obesity (e.g. body mass index) and specifically, central obesity (e.g. waist circumference and waist-to-height ratio) (157, 164-167). This is attributed to adipose tissue, and especially visceral adipose tissue, being a major source of PAI-1 production (168, 169). Furthermore, triglycerides are

believed to upregulate PAI-1 expression (170) *via* a very low-density lipoprotein (VLDL)-response element in the promoter area of the PAI-1 (*SERPINE1*) gene (171).

#### *Ethnicity, sex and age*

PAI-1 levels are also known to be influenced by non-modifiable factors, with higher PAI-1 generally associated with higher age (157, 158, 172) and male sex (157, 158, 173). Furthermore, a sex-specific biology for PAI-1 has been suggested (83, 173). PAI-1 levels also differ between ethnicities (174) and are known to be higher in White than Black South Africans (70, 175).

#### *The PAI-1 4G/5G polymorphism*

The 4G/5G polymorphism in the promoter region of the *SERPINE1* gene is known to influence PAI-1 expression, with the 4G/4G genotype generally being associated with higher PAI-1 (85, 158, 176-178). The 4G/5G polymorphism is, however, considered a response polymorphism (82), which implies that the genotype's effect on PAI-1 levels becomes more evident when combined with environmental factors and/or disease states that influence PAI-1 expression. For example, the polymorphism has been shown to influence PAI-1 levels in individuals with central obesity but not in lean controls (178). In a Black South African population, as investigated in the South African arm of the Prospective Urban and Rural Epidemiological (PURE) study, 72.5% of participants carried the 5G/5G genotype, 24.9% the 4G/5G genotype and 2.6% the 4G/4G genotype (n=1799) (85). In this study population, 5G/5G carriers presented with the lowest PAI-1 levels, 4G/5G carriers with intermediate and 4G/4G carriers with the highest (85).

### **1.4. Black South Africans display unique cardiovascular and haemostatic profiles**

Individuals of African descent are known to display higher cardiovascular risk, as reflected by their higher risk of stroke and higher diabetes and hypertension rates, compared with Europeans (179). This is likely partially attributed to the known differences in cardiovascular and haemostatic profiles between ethnicities. For instance, retinal vascular calibres (142, 180, 181), carotid artery diameter (182), carotid wall thickness (183-186), arterial stiffness (182, 184, 187, 188), and retinal vascular function (189) have been shown to differ between ethnic and/or geographic populations. Likewise, ethnic variance is also seen in haemostatic profiles, including PAI-1 levels (142, 174, 176, 190).

Multiple studies have also reported differences in vascular structure and function between Black and White South Africans, which is evident across the entire life course (63-69). Black individuals generally display increased arterial stiffness (66), greater carotid wall thickness

(63), higher total peripheral resistance (TPR) (68), higher blood pressure (office brachial systolic [SBP] and diastolic blood pressure [DBP] (66), 24-hour SBP, DBP and pulse pressure [PP] (69), and central SBP (67)), smaller retinal arteriolar (67) and larger venular calibres (69) than their White counterparts. Furthermore, a study by Pienaar *et al.* (65) showed higher endothelium-independent microvascular function in young, apparently healthy, White individuals compared with African or mixed-ancestry counterparts. Already at a young age, African boys (aged 6-8 years) presented with stiffer arteries, higher brachial DBP and mean arterial pressure (MAP), higher TPR, and greater carotid intima-media thickness (cIMT) than White boys (64). Despite their generally detrimental cardiovascular profiles, Africans, however, display greater retinal vasodilatory responses to flicker light provocation than Whites (67, 69).

Furthermore, there are definite differences between the haemostatic profiles of Black and White South Africans, which includes lower PAI-1 activity amongst the Black populations (70, 175). These ethnic differences in vascular and haemostatic profiles probably all contribute to the higher blood pressure of African populations (66, 67, 69) and their tendency to develop hypertension (61, 62). PAI-1's contribution to hypertension and vascular function is currently unclear – especially in the South African setting, where Africans are regarded as an understudied population in terms of CVD aetiology (42).

### **1.5. Problem statement and motivation**

Although there is no doubt that PAI-1 is linked to endothelial dysfunction and the vascular alterations that are associated with hypertension, the debate as to whether elevated PAI-1 levels result from or precede (and possibly also contribute to) elevated blood pressure still continues. Studies of these associations in ethnic groups other than those from European or American descent are limited. Furthermore, increasing evidence suggests that CVD originates as early as during childhood (191-193) and that microvascular abnormalities (as assessed in, for example, the retinal microvasculature) accompany and often precede the development and progression of macrovascular and cardiovascular disease (194-196). This warrants the investigation of early markers of vascular dysfunction in apparently healthy individuals, which will enable us to better characterise the relationships between microvascular and macrovascular function in the absence of existing pathology. Furthermore, prospective studies on the association of blood pressure with PAI-1, which may potentially shed light on the controversial direction of the blood pressure-PAI-1 association, are limited (21, 41, 42).

Black South Africans display unique vascular and haemostatic profiles compared with their white counterparts, with Black individuals generally displaying profiles of increased risk, including being prone to hypertension development (61, 62). Yet there are limited studies investigating CVD aetiology in the African population (42). The investigation of the associations of PAI-1 with blood pressure and microvascular function (as indicators of endothelial function and vascular health) in this population, as well as identification of modifiable and non-modifiable factors that may influence these associations (such as sex, ethnicity, central adiposity, smoking and alcohol use), will consequently contribute to a better understanding of the underlying mechanisms. Furthermore, the evaluation of 24-hour (24 h) ambulatory (ABPM) and central blood pressure (in addition to conventional office brachial blood pressure) associations with PAI-1 would be of importance, since ABPM and central blood pressure have shown a stronger relation to cardiovascular risk factors than brachial blood pressure (197, 198).

In order to determine the associations of PAI-1 with the above-mentioned vascular markers, data from two studies were used. Data obtained from young, healthy Black and White South Africans participating in the African-PREDICT (Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension) study were used in cross-sectional analysis to determine the association of PAI-1 with both microvascular function as well as blood pressure. Data obtained from an older cohort of Black adults participating in the South African arm of the Prospective Urban and Rural Epidemiological (PURE) study were used for investigating the prospective association of PAI-1 and the 4G/5G polymorphism with blood pressure in an older population already presenting with hypertension and CVD progression.

## **1.6. Aims, objectives and hypotheses**

This study aimed to elucidate the associations of blood pressure and microvascular function with PAI-1 in Black South Africans, and to investigate the influences of selected modifiable and non-modifiable factors on these associations. The different objectives were addressed in three research articles:

### **Article 1:**

#### ***Objectives:***

- To investigate the associations of retinal vasodilatory responses to flicker light provocation with PAI-1 in young and healthy Black and White men and women.

***Hypothesis:***

- Maximal retinal arteriolar and venular dilation to flicker light will be negatively associated with PAI-1. Owing to known sex and ethnic differences in PAI-1 biology and cardiovascular profiles, these associations will likely differ in men and women and be ethnic-specific.

**Article 2:*****Objectives:***

- To investigate whether an association between 24 h blood pressure and PAI-1 already exists in a young, healthy cohort of Black and White men and women, in the absence of overt hypertension.
- To investigate the influence of smoking and alcohol consumption on the association between 24 h blood pressure and PAI-1.

***Hypotheses:***

- A positive association will exist between 24 h blood pressure and PAI-1.
- Smoking and alcohol consumption will likely alter this association. Owing to reported differences in PAI-1 biology and cardiovascular function between men and women and between different ethnic groups, associations may likely differ according to these subgroups.

**Article 3:*****Objectives:***

- To investigate the associations of blood pressure (brachial and central) and hypertension status with PAI-1 and the 4G/5G polymorphism in prospective analysis over a 10-year period (conducted in an older cohort of Black South Africans).
- To investigate the influence of central obesity on these associations based on the reported interplay between central obesity, PAI-1 and the 4G/5G polymorphism.

***Hypotheses:***

- A positive association will exist between blood pressure (brachial and central) and PAI-1.
- In prospective analysis, elevations in PAI-1 will precede elevations in blood pressure and will increase the odds of developing hypertension.

- Blood pressure and hypertension status will be associated with the 4G/5G polymorphism, with 4G/4G carriers presenting with higher blood pressure and having increased odds of developing hypertension.
- The associations of blood pressure with PAI-1 and the 4G/5G polymorphism will be dependent on the degree of central obesity.

## 1.7. References

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# **CHAPTER 2**

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## **Study design and methodology**

## 2.1. Study design

This thesis made use of data collected in two studies, namely the African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT) and the South African arm of the Prospective Urban and Rural Epidemiological (PURE) study. The African-PREDICT study is an ongoing study, while the last data collection of PURE-SA took place in 2015. The data collection of both studies took place in the North West Province of South Africa (Figure 1).

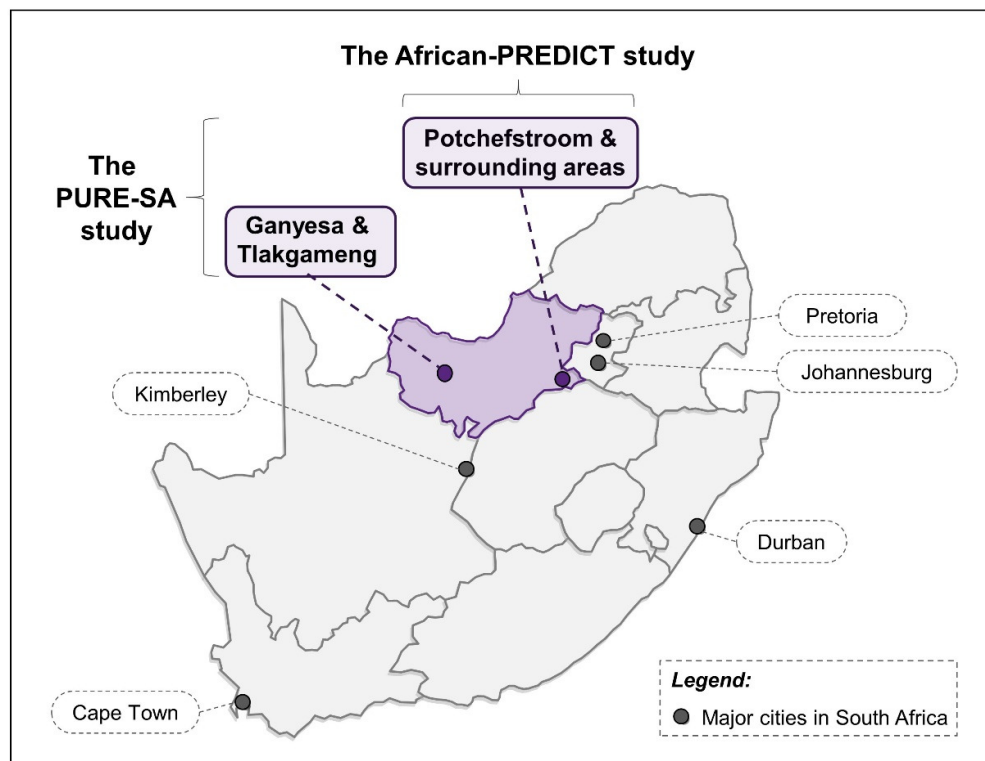


**Figure 1.** Map of South Africa indicating the geographical locations where data collection for the African-PREDICT and PURE-SA studies took place

The African-PREDICT study is a prospective study aimed at identifying early markers in the development of cardiovascular disease (CVD). At baseline, the study population comprised young, normotensive Black and White South Africans aged 20–30 years who are subjected to a series of assessments every 5 years. Collection of baseline data took place from 2013 to 2017, and a total of 1202 Black ( $n=606$ ) and White ( $n=596$ ) women ( $n=624$ ) and men ( $n=578$ ) were enrolled (1). The African-PREDICT study complied with all applicable requirements of international regulations, in particular the Declaration of Helsinki for research involving human participants. The African-PREDICT study was endorsed by the National Department of Health and was approved by the Health Research Ethics Committee

(HREC) of the North-West University (NWU) (NWU-00001-12-A1) (**Appendix A**). The study is also registered at ClinicalTrials.gov (identifier: NCT03292094). All participants gave voluntary written informed consent (**Appendix B**). This PhD study made use of data collected as part of the study's baseline phase.

The international PURE study is a large-scale epidemiological study conducted across 27 low-, middle- and high-income countries, including South Africa (2). The main aim of the study was to prospectively investigate lifestyle behaviours, cardiovascular risk factors and chronic non-communicable disease (NCD) incidence within different communities. Black adults between 30 and 70 years of age residing in rural and urban communities in the North West province, South Africa, were targeted for recruitment. The goal was to collect follow-up data every 5 years for at least 10 years. In South Africa, the baseline data were collected in 2005 (n=2010), with follow-up data collection in 2010 (n=1288) and 2015 (n=926). The South-African arm of the PURE study was approved by the NWU HREC for the time period from 2005 to 2009 (04M10) (**Appendix C**) as well as 2010 to 2015 and 2015 to 2020 (NWU-00016-10-A1) (**Appendix D**). Additionally, NWU HREC approval has also been obtained for the PURE-affiliated study titled "Characterisation of the haemostatic profile of a black South African population" (NWU-00034-17-A1) (**Appendix E**). Participants gave written voluntary informed consent prior to each data collection of the PURE study (**Appendix F and G**). All measurements and sample collection were performed by appropriately trained personnel in an ethical manner in accordance with the Declaration of Helsinki for research involving human participants. Data collection and feedback procedures were similar during the 2005, 2010 and 2015 time points.

This PhD study made use of existing data collected as part of the African-PREDICT study (baseline data) and the South African arm of the PURE study (baseline and follow-up data), and no additional measurements or data collection were required. This PhD study was approved by the Scientific Committee of the Hypertension in Africa Research Team (HART), NWU, as well as by the NWU HREC (NWU-00105-17-A1) (**Appendix H**). The methodologies of the African-PREDICT and PURE studies applicable to this PhD are described in the following sections.

## **2.2. Methodology pertaining to the African-PREDICT study**

### **2.2.1. Participant recruitment**

Participants were recruited by advertising in local newspapers and on billboards, at public health screening events, or by direct recruitment in the workplace. Trained fieldworkers and registered nurses (excluding the principal investigator) acted as recruitment mediators.

Recruited participants were first subjected to a screening process to determine their eligibility for participation in the study (see the inclusion and exclusion criteria in the following section).

### 2.2.2. Screening for eligibility

In order to participate in the African-PREDICT study, certain eligibility criteria had to be met (Table 1). The screening procedures included basic measurements of office blood pressure, blood glucose and lipids (finger prick), a urine sample test (dipstick), and body composition measurements performed by trained research staff and postgraduate students. HIV testing was also performed by trained counsellors in a private room and included pre- and post-test counselling. Upon completion of the screening measurements, participants who met the inclusion criteria of the study were invited to return for advanced research measurements. Participation was based on a balanced selection according to ethnicity, sex and socio-economic status. Figure 2 provides a summary of the participant screening and inclusion in the baseline data collection of the African-PREDICT study.

**Table 1.** Eligibility criteria for participants to be included in the African-PREDICT study

| Inclusion criteria                        | Justification                                                                                                                                                                                                                                                                                                                                                                                                                                         |
|-------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1) Self-reported Black or White ethnicity | It is well known that Black individuals display more adverse cardiovascular profiles and are more prone to hypertension development than White individuals, even from a young age (3-10). As per the aims of the African-PREDICT study, cardiovascular and biochemical profiles of Black and White individuals were compared in order to enhance our understanding of the mechanisms underlying the early development of CVD (inclusion criterion 1). |
| 2) Women and men                          | Both women and men were included to identify potential sex differences (inclusion criterion 2).                                                                                                                                                                                                                                                                                                                                                       |

| <p>3) 20–30 years of age</p> <p>4) Apparently healthy</p> <p>5) Office brachial systolic blood pressure (SBP) &lt;140mmHg and diastolic blood pressure (DBP) &lt;90mmHg</p>                                                                                                                                                                                                                                                                                                                 | <p>Since the aim of the African-PREDICT study is to investigate the early phases of hypertension and CVD development, young and healthy adults at a stage prior to cardiovascular deterioration were considered the ideal target study population at study initiation (inclusion criteria 3-5).</p>                                                                                                                                                                                                                                                                                  |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Exclusion criteria                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | Justification                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <p>1) Not a permanent resident of Potchefstroom or surrounding area</p> <p>2) Fever (internal ear temperature &gt;37.5°C on the research day)</p> <p>3) Self-reported and previously diagnosed chronic disease (e.g. liver disease, renal disease, cancer, tuberculosis, diabetes or CVD)</p> <p>4) Medication use for chronic disease (e.g. anti-hypertensive, anti-diabetic, or anti-retroviral medication)</p> <p>5) HIV positive</p> <p>6) Self-reported pregnancy or breastfeeding</p> | <p>Owing to the longitudinal nature of the study, residence in the Potchefstroom area was preferred as it increased the likelihood of follow-up (exclusion criterion 1).</p> <p>Since the study aimed at investigating the development of CVD in an initially healthy study population, individuals with any known diseases or risk factors that might influence cardiovascular health were excluded (exclusion criteria 2-5).</p> <p>Because of the known effects of hormones on cardiovascular health, pregnant and lactating women were not included (exclusion criterion 6).</p> |

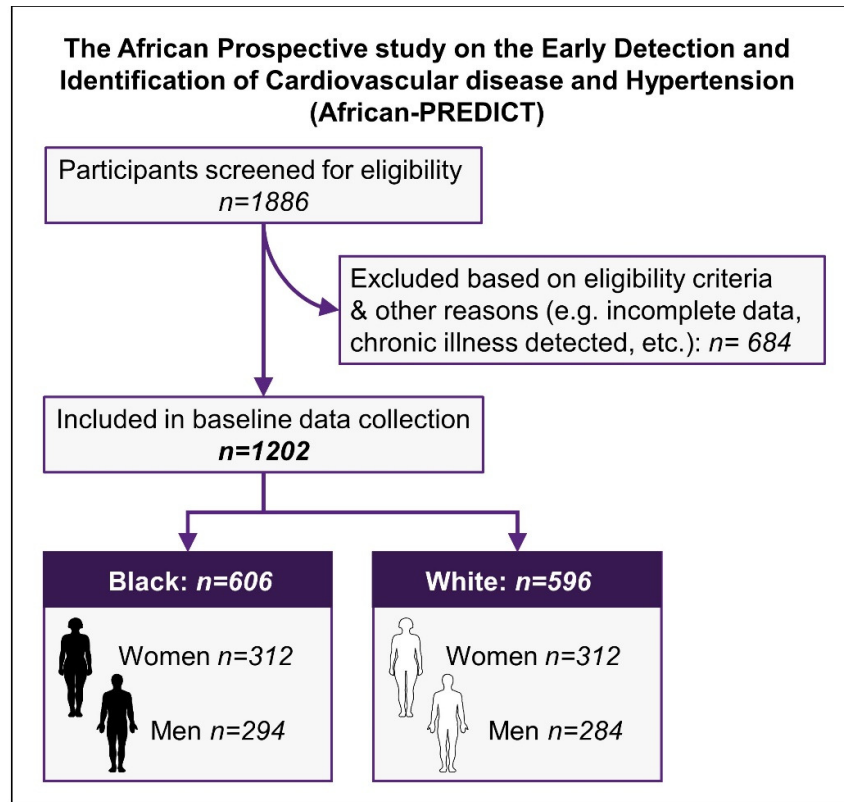


**Figure 2.** Summary of the screening and inclusion of participants in the baseline data collection of the African-PREDICT study

### 2.2.3. Questionnaire data

General health and demographic questionnaires were completed, and for the purpose of this PhD study, the following data obtained from this questionnaire were used: self-reported ethnicity, sex, age, current smoking status (no/yes), alcohol use (no/yes), and hormonal contraceptive use (women, no/yes). Household income and skill and education levels, also obtained using this questionnaire, were used to calculate a socio-economic status (SES) score. For this point-system SES scoring, Kuppuswamy's Socioeconomic Status Scale (11) was used, adapted to the South African context (1). The SES scores were consequently used to categorise participants into low-, middle- and high-SES classes.

### 2.2.4. Anthropometric measurements

Using standard procedures, an anthropometrist measured in triplicate body height (cm) using a SECA 213 Stadiometer (SECA, Hamburg, Germany), body weight (kg) using a SECA 813 electronic scale (SECA, Hamburg, Germany) and waist circumference (WC) (cm) using a Lufkin W606PM steel anthropometric tape (Lufkin, Apex, USA). Using these data, the waist-to-height ratio (WHtR) was calculated, as well as the body mass index (BMI)

(kg/m<sup>2</sup>), using the standard formula weight (kg) / height (m)<sup>2</sup>. All anthropometric measurements were performed according to guidelines described by the International Society for the Advancement of Kinanthropometry (12).

### *2.2.5. Cardiovascular measurements*

#### *Office blood pressure*

Office brachial blood pressure was measured after a 10-minute rest period using the validated Dinamap® Procare 100 device (GE Medical Systems, Milwaukee, USA) with GE Critikon latex-free Dura-Cuffs (medium and large) whilst participants were in a seated, relaxed state. Duplicate measurements were taken on each arm at 5-minute intervals. Systolic (SBP) and diastolic blood pressure (DBP) were measured, and the pulse pressure (PP) and mean arterial pressure (MAP) subsequently calculated.

#### *24-hour (24 h) ambulatory blood pressure*

Card(X)plore devices (Meditech, Budapest, Hungary) were used for the measurement of 24 h ambulatory blood pressure (ABPM). An appropriate-sized cuff was fitted to the participant's non-dominant arm and instructions were given on how to ensure successful inflations over the 24 h time period. The ABPM apparatus was programmed to measure blood pressure every 30 minutes during the day (06:00-22:00) and every hour during the night (22:00-06:00). Measurements were repeated if not successful, and an ambulatory diary card was completed by participants during the 24 h period. Measurements included mean 24 h SBP, DBP, PP and MAP.

#### *Dynamic retinal vessel analysis*

Retinal vessel analysis was performed by three trained researchers. Mydriatic conditions were induced by administration of an eye drop (Tropicamide, 1% from Alcon (Alcon laboratories, Bryanston, South Africa)) into the right eye 30 minutes before the measurement. In the event of an injury to the right eye, the left eye was used. A retinal vessel analyser (RVA) (IMEDOS Systems GmbH, Jena, Germany) equipped with a Zeiss Fundus FF-450<sup>Plus</sup> camera (Carl Zeiss, Meditech, Jena, Germany) was used for dynamic retinal vessel analysis, as described previously (13). A standard flicker protocol by IMEDOS Systems (14) was used, and data were captured using RVA 4.50 software.

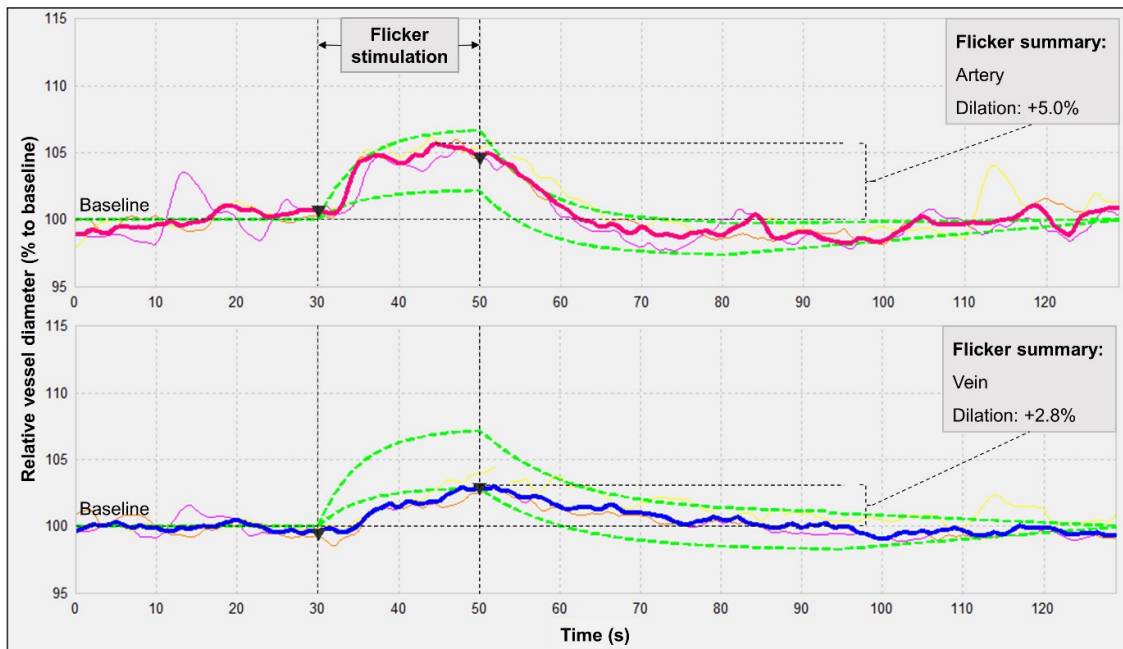


**Figure 3.** An example of retinal arteriolar (red line) and venular (blue line) dilatory responses to flicker light provocation; maximal vessel dilation expressed as a percentage of the baseline

During the flicker assessment, the baseline phase lasted for 50 seconds, followed by three 20-second flicker provocation cycles and an 80-second recovery/second baseline period after each provocation. In total, the measurement lasted 350 seconds. With the camera set at a 30-degree angle and the participant focusing on the tip of a fixation rod, a small arteriolar and a venular segment (as long as possible) were selected in the upper (mostly) or lower temporal quadrant of the fundus image. As indicated by the RVA software manual, vessels were selected if they were at least between 0.5–2.0 optic disc diameters away from the optic disc margin. The raw numerical data generated from the measurement were exported to an Excel (Microsoft) sheet with built-in macros for further filtering, processing and analysis of data, as previously described (13). The absolute baseline diameters of the selected segments were calculated individually as the median value over the last 30 seconds of the first baseline period prior to flicker provocation. A smoothed average flicker response curve was used to determine the maximal arteriolar and venular dilation (the absolute maximum of the reaction curve between the start of the flicker stimulation and 30 seconds after flicker initiation, expressed as a percentage of baseline).

#### 2.2.6. Biological sampling and biochemical measurements

In order to obtain early morning fasting blood samples, participants were requested to fast overnight for a period of 8–10 hours. Blood samples were collected in serum, EDTA, citrate,

sodium fluoride and Stabilyte (acidified citrated) blood collection tubes. Preparation of the blood samples entailed centrifugation of serum tubes at  $1602 \times g$  at room temperature for 30 minutes, EDTA tubes at  $2500 \times g$  at  $4^{\circ}\text{C}$  for 10 minutes, citrate tubes at  $1602 \times g$  at room temperature for 15 minutes, sodium fluoride tubes at  $2307 \times g$  at  $4^{\circ}\text{C}$  for 10 minutes, and Stabilyte tubes at  $1602 \times g$  at  $20^{\circ}\text{C}$  for 15 minutes. Samples were aliquoted and stored in cryovials at  $-80^{\circ}\text{C}$  until analysis. Biochemical analyses were performed by or under the supervision of a qualified biochemist according to standardised methodology and internationally recognised biochemical procedures, using calibrated instruments.

PAI-1 activity ( $\text{PAI-1}_{\text{act}}$ ) was measured in citrated plasma samples (collected in Stabilyte tubes) using the Technozym® PAI-1 Actibind® enzyme-linked immunosorbent assay (ELISA) kit (Technoclone GmbH, Vienna, Austria). The Cobas Integra® 400 Plus analyzer (Roche, Basel, Switzerland) was used for the measurement of C-reactive protein (hs-CRP), gamma-glutamyl transferase (GGT), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides in serum samples, and glucose in sodium fluoride plasma samples. Interleukin-6 (hs-IL-6) in serum samples and monocyte chemoattractant protein-1 (MCP-1) in EDTA plasma samples were measured using Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA). Serum cotinine was measured using a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany), and serum insulin using an electrochemiluminescence method on the E411 analyzer (Roche, Basel, Switzerland). An ELISA kit (DAKO, Glostrup, Denmark) was used for the measurement of von Willebrand factor antigen ( $\text{vWF}_{\text{ag}}$ ) in citrated plasma samples. Polyclonal rabbit anti-vWF antibody and rabbit anti-vWF-horseradish peroxidase (HRP) 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.

## **2.3. Methodology pertaining to the South African arm of the PURE study**

### **2.3.1. Participant recruitment and follow-up of the PURE study**

Permission for access to and recruitment in the community was granted on a judicial (national) and goodwill (tribal/community and household) basis by the South African Department of Health, the city mayors of Potchefstroom and Ganyesa, tribal chiefs, community leaders (acting as gatekeepers), and the heads of households, respectively. Consent was granted for data collection during 2005, 2010 and 2015. Nevertheless, tribal/community permission was obtained prior to each follow-up data collection as a gesture of respect for each community's leadership structure.

In an effort to reach the goal of 2000 or more participants (based on a power calculation published by Vorster (15)), 6000 households were approached during recruitment. Trained fieldworkers, who were selected from the relevant communities, used a door-to-door approach of randomly selected addresses in each community and spoke to each potential participant individually. After being supplied with information about the study and being allowed time to contemplate their participation, all potential participants gave written voluntary informed consent. Upon signing the voluntary informed consent forms, participants were asked to complete a validated questionnaire regarding their health (physical and psychological), lifestyle, socio-economic status and background. Participants that took part in the baseline data collection were recruited for subsequent data collection by follow-up visits by the fieldworkers. Of the 3750 participants who completed questionnaires upon initial informed consent,  $n=2010$  took part in the baseline data collection. Figure 4 shows the number of participants included at baseline and at each follow-up data collection point.

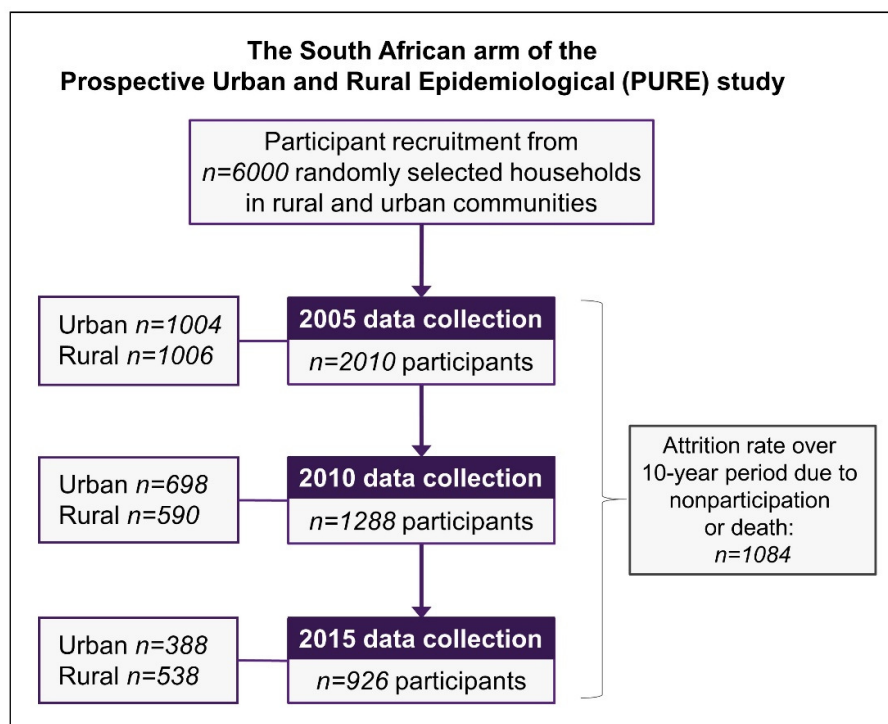


**Figure 4.** Number of participants included in the 2005 baseline data collection, and in the 5- and 10-year follow-up data collections of the PURE-SA study

### 2.3.2. Screening for eligibility

In an effort to equally represent urban, urban-rural (transitional), and rural areas of the North West province in South Africa, Tswana-speaking individuals from Ikageng near Potchefstroom (Zonderwater, Extension 7 or Extension 11 (urban settlements)), Ganyesa

(urban-rural town) or Tlaskgameng (rural area) were recruited for participation. Potential participants had to meet certain eligibility criteria in order to participate in the study (Table 2). In view of the study's longitudinal design, participants had to be willing to take part in follow-up data collections spanning 10 years, and hence had to intend residing in the area for this period of time. Eligibility to participate was ultimately determined by the individual's voluntary willingness to participate, and by his/her ability to comprehend fully the information given prior to signing the voluntary written informed consent.

**Table 2.** Eligibility criteria for participants to be included in the PURE study

| Inclusion criteria                           | Justification                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|----------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1) Women and men aged 30 – 70 years          | <p>The aim of the PURE study is to investigate the influence of urbanisation on NCD development. Therefore, participants were selected in the older age range, as NCDs are less prevalent in the young.</p> <p>Furthermore, since the PURE study is a longitudinal study that aimed to collect follow-up data over a minimum period of 10 years, participants had to be of an age that allowed long-term follow-up, e.g. when they were likely to be stable regarding residency in the area (inclusion criterion 1).</p> |
| 2) Apparently healthy (no reported diseases) | <p>In order to investigate the incidence of NCDs, participants had to be apparently healthy at study initiation to allow comparison of those who developed NCDs over the 10-year time frame with those who did not (inclusion criteria 2-3).</p>                                                                                                                                                                                                                                                                         |
| 3) Not using acute or chronic medication     |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |

| 4) Permanent resident of Ikageng (Zonderwater, Extension 7, Extension 11), Ganyesa or Tlakgameng and intended to remain a resident for the duration of the study | The PURE study investigates the effects of urbanisation and, therefore, the communities were selected based on the degree of urbanisation. Migration stability within the chosen communities is important to ensure follow-up in this longitudinal study (inclusion criterion 4). |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 5) No acute illness (body temperature > 37°C)                                                                                                                    | Fever may be indicative of an acute or chronic infection which can significantly influence many of the outcome variables (inclusion criterion 5).                                                                                                                                 |
| Exclusion criteria                                                                                                                                               | Justification                                                                                                                                                                                                                                                                     |
| 1) If an individual was inebriated, or incapable of being fully informed at the time of consent                                                                  | Participation was prohibited if an individual was not in a state of mind to be fully informed at the time of consent. This was to ensure that voluntary written informed consent was obtained in an ethical manner (exclusion criterion 1).                                       |

### 2.3.3. Questionnaire data

Standardised questionnaires were used to obtain self-reported information regarding rural/urban status, sex, age, smoking and alcohol consumption habits (never, current, former), use of any medication (no/yes, and name of medication) and female hormonal contraceptives (no/yes).

### 2.3.4. Anthropometric measurements

Anthropometric measurements were performed by trained anthropometrists using standard procedures whilst participants were wearing minimal clothing. Waist circumference (WC, in cm) measurements were used in this PhD study, which were measured in triplicate using a Holtain unstretchable metal tape (Crymych, UK) during 2005 and 2010 data collection, and a Lufkin steel tape (Cooper Tools; Apex, North Carolina, USA) for 2015 measurements.

### 2.3.5. Cardiovascular measurements

After a 10-minute rest period, duplicate measurements of blood pressure were taken on the right arm. Brachial SBP (bSBP) and DBP (bDBP) were measured with the Omron HEM-757 device in 2005 and 2010, and with the Omron M6 device (Omron Healthcare, Kyoto, Japan) in 2015. Brachial PP (bPP) was calculated as the difference between bSBP and bDBP. In 2015, central SBP (cSBP) and PP (cPP) were determined by performing pulse wave analysis using the SphygmoCor® XCEL System (AtCor Medical Pty. Ltd., Sydney, Australia). Hypertension was defined according to the 2018 European Society of Cardiology (ESC) and the European Society of Hypertension (ESH) guidelines (16) as bSBP  $\geq$  140 mmHg and/or bDBP  $\geq$  90 mmHg, or the use of anti-hypertensive medication.

### 2.3.6. Biological sampling and biochemical measurements

Blood samples were collected in the early morning (07:00 AM to 11:00 AM) after participants had fasted for a period of 10 hours. Blood intended for the analysis of PAI-1<sub>act</sub> was collected in 3.2% sodium citrate tubes. For serum total cholesterol (TC), HDL-C, triglycerides, GGT, and CRP, tubes that did not contain anti-coagulants were used. Upon collection, samples were carried on ice to the on-site laboratory for centrifugation and storage. Samples were centrifuged at 2000  $\times$  g for 15 minutes within 30 minutes after collection. Following the removal of serum and plasma fractions from the blood collection tubes, the buffy coat was removed for the purpose of DNA isolation. Samples were stored at -80°C until analysis.

Plasma PAI-1<sub>act</sub> was measured by an indirect enzymatic method (Spectrolyze PAI-1; Trinity Biotech, Bray, Ireland). Serum TC, HDL-C, triglycerides, GGT and hs-CRP were quantified using the Konelab20i™ auto-analyser (Thermo Fisher Scientific, Vantaa, Finland), and LDL-C was calculated using the formula described by Sathiyakumar *et al.* (17).

Genomic DNA was isolated from the buffy coat fractions of blood samples collected in 2005 using QIAGEN Flexigene DNA extraction kits (QIAGEN, Valencia, California, USA). A NanoDrop spectrophotometer (ND-1000, Wilmington, Delaware, USA) was used to monitor the quality and determine the concentration of the DNA. Re-isolations were performed with Maxwell® blood DNA purification kits (Promega Corporation, Madison, Wisconsin, USA) when the output of the NanoDrop was unsatisfactory. The 4G/5G polymorphism in the promoter area of the PAI-1 (*SERPINE1*) gene was genotyped (18) with TaqMan-based assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as previously described (19), using the MyIQ5 BioRad real-time polymerase chain reaction (PCR) thermal cycler (Bio-Rad Laboratories Inc., Hercules, California, USA).

A First Response HIV 1-2-0 test (Premier Medical Corporation Ltd, Daman, India) was used for initial HIV testing on whole blood samples, after which confirmatory tests were performed with the Pareeshak card test (BHAT Bio-tech, Bengaluru, Karnataka, India), the SD BIOLINE HIV 1/2 3.0 card test (Standard Diagnostics Inc., Gyeonggi-do, Korea) and an ABON HIV test (Biopharm Corporation Limited, Hangzhou, China) in 2005, 2010 and 2015, respectively.

#### **2.4. PhD candidate's contributions to the African-PREDICT and PURE-SA studies**

The PhD candidate contributed to data collection and analysis of both the African-PREDICT and PURE studies. Regarding the African-PREDICT study, the PhD candidate was involved in the logistics of blood and urine sample storage and organisation at the Centre of Excellence for Nutrition (CEN), Potchefstroom campus, NWU. He performed carotid-femoral pulse wave velocity (cfPWV) measurements (using the Sphygmocor® XCEL device) at the Hypertension Research and Training Clinic, Potchefstroom campus, NWU, as part of data collection in the African-PREDICT study. In the laboratory, the PhD candidate and a colleague performed all measurements of plasma PAI-1<sub>act</sub>, which is the main variable of this PhD study.

Regarding the South African arm of the PURE study, the PhD candidate was involved in the 2015 follow-up data collection and analysis. He was in charge of the temporary field laboratory that was set up in Ganyesa (an urban-rural town in South Africa) where the collected biological samples were prepared and analysed. In addition to organisational procedures, this entailed centrifugation, aliquoting, freezing and transportation of blood and urine samples (for future analysis), and basic on-site biochemical analyses (e.g. blood haemoglobin and glycated haemoglobin [HbA1c] determination). Furthermore, he was responsible for the determination of the fatty acid composition of blood samples collected in the PURE study, using gas chromatography-mass spectrometry (GC-MS).

The PhD candidate assisted in data cleaning for both the African-PREDICT and PURE-SA studies, and performed the majority of the statistical analyses and data interpretation for this thesis. He also wrote the PhD proposal following an in-depth literature review, and submitted the ethics application to the NWU HREC for approval.

#### **2.5. Statistical analyses**

SPSS versions 25 and 26 (IBM Corp. Released 2017; 2019. IBM SPSS Statistics for Windows, Version 25.0; 26.0. Armonk, NY: IBM Corp.) software packages were used for statistical analyses, and GraphPad Prism version 5.03 (GraphPad Software Inc., CA, USA)

for graphical representations, of research articles 1 and 2 (Chapters 3 and 4). SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA) was used for the statistical analyses of research article 3 (Chapter 5).

Statistical analyses included separate analyses in women in men owing to known sex differences in blood pressure regulation (20) and retinal microvascular function (21), and also because a sex-specific biology for PAI-1 has been suggested (22, 23). The African-PREDICT study population (research articles 1 and 2) were additionally stratified according to ethnicity because of known differences in blood pressure (24), retinal microvascular function (25), and haemostatic profiles (26) between different ethnicities.

In this PhD study, both cross-sectional (African-PREDICT study data: research articles 1 and 2) and prospective (PURE study data over a 10-year period: research article 3) statistical analyses were performed. In-depth descriptions of the statistical analyses used are provided in the respective research articles (Chapters 3–5).

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# CHAPTER 3

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Research article 1:

**Retinal vasodilatory responses are inversely associated with plasminogen activator inhibitor-1: The African-PREDICT study**

This article is currently under review at *Microvascular Research*.



# Microvascular Research

|                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
|----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Impact factor</b> | 2.730                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| <b>Scope</b>         | <p><i>Microvascular Research</i> is dedicated to the dissemination of fundamental information related to the <b>microvascular</b> field. Full-length articles presenting the results of original research and brief communications are featured.</p> <p>Research areas include: angiogenesis, biochemistry, bioengineering, biomathematics, biophysics, cancer, circulatory homeostasis, comparative physiology, drug delivery, neuropharmacology, microvascular pathology, rheology, and tissue engineering.</p> |

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|--------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Title</b>                         | ≤ 100 characters; should capture and communicate the key message of the research to a broader audience; abbreviations, unless familiar to a broad audience, should be avoided.                                                                                |
| <b>Author names and affiliations</b> | Indicate affiliations with a lower-case superscript letter after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including country name and, if available, the e-mail address of each author. |
| <b>Corresponding author</b>          | E-mail address and full postal address.                                                                                                                                                                                                                       |
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| <b>Keywords</b>                      | ≤ 10 keywords, immediately after the abstract.                                                                                                                                                                                                                |
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**Title:** Retinal vasodilatory responses are inversely associated with plasminogen activator inhibitor-1: The African-PREDICT study

**Authors:**

Adriaan JACOBS<sup>a,b</sup>, Marlien PIETERS<sup>b,c</sup>, Wayne SMITH<sup>a,b</sup>, Aletta E. SCHUTTE<sup>a,b,d\*</sup>

**Affiliations:**

<sup>a</sup>Hypertension in Africa Research Team (HART), North-West University, Private bag X1290, Potchefstroom, South Africa

<sup>b</sup>Medical Research Council Unit for Hypertension and Cardiovascular Disease, North-West University, Private bag X6001, Potchefstroom, South Africa

<sup>c</sup>Centre of Excellence for Nutrition (CEN), North-West University, Private bag X6001, Potchefstroom, South Africa

<sup>d</sup>School of Population Health, University of New South Wales; The George Institute for Global Health, Sydney, NSW 2052, Australia

**Name and address of corresponding author:**

\*Professor Aletta E. Schutte

E-mail: [a.schutte@unsw.edu.au](mailto:a.schutte@unsw.edu.au)

School of Population Health

Tel: +61 (0)450 315 918

University of New South Wales

Twitter: @alta\_schutte

Sydney, NSW 2052

Australia

**Email addresses of other authors:**

Adriaan Jacobs: [Adriaan.Jacobs@nwu.ac.za](mailto:Adriaan.Jacobs@nwu.ac.za)

Wayne Smith: [Wayne.Smith@nwu.ac.za](mailto:Wayne.Smith@nwu.ac.za)

Marlien Pieters: [Marlien.Pieters@nwu.ac.za](mailto:Marlien.Pieters@nwu.ac.za)

**Competing interests:** none

**Disclosure:** All authors substantially contributed to this research and the article preparation, and also approved the final article for submission.

**Abstract word count:** 199

**Text word count:** 3854 (excluding abstract, figure, tables and references)

**Number of references:** 51

**Number of figures:** 1

**Number of tables:** 2

## ABSTRACT

**Aims:** Plasminogen activator inhibitor-1 (PAI-1), traditionally associated with fibrinolysis, is increasingly implicated in impaired vascular function. However, studies on its association with microvascular function are limited to the cutaneous and coronary microvascular beds in older and diseased individuals. To better understand its potential involvement in the early stages of disease development, we investigated the associations of retinal vasodilatory responses to flicker light with PAI-1 activity (PAI-1<sub>act</sub>) in young and healthy individuals.

**Methods:** We included healthy Black and White women and men (n=518; aged 20–30 years), and measured plasma PAI-1<sub>act</sub> and retinal vasodilatory responses to flicker light provocation. We also collected demographic and lifestyle data, measured blood pressure, anthropometry, blood lipids, inflammatory and other biomarkers.

**Results:** In multivariate regression analyses, maximal retinal venular dilation associated independently and inversely with PAI-1<sub>act</sub> (Adj.  $R^2=0.11$ ;  $\beta=-0.15$ ;  $p=0.001$ ) in the total group. In exploratory subgroup analyses, this association remained in White women (Adj.  $R^2=0.07$ ;  $\beta=-0.23$ ;  $p=0.005$ ), and was more robust with younger age and lower blood pressure and in non-smokers, but also with greater central adiposity, higher low-density lipoprotein cholesterol and inflammation (all  $p<0.05$ ).

**Conclusions:** Our data suggest that in young individuals, PAI-1 may already be associated with subclinical microvascular dysfunction.

## KEYWORDS

Plasminogen activator inhibitor-1; dynamic vessel analysis; retinal vascular function; endothelial dysfunction; inflammation

**ABBREVIATIONS**

AVR, Arteriolar-to-venular ratio

BMI, Body mass index

CRP, C-reactive protein

CVD, Cardiovascular disease

DBP, Diastolic blood pressure

EDTA, Ethylenediaminetetraacetic acid

ELISA, Enzyme-linked immunosorbent assay

eNOS, Endothelial nitric oxide synthase

FMD, Flow-mediated dilation

GGT, Gamma-glutamyltransferase

HDL-C, High-density lipoprotein cholesterol

HIV, Human immunodeficiency virus

IL-6, Interleukin-6

LDL-C, Low-density lipoprotein cholesterol

MAP, Mean arterial pressure

MCP-1, Monocyte chemoattractant protein-1

NO, Nitric oxide

PAI-1, Plasminogen activator inhibitor-1

PAI-1<sub>act</sub>, Plasminogen activator inhibitor-1 activity

PREDICT, Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension

RVA, Retinal vessel analyser

SBP, Systolic blood pressure

SES, Socioeconomic status

t-PA, Tissue-type plasminogen activator

WC, Waist circumference

WHtR, Waist-to-height ratio

## INTRODUCTION

Cardiovascular disease (CVD) continues to be a major health burden, with an estimated 423 million CVD cases and 18 million CVD deaths globally in 2015 (Roth et al., 2017). Assessment of the microcirculation may provide an increased understanding on the early phases of diabetes and CVD development, since it has been reported that microvascular dysfunction not only accompanies, but also possibly precedes, macrovascular dysfunction and organ damage (Houben et al., 2017).

Since the retinal microvasculature can be assessed easily and directly (Heitmar and Summers, 2012; Ikram et al., 2013), this vascular bed represents an ideal target for assessing the microcirculation. Adverse changes in retinal vessel calibres (determined from fundus images) have been independently associated with diabetes (Nguyen et al., 2008), cardiovascular risk factors, development of CVD (Ikram et al., 2013), and higher long-term risk of ischaemic stroke and mortality in both sexes (Seidemann et al., 2016). More recently, the assessment of retinal vascular function has also been made possible by evaluating arteriolar and venular dilation responses to a stressor, namely flicker light provocation (Heitmar and Summers, 2012). Impaired arteriolar or venular function, based on this method, has also been associated with cardiovascular risk factors and diseases such as obesity (Kotliar et al., 2011), diabetes (Bettermann et al., 2017; Nguyen et al., 2009), coronary artery disease (Al-Fiadh et al., 2015), and chronic heart failure (Nägele et al., 2018). Besides these studies, impaired retinal venular dilation has recently been shown to independently predict all-cause mortality in end-stage renal disease patients (Günthner et al., 2019). The value of dynamic vessel analysis is that it provides an *in vivo* assessment of microvascular endothelial function (Heitmar and Summers, 2012) and can be used in addition to more traditional measures of endothelial function such as flow-mediated dilation (FMD) in larger vessels, or the measurement of circulating biomarkers of endothelial dysfunction.

Plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor that plays a classical role in fibrinolysis (Agirbasli, 2005), has in recent years gained attention for its non-fibrinolytic involvement in CVDs such as hypertension (Jacobs et al., 2019). This association has been attributed partly to PAI-1's alternative role in vasculopathy, and specifically, endothelial dysfunction (Paulus et al., 2011) and vascular remodelling (Diebold et al., 2008). Furthermore, PAI-1 is also an acute-phase protein that is upregulated during inflammation (Paulus et al., 2011).

Only a limited number of studies have explored the association of microvascular function with PAI-1 thus far (Lee et al., 2001; Naya et al., 2007). These studies, conducted in middle-aged hypertensive patients (Naya et al., 2007) and normoglycaemic individuals with and

without a family history of diabetes (Lee et al., 2001), demonstrated that PAI-1 was associated with impaired coronary (Naya et al., 2007) and cutaneous microvascular function (Lee et al., 2001). Retinal vascular function is also associated with inflammatory mediators. In patients with end-stage renal disease, maximal retinal venular dilation associated inversely with interleukin-6 (IL-6) and C-reactive protein (CRP) (Günthner et al., 2019), whilst the retinal arterial response to flicker stimulation correlated inversely with CRP and tumour necrosis factor-alpha in patients with hypertension (Machalińska et al., 2018).

To improve our understanding of the potential role of PAI-1 in the early phases of CVD development, we investigated whether retinal vasodilatory responses to flicker light provocation are already negatively associated with PAI-1 activity (PAI-1<sub>act</sub>) in young, healthy adults. We also explored whether such associations are sex- or ethnic-specific. This may potentially shed light on the link between PAI-1 and adverse cardiovascular outcomes later in life.

## **MATERIALS AND METHODS**

### ***Study population***

Data were collected as part of the baseline phase of the African-PREDICT (Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension) study. This is an ongoing longitudinal study that was designed to track the early pathophysiological changes associated with CVD development in young, apparently healthy adults (Schutte et al., 2019). The study complied with all applicable requirements of the international regulations for research involving human participants, in particular the Declaration of Helsinki, and was approved by the Health Research Ethics Committee of the North-West University, Potchefstroom Campus (NWU-00001-12-A1). All participants gave written informed consent prior to participation.

Briefly, apparently healthy Black and White men and women (aged 20–30 years) were recruited from Potchefstroom and the surrounding areas, a region situated in the North West province of South Africa. Potential participants were first subjected to a screening process during which eligibility was determined. Participants who were classified as clinic normotensive (systolic blood pressure [SBP] <140mmHg and diastolic blood pressure [DBP] <90mmHg), HIV-negative, had no previously diagnosed chronic diseases and, in the case of women, were not pregnant or breastfeeding during the time of screening, were eligible for participation. At baseline, data were collected from n=1202 Black (n=606) and White (n=596) men and women (Schutte et al., 2019). For this sub-study, only cases containing data for the

main predictor (PAI-1<sub>act</sub>) and dependent variables (dynamic retinal vessel parameters) were included (total n=518).

### ***Questionnaires and anthropometry***

Self-reported ethnicity, sex, age, current smoking status (no/yes), and hormonal contraceptive use (women, no/yes) data were obtained using a general health and demographic questionnaire. Skill and education levels, as well as household income, also obtained using this questionnaire, were used to calculate a socioeconomic status (SES) score based on Kuppuswamy's Socioeconomic Status Scale, adapted for a South African environment (Schutte et al., 2019). The SES scores were used to categorise participants into low, middle and high SES classes. Waist circumference (WC) and body height were measured by standard procedures using a Lufkin steel anthropometric tape (W606PM, Lufkin, Apex, USA) and SECA 213 Stadiometer (SECA, Hamburg, Germany), respectively, after which the waist-to-height ratio (WHtR) was calculated. Body weight was measured using a SECA 813 electronic scale (SECA, Hamburg, Germany) and body mass index (BMI) calculated as weight (kg) / height (m)<sup>2</sup>.

### ***Retinal vessel measurements***

Retinal vessel responses to flicker light-induced provocation were analysed as previously described (Smith et al., 2020) by using a retinal vessel analyser (RVA) (IMEDOS Systems GmbH, Jena, Germany) equipped with a Zeiss Fundus FF-450<sup>Plus</sup> camera (Carl Zeiss, Mediatech, Jena, Germany). Briefly, a drop of Tropicamide (1% Alcon) was administered to (predominantly) the right eye 30 minutes prior to measurements to induce mydriatic conditions. A standard flicker protocol by IMEDOS Systems (Kotliar et al., 2017) was used, and data were captured using RVA 4.50 software. The flicker assessments consisted of a baseline phase (50 seconds), followed by flicker light provocation (20 seconds) and a recovery/second baseline period (80 seconds). Three flicker light provocation cycles were conducted, the entire measurement lasting 350 seconds. With the camera set at a 30° angle and the participant focusing on the tip of a fixation rod, a small arteriolar and a venular segment were selected in the upper (mostly) or lower temporal quadrant of the fundus image, between 0.5–2.0 optic disc diameters away from the optic disc margin. The raw numerical data generated with the RVA were exported to an Excel template (Microsoft) with built-in macros to enable further filtering, processing and analysis of data, as previously described (Smith et al., 2020). Absolute vessel baseline diameters for the selected segments were calculated individually as the median value over the last 30 seconds of the first baseline period before flicker stimulation. The following parameters were determined from a smoothed average flicker response curve: maximal arteriolar and venular dilation (the

absolute maximum of the reaction curve taken between the start of the flicker stimulation and 30 seconds after flicker initiation, expressed as a percentage of baseline).

### **Blood pressure measurements**

Clinic brachial blood pressure was measured after 10 min rest using the Dinamap® Procare 100 device (GE Medical Systems, Milwaukee, USA) while participants were in a seated, relaxed state. Measurements were taken in duplicate on each arm with a 5-minute interval between measurements. Systolic (SBP) and diastolic blood pressure (DBP) were calculated as the means of the left and right arm second measurements, after which mean arterial pressure (MAP) was calculated.

### **Biochemical sampling and analyses**

Early morning, fasting blood samples were collected in Stabilyte (acidified citrated), EDTA, serum and sodium fluoride blood collection tubes. Samples were aliquoted after centrifugation and stored at -80°C until analysis. PAI-1 activity (PAI-1<sub>act</sub>) was measured in citrated plasma samples (collected in Stabilyte tubes) using the Technozym® PAI-1 Actibind® ELISA kit (Technoclone, Vienna, Austria). Monocyte chemoattractant protein-1 (MCP-1) was measured in EDTA plasma samples and interleukin-6 (hs-IL-6) in serum samples using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). The Cobas Integra® 400 Plus analyzer (Roche, Basel, Switzerland) was used for the measurement of C-reactive protein (hs-CRP), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, and gamma-glutamyltransferase (GGT) from serum samples, and glucose from sodium fluoride plasma samples. Insulin was measured in serum samples using an electrochemiluminescence method on the E411 analyzer (Roche, Basel, Switzerland).

### **Statistical analysis**

G\*Power version 3.1.9.4 software (Faul et al., 2007) was used *a priori* to determine the required sample sizes of the total group and for subgroups stratified by sex and ethnicity. For multiple linear regression analysis set at 80% power and  $\alpha=0.05$ , a sample size between  $n=61$  and  $n=127$  was required for the total group (12 predictor variables) for a medium to large effect size ( $f^2 = 0.15 - 0.35$ ). For women, a sample size between  $n=59$  and  $n=123$  was required (11 predictor variables), and a sample size between  $n=57$  and  $n=118$  for men (10 predictor variables).

Data were evaluated for normality using histograms, and non-Gaussian distributed variables were logarithmically transformed to improve normality. Descriptive statistics are presented in **Table 1** as arithmetic means  $\pm$  standard deviations, medians and interquartile ranges, or

counts and percentages. Differences between groups were determined by independent t-tests and Chi-square tests in the case of continuous and categorical data, respectively.

To evaluate the associations of retinal vessel dilatory responses with PAI-1<sub>act</sub>, multivariate backward stepwise regression analyses were performed with either maximal retinal arteriolar or venular dilation as the dependent variable (**Table 2**). Basic models were constructed in the total group with PAI-1<sub>act</sub> as the predictor, with ethnicity, sex, and age as potential covariates. Full models additionally included SES score, smoking status, WHtR, clinic MAP, MCP-1 (as a marker of endothelial dysfunction and inflammation (Storch et al., 2017)), LDL-C, GGT, and glucose as co-variables. We used bivariate correlations of potential covariates with the independent and dependent variables to identify covariates to be included in the final regression models. Owing to known differences in PAI-1 levels and retinal vascular function between women and men (Asselbergs et al., 2007; Seshadri et al., 2016) and between different ethnicities (Lutsey et al., 2006; Patel et al., 2011), including those in our study population (Ahianti et al., 2020; Jacobs et al., 2020; Smith et al., 2020), the backward stepwise regression analyses were repeated in groups stratified by ethnicity and sex (**Supplement Tables S1 and S2**). Fully-adjusted models in women additionally contained hormonal contraceptive use as a predictor.

The IBM SPSS Statistics version 26 (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp.) software package was used for statistical analyses, and GraphPad Prism version 5.03 (GraphPad Software Inc., CA, USA) for graphical representations.

## RESULTS

The study population, with a mean age of  $25.0 \pm 3.14$  years, was equally distributed in terms of Black and White ethnicity (48% vs 52%) with fewer men than women included (41% men vs 59% women) (**Table 1**). The White groups (both women and men) displayed smaller light flicker-induced maximal retinal arteriolar and venular dilation than their Black counterparts (all  $p < 0.001$ ). White women had a larger arteriolar segment diameter and smaller venular segment diameter than Black women ( $p < 0.05$ ). Median PAI-1<sub>act</sub> levels of Black women were 5.03 U/mL compared with 3.49 U/mL in White women ( $p = 0.054$ ), whilst White men had higher PAI-1<sub>act</sub> than Black men (16.8 vs 3.36 U/mL,  $p < 0.001$ ).

Using multivariate-adjusted regression models, we determined whether associations exist between retinal vessel dilatory responses to light flicker provocation and PAI-1<sub>act</sub>. Backward stepwise regression analysis in the total group ( $n = 518$ ) revealed a negative association of maximal retinal venular dilation with PAI-1<sub>act</sub> (Adj.  $R^2 = 0.09$ ,  $\beta = -0.12$ ,  $p = 0.005$ ) in the basic

model, adjusted for ethnicity, sex and age (**Table 2**). This association remained significant (Adj.  $R^2=0.11$ ,  $\beta= -0.15$ ,  $p=0.001$ ) after additionally adjusting for SES score, smoking, WHtR, clinic MAP, MCP-1, LDL-C, GGT, and glucose. Other covariates that contributed to the variance in retinal vessel dilatory responses in full models were ethnicity, SES score, and WHtR ( $p<0.05$ ).

When stratified by ethnicity and sex, maximal retinal arteriolar dilation (Adj.  $R^2=0.08$ ,  $\beta= -0.18$ ,  $p=0.026$ ) (**Supplement Table S1**) and venular dilation (Adj.  $R^2=0.07$ ,  $\beta= -0.23$ ,  $p=0.005$ ) (**Supplement Table S2**) associated negatively with PAI-1<sub>act</sub> in the fully-adjusted models in White women only ( $n=156$ ). Whilst recognising the smaller sample sizes of these subgroups, we found no associations in men or Black women. Other covariates that contributed to the variance in retinal vessel dilatory responses in full models were age, smoking, hormonal contraceptive use, WHtR, clinic MAP, MCP-1, LDL-C, and glucose ( $p<0.05$ ).

The significance of PAI-1<sub>act</sub> in fully-adjusted models obtained using backward stepwise regression analysis was confirmed using forward stepwise regression analysis.

To gain a better understanding of the contributions of different potential confounders on the associations between retinal vasodilatory responses and PAI-1<sub>act</sub>, we performed subgroup analyses, stratified according to categorical variables (ethnicity, sex, smoking status and SES class) and median-split for continuous variables (age, WHtR, clinic MAP, MCP-1, LDL-C, GGT and glucose) (maximal retinal venular dilation in **Figure 1** and maximal retinal arteriolar dilation in **Supplement Figure S1**). Although MCP-1 (as a marker of endothelial dysfunction and inflammation (Storch et al., 2017)) was included as a covariate in multivariate regression models, we also performed analyses in categories based on median-split CRP to further evaluate the association from a systemic inflammation perspective. For maximal retinal venular dilation, the independent negative association with PAI-1<sub>act</sub> was most robust in White women, those aged < 25 years, of high SES class, non-smoking, and with lower clinic MAP but with higher WHtR, LDL-C and CRP. The independent negative association of maximal retinal arteriolar dilation with PAI-1<sub>act</sub> was most robust in individuals of high SES class and with low MCP-1.

**Table 1.** Characteristics of the study population

|                                                |               | Total group (n=518) |  |  | Women (n=308)    |                  |        | Men (n=210)      |                  |        |
|------------------------------------------------|---------------|---------------------|--|--|------------------|------------------|--------|------------------|------------------|--------|
|                                                |               |                     |  |  | Black (n=152)    | White (n=156)    | p *    | Black (n=97)     | White (n=113)    | p †    |
| Ethnicity (Black), n (%)                       |               | 249 (48.1)          |  |  | -                | -                | -      | -                | -                | -      |
| Sex (men), n (%)                               |               | 210 (40.5)          |  |  | -                | -                | -      | -                | -                | -      |
| Age (years)                                    |               | 25.0 ± 3.14         |  |  | 24.8 ± 3.40      | 25.4 ± 2.73      | 0.10   | 24.0 ± 3.26      | 25.6 ± 2.99      | <0.001 |
| Socio-economic status score                    |               | 21.4 ± 6.67         |  |  | 18.0 ± 5.87      | 25.4 ± 4.38      | <0.001 | 16.5 ± 5.89      | 24.8 ± 5.74      | <0.001 |
| SES class                                      | Low, n (%)    | 179 (34.6)          |  |  | 83 (54.6)        | 14 (9.0)         | <0.001 | 66 (68.0)        | 16 (14.2)        | <0.001 |
|                                                | Middle, n (%) | 119 (23.0)          |  |  | 43 (28.3)        | 36 (23.1)        |        | 18 (18.6)        | 22 (19.5)        |        |
|                                                | High, n (%)   | 220 (42.5)          |  |  | 26 (17.1)        | 106 (67.9)       |        | 13 (13.4)        | 75 (66.4)        |        |
| Smoking, n (%) <sup>a</sup>                    |               | 126 (24.3)          |  |  | 18 (11.8)        | 23 (14.7)        | 0.45   | 52 (53.6)        | 33 (29.2)        | <0.001 |
| Hormonal contraceptive use, n (%) <sup>a</sup> |               | 136 (44.2)          |  |  | 72 (47.4)        | 64 (41.0)        | 0.18   | -                | -                | -      |
| Body mass index (kg/m <sup>2</sup> )           |               | 24.3 (21.1-28.0)    |  |  | 25.9 (21.9-29.9) | 23.1 (21.0-26.0) | 0.001  | 20.9 (19.2-23.9) | 26.6 (24.2-29.8) | <0.001 |
| Waist-to-height ratio                          |               | 0.46 (0.42-0.52)    |  |  | 0.49 (0.43-0.56) | 0.43 (0.41-0.48) | <0.001 | 0.42 (0.40-0.47) | 0.50 (0.45-0.53) | <0.001 |
| <b>Retinal measurements</b>                    |               |                     |  |  |                  |                  |        |                  |                  |        |
| Maximal retinal arteriolar dilation (%)        |               | 4.53 ± 2.22         |  |  | 5.72 ± 1.83      | 3.30 ± 1.93      | <0.001 | 5.39 ± 2.03      | 3.91 ± 2.13      | <0.001 |
| Arteriolar segment diameter (MU)               |               | 121 ± 15.3          |  |  | 119 ± 15.0       | 124 ± 14.3       | 0.009  | 118 ± 16.3       | 120 ± 15.7       | 0.45   |
| Maximal retinal venular dilation (%)           |               | 4.10 (2.90-5.70)    |  |  | 4.60 (3.20-6.40) | 3.60 (2.80-5.10) | <0.001 | 4.80 (3.50-6.65) | 3.40 (2.40-4.60) | <0.001 |
| Venular segment diameter (MU)                  |               | 152 ± 20.0          |  |  | 155 ± 19.7       | 150 ± 20.1       | 0.046  | 155 ± 20.5       | 150 ± 19.5       | 0.10   |
| <b>Blood pressure</b>                          |               |                     |  |  |                  |                  |        |                  |                  |        |
| Clinic SBP (mmHg)                              |               | 116 ± 11.8          |  |  | 114 ± 9.84       | 109 ± 10.0       | <0.001 | 123 ± 11.6       | 123 ± 9.34       | 0.69   |
| Clinic DBP (mmHg)                              |               | 78.1 ± 8.20         |  |  | 78.3 ± 7.49      | 74.9 ± 7.39      | <0.001 | 81.4 ± 8.73      | 79.4 ± 8.26      | 0.09   |
| Clinic MAP (mmHg)                              |               | 93.3 ± 8.82         |  |  | 92.5 ± 7.68      | 88.6 ± 7.89      | <0.001 | 97.9 ± 8.93      | 97.0 ± 7.81      | 0.40   |
| <b>Biochemical measurements</b>                |               |                     |  |  |                  |                  |        |                  |                  |        |
| PAI-1 <sub>act</sub> (U/mL)                    |               | 5.15 (2.07-11.8)    |  |  | 5.03 (2.08-9.70) | 3.49 (1.47-8.48) | 0.054  | 3.36 (1.76-7.18) | 16.8 (6.77-27.5) | <0.001 |

|                        |                  |                  |                  |                  |                  |                  |                  |
|------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| MCP-1 (pg/mL)          | 137 (118-168)    | 150 (123-173)    | 120 (101-137)    | <b>&lt;0.001</b> | 169 (146-197)    | 133 (117-157)    | <b>&lt;0.001</b> |
| CRP (mg/L)             | 1.04 (0.39-2.88) | 1.82 (0.73-4.06) | 0.82 (0.35-3.14) | <b>0.021</b>     | 0.66 (0.25-1.38) | 0.97 (0.35-2.31) | <b>0.026</b>     |
| IL-6 (pg/mL)           | 0.91 (0.59-1.41) | 1.16 (0.76-1.80) | 0.73 (0.47-1.25) | <b>&lt;0.001</b> | 0.92 (0.56-1.24) | 0.86 (0.58-1.34) | 0.60             |
| HDL-C (mmol/L)         | 1.31 (1.09-1.58) | 1.22 (1.03-1.51) | 1.53 (1.32-1.79) | <b>&lt;0.001</b> | 1.32 (1.13-1.58) | 1.13 (0.94-1.29) | <b>&lt;0.001</b> |
| LDL-C (mmol/L)         | 2.67 (2.07-3.34) | 2.39 (1.88-2.94) | 2.84 (2.32-3.40) | <b>&lt;0.001</b> | 2.13 (1.69-2.72) | 3.33 (2.74-3.98) | <b>&lt;0.001</b> |
| Triglycerides (mmol/L) | 0.83 (0.62-1.13) | 0.69 (0.54-0.90) | 0.90 (0.66-1.23) | <b>&lt;0.001</b> | 0.80 (0.62-1.04) | 1.11 (0.77-1.71) | <b>&lt;0.001</b> |
| GGT (U/L)              | 20.7 (13.6-30.4) | 21.4 (15.0-33.7) | 12.9 (9.83-20.4) | <b>&lt;0.001</b> | 26.7 (19.2-35.8) | 26.4 (18.1-33.8) | 0.66             |
| Glucose (mmol/L)       | 4.60 ± 0.75      | 4.35 ± 0.78      | 4.74 ± 0.53      | <b>&lt;0.001</b> | 4.17 ± 0.86      | 5.11 ± 0.49      | <b>&lt;0.001</b> |
| Insulin (μU/mL)        | 8.84 (6.00-13.2) | 11.5 (8.52-16.3) | 7.91 (5.55-10.8) | <b>&lt;0.001</b> | 7.37 (4.83-10.0) | 8.64 (5.92-13.6) | <b>0.015</b>     |

Abbreviations: SES, socio-economic status; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MAP, mean arterial pressure; PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; MCP-1, monocyte chemoattractant protein-1; CRP, C-reactive protein; IL-6, interleukin-6; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase.

Notes: data expressed as arithmetic mean ± standard deviation, median and interquartile range, or count and percentage.

Bold text indicates p-values < 0.05.

<sup>a</sup> Self-reported smoking and hormonal contraceptive use.

\* Difference between Black and White women; † Difference between Black and White men.

**Table 2.** Multivariate backward linear regression analysis with maximal retinal arteriolar and venular dilation as dependent variables (n=518), with PAI-1<sub>act</sub> as main independent variable

|                             | Maximal retinal arteriolar dilation  |                  | Maximal retinal venular dilation     |                  |
|-----------------------------|--------------------------------------|------------------|--------------------------------------|------------------|
|                             | $\beta$ (95% CI)                     | p                | $\beta$ (95% CI)                     | p                |
| <b>Model 1</b>              | <i>Adjusted R<sup>2</sup>=0.21 *</i> |                  | <i>Adjusted R<sup>2</sup>=0.09 *</i> |                  |
| PAI-1 <sub>act</sub> (U/mL) | -                                    | -                | -0.12 (-0.20; -0.04)                 | <b>0.005</b>     |
| Ethnicity <sup>a</sup>      | -0.46 (-0.54; -0.38)                 | <b>&lt;0.001</b> | -0.23 (-0.31; -0.14)                 | <b>&lt;0.001</b> |
| Age (years)                 | -                                    | -                | -0.12 (-0.20; -0.04)                 | <b>0.005</b>     |
| <b>Model 2</b>              | <i>Adjusted R<sup>2</sup>=0.24 *</i> |                  | <i>Adjusted R<sup>2</sup>=0.11 *</i> |                  |
| PAI-1 <sub>act</sub> (U/mL) | -                                    | -                | -0.15 (-0.24; -0.06)                 | <b>0.001</b>     |
| Ethnicity <sup>a</sup>      | -0.45 (-0.53; -0.37)                 | <b>&lt;0.001</b> | -0.18 (-0.29; -0.08)                 | <b>0.001</b>     |
| Age (years)                 | -                                    | -                | -0.09 (-0.19; 0.01)                  | 0.082            |
| SES score                   | -                                    | -                | -0.12 (-0.24; -0.004)                | <b>0.043</b>     |
| WHtR                        | 0.16 (0.08; 0.23)                    | <b>&lt;0.001</b> | 0.11 (0.02; 0.20)                    | <b>0.023</b>     |
| Clinic MAP (mmHg)           | 0.08 (-0.004; 0.15)                  | 0.063            | -                                    | -                |
| GGT (U/L)                   | -                                    | -                | -0.09 (-0.18; 0.004)                 | 0.059            |

Model 1 predictor variables included: PAI-1<sub>act</sub>, ethnicity, sex, and age; Model 2 predictor variables included all model 1 predictor variables plus SES score, smoking, WHtR, clinic MAP, MCP-1, LDL-C, GGT, and glucose.

Abbreviations: PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; SES, socio-economic status; WHtR, waist-to-height ratio; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase.

Notes: <sup>a</sup> Ethnicity coded as 0, Black and 1, White.

Bold text indicates p-values < 0.05.

- Indicates predictor variables not entered into final backward stepwise regression models.

\* Model p<0.05.

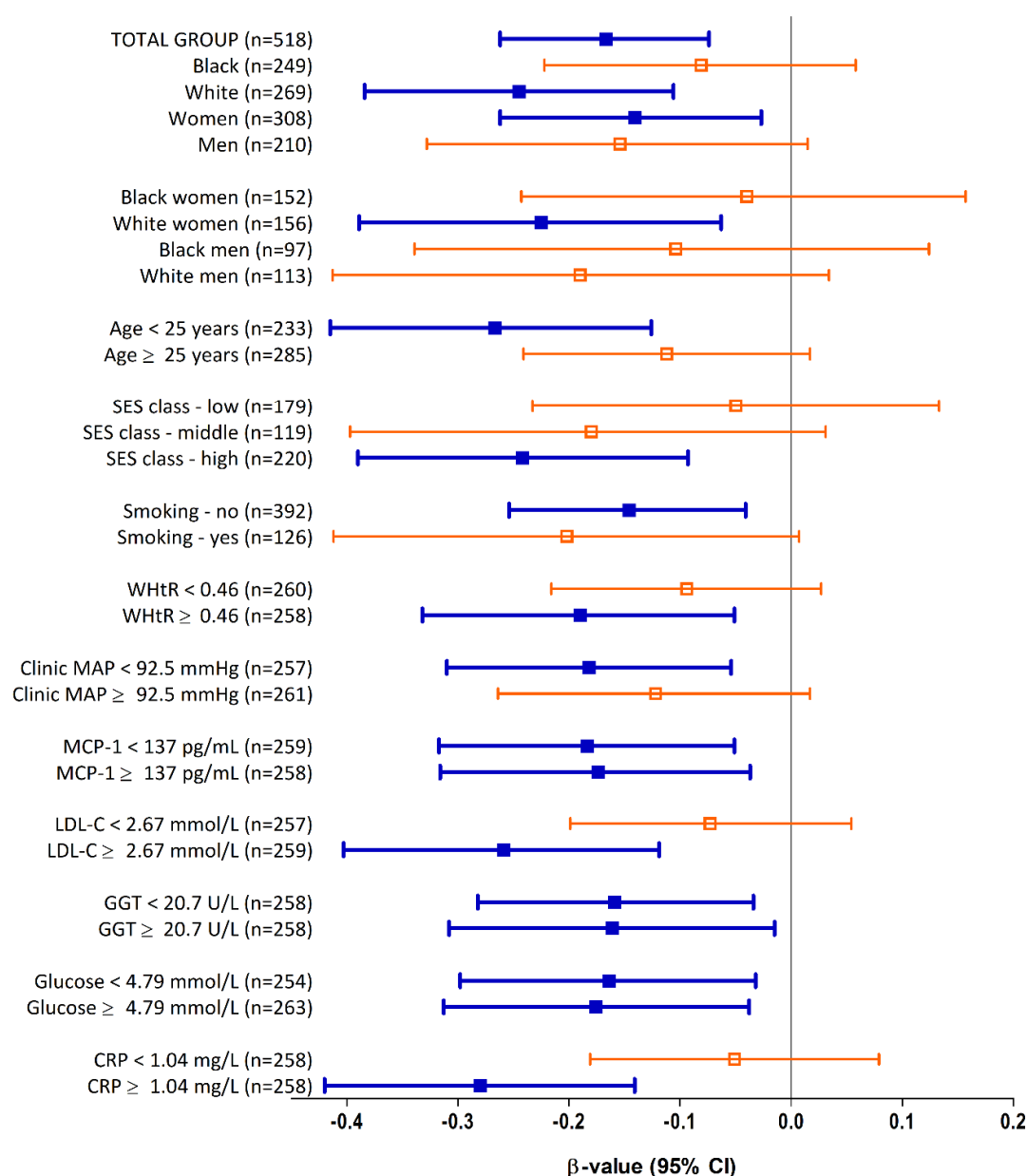


**Figure 1.** Multivariate linear regression analysis with maximal retinal venular dilation as dependent variable and PAI-1<sub>act</sub> as main independent variable, according to categorical variables and median split values for continuous variables.

Adjusted for ethnicity, sex, age, SES score, smoking, WHtR, clinic MAP, MCP-1, LDL-C, GGT, glucose and contraceptive use (women only).

Abbreviations: SES, socio-economic status; WHtR, waist-to-height ratio; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase; CRP, C-reactive protein.

## DISCUSSION

We determined whether retinal vessel vasodilatory responses to flicker light provocation are associated with PAI-1<sub>act</sub> in young healthy adults. We found an independent inverse association of maximal retinal venular dilation with PAI-1<sub>act</sub> in the total group.

Our findings suggest that PAI-1 may already be involved in modulating microvascular function in individuals without any overt CVD at an early stage of their lives. To our knowledge, this is the first study to report an association of flicker-induced retinal vessel dilation with PAI-1, a biomarker representative of endothelial dysfunction and inflammation. A limited number of studies have investigated the associations of static retinal vessel calibres with PAI-1, with diverse findings being reported (Brazionis et al., 2010; Lammertyn et al., 2015; Wong et al., 2006). No associations were found in a group of Black and White South Africans (mean ages 48 and 49 years, respectively) (Lammertyn et al., 2015), whilst a weak positive association between PAI-1 and retinal venular calibre was found in individuals (mean age 62 years) free from clinical CVD (Wong et al., 2006). Conversely, in patients with Type 2 diabetes (mean age 64 years), PAI-1 associated negatively with retinal venular calibre and positively with retinal arteriolar calibre and arteriolar-to-venular ratio (AVR) (Brazionis et al., 2010). From these studies it is clear that a relationship exists between the retinal vessel calibres and PAI-1, but that this association is likely influenced by the participant selection and characteristics. As the calibres largely reflect the structure of the vessels, additional information on this relationship may be gained when the retinal microvascular bed is challenged, as with the light flicker test.

Associations between PAI-1 and microvascular responses in other vascular beds, i.e. coronary (Naya et al., 2007) and cutaneous (Lee et al., 2001), have been investigated in relatively small studies. These studies, conducted in patients with hypertension (mean age 52.8 years; n=29) (Naya et al., 2007) and normoglycaemic individuals with (mean age 42 years; n=21) and without family history of diabetes (mean age 40 years; n=21) (Lee et al., 2001), also reported that PAI-1 was associated with impaired coronary (Naya et al., 2007) and cutaneous microvascular function (Lee et al., 2001). Patients with Type 2 diabetes with higher PAI-1 levels compared with controls also showed impairment of both endothelium-dependent and -independent vasodilation of the forearm skin microvasculature (Beer et al., 2008). Higher PAI-1 was also associated with diabetic retinopathy (Azad et al., 2014). These findings, based on the microvasculature, confirm previous reports in the macrovasculature where PAI-1 associated negatively with FMD (Eržen and Šabovič, 2013).

Our main finding is that maximal retinal venular dilation was inversely associated with PAI-1<sub>act</sub> in a young healthy cohort. Maximal retinal venular dilation has also been inversely associated with leptin levels (Ahiente et al., 2020), and impaired retinal venular dilatory responses have been associated with cardiovascular disease, risk factors and metabolic disturbances, such as chronic heart failure (Nägele et al., 2018), hypercholesterolaemia (Reimann et al., 2009), obesity (Kotliar et al., 2011), diabetes (Nguyen et al., 2009) and diabetic retinopathy (Lim et al., 2014; Nguyen et al., 2009). In patients with end-stage renal disease, impaired retinal venular dilation emerged as an independent predictor of all-cause mortality (Günthner et al., 2019). In this study, maximal retinal venular dilation was also inversely associated with CRP and IL-6 (Günthner et al., 2019). In patients with hypercholesterolaemia, the retinal venular dilatory response to flicker light improved following LDL-apheresis, suggesting differential responses of arterioles and venules to metabolic perturbations as there was no improvement in the retinal arteriolar dilatory response (Reimann et al., 2009). The authors attributed this improvement in venular dilation to a reduction in retinal microcirculatory oxidative stress and not to decreased serum lipids *per se* (Reimann et al., 2009). However, the possibility exists that a reduction in PAI-1 may have contributed to the improved venular dilatory response, since LDL-apheresis is also known to decrease haemostatic factors, including PAI-1 levels (Hovland et al., 2010). This is supported by our observation of a stronger association of maximal retinal venular dilation with PAI-1<sub>act</sub> in the presence of higher LDL-C.

Exploratory analysis indicated that the inverse association of maximal retinal venular dilation with PAI-1<sub>act</sub> was most prominent in White participants, in women, in those aged < 25 years, in non-smokers and in those with lower blood pressure, but with higher central adiposity, LDL-C and CRP. Given the inverse association of maximal retinal venular dilation with PAI-1<sub>act</sub> observed in the total group, the possibility exists that these associations may also be present in other subgroups, but that the limited sample sizes hampered the statistical significance of the associations in these groups. Nevertheless, we speculate that both ethnic and sex differences may play a role in the investigated associations (Hao et al., 2010; Patel et al., 2011; Seshadri et al., 2016), the latter being supported by a sex-specific biology for PAI-1 (Asselbergs et al., 2007; Björck et al., 2011). Greater central adiposity is generally accompanied by higher PAI-1 levels, since visceral adipose tissue is a major source of PAI-1 production (Barnard et al., 2016). LDL-C and oxidised LDL-C are believed to stimulate PAI-1 expression and release (Jovin et al., 2003; Song et al., 2005) and expression of PAI-1, an acute-phase protein, is also upregulated by CRP (Chen et al., 2008), which underscores the strong link of PAI-1 to inflammation. Reduced retinal vessel dilation has been associated

with smoking (Garhöfer et al., 2011), ageing (Seshadri et al., 2016) and hypertension (Nagel et al., 2004), the former demonstrated in retinal venules and the latter two in arterioles. We speculate that the associations of these factors with impaired retinal venular dilation may be of such an extent that the more subtle inverse association of maximal venular dilation with PAI-1<sub>act</sub> is partly obscured. The latter association thus becomes more evident in non-smokers, and with younger age and lower blood pressure, as demonstrated by our results. It is notable that we found this result specifically in White women as a subgroup. Furthermore, we also found a weak negative association of maximal retinal arteriolar dilation with PAI-1<sub>act</sub> in White women, and this association was more robust in individuals of high SES class and with low MCP-1. The reason for this finding in White women specifically is not clear and more information will be gained with future follow-up work in this subgroup.

The mechanism that underlies the inverse association of maximal retinal vessel dilation with PAI-1<sub>act</sub> remains a matter of speculation. Tissue-type plasminogen activator (t-PA), one of the substrates of PAI-1, has been shown to promote vasodilation during neurovascular coupling (Anfray et al., 2019; Park et al., 2008). It is thus conceivable that PAI-1, as the inhibitor of t-PA, is capable of attenuating this vasodilatory effect of t-PA. Indeed, Park *et al.* (2008) demonstrated that PAI-1 attenuated the t-PA-mediated blood flow increase upon neuronal stimulation. A second mechanism by which PAI-1 may relate to impaired vasodilation involves PAI-1's role in endothelial dysfunction, specifically in relation to NO bioavailability. Nitric oxide has been shown to play a role in flicker-induced retinal vasodilation (Dorner et al., 2003) and PAI-1 has recently been shown to directly inhibit eNOS activity (Garcia et al., 2020), thereby reducing NO synthesis.

To our knowledge, this was the first study to investigate the associations of retinal vessel dilatory responses with PAI-1. Our findings should be interpreted within the context of certain limitations. The cross-sectional study design lent itself to the investigation of associations, but cause-and-effect could not be established. Furthermore, inclusion of participants in the African-PREDICT study was based on selected criteria to ensure a young population free of overt cardiovascular disease, and this may consequently limit generalisation of our findings to the larger population.

In conclusion, maximal retinal venular dilation in response to flicker light was independently and inversely associated with PAI-1<sub>act</sub> levels in young healthy individuals. This adds supporting evidence to the association of PAI-1 with vasculopathy and, specifically, impaired microvascular function, possibly due to PAI-1's mechanistic link to inflammation and endothelial dysfunction.

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## SUPPLEMENTARY MATERIAL

**Supplement Table S1.** Multivariate backward linear regression analysis with maximal retinal arteriolar dilation as dependent variable, according to ethnicity and sex

|                             | Black women (n=152)                  |              | White women (n=156)                  |              | Black men (n=97) |   | White men (n=113)                    |              |
|-----------------------------|--------------------------------------|--------------|--------------------------------------|--------------|------------------|---|--------------------------------------|--------------|
|                             | $\beta$ (95% CI)                     | p            | $\beta$ (95% CI)                     | p            | $\beta$ (95% CI) | p | $\beta$ (95% CI)                     | p            |
| <b>Model 1</b>              |                                      |              | <i>Adjusted R<sup>2</sup>=0.02</i>   |              |                  |   | <i>Adjusted R<sup>2</sup>=0.03</i>   |              |
| PAI-1 <sub>act</sub> (U/mL) | -                                    | -            | -0.15 (-0.31; 0.003)                 | 0.055        | -                | - | 0.18 (-0.002; 0.37)                  | 0.052        |
| <b>Model 2</b>              | <i>Adjusted R<sup>2</sup>=0.10 *</i> |              | <i>Adjusted R<sup>2</sup>=0.08 *</i> |              |                  |   | <i>Adjusted R<sup>2</sup>=0.09 *</i> |              |
| PAI-1 <sub>act</sub> (U/mL) | -                                    | -            | -0.18 (-0.34; -0.02)                 | <b>0.026</b> | -                | - | -                                    | -            |
| Age (years)                 | -                                    | -            | -                                    | -            | -                | - | -0.20 (-0.39; -0.01)                 | <b>0.036</b> |
| Contraceptives <sup>a</sup> | 0.16 (0.01; 0.32)                    | <b>0.044</b> | 0.17 (0.01; 0.32)                    | <b>0.037</b> | -                | - | -                                    | -            |
| WtHR                        | 0.24 (0.08; 0.39)                    | <b>0.003</b> | -                                    | -            | -                | - | -                                    | -            |
| Clinic MAP (mmHg)           | -                                    | -            | 0.23 (0.07; 0.39)                    | <b>0.005</b> | -                | - | 0.20 (0.02; 0.38)                    | <b>0.034</b> |
| MCP-1 (pg/mL)               | -                                    | -            | -                                    | -            | -                | - | 0.22 (0.04; 0.40)                    | <b>0.017</b> |
| Glucose (mmol/L)            | -0.18 (-0.33; -0.02)                 | <b>0.027</b> | -                                    | -            | -                | - | -                                    | -            |

Model 1 predictor variables included: PAI-1<sub>act</sub> and age; Model 2 predictor variables included all model 1 predictor variables plus SES score, smoking, hormonal contraceptive use (women only), WtHR, clinic MAP, MCP-1, LDL-C, GGT, and glucose.

Abbreviations: PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; SES, socio-economic status; WtHR, waist-to-height ratio; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase.

Notes: <sup>a</sup> Contraceptive use coded as 0, no and 1, yes.

Bold text indicates p-values < 0.05.

- Indicates predictor variables not entered into final backward stepwise regression models.

\* Model p<0.05.

**Supplement Table S2.** Multivariate backward linear regression analysis with maximal retinal venular dilation as dependent variable, according to ethnicity and sex

|                             | Black women (n=152)                  |              | White women (n=156)                  |              | Black men (n=97)                     |              | White men (n=113)                    |              |
|-----------------------------|--------------------------------------|--------------|--------------------------------------|--------------|--------------------------------------|--------------|--------------------------------------|--------------|
|                             | $\beta$ (95% CI)                     | p            | $\beta$ (95% CI)                     | p            | $\beta$ (95% CI)                     | p            | $\beta$ (95% CI)                     | p            |
| <b>Model 1</b>              | <i>Adjusted R<sup>2</sup>=0.03 *</i> |              | <i>Adjusted R<sup>2</sup>=0.03 *</i> |              |                                      |              |                                      |              |
| PAI-1 <sub>act</sub> (U/mL) | -                                    | -            | -0.18 (-0.34; -0.02)                 | <b>0.024</b> | -                                    | -            | -                                    | -            |
| Age (years)                 | -0.20 (-0.36; -0.04)                 | <b>0.014</b> | -                                    | -            | -                                    | -            | -                                    | -            |
| <b>Model 2</b>              | <i>Adjusted R<sup>2</sup>=0.03 *</i> |              | <i>Adjusted R<sup>2</sup>=0.07 *</i> |              | <i>Adjusted R<sup>2</sup>=0.06 *</i> |              | <i>Adjusted R<sup>2</sup>=0.05 *</i> |              |
| PAI-1 <sub>act</sub> (U/mL) | -                                    | -            | -0.23 (-0.38; -0.07)                 | <b>0.005</b> | -                                    | -            | -                                    | -            |
| Age (years)                 | -0.19 (-0.35; -0.03)                 | <b>0.023</b> | -0.14 (-0.29; 0.02)                  | 0.088        | -                                    | -            | -                                    | -            |
| SES score                   | -                                    | -            | -                                    | -            | -                                    | -            | -0.16 (-0.35; 0.02)                  | 0.079        |
| Smoking <sup>a</sup>        | -                                    | -            | -                                    | -            | 0.26 (0.06; 0.46)                    | <b>0.012</b> | -                                    | -            |
| WHtR                        | -                                    | -            | 0.20 (0.03; 0.37)                    | <b>0.019</b> | -                                    | -            | -                                    | -            |
| LDL-C (mmol/L)              | -                                    | -            | -                                    | -            | -                                    | -            | 0.21 (0.02; 0.39)                    | <b>0.028</b> |
| GGT (U/L)                   | -                                    | -            | -0.15 (-0.31; 0.02)                  | 0.080        | -                                    | -            | -                                    | -            |

Model 1 predictor variables included: PAI-1<sub>act</sub> and age; Model 2 predictor variables included all model 1 predictor variables plus SES score, smoking, hormonal contraceptive use (women only), WHtR, clinic MAP, MCP-1, LDL-C, GGT, and glucose.

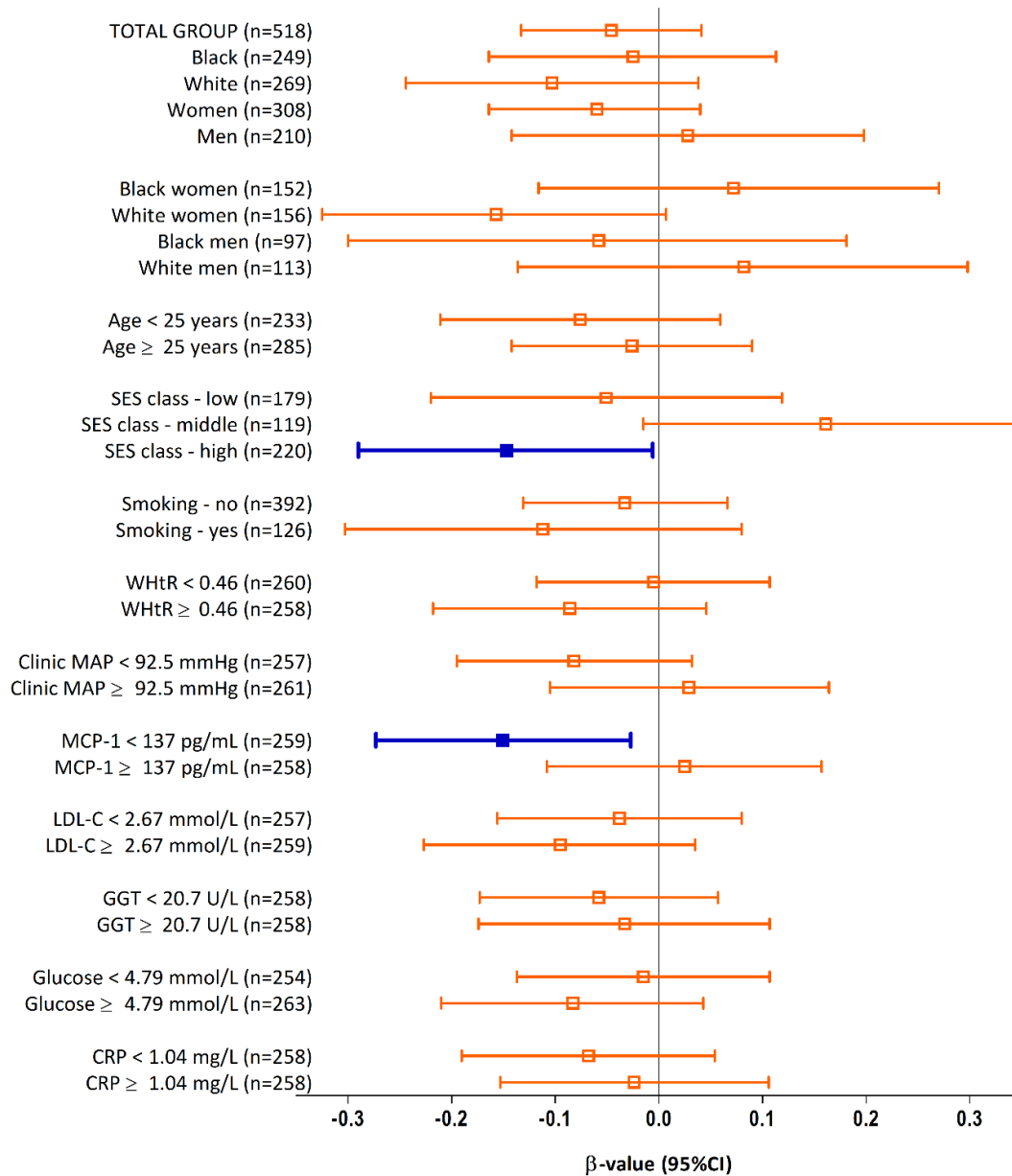
Abbreviations: PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; SES, socio-economic status; WHtR, waist-to-height ratio; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase.

Notes: <sup>a</sup> Current smoking coded as 0, no and 1, yes.

Bold text indicates p-values < 0.05.

- Indicates predictor variables not entered into final backward stepwise regression models.

\* Model p<0.05.



**Supplement Figure S1.** Multivariate linear regression analysis with maximal retinal arteriolar dilation as dependent variable and PAI-1<sub>act</sub> as main independent variable, according to categorical variables and median split values for continuous variables.

Adjusted for ethnicity, sex, age, SES score, smoking, WHtR, clinic MAP, MCP-1, LDL-C, GGT, glucose and contraceptive use (women only).

Abbreviations: SES, socio-economic status; WHtR, waist-to-height ratio; MAP, mean arterial pressure; MCP-1, monocyte chemoattractant protein-1; LDL-C, low-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase; CRP, C-reactive protein.

**HIGHLIGHTS**

- Maximal retinal venular dilation associated inversely with PAI-1 in young adults
- This association was most robust in White women
- Conditions e.g. central obesity and increased inflammation influenced this association
- Maximal arteriolar dilation weakly associated inversely with PAI-1 in White women

# CHAPTER 4

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Research article 2:

**The association of PAI-1 with 24 h blood pressure in young healthy adults is influenced by smoking and alcohol use:  
The African-PREDICT study**

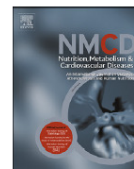
**This article was published in *Nutrition, Metabolism & Cardiovascular Diseases*.**

*Jacobs A, Pieters M, Schutte AE. The association of PAI-1 with 24 h blood pressure in young healthy adults is influenced by smoking and alcohol use: The African-PREDICT study. Nutrition, Metabolism and Cardiovascular Diseases. 2020;30:2063-71; doi.org/10.1016/j.numecd.2020.07.003*

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## Nutrition, Metabolism & Cardiovascular Diseases

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|                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|-----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Impact factor</b>  | 3.700                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| <b>Aims and scope</b> | <i>Nutrition, Metabolism &amp; Cardiovascular Diseases</i> is a forum designed to focus on the powerful interplay between nutritional and metabolic alterations, and cardiovascular disorders. It aims to be a highly qualified tool to help refine strategies against the nutrition-related epidemics of metabolic and cardiovascular diseases. By presenting original clinical and experimental findings, it introduces readers and authors into a rapidly developing area of clinical and preventive medicine, including also vascular biology. Of particular concern are the origins, the mechanisms and the means to prevent and control diabetes, atherosclerosis, hypertension, and other nutrition-related diseases. |

### INSTRUCTIONS FOR AUTHORS:

| General and technical considerations                                                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                                                         |                 |               |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|---------------|
| <ul style="list-style-type: none"> <li>Language: English (American or British usage is accepted, but not a mixture of these).</li> <li>Use decimal points (not decimal commas); use a space for thousands (10 000 and above).</li> <li>Do not use the word processor's options to justify text or to hyphenate words.</li> </ul> |                                                                                                                                                                                                                                                                                         |                 |               |
| Title page information                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                                                         |                 |               |
| <b>Title</b>                                                                                                                                                                                                                                                                                                                     | ≤ 120 characters, informative and focused on innovative contents of study.                                                                                                                                                                                                              |                 |               |
| <b>Author names and affiliations</b>                                                                                                                                                                                                                                                                                             | <ul style="list-style-type: none"> <li>First and last names spelt out in full, middle names shown as initial(s).</li> <li>Affiliations below authors' names, indicated with a lower-case superscript letter after the author's name and in front of the appropriate address.</li> </ul> |                 |               |
| <b>Corresponding author</b>                                                                                                                                                                                                                                                                                                      | <ul style="list-style-type: none"> <li>Telephone number, e-mail address and complete postal address.</li> </ul>                                                                                                                                                                         |                 |               |
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| <b>Number of references, figures and tables</b>                                                                                                                                                                                                                                                                                  | ≤ 60 references.<br>≤ 6 figures/tables.                                                                                                                                                                                                                                                 | <b>Keywords</b> | 3-8 keywords. |
| Abstract                                                                                                                                                                                                                                                                                                                         |                                                                                                                                                                                                                                                                                         |                 |               |
| <ul style="list-style-type: none"> <li>Typed double spaced on a separate page.</li> <li>Structured under headings (1) Background and Aims, (2) Methods and Results, (3) Conclusion.</li> </ul>                                                                                                                                   |                                                                                                                                                                                                                                                                                         |                 |               |

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| <b>Text (body)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| Subheads that should be included in all research articles: Introduction, Methods, Results, Discussion, Acknowledgements, References, Appendices, Tables, Figure Legends.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| <b>Methods section</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
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| <b>Tables</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| <ul style="list-style-type: none"> <li>• Tables should include titles, description, and footnotes.</li> <li>• Can be placed either next to the relevant text in the article, or on separate page(s) at the end.</li> <li>• Number tables consecutively in accordance with their appearance in the text.</li> </ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| <b>Figures/electronic artwork</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
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| List funding sources in the standard way to facilitate compliance to funder's requirements.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| <b>Declaration of interest</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
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**Title:** The association of PAI-1 with 24 h blood pressure in young healthy adults is influenced by smoking and alcohol use: The African-PREDICT study

**Authors:**

Adriaan JACOBS<sup>ab</sup>, Marlien PIETERS<sup>a</sup>, Aletta E. SCHUTTE<sup>bc\*</sup>

**Affiliations:**

<sup>a</sup>Centre of Excellence for Nutrition (CEN), North-West University, Potchefstroom, South Africa

<sup>b</sup>Hypertension in Africa Research Team (HART), Medical Research Council Unit for Hypertension and Cardiovascular Disease, North-West University, Potchefstroom, South Africa

<sup>c</sup>School of Population Health, University of New South Wales; The George Institute for Global Health, Sydney, Australia

**Name and address of corresponding author:**

\*Professor Aletta E. Schutte

E-mail: a.schutte@unsw.edu.au

School of Population Health

Tel: +61 (0)450 315 918

University of New South Wales

Twitter: @alta\_schutte

Sydney, NSW 2052

Australia

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**Keywords:** Ambulatory blood pressure; Tobacco; Drinking; Young adults; Healthy; Endothelial dysfunction

## ABSTRACT

**Background and Aims:** The association between plasminogen activator inhibitor-1 (PAI-1) and blood pressure is well established, but it is debatable whether raised PAI-1 levels precede or result from raised blood pressure. Furthermore, it is unclear whether this association already exists in the absence of overt hypertension and to what degree it is influenced by health behaviours. Our aim was to investigate the association of 24 h blood pressure with PAI-1 activity (PAI-1<sub>act</sub>) in a young, healthy cohort, and to assess the influence of alcohol consumption and smoking on these associations.

**Methods and Results:** Healthy black and white men and women (aged 20-30 years, n=1156) were cross-sectionally analysed. Statistical analysis was performed first split by ethnicity and sex and then by alcohol consumption and smoking. Regression analyses adjusted for age revealed positive associations of 24 h blood pressure with PAI-1<sub>act</sub> in most groups ( $p<0.05$ ). In multivariate-adjusted analyses, significance was lost in all groups except black men, who also had higher monocyte chemoattractant protein-1 (MCP-1) and von Willebrand factor antigen (vWF<sub>ag</sub>) compared to white men (both  $p<0.001$ ). Analyses in black men, split by self-reported alcohol use and smoking, revealed 24 h blood pressure-PAI-1<sub>act</sub> associations only in alcohol users (24 h SBP [B=4.22,  $p<0.001$ ], DBP [B=2.04,  $p=0.015$ ] and PP [B=2.18,  $p=0.013$ ]) and smokers (24 h SBP [B=6.10,  $p<0.001$ ] and PP [B=4.33,  $p=0.001$ ]).

**Conclusion:** Our findings support a positive association between 24 h blood pressure and PAI-1, particularly in individuals with higher MCP-1 and vWF<sub>ag</sub> levels. Furthermore, smoking and alcohol consumption play an important role in modifying the association between blood pressure and PAI-1.

## INTRODUCTION

Worldwide, raised systolic blood pressure was the leading cardiovascular risk factor contributing to 10.4 million deaths and 218 million disability-adjusted life-years (DALYs) in 2017 [1]. In South Africa, 78% of people aged 50 years and older present with hypertension [2], which reiterates the tendency of black populations to develop hypertension [3, 4]. In order to curb this trend, an understanding of the early pathophysiological mechanisms and the identification of health behaviours and early markers that contribute to the development of hypertension is essential.

Plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor that plays a classical role in fibrinolysis [5] and alternative roles in endothelial dysfunction [6] and vascular remodelling [7], has consistently been associated with hypertension [8, 9]. The debate as to whether elevated PAI-1 levels precede or result from raised blood pressure still continues, since the former [9-12] as well as the latter [13, 14] have been suggested. PAI-1 levels are also adversely influenced by health behaviours such as smoking [15, 16], alcohol consumption [17, 18] and obesity (specifically central obesity) [19].

There is a paucity of data on the associations between blood pressure and PAI-1 in young and healthy humans in the absence of overt hypertension, which may potentially shed light on the sequence of events. Hence, our study investigated the associations of 24 h ambulatory blood pressure with PAI-1 in young and healthy black and white South Africans.

Risky health behaviour such as smoking and alcohol consumption are known modifiable risk factors that are of major health concern in South Africa [20], with smoking being considered the second highest risk factor for risk-attributable DALYs globally [1]. Both smoking [21] and alcohol consumption [22] are considered to contribute to raised blood pressure. As is the case with PAI-1 [6], both smoking and alcohol consumption are associated with endothelial dysfunction and inflammation on a vascular level [21, 22]. Hence, the effects of alcohol consumption and smoking on the associations between 24 h blood pressure and PAI-1 were also investigated. The objective of our study was therefore to investigate the associations of 24 h blood pressure with PAI-1 activity in apparently healthy black and white men and women, first split by ethnicity and sex and then by alcohol consumption and smoking.

## METHODS

### *Study population*

This study made use of baseline data that was collected as part of the African-PREDICT (Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension) study, which is a longitudinal study aimed at identifying early markers for the development of cardiovascular disease (CVD) in a young, apparently healthy population [23]. The study complied with all applicable requirements of the international regulations for research involving human participants, in particular the Declaration of Helsinki, and was approved by the Health Research Ethics Committee of the North-West University, Potchefstroom Campus (NWU-00001-12-A1). Written informed consent was obtained from all participants.

Briefly, young (aged 20-30 years), apparently healthy black and white men and women were recruited from Potchefstroom and the surrounding areas in South Africa and were first subjected to a screening process to determine their eligibility for participation in the African-PREDICT study. Those with clinic systolic blood pressure (SBP) <140mmHg and diastolic blood pressure (DBP) <90mmHg, who were HIV-negative, not pregnant or breastfeeding, and had no previously diagnosed chronic diseases at screening, were eligible to participate in the study and were invited to return for follow-up measurements. Data were collected from n=1202 participants comprising n=294 black men, n=312 black women, n=284 white men and n=312 white women.

### *Questionnaires and anthropometry*

Smoking, alcohol and hormonal contraceptive use (yes/no answers based on current smoking and alcohol consumption habits, as well as hormonal contraceptive use) were reported using a general health and demographic questionnaire. Three categories in this questionnaire (household income and skill and education levels) were used to calculate a socio-economic status (SES) score. For this point-system SES scoring, Kuppuswamy's Socioeconomic Status Scale [24] was adapted for a South African environment [23]. The SES scores were used to categorise participants into low, middle and high socioeconomic classes. Detailed information on the education level, employment status and household income of the African-PREDICT study participants has been described previously [23]. Height and waist circumference (WC) measurements were performed and waist-to-height ratio (WHtR) calculated using standard equipment and procedures.

### ***Cardiovascular measurements***

After a 10-minute rest period, office systolic (SBP) and diastolic blood pressure (DBP) were measured in duplicate on each arm with 5-minute intervals whilst in a seated position (Dinamap Procare 100, GE Medical Systems, Milwaukee, USA), after which the averages of the right arm measurements were calculated. Card(X)plore devices (Meditech, Budapest, Hungary) with appropriate sized cuffs were used for 24 h ambulatory blood pressure monitoring (ABPM). Recordings were taken every 30 minutes during the daytime period (06:00-22:00) and every hour during the nighttime period (22:00-06:00). The mean successful inflation rate of participants included in this study was  $88.2 \pm 12.2$  %. Measurements included mean 24 h SBP and DBP, with mean 24 h pulse pressure (PP) being calculated as the difference between SBP and DBP.

### ***Biochemical sampling and analyses***

Blood samples were collected early morning (on the same day that the ABPM devices were fitted), after 8-10 hours of fasting, in serum, EDTA, citrate, sodium fluoride and Stabilyte (acidified citrated) blood collection tubes. Preparation of the blood samples entailed centrifugation of Stabilyte tubes at  $1602 \times g$  at  $20^{\circ}\text{C}$  for 15 minutes, citrate tubes at  $1602 \times g$  at room temperature for 15 minutes, serum tubes at  $1602 \times g$  at room temperature for 30 minutes, EDTA tubes at  $2500 \times g$  at  $4^{\circ}\text{C}$  for 10 minutes, and sodium fluoride tubes at  $2307 \times g$  at  $4^{\circ}\text{C}$  for 10 minutes. Samples were kept at  $-80^{\circ}\text{C}$  until analysis. The Technozym® PAI-1 Actibind® ELISA kit (Technoclone GmbH, Vienna, Austria) was used for the measurement of plasminogen activator inhibitor-1 activity (PAI-1<sub>act</sub>) in samples that were collected in Stabilyte tubes. High-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, C-reactive protein (hs-CRP), and  $\gamma$ -glutamyl transferase (GGT) were measured in serum samples and glucose was measured in sodium fluoride plasma samples using the Cobas Integra 400plus (Roche, Basel, Switzerland). Serum insulin was measured using an electrochemiluminescence method on the E411 (Roche, Basel, Switzerland) and serum cotinine using a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA) were used to measure interleukin-6 (hs-IL-6) in serum samples and monocyte chemoattractant protein-1 (MCP-1) in EDTA plasma samples. Citrated plasma samples were used for the analysis of von Willebrand factor antigen (vWF<sub>ag</sub>), which was determined using an ELISA kit (DAKO, Glostrup, Denmark). Polyclonal rabbit anti-vWF antibody and rabbit anti-vWF-horseradish peroxidase (HRP) 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.

### **Statistical analysis**

Data were evaluated for normality using histograms, and non-Gaussian distributed variables were logarithmically transformed to improve normality. Descriptive statistics are presented in **Table 1** as arithmetic means  $\pm$  standard deviations, medians and interquartile ranges, or counts and percentages. Differences between groups were determined by independent t-tests and Chi-square tests in the case of continuous and categorical data, respectively. Interactions of sex and ethnicity on associations between 24 h SBP, DBP and PP, and PAI-1<sub>act</sub> were tested using generalized linear models (**Supplement, Table S1**). Interactions with sex were observed for the association between 24 h SBP and PAI-1<sub>act</sub> ( $p=0.011$ ), and with ethnicity for the associations of 24 h SBP and PP with PAI-1<sub>act</sub> ( $p=0.004$  and  $p=0.002$ , respectively). Since several studies have also reported differences in PAI-1 levels and blood pressure between men and women [25, 26] and between different ethnicities [27, 28], statistical analyses were performed separately in black and white men and women.

To evaluate the association of 24 h blood pressure with PAI-1, multivariate linear regression analyses were performed with 24 h SBP, DBP and PP as outcomes (**Table 2 and Supplement, Table S2**). Basic models were constructed with PAI-1<sub>act</sub> as the predictor, adjusted for age. Full models were additionally adjusted for smoking status (no/yes), hormonal contraceptive use (no/yes, women only), SES score, WHtR (a recognised marker of central obesity [29]), HDL-C, triglycerides, GGT (a conventional marker of alcohol use), insulin, and MCP-1, an inflammatory marker that was selected based on its known associations with endothelial dysfunction, inflammation [30] and hypertension [31]. Pearson correlations with the predictor and outcome variables aided in determining which of the potential covariates to include in the final regression models.

To evaluate the potential role of alcohol consumption and smoking in the associations between 24 h blood pressure and PAI-1<sub>act</sub>, regression analyses were repeated, split by alcohol consumption and smoking status (**Table 3, Figure 1 and Supplement, Table S3**).

Only cases containing data for the main predictor variable (PAI-1<sub>act</sub>) were included in this study ( $n=1156$ ). Furthermore, cases with missing data for covariates (low and random occurrence) were excluded from regression models in a listwise manner. SPSS version 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) software package was used for statistical analyses, and GraphPad Prism version 5.03 (GraphPad Software Inc., CA, USA) for graphical representations.

## RESULTS

The study population was equally distributed in terms of sex (52.3% women vs 47.7% men) and ethnicity (51.1% black vs 49.9% white) and had a mean age of  $24.5 \pm 3.11$  years (**Table 1**). Black men had lower PAI-1<sub>act</sub> levels, smaller WCs and WHtRs, and lower 24 h SBP and PP compared to white men, while self-reported smoking, and MCP-1 and vWF<sub>ag</sub> levels were higher in black than white men. Black women had greater WCs and WHtRs, higher MCP-1 and vWF<sub>ag</sub> levels, and lower self-reported smoking compared to white women.

Multivariate linear regression analyses showing the associations of 24 h SBP (**Table 2**), DBP and PP (**Supplement, Table S2**) with PAI-1<sub>act</sub>, indicated that PAI-1<sub>act</sub> was a significant predictor of 24 h SBP, DBP (except in white women) and PP (except in black women and white men) in basic models adjusted for age. In fully adjusted models for 24 h SBP and PP, significant positive associations with PAI-1 remained in black men only ( $R^2=0.19$ ,  $B=2.72$ ,  $p=0.003$  and  $R^2=0.16$ ,  $B=1.66$ ,  $p=0.02$ , respectively), whilst for 24 h DBP, significance was lost in all groups. Of note, black men was the only group in which WHtR, a measure of central adiposity (a major production site of PAI-1), did not significantly contribute to the prediction models for 24 h SBP, DBP or PP. Other covariates that contributed to the variance in 24 h BP in the different groups were age, SES score, smoking, GGT, insulin, HDL-C, triglyceride and MCP-1 levels.

In order to evaluate the effects of alcohol and tobacco use on the relationship between 24 h BP and PAI-1<sub>act</sub>, we performed multivariate linear regression analyses in black men split by self-reported alcohol use and smoking (**Table 3**, **Figure 1** and **Supplement, Table S3**). 24 h BP associated with PAI-1<sub>act</sub> in fully adjusted models in alcohol users (24 h SBP, DBP and PP) and smokers (24 h SBP and PP) only. Other covariates that contributed to the models in conjunction with PAI-1<sub>act</sub> were MCP-1 (24 h SBP and DBP in alcohol users and 24 h PP in smokers), SES score (24 h DBP in alcohol users and 24 h PP in smokers), GGT levels (24 h DBP in alcohol users), and age (24 h PP in alcohol users and smokers). When performing similar analyses in black women and the white groups, no associations were found except for a single association between 24 h DBP and PAI-1<sub>act</sub> in white men who consumed alcohol ( $R^2=0.26$ ,  $B=2.33$ ,  $p=0.04$ ).

Due to the different associations of 24 h BP with PAI-1<sub>act</sub> between drinkers and non-drinkers and between smokers and non-smokers, we compared the characteristics of black men, split by alcohol use and smoking (**Supplement, Table S4**). More drinkers also smoked and *vice versa* compared to non-drinkers and non-smokers. Smokers had lower WC, WHtR and vWF<sub>ag</sub> levels compared to non-smokers. Drinkers had higher triglycerides, HDL-C, GGT and PAI-1<sub>act</sub> levels compared to non-drinkers ( $p<0.05$  for all).

**Table 1.** Characteristics of the study population (n=1156)

|                                                |               | Women (n=605)    |                  |        | Men (n=551)      |                  |        |
|------------------------------------------------|---------------|------------------|------------------|--------|------------------|------------------|--------|
|                                                |               | Black (n=300)    | White (n=305)    | p      | Black (n=279)    | White (n=272)    | p      |
| Age (years)                                    |               | 24.6 ± 3.30      | 24.4 ± 3.07      | 0.43   | 24.3 ± 3.05      | 24.8 ± 3.01      | 0.09   |
| Smoking, n (%) <sup>a</sup>                    |               | 29 (9.7)         | 49 (16.1)        | 0.019  | 118 (42.3)       | 76 (27.9)        | <0.001 |
| Alcohol use, n (%) <sup>a</sup>                |               | 149 (49.7)       | 162 (53.1)       | 0.40   | 173 (62.0)       | 161 (59.2)       | 0.50   |
| Hormonal contraceptive use, n (%) <sup>a</sup> |               | 141 (47.0)       | 125 (41.0)       | 0.14   | -                | -                | -      |
| SES score                                      |               | 18.2 ± 5.67      | 23.3 ± 5.30      | <0.001 | 17.3 ± 5.48      | 23.8 ± 5.44      | <0.001 |
| SES class                                      | Low, n (%)    | 161 (53.7)       | 63 (20.7)        | <0.001 | 177 (63.4)       | 51 (18.8)        | <0.001 |
|                                                | Middle, n (%) | 90 (30.0)        | 92 (30.2)        | 0.96   | 67 (24.0)        | 84 (30.9)        | 0.07   |
|                                                | High, n (%)   | 49 (16.3)        | 150 (49.2)       | <0.001 | 35 (12.5)        | 137 (50.4)       | <0.001 |
| WC (cm)                                        |               | 77.3 (70.3-86.9) | 73.4 (68.6-80.2) | 0.004  | 75.0 (69.8-81.6) | 87.5 (81.2-94.5) | <0.001 |
| WHtR                                           |               | 0.49 (0.44-0.55) | 0.44 (0.41-0.49) | <0.001 | 0.44 (0.41-0.48) | 0.49 (0.45-0.53) | <0.001 |
| <b>Cardiovascular measurements</b>             |               |                  |                  |        |                  |                  |        |
| Office SBP (mmHg)                              |               | 115 ± 10.3       | 112 ± 9.81       | <0.001 | 124 ± 11.9       | 126 ± 9.83       | 0.07   |
| Office DBP (mmHg)                              |               | 77.9 ± 7.30      | 75.5 ± 6.97      | <0.001 | 81.4 ± 8.83      | 79.4 ± 7.49      | 0.004  |
| 24 h SBP (mmHg)                                |               | 113 ± 8.27       | 113 ± 8.52       | 0.93   | 119 ± 8.36       | 124 ± 7.32       | <0.001 |
| 24 h DBP (mmHg)                                |               | 68.1 ± 5.50      | 67.5 ± 5.55      | 0.17   | 69.5 ± 6.29      | 69.7 ± 5.84      | 0.71   |
| 24 h PP (mmHg)                                 |               | 44.6 ± 5.59      | 45.1 ± 5.72      | 0.22   | 49.8 ± 6.64      | 54.0 ± 6.66      | <0.001 |
| <b>Biochemical measurements</b>                |               |                  |                  |        |                  |                  |        |
| PAI-1 <sub>act</sub> (IU/mL)                   |               | 5.27 (2.19-9.80) | 4.88 (2.00-11.1) | 0.75   | 3.17 (1.59-7.92) | 12.6 (5.82-26.4) | <0.001 |
| HDL-C (mmol/L)                                 |               | 1.18 (0.92-1.40) | 1.32 (0.99-1.65) | <0.001 | 1.07 (0.81-1.34) | 0.94 (0.71-1.17) | <0.001 |
| LDL-C (mmol/L)                                 |               | 2.34 (1.88-2.94) | 2.42 (1.89-3.18) | 0.042  | 1.96 (1.49-2.53) | 2.63 (1.85-3.52) | <0.001 |
| Trig (mmol/L)                                  |               | 0.63 (0.49-0.86) | 0.75 (0.53-1.09) | <0.001 | 0.65 (0.46-0.89) | 0.85 (0.59-1.26) | <0.001 |
| CRP (mg/L)                                     |               | 1.92 (0.74-4.80) | 0.84 (0.32-3.10) | <0.001 | 0.56 (0.20-1.42) | 0.63 (0.24-1.58) | 0.36   |
| GGT (U/L)                                      |               | 20.4 (14.5-29.8) | 11.5 (8.10-16.9) | <0.001 | 23.1 (15.6-34.4) | 18.9 (11.3-29.6) | <0.001 |
| Glucose (mmol/L)                               |               | 4.20 ± 0.91      | 4.23 ± 0.96      | 0.68   | 3.69 ± 1.06      | 4.31 ± 1.13      | <0.001 |
| Insulin (μU/mL)                                |               | 11.6 (8.25-15.7) | 7.96 (5.87-10.8) | <0.001 | 6.91 (4.85-10.6) | 8.05 (5.90-11.4) | 0.012  |
| IL-6 (pg/mL)                                   |               | 1.36 (0.94-2.11) | 0.85 (0.59-1.42) | <0.001 | 0.97 (0.66-1.38) | 0.87 (0.61-1.39) | 0.06   |
| MCP-1 (pg/mL)                                  |               | 153 (130-175)    | 122 (107-140)    | <0.001 | 169 (146-199)    | 140 (123-164)    | <0.001 |
| vWF <sub>ag</sub> (%)                          |               | 94.0 (69.0-128)  | 78.5 (59.0-102)  | <0.001 | 90.5 (69.0-120)  | 71.0 (56.3-95.5) | <0.001 |

Abbreviations: SES, socio-economic status; WC, waist circumference; WHtR, waist-to-height ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Trig, triglycerides; CRP, C-reactive protein; GGT, gamma-glutamyltransferase; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; vWF<sub>ag</sub>, von Willebrand factor antigen.

Notes: data expressed as arithmetic mean ± standard deviation, median and interquartile range, or count and percentage.

<sup>a</sup>Self-reported smoking, alcohol and hormonal contraceptive use.

**Table 2.** Multivariate linear regression analysis with 24 h systolic blood pressure as dependent variable

| Variable                            | Black women (n=276)  |                  | White women (n=293)  |                  | Black men (n=260)    |                  | White men (n=268)     |                  |
|-------------------------------------|----------------------|------------------|----------------------|------------------|----------------------|------------------|-----------------------|------------------|
|                                     | B (95% CI)           | p                | B (95% CI)           | p                | B (95% CI)           | p                | B (95% CI)            | p                |
| <b>Model 1</b>                      |                      |                  |                      |                  |                      |                  |                       |                  |
| PAI-1 <sub>act</sub> (IU/mL)        | 2.714 (0.97; 4.46)   | <b>0.002</b>     | 2.307 (0.83; 3.78)   | <b>0.002</b>     | 3.992 (2.43; 5.56)   | <b>&lt;0.001</b> | 2.801 (1.20; 4.40)    | <b>0.001</b>     |
| Age (years)                         | 0.311 (0.03; 0.59)   | <b>0.029</b>     | 0.354 (0.05; 0.66)   | <b>0.024</b>     | 0.050 (-0.26; 0.36)  | 0.75             | 0.135 (-0.15; 0.42)   | 0.35             |
| <b>Model 2</b>                      |                      |                  |                      |                  |                      |                  |                       |                  |
| PAI-1 <sub>act</sub> (IU/mL)        | -0.984 (-2.85; 0.88) | 0.30             | 0.204 (-1.11; 1.52)  | 0.76             | 2.721 (0.94; 4.50)   | <b>0.003</b>     | 0.583 (-1.31; 2.48)   | 0.55             |
| Age (years)                         | -0.163 (-0.46; 0.14) | 0.29             | 0.063 (-0.25; 0.38)  | 0.69             | -0.158 (-0.50; 0.18) | 0.37             | -0.091 (-0.45; 0.27)  | 0.62             |
| Smoking (no) <sup>a</sup>           | 0.232 (-2.82; 3.28)  | 0.88             | -0.249 (-2.51; 2.01) | 0.83             | 0.489 (-1.52; 2.49)  | 0.63             | 1.493 (-0.42; 3.40)   | 0.13             |
| Contraceptive use (no) <sup>a</sup> | -0.001 (-1.87; 1.87) | 1.00             | -0.063 (-1.89; 1.76) | 0.95             | -                    | -                | -                     | -                |
| SES score                           | 0.104 (-0.06; 0.27)  | 0.23             | 0.134 (-0.04; 0.31)  | 0.14             | 0.114 (-0.09; 0.32)  | 0.27             | 0.066 (-0.12; 0.26)   | 0.50             |
| WHtR                                | 50.364 (33.6; 67.2)  | <b>&lt;0.001</b> | 60.340 (43.0; 77.7)  | <b>&lt;0.001</b> | 23.702 (-2.22; 49.6) | 0.07             | 33.995 (17.9; 50.1)   | <b>&lt;0.001</b> |
| HDL-C (mmol/L)                      | -2.264 (-9.08; 4.55) | 0.52             | 1.084 (-5.53; 7.70)  | 0.75             | -0.640 (-7.79; 6.51) | 0.86             | -6.905 (-12.8; -1.02) | <b>0.022</b>     |
| Triglycerides (mmol/L)              | -1.804 (-7.45; 3.84) | 0.53             | 2.867 (-1.67; 7.41)  | 0.22             | -1.651 (-6.79; 3.49) | 0.53             | -0.589 (-4.68; 3.50)  | 0.78             |
| GGT (U/L)                           | 4.839 (1.14; 8.54)   | <b>0.010</b>     | 0.349 (-3.38; 4.08)  | 0.85             | 3.329 (-0.81; 7.47)  | 0.12             | 4.220 (0.02; 8.42)    | <b>0.049</b>     |
| Insulin (μU/mL)                     | 2.946 (-1.73; 7.62)  | 0.22             | 6.359 (1.68; 11.0)   | <b>0.008</b>     | 3.958 (-0.001; 7.92) | 0.05             | -0.075 (-4.63; 4.48)  | 0.97             |
| MCP-1 (pg/mL)                       | 4.497 (-3.80; 12.8)  | 0.29             | 6.257 (-0.89; 13.4)  | 0.09             | 12.226 (3.12; 21.3)  | <b>0.008</b>     | 1.608 (-6.41; 9.63)   | 0.69             |

Abbreviations: PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; SES, socio-economic status; WHtR, waist-to-height ratio; HDL-C, high-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase; MCP-1, monocyte chemoattractant protein-1.

Notes: Model 1, basic model including only age as covariate; Model 2, fully adjusted model.

<sup>a</sup>Smoking (yes) and hormonal contraceptive use (yes) used as references.

Bold text indicates p-values < 0.05.

**Table 3.** Multivariate linear regression analysis with 24 h SBP as dependent variable performed in black men, split by self-reported alcohol use and smoking

| Variable                               | No alcohol (n=96)    |              | Alcohol (n=162)      |                  | No smoking (n=149)   |              | Smoking (n=109)      |                  |
|----------------------------------------|----------------------|--------------|----------------------|------------------|----------------------|--------------|----------------------|------------------|
|                                        | B (95% CI)           | p            | B (95% CI)           | p                | B (95% CI)           | p            | B (95% CI)           | p                |
| <b>Model 1</b>                         |                      |              |                      |                  |                      |              |                      |                  |
| PAI-1 <sub>act</sub> (IU/mL)           | 2.306 (-0.38; 4.99)  | 0.09         | 5.156 (3.21; 7.11)   | <b>&lt;0.001</b> | 2.863 (0.95; 4.77)   | <b>0.003</b> | 6.305 (3.66; 8.95)   | <b>&lt;0.001</b> |
| Age (years)                            | -0.039 (-0.56; 0.48) | 0.88         | 0.079 (-0.31; 0.47)  | 0.69             | -0.036 (-0.45; 0.38) | 0.86         | 0.065 (-0.41; 0.54)  | 0.79             |
| <b>Model 2</b>                         |                      |              |                      |                  |                      |              |                      |                  |
| PAI-1 <sub>act</sub> (IU/mL)           | 0.424 (-2.56; 3.41)  | 0.78         | 4.219 (2.00; 6.44)   | <b>&lt;0.001</b> | 1.184 (-0.98; 3.35)  | 0.28         | 6.099 (2.93; 9.27)   | <b>&lt;0.001</b> |
| Age (years)                            | -0.193 (-0.70; 0.32) | 0.46         | -0.210 (-0.67; 0.25) | 0.38             | -0.363 (-0.82; 0.09) | 0.12         | -0.105 (-0.64; 0.43) | 0.70             |
| Smoking <sup>b</sup> (no) <sup>a</sup> | -0.556 (-4.17; 3.06) | 0.76         | 1.235 (-1.18; 3.65)  | 0.32             | -                    | -            | -                    | -                |
| Alcohol <sup>c</sup> (no) <sup>a</sup> | -                    | -            | -                    | -                | -1.322 (-3.86; 1.22) | 0.31         | 0.677 (-2.89; 4.24)  | 0.71             |
| SES score                              | 0.097 (-0.23; 0.43)  | 0.56         | 0.132 (-0.13; 0.40)  | 0.33             | 0.221 (-0.05; 0.49)  | 0.11         | -0.059 (-0.37; 0.26) | 0.71             |
| WHTR                                   | 63.298 (21.1; 105)   | <b>0.003</b> | 1.623 (-30.8; 34.0)  | 0.92             | 29.853 (-4.50; 64.2) | 0.09         | 14.170 (-25.1; 53.5) | 0.48             |
| HDL-C (mmol/L)                         | 3.381 (-8.58; 15.3)  | 0.58         | -3.949 (-13.0; 5.09) | 0.39             | 2.722 (-6.39; 11.8)  | 0.56         | -8.593 (-20.8; 3.66) | 0.17             |
| Triglycerides (mmol/L)                 | -4.178 (-13.5; 5.15) | 0.38         | -0.789 (-7.11; 5.53) | 0.81             | -0.775 (-7.00; 5.45) | 0.81         | -1.375 (-10.4; 7.66) | 0.77             |
| GGT (U/L)                              | 0.279 (-6.83; 7.39)  | 0.94         | 3.977 (-1.16; 9.12)  | 0.13             | 2.888 (-2.68; 8.46)  | 0.31         | 3.423 (-2.84; 9.68)  | 0.28             |
| Insulin (μU/mL)                        | 5.348 (-0.89; 11.6)  | 0.09         | 2.921 (-2.31; 8.15)  | 0.27             | 6.125 (1.32; 10.9)   | <b>0.012</b> | 0.058 (-6.63; 6.75)  | 0.99             |
| MCP-1 (pg/mL)                          | 5.798 (-10.3; 21.9)  | 0.48         | 13.460 (2.40; 24.5)  | <b>0.017</b>     | 12.900 (1.43; 24.4)  | <b>0.027</b> | 10.485 (-4.63; 25.6) | 0.17             |

Abbreviations: PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; SES, socio-economic status; WHTR, waist-to-height ratio; HDL-C, high-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase; MCP-1, monocyte chemoattractant protein-1.

Notes: Model 1, basic model including only age as covariate; Model 2, fully adjusted model.

<sup>a</sup>Smoking (yes) and alcohol use (yes) used as references.

<sup>b</sup>Smoking not included in models split for smoking; <sup>c</sup>Alcohol not included in models split for alcohol use.

Bold text indicates p-values < 0.05.

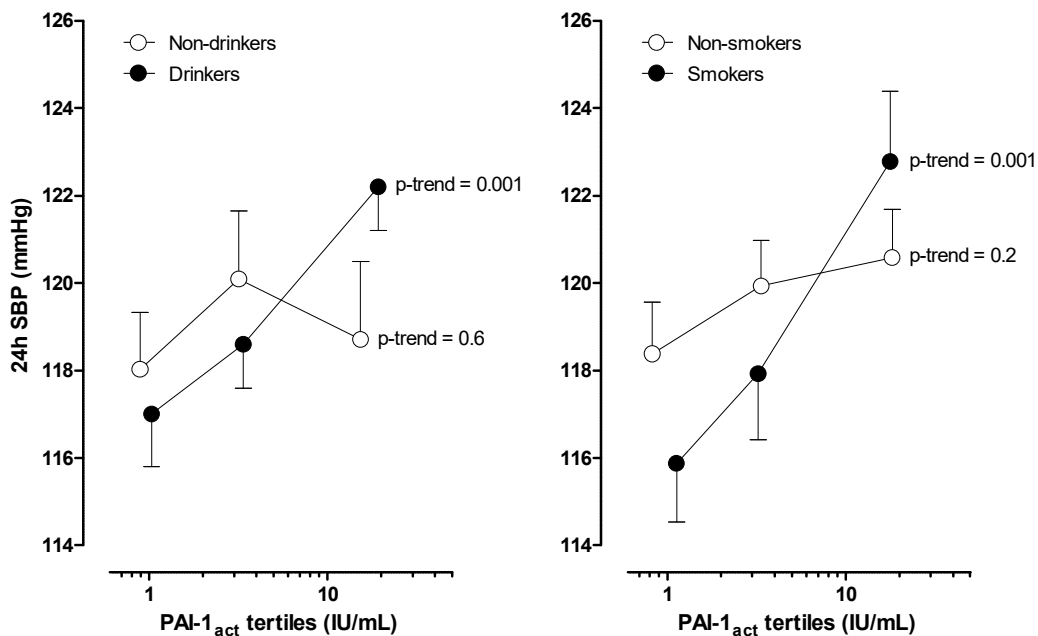


**Figure 1.** 24 h SBP (adjusted means  $\pm$  SEM) of black men plotted against tertiles of PAI-1<sub>act</sub> for drinkers vs. non-drinkers and smokers vs. non-smokers

Adjusted for age, smoking (in the case of drinkers and non-drinkers), alcohol consumption (in the case of smokers and non-smokers), socio-economic status score, waist-to-height ratio, high-density lipoprotein, triglyceride, gamma-glutamyltransferase, insulin, and monocyte chemoattractant protein-1

Abbreviations: SBP, systolic blood pressure; PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity

## DISCUSSION

We determined whether 24 h blood pressure is already associated with PAI-1<sub>act</sub> levels in a young, healthy cohort of black and white adults in the absence of overt hypertension. An independent positive association between 24 h ambulatory blood pressure and PAI-1<sub>act</sub> was found only in black men, who also displayed a degree of endothelial dysfunction and vascular inflammation as reflected by their high MCP-1 and vWF<sub>ag</sub> levels. Furthermore, these associations were specifically found in black men who smoked and consumed alcohol, suggesting that these health behaviours play an important role in modifying the association between blood pressure and PAI-1<sub>act</sub>.

Although 24 h BP was significantly associated with PAI-1<sub>act</sub> in most groups in basic models adjusted for age, significance was lost in black women and whites in fully adjusted models. This was largely due to the significant contribution of central adiposity to 24 h BP in these groups, which likely obscured the association of 24 h BP with PAI-1<sub>act</sub>. The strong link between PAI-1 and central adiposity is mechanistically driven by visceral adipose tissue being a major source of PAI-1 production [19]. It is well known that obesity, including central obesity, contributes to hypertension development through numerous mechanisms [32].

Black men was the only group in which central adiposity did not contribute to 24 h BP, and in fully adjusted models the associations of 24 h BP with PAI-1<sub>act</sub> was confirmed in this group. This finding suggests that the 24 h BP and PAI-1<sub>act</sub> associations are not merely a reflection of the association of 24 h BP with central adiposity in black men. The lack of associations between 24 h BP and central adiposity in this group is unlikely to be explained by a smaller WHtR in black men, since black men and white women had comparable waist circumferences and WHtRs. Furthermore, other cardiometabolic risk factors typically associated with central obesity, e.g. metabolic syndrome components (raised triglyceride or fasting glucose levels, or reduced HDL-C levels [33]) cannot account for the unique associations observed in black men, since these parameters were within the same ranges (some even lower) than the other three groups.

Based on the known relationships of smoking and alcohol consumption with PAI-1 and blood pressure [15-18, 21, 22], we consequently investigated their effects on the association between 24 h BP and PAI-1<sub>act</sub> in black men. We found that 24 h BP only associated with PAI-1<sub>act</sub> in smokers and alcohol consumers. The contribution of central adiposity to 24 h BP was again comparatively larger in non-smokers and non-drinkers, despite there being no difference in WHtR between drinkers and non-drinkers and both smokers and non-smokers having a mean WHtR indicative of the absence of central obesity. Hence, the results suggest

that alcohol consumption and smoking diminish the association of 24 h BP with central adiposity in black men, thereby revealing the role of PAI-1<sub>act</sub>.

It is well known that black Africans display less favourable cardiovascular profiles compared to their white counterparts [34-39]. In multiple studies conducted in South African populations, black men specifically had higher blood pressure [34, 35, 37], stiffer vasculature [34, 37], higher resting total peripheral resistance, and increased vascular resistance reactivity upon challenging the cardiovascular system [34] compared to white men. Even from a young age, black boys (6-8 years) presented with greater arterial stiffness and carotid intima-media thickness, as well as higher brachial DBP than white boys [36]. Despite the inclusion criteria (being young and apparently healthy) of the African-PREDICT study, the black men also presented with higher cardiovascular risk based on higher central SBP, higher total peripheral resistance [38] and smaller retinal arteriolar calibers [39] compared to white men. In addition, in our study, black men had higher GGT and MCP-1 levels compared to black women and whites. Their high MCP-1 levels, as well as the higher vWF<sub>ag</sub> levels of blacks compared to whites, indicate a degree of endothelial dysfunction and vascular inflammation [30, 40].

A high percentage of the black men in our study population consumed alcohol and smoked. There is strong evidence that heavy alcohol consumption contributes to raised blood pressure by various possible mechanisms. These include increased oxidative stress and endothelial dysfunction, activation of the sympathetic nervous system, and involvement of the renin-angiotensin-aldosterone system [22]. Smoking acutely raises blood pressure mainly by activation of the sympathetic nervous system. On the other hand, the effect of chronic smoking on blood pressure is unclear since inconsistent results have been reported [21]. Nevertheless, smoking increases cardiovascular risk by promoting oxidative stress, endothelial dysfunction, inflammation, and thrombosis, among other mechanisms [21]. PAI-1 also plays a role in endothelial dysfunction [6] and in the presence of a vulnerable endothelium (as reflected by high MCP-1 and vWF<sub>ag</sub> levels in the case of the black men), further compromised by smoking and alcohol consumption, 24 h BP increased with increasing PAI-1<sub>act</sub> levels. Potential mechanisms by which PAI-1 may contribute to raised blood pressure include PAI-1's suggested alternative roles in endothelial dysfunction [6] and vascular remodelling [7], which likely becomes more prominent in the presence of an already compromised endothelium.

A previous study conducted in Japanese hypertensive patients relates to our findings [16]. In this study, both smoking and high plasma PAI-1 antigen levels increased the risk for CVD

events. More importantly, the risk for CVD events was significantly higher when smoking was accompanied by high (as compared to low) PAI-1 antigen levels [16].

To our knowledge, this is the first study to investigate the associations of 24 h BP with PAI-1 in a cohort of young, healthy adults in the absence of overt hypertension. A strength of our study is its bi-ethnic design, since black and white South Africans are known to display different cardiovascular [34-39] and haemostatic [41] profiles. A limitation of this study is that due to the cross-sectional study design, causality or the controversial direction of the blood pressure-PAI-1 association cannot be established. Although potential confounders were taken into consideration, the possibility of residual confounding cannot be excluded. Furthermore, dietary data not being included in statistical analyses may be considered a potential limitation, and is recommended for future studies.

In conclusion, in the absence of overt hypertension, PAI-1<sub>act</sub> levels were positively and independently associated with 24 h ambulatory blood pressure in young, healthy black men who smoked and consumed alcohol. This adds supporting evidence that PAI-1 may contribute to hypertension development rather than solely being a consequence thereof, particularly in individuals with higher MCP-1 and vWF<sub>ag</sub> levels. Our data furthermore provides insight into the potential biological pathways by which alcohol use and smoking modify cardiovascular risk already from early on in life.

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## **COMPETING INTERESTS**

The authors have no conflict of interest to declare.

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## APPENDICES

None

## SUPPLEMENTARY MATERIAL

**Supplement Table 1.** Significance of interaction terms with 24 h SBP, DBP and PP as dependent variables

| Dependent variable | Sex-PAI-1<br>interaction (p) | Ethnicity-PAI-1<br>interaction (p) |
|--------------------|------------------------------|------------------------------------|
| 24 h SBP (mmHg)    | <b>0.011</b>                 | <b>0.004</b>                       |
| 24 h DBP (mmHg)    | 0.07                         | 0.42                               |
| 24 h PP (mmHg)     | 0.10                         | <b>0.002</b>                       |

Abbreviations: PAI-1, plasminogen activator inhibitor-1; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure.

Notes: Interaction terms p-values calculated using multiple linear regression analyses.

Bold text indicates p-values < 0.05.

**Supplement Table 2.** Multivariate linear regression analysis with 24 h diastolic and pulse pressures as dependent variables

| Variable                            | Black women (n=276)  |              | White women (n=293)  |              | Black men (n=260)     |                  | White men (n=268)     |                  |
|-------------------------------------|----------------------|--------------|----------------------|--------------|-----------------------|------------------|-----------------------|------------------|
|                                     | B (95% CI)           | p            | B (95% CI)           | p            | B (95% CI)            | p                | B (95% CI)            | p                |
| <b>24 h DBP</b>                     |                      |              |                      |              |                       |                  |                       |                  |
| <b>Model 1</b>                      |                      |              |                      |              |                       |                  |                       |                  |
| PAI-1 <sub>act</sub> (IU/mL)        | 1.484 (0.33; 2.64)   | <b>0.012</b> | 0.937 (-0.03; 1.91)  | 0.06         | 1.855 (0.71; 3.00)    | <b>0.002</b>     | 2.924 (1.71; 4.13)    | <b>&lt;0.001</b> |
| Age (years)                         | 0.323 (0.14; 0.51)   | <b>0.001</b> | 0.253 (0.05; 0.46)   | <b>0.014</b> | 0.620 (0.39; 0.85)    | <b>&lt;0.001</b> | 0.530 (0.32; 0.75)    | <b>&lt;0.001</b> |
| <b>Model 2</b>                      |                      |              |                      |              |                       |                  |                       |                  |
| PAI-1 <sub>act</sub> (IU/mL)        | -0.089 (-1.41; 1.24) | 0.90         | 0.303 (-0.66; 1.27)  | 0.54         | 1.061 (-0.24; 2.37)   | 0.11             | 0.877 (-0.59; 2.34)   | 0.24             |
| Age (years)                         | 0.112 (-0.10; 0.33)  | 0.30         | 0.065 (-0.17; 0.30)  | 0.58         | 0.331 (0.08; 0.58)    | <b>0.010</b>     | 0.427 (0.15; 0.70)    | <b>0.002</b>     |
| Smoking (no) <sup>a</sup>           | -0.250 (-2.41; 1.91) | 0.82         | -0.375 (-2.03; 1.28) | 0.66         | 0.392 (-1.08; 1.86)   | 0.60             | -1.026 (-2.50; 0.45)  | 0.17             |
| Contraceptive use (no) <sup>a</sup> | -0.494 (-1.82; 0.83) | 0.47         | -0.193 (-1.53; 1.14) | 0.78         | -                     | -                | -                     | -                |
| SES score                           | 0.051 (-0.07; 0.17)  | 0.40         | 0.119 (-0.01; 0.25)  | 0.07         | 0.171 (0.02; 0.32)    | <b>0.025</b>     | 0.048 (-0.10; 0.19)   | 0.52             |
| WHtR                                | 17.582 (5.67; 29.5)  | <b>0.004</b> | 17.806 (5.10; 30.5)  | <b>0.006</b> | 10.223 (-8.80; 29.2)  | 0.29             | 13.592 (1.14; 26.0)   | <b>0.032</b>     |
| HDL-C (mmol/L)                      | 0.346 (-4.49; 5.18)  | 0.89         | 2.868 (-1.97; 7.71)  | 0.25         | 0.365 (-4.88; 5.61)   | 0.89             | -1.618 (-6.16; 2.93)  | 0.49             |
| Triglycerides (mmol/L)              | 1.478 (-2.53; 5.48)  | 0.47         | 4.309 (0.99; 7.63)   | <b>0.011</b> | -0.244 (-4.02; 3.53)  | 0.90             | -0.959 (-4.12; 2.20)  | 0.55             |
| GGT (U/L)                           | 2.357 (-0.27; 4.98)  | 0.08         | -0.391 (-3.12; 2.34) | 0.78         | 4.865 (1.83; 7.90)    | <b>0.002</b>     | 3.150 (-0.09; 6.39)   | 0.06             |
| Insulin (μU/mL)                     | -0.997 (-4.31; 2.32) | 0.56         | 4.590 (1.17; 8.01)   | <b>0.009</b> | -0.410 (-3.32; 2.50)  | 0.78             | 3.609 (0.09; 7.12)    | <b>0.044</b>     |
| MCP-1 (pg/mL)                       | 4.671 (-1.21; 10.6)  | 0.12         | 1.792 (-3.44; 7.02)  | 0.50         | 8.369 (1.69; 15.1)    | <b>0.014</b>     | -2.777 (-8.96; 3.41)  | 0.38             |
| <b>24 h PP</b>                      |                      |              |                      |              |                       |                  |                       |                  |
| <b>Model 1</b>                      |                      |              |                      |              |                       |                  |                       |                  |
| PAI-1 <sub>act</sub> (IU/mL)        | 1.158 (-0.04; 2.36)  | 0.06         | 1.370 (0.37; 2.37)   | <b>0.007</b> | 2.137 (0.91; 3.37)    | <b>0.001</b>     | -0.123 (-1.59; 1.35)  | 0.87             |
| Age (years)                         | -0.014 (-0.21; 0.18) | 0.88         | 0.101 (-0.11; 0.31)  | 0.34         | -0.570 (-0.82; -0.32) | <b>&lt;0.001</b> | -0.395 (-0.66; -0.14) | <b>0.003</b>     |

**Model 2**

|                                     |                       |                  |                      |                  |                       |              |                       |              |
|-------------------------------------|-----------------------|------------------|----------------------|------------------|-----------------------|--------------|-----------------------|--------------|
| PAI-1 <sub>act</sub> (IU/mL)        | -0.976 (-2.27; 0.32)  | 0.14             | -0.099 (-1.02; 0.82) | 0.83             | 1.660 (0.22; 3.10)    | <b>0.024</b> | -0.293 (-2.08; 1.50)  | 0.75         |
| Age (years)                         | -0.275 (-0.48; -0.07) | <b>0.010</b>     | -0.002 (-0.22; 0.22) | 0.99             | -0.489 (-0.77; -0.21) | <b>0.001</b> | -0.517 (-0.86; -0.18) | <b>0.003</b> |
| Smoking (no) <sup>a</sup>           | 0.479 (-1.63; 2.59)   | 0.66             | 0.126 (-1.46; 1.71)  | 0.88             | 0.097 (-1.53; 1.73)   | 0.91         | 2.519 (0.72; 4.32)    | <b>0.006</b> |
| Contraceptive use (no) <sup>a</sup> | 0.458 (-0.84; 1.75)   | 0.49             | 0.130 (-1.14; 1.41)  | 0.84             | -                     | -            | -                     | -            |
| SES score                           | 0.051 (-0.07; 0.17)   | 0.39             | 0.015 (-0.11; 0.14)  | 0.81             | -0.057 (-0.22; 0.11)  | 0.50         | 0.018 (-0.16; 0.20)   | 0.85         |
| WHtR                                | 32.391 (20.8; 44.0)   | <b>&lt;0.001</b> | 42.534 (30.4; 54.7)  | <b>&lt;0.001</b> | 13.479 (-7.57; 34.5)  | 0.21         | 20.403 (5.16; 35.6)   | <b>0.009</b> |
| HDL-C (mmol/L)                      | -2.700 (-7.42; 2.02)  | 0.26             | -1.784 (-6.41; 2.84) | 0.45             | -1.006 (-6.81; 4.80)  | 0.73         | -5.288 (-10.9; 0.28)  | 0.06         |
| Triglycerides (mmol/L)              | -3.175 (-7.08; 0.73)  | 0.11             | -1.443 (-4.62; 1.73) | 0.37             | -1.407 (-5.58; 2.77)  | 0.51         | 0.370 (-3.50; 4.24)   | 0.85         |
| GGT (U/L)                           | 2.469 (-0.10; 5.03)   | 0.06             | 0.740 (-1.87; 3.35)  | 0.58             | -1.536 (-4.89; 1.82)  | 0.37         | 1.070 (-2.90; 5.04)   | 0.60         |
| Insulin (μU/mL)                     | 3.999 (0.76; 7.24)    | <b>0.015</b>     | 1.769 (-1.50; 5.04)  | 0.29             | 4.369 (1.15; 7.58)    | <b>0.008</b> | -3.684 (-7.99; 0.62)  | 0.09         |
| MCP-1 (pg/mL)                       | -0.207 (-5.95; 5.54)  | 0.94             | 4.466 (-0.53; 9.46)  | 0.08             | 3.857 (-3.54; 11.3)   | 0.31         | 4.385 (-3.19; 12.0)   | 0.26         |

Abbreviations: DBP, diastolic blood pressure; PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; SES, socio-economic status; WHtR, waist-to-height ratio; HDL-C, high-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase; MCP-1, monocyte chemoattractant protein-1; PP, pulse pressure.

Notes: Model 1, basic model including only age as covariate; Model 2, fully adjusted model.

<sup>a</sup>Smoking (yes) and hormonal contraceptive use (yes) used as references.

Bold text indicates p-values < 0.05.

**Supplement Table 3.** Multivariate linear regression analysis with 24 h diastolic and pulse pressures as dependent variables performed in black men, split by self-reported alcohol use and smoking

| Variable                               | No alcohol (n=96)     |              | Alcohol (n=162)       |                  | No smoking (n=149)    |              | Smoking (n=109)       |                  |
|----------------------------------------|-----------------------|--------------|-----------------------|------------------|-----------------------|--------------|-----------------------|------------------|
|                                        | B (95% CI)            | p            | B (95% CI)            | p                | B (95% CI)            | p            | B (95% CI)            | p                |
| <b>24 h DBP</b>                        |                       |              |                       |                  |                       |              |                       |                  |
| <b>Model 1</b>                         |                       |              |                       |                  |                       |              |                       |                  |
| PAI-1 <sub>act</sub> (IU/mL)           | 0.537 (-1.28; 2.35)   | 0.56         | 2.715 (1.22; 4.21)    | <b>&lt;0.001</b> | 1.537 (0.17; 2.90)    | <b>0.028</b> | 2.510 (0.48; 4.54)    | <b>0.016</b>     |
| Age (years)                            | 0.439 (0.09; 0.79)    | <b>0.014</b> | 0.721 (0.42; 1.02)    | <b>&lt;0.001</b> | 0.503 (0.21; 0.80)    | <b>0.001</b> | 0.733 (0.37; 1.10)    | <b>&lt;0.001</b> |
| <b>Model 2</b>                         |                       |              |                       |                  |                       |              |                       |                  |
| PAI-1 <sub>act</sub> (IU/mL)           | -0.545 (-2.65; 1.56)  | 0.61         | 2.043 (0.39; 3.70)    | <b>0.015</b>     | 0.837 (-0.71; 2.39)   | 0.29         | 1.765 (-0.65; 4.18)   | 0.15             |
| Age (years)                            | 0.298 (-0.06; 0.66)   | 0.10         | 0.319 (-0.03; 0.67)   | 0.07             | 0.175 (-0.15; 0.50)   | 0.29         | 0.542 (0.13; 0.95)    | <b>0.010</b>     |
| Smoking* (no) <sup>a</sup>             | -0.731 (-3.28; 1.81)  | 0.57         | 0.807 (-0.99; 2.60)   | 0.38             | -                     | -            | -                     | -                |
| Alcohol <sup>†</sup> (no) <sup>a</sup> | -                     | -            | -                     | -                | -0.920 (-2.74; 0.90)  | 0.32         | 1.178 (-1.54; 3.90)   | 0.40             |
| SES score                              | 0.044 (-0.19; 0.28)   | 0.71         | 0.294 (0.10; 0.49)    | <b>0.004</b>     | 0.161 (-0.03; 0.36)   | 0.11         | 0.208 (-0.03; 0.45)   | 0.09             |
| WhtR                                   | 43.459 (13.7; 73.2)   | <b>0.004</b> | -10.889 (-35.0; 13.2) | 0.38             | 14.219 (-10.4; 38.8)  | 0.26         | 6.201 (-23.7; 36.1)   | 0.69             |
| HDL-C (mmol/L)                         | 5.954 (-2.48; 14.4)   | 0.17         | -2.313 (-9.04; 4.41)  | 0.50             | 3.415 (-3.11; 9.94)   | 0.31         | -0.659 (-9.99; 8.67)  | 0.89             |
| Triglycerides (mmol/L)                 | -0.060 (-6.63; 6.51)  | 0.99         | -0.889 (-5.60; 3.82)  | 0.71             | 0.168 (-4.29; 4.63)   | 0.94         | -1.720 (-8.60; 5.16)  | 0.62             |
| GGT (U/L)                              | 3.448 (-1.56; 8.46)   | 0.18         | 5.554 (1.72; 9.38)    | <b>0.004</b>     | 4.113 (0.12; 8.10)    | <b>0.043</b> | 4.934 (0.17; 9.70)    | <b>0.043</b>     |
| Insulin (μU/mL)                        | -2.796 (-7.19; 1.60)  | 0.21         | 1.831 (-2.06; 5.72)   | 0.36             | 0.410 (-3.03; 3.85)   | 0.82         | -1.488 (-6.58; 3.61)  | 0.57             |
| MCP-1 (pg/mL)                          | 5.864 (-5.50; 17.2)   | 0.31         | 9.691 (1.46; 17.9)    | <b>0.021</b>     | 14.176 (5.96; 22.4)   | <b>0.001</b> | -1.362 (-12.9; 10.2)  | 0.82             |
| <b>24 h PP</b>                         |                       |              |                       |                  |                       |              |                       |                  |
| <b>Model 1</b>                         |                       |              |                       |                  |                       |              |                       |                  |
| PAI-1 <sub>act</sub> (IU/mL)           | 1.769 (-0.53; 4.06)   | 0.13         | 2.441 (0.98; 3.90)    | <b>0.001</b>     | 1.326 (-0.19; 2.84)   | 0.09         | 3.795 (1.71; 5.88)    | <b>&lt;0.001</b> |
| Age (years)                            | -0.479 (-0.92; -0.04) | <b>0.033</b> | -0.643 (-0.94; -0.35) | <b>&lt;0.001</b> | -0.539 (-0.87; -0.21) | <b>0.001</b> | -0.669 (-1.05; -0.29) | <b>0.001</b>     |

|                                        |                       |              |                       |              |                       |              |                       |              |
|----------------------------------------|-----------------------|--------------|-----------------------|--------------|-----------------------|--------------|-----------------------|--------------|
| <b>Model 2</b>                         |                       |              |                       |              |                       |              |                       |              |
| PAI-1 <sub>act</sub> (IU/mL)           | 0.969 (-1.65; 3.59)   | 0.47         | 2.176 (0.47; 3.89)    | <b>0.013</b> | 0.348 (-1.45; 2.15)   | 0.71         | 4.334 (1.88; 6.79)    | <b>0.001</b> |
| Age (years)                            | -0.491 (-0.94; -0.04) | <b>0.031</b> | -0.529 (-0.89; -0.17) | <b>0.004</b> | -0.538 (-0.92; -0.16) | <b>0.005</b> | -0.647 (-1.06; -0.23) | <b>0.002</b> |
| Smoking* (no) <sup>a</sup>             | 0.175 (-3.00; 3.35)   | 0.91         | 0.428 (-1.43; 2.29)   | 0.65         | -                     | -            | -                     | -            |
| Alcohol <sup>†</sup> (no) <sup>a</sup> | -                     | -            | -                     | -            | -0.403 (-2.52; 1.71)  | 0.71         | -0.502 (-3.26; 2.26)  | 0.72         |
| SES score                              | 0.053 (-0.24; 0.34)   | 0.72         | -0.162 (-0.37; 0.04)  | 0.12         | 0.060 (-0.17; 0.29)   | 0.60         | -0.267 (-0.51; -0.02) | <b>0.031</b> |
| WHtR                                   | 19.838 (-17.2; 56.9)  | 0.29         | 12.512 (-12.4; 37.4)  | 0.33         | 15.634 (-13.0; 44.2)  | 0.28         | 7.969 (-22.4; 38.4)   | 0.61         |
| HDL-C (mmol/L)                         | -2.572 (-13.1; 7.94)  | 0.63         | -1.636 (-8.60; 5.32)  | 0.65         | -0.694 (-8.28; 6.89)  | 0.86         | -7.933 (-17.4; 1.55)  | 0.10         |
| Triglycerides (mmol/L)                 | -4.118 (-12.3; 4.08)  | 0.33         | 0.101 (-4.77; 4.97)   | 0.97         | -0.943 (-6.12; 4.24)  | 0.72         | 0.345 (-6.65; 7.34)   | 0.92         |
| GGT (U/L)                              | -3.169 (-9.42; 3.08)  | 0.32         | -1.577 (-5.54; 2.38)  | 0.44         | -1.225 (-5.86; 3.41)  | 0.61         | -1.511 (-6.35; 3.33)  | 0.54         |
| Insulin (μU/mL)                        | 8.144 (2.66; 13.6)    | <b>0.004</b> | 1.090 (-2.94; 5.12)   | 0.60         | 5.715 (1.71; 9.72)    | <b>0.005</b> | 1.545 (-3.63; 6.72)   | 0.56         |
| MCP-1 (pg/mL)                          | -0.066 (-14.2; 14.1)  | 0.99         | 3.769 (-4.75; 12.3)   | 0.39         | -1.276 (-10.8; 8.28)  | 0.79         | 11.846 (0.15; 23.5)   | <b>0.047</b> |

Abbreviations: DBP, diastolic blood pressure; PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; SES, socio-economic status; WHtR, waist-to-height ratio; HDL-C, high-density lipoprotein cholesterol; GGT, gamma-glutamyltransferase; MCP-1, monocyte chemoattractant protein-1; PP, pulse pressure.

Notes: Model 1, basic model including only age as covariate; Model 2, fully adjusted model.

\*Smoking not included in models split for smoking; <sup>†</sup>Alcohol not included in models split for alcohol use.

<sup>a</sup>Smoking (yes) and alcohol use (yes) used as references.

Bold text indicates p-values < 0.05.

**Supplement Table 4.** Characteristics of black men, split by alcohol use and smoking

|                                 | <b>Non-drinkers</b><br>(n=104) | <b>Drinkers</b><br>(n=173) | <b>Non-smokers</b><br>(n=161) | <b>Smokers</b><br>(n=118) |   |
|---------------------------------|--------------------------------|----------------------------|-------------------------------|---------------------------|---|
| Age (years)                     | 24.4 ± 3.22                    | 24.3 ± 2.94                | 24.8 ± 3.01                   | 23.7 ± 3.01               | * |
| Smoking, n (%) <sup>a</sup>     | 28 (27)                        | 90 (52) *                  |                               |                           |   |
| Alcohol use, n (%) <sup>a</sup> |                                |                            | 83 (52)                       | 90 (76) *                 |   |
| SES score                       | 18.8 ± 5.55                    | 16.4 ± 5.17 **             | 18.8 ± 5.33                   | 15.3 ± 5.01 ***           |   |
| WC (cm)                         | 76.4 (71.1-82.2)               | 73.6 (68.5-81.4)           | 77.6 (71.5-83.0)              | 72.0 (67.6-78.9) *        |   |
| WHtR                            | 0.45 (0.42-0.48)               | 0.44 (0.41-0.48)           | 0.45 (0.42-0.49)              | 0.42 (0.40-0.46) *        |   |
| 24 h SBP (mmHg)                 | 119 ± 8.58                     | 120 ± 8.26                 | 120 ± 8.20                    | 119 ± 8.56                |   |
| 24 h DBP (mmHg)                 | 69.1 ± 5.91                    | 69.6 ± 6.51                | 70.0 ± 6.02                   | 68.8 ± 6.61               |   |
| 24 h PP (mmHg)                  | 49.6 ± 7.46                    | 50.0 ± 6.17                | 49.8 ± 6.58                   | 49.9 ± 6.75               |   |
| PAI-1 <sub>act</sub> (IU/mL)    | 2.44 (0.83-7.23)               | 3.59 (1.87-8.37) *         | 3.32 (1.62-8.60)              | 2.90 (1.50-7.32)          |   |
| HDL-C (mmol/L)                  | 1.00 (0.79-1.21)               | 1.14 (0.81-1.41) *         | 1.03 (0.82-1.33)              | 1.13 (0.81-1.41)          |   |
| LDL-C (mmol/L)                  | 1.88 (1.48-2.48)               | 2.00 (1.52-2.57)           | 1.97 (1.48-2.66)              | 1.96 (1.57-2.38)          |   |
| Triglycerides (mmol/L)          | 0.54 (0.44-0.74)               | 0.71 (0.52-0.97) **        | 0.60 (0.45-0.89)              | 0.66 (0.51-0.91)          |   |
| CRP (mg/L)                      | 0.60 (0.22-1.47)               | 0.50 (0.19-1.36)           | 0.60 (0.23-1.44)              | 0.46 (0.19-1.39)          |   |
| GGT (U/L)                       | 20.3 (13.9-28.0)               | 25.6 (16.9-37.2) **        | 23.4 (15.6-35.1)              | 23.0 (15.8-32.7)          |   |
| Glucose (mmol/L)                | 3.81 ± 1.10                    | 3.61 ± 1.03                | 3.82 ± 1.15                   | 3.53 ± 0.91               |   |
| Insulin (μU/mL)                 | 6.94 (5.10-11.4)               | 6.85 (4.80-10.3)           | 7.30 (5.33-10.9)              | 6.39 (4.42-9.42) *        |   |
| IL-6 (pg/mL)                    | 1.00 (0.73-1.48)               | 0.95 (0.63-1.33)           | 1.01 (0.74-1.39)              | 0.90 (0.62-1.37)          |   |
| MCP-1 (pg/mL)                   | 168 (145-201)                  | 169 (146-199)              | 165 (145-199)                 | 174 (148-202)             |   |
| vWF <sub>ag</sub> (%)           | 92.0 (68.5-120)                | 90.0 (69.0-120)            | 95.0 (69.5-126)               | 85.0 (68.0-117) *         |   |
| Cotinine (ng/mL)                | 1.00 (1.00-1.00)               | 18.8 (1.00-201) ***        | 1.00 (1.00-1.00)              | 210 (77.4-324) ***        |   |
| Cotinine, smokers only (ng/mL)  | 236 (77.2-342)                 | 181 (77.5-306)             |                               |                           |   |

Abbreviations: SES, socio-economic status; WC, waist circumference; WHtR, waist-to-height ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; PAI-1<sub>act</sub>, plasminogen activator inhibitor-1 activity; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; GGT, gamma-glutamyltransferase; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; vWF<sub>ag</sub>, von Willebrand factor antigen.

Notes: data expressed as arithmetic mean ± standard deviation, median and interquartile range, or count and percentage.

<sup>a</sup>Self-reported smoking and alcohol use.

\*p < 0.05, \*\*p < 0.001, \*\*\*p < 0.0001 for no alcohol vs alcohol and no smoking vs smoking comparisons.

**HIGHLIGHTS**

- In young healthy adults, PAI-1 relates positively with BP
- The relationship is confounded largely by obesity
- In black men with endothelial dysfunction, PAI-1 associated independently with BP
- Alcohol use and smoking prominently influence PAI-1's relationship with BP

# CHAPTER 5

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Research article 3:

**Plasminogen activator inhibitor-1 activity and the  
4G/5G polymorphism are prospectively associated  
with blood pressure and hypertension status**

**This article was published in *Journal of Hypertension*.**

*Jacobs A, Schutte AE, Ricci C, Pieters M. Plasminogen activator inhibitor-1 activity and the 4G/5G polymorphism are prospectively associated with blood pressure and hypertension status. Journal of Hypertension. 2019 Dec;37(12):2361-2370; doi: 10.1097/HJH.0000000000002204.*

[https://journals.lww.com/jhypertension/Fulltext/2019/12000/Plasminogen\\_activator\\_inhibitor\\_1\\_activity\\_and\\_the.8.aspx](https://journals.lww.com/jhypertension/Fulltext/2019/12000/Plasminogen_activator_inhibitor_1_activity_and_the.8.aspx)

# Journal of Hypertension

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**Title:** Plasminogen activator inhibitor-1 activity and the 4G/5G polymorphism are prospectively associated with blood pressure and hypertension status

**Short title:** Plasminogen activator inhibitor-1 and blood pressure

Adriaan JACOBS<sup>ab</sup>, Aletta E. SCHUTTE<sup>b\*</sup>, Cristian RICCI<sup>a</sup>, Marlien PIETERS<sup>a</sup>

<sup>a</sup>Centre of Excellence for Nutrition (CEN), North-West University, Potchefstroom, South Africa

<sup>b</sup>Hypertension in Africa Research Team (HART), Medical Research Council Unit for Hypertension and Cardiovascular Disease, North-West University, Potchefstroom, South Africa

**Corresponding author:**

\*Professor AE Schutte

E-mail: Alta.Schutte@nwu.ac.za

Hypertension in Africa Research Team

Tel: +27 (0)18 299 2444

North-West University

Fax: +27 (0)18 285 2432

Private Bag X1290

Potchefstroom 2520, South Africa

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## ABSTRACT

**Objectives:** Plasminogen activator inhibitor-1 (PAI-1) has consistently shown positive associations with blood pressure (BP). Whether elevations in PAI-1 levels precede or result from raised BP is still under debate and data on prospective studies are limited. Hence, we investigated the prospective associations of PAI-1 and the 4G/5G polymorphism with brachial and central BP and pulse pressure (PP) over a 10-year period.

**Methods:** Black South Africans aged 30 years and older were included. Baseline data collection commenced in 2005 (n=2010) with follow-up data collection in 2010 (n=1288) and 2015 (n=926). Plasma PAI-1 activity (PAI-1<sub>act</sub>), 4G/5G polymorphism genotyping, waist circumference and BP measurements were performed and analysed using sequential regression and mixed models.

**Results:** In multivariable adjusted analyses, PAI-1<sub>act</sub> and the 4G/4G (vs. the 5G/5G) genotype increased the odds of developing hypertension in the total group (1.04 (1.01; 1.08) and 1.82 (1.07; 3.12) respectively). Furthermore, PAI-1<sub>act</sub> was prospectively associated with brachial SBP ( $r = 0.0815$ ) and PP ( $r = 0.0832$ ) in the total group, and with central PP in women ( $r = 0.1125$ ; all  $p < 0.05$ ). Addition of waist circumference to the models either decreased or nullified the contribution of PAI-1<sub>act</sub> to BP and hypertension development.

**Conclusions:** PAI-1<sub>act</sub> and the 4G/4G (vs. the 5G/5G) genotype increased the odds of developing hypertension. Furthermore, PAI-1<sub>act</sub> associated prospectively with both brachial and central BP. These associations were mediated in part by central adiposity. The study supports the hypothesis that PAI-1 also contributes to hypertension development rather than solely being a consequence thereof.

## CONDENSED ABSTRACT

Associations between plasminogen activator inhibitor-1 activity (PAI-1<sub>act</sub>), the 4G/5G polymorphism and blood pressure were investigated in black South Africans in a prospective epidemiological study in order to address the controversy as to whether elevated PAI-1 levels precede or result from hypertension. The 4G/4G (vs. the 5G/5G) genotype and PAI-1<sub>act</sub> increased the odds of developing hypertension. PAI-1<sub>act</sub> furthermore associated prospectively with both brachial and central blood pressure. The contribution of PAI-1 to raised blood pressure was mediated in part by central adiposity. Our results support the hypothesis that PAI-1 also contributes to hypertension rather than solely being a consequence thereof.

## KEY WORDS

Plasminogen activator inhibitor-1, 4G/5G polymorphism, brachial blood pressure, central blood pressure, hypertension

## ABBREVIATIONS AND SYMBOLS

bDBP, brachial diastolic blood pressure  
 BMI, body mass index  
 bPP, brachial pulse pressure  
 bSBP, brachial systolic blood pressure  
 cPP, central pulse pressure  
 CRP, C-reactive protein  
 cSBP, central systolic blood pressure  
 CVD, cardiovascular disease  
 DALY, disability-adjusted life year  
 GGT,  $\gamma$ -glutamyl transferase  
 HDL-C, high-density lipoprotein cholesterol  
 HIV, human immunodeficiency virus  
 LDL-C, low-density lipoprotein cholesterol  
 PA, plasminogen activator  
 PAI-1, plasminogen activator inhibitor-1  
 PAI-1<sub>act</sub>, PAI-1 activity  
 PAI-1<sub>ag</sub>, PAI-1 antigen  
 PCR, polymerase chain reaction  
 TC, total cholesterol  
 VLDL, very low-density lipoprotein  
 WC, waist circumference

## INTRODUCTION

The assessment of risk factors for the Global Burden of Disease Study estimated high SBP to be the leading risk factor for risk-attributable global disability-adjusted life years (DALYs), accounting for 10.4 million deaths and 218 million DALYs globally [1]. Several circulating biomarkers are associated with raised blood pressure, one of these biomarkers being plasminogen activator inhibitor-1 (PAI-1) [2, 3], a known cardiovascular disease (CVD) risk marker [4]. PAI-1 is a serine protease inhibitor (serpin) that plays a classical role in fibrinolysis wherein it regulates fibrin breakdown by inhibition of plasminogen activators [5]. PAI-1's association with hypertension is presumably due to its suggested alternative roles in endothelial dysfunction [6] and vascular remodelling [7], both processes that are known to be associated with hypertension [8,9]. On a genetic level, associations between blood pressure and the 4G/5G polymorphism in the promoter region of the PAI-1 gene have also been found [10, 11].

Numerous cross-sectional studies [12-15] have reported positive associations between PAI-1 and blood pressure or hypertension, but prospective data [3, 16, 17] are limited. Whether elevated PAI-1 levels precede or result from elevated blood pressure is still under debate, as the former [3, 16, 17] as well as the latter [14, 15] have both been suggested. Results from experimental studies in mice, however, suggest that PAI-1 may play a causal role in the development of hypertension [18, 19]. The aim of this study was to investigate the 10-year prospective associations of PAI-1 and the 4G/5G PAI-1 polymorphism with hypertensive status and brachial and central blood pressure measures, with central pressure recently receiving much attention as a stronger predictor of cardiovascular risk [20] than brachial blood pressure. These associations were investigated in an epidemiological setting in black South Africans who are prone to the development of hypertension [21, 22] and display unique vascular [23-28] and haemostatic [23, 26, 29, 30] profiles compared with their white counterparts. A particular characteristic of the black South African population is the high degree of obesity, especially among women [31]. Obesity is not only known to be associated with hypertension [32], but there is also a strong link between obesity (in particular central obesity) and PAI-1 [33]. Hence, the influence of central obesity on the investigated relationships between PAI-1, the 4G/5G polymorphism and blood pressure was also evaluated.

## **METHODS**

### ***Study design***

Baseline and prospective data were obtained from black South Africans enrolled in the Prospective Urban and Rural Epidemiological (PURE) study. The South African arm of the PURE study was conducted in accordance with the overarching PURE study guidelines as previously described [34]. In short, the PURE study is a large-scale epidemiological study aimed at investigating the relationship between lifestyle behaviours, risk factors and the incidence of noncommunicable diseases in rural and urban communities across 27 low, middle and high-income countries. In South Africa, baseline data collection commenced in 2005 with follow-up data collection taking place every 5 years (2010, 2015). Study participants were recruited from 6000 randomly selected households in the rural and urban communities in the North West province, South Africa. Apparently healthy black men and women older than 30 years participated in the study, provided that they intended to reside in the community for a minimum of 4 years in order to increase long-term follow-up feasibility. Baseline data were collected from n=2010 enrolled participants, of whom n=1288 participated at 5-year follow-up and n=926 participated at 10-year follow-up. The attrition rates over the 10-year period are due to nonparticipation (n=726) or death (n=358). All participants gave voluntary written informed consent prior to each data collection. Data were dealt with in a confidential manner and all subsequent analyses were performed on anonymised, coded data. All aspects of data collection complied with the Declaration of Helsinki of 1975 (as revised in 2013) for research involving human participants. The South African arm of the PURE study was approved by the Health Research Ethics Committee of the North-West University, South Africa (NWU-00016-10-A1).

### ***Questionnaire data***

Information regarding rural/urban status, self-reported smoking and alcohol consumption habits, and anti-hypertensive medication and contraceptive use was recorded using standardised questionnaires.

### ***Anthropometric measurements***

Waist circumference was measured using a Holtain unstretchable metal tape (Crymych, UK) in 2005 and 2010, and a Lufkin steel tape (Cooper Tools; Apex, North Carolina, USA) for 2015 measurements.

### **Cardiovascular measurements**

The Omron HEM-757 device was used in 2005 and 2010 and the OMRON M6 device (Omron Healthcare, Kyoto, Japan) in 2015 for brachial SBP (bSBP) and DBP (bDBP) measurements. Brachial pulse pressure (bPP) was calculated as the difference between bSBP and bDBP. By performing pulse wave analysis using the SphygmoCor XCEL System (AtCor Medical Pty. Ltd., Sydney, Australia), we were able to determine central SBP (cSBP) and central pulse pressure (cPP) in 2015. Hypertension was defined according to the 2018 ESC/ESH guidelines [35] as a bSBP and/or bDBP greater than or equal to 140/90 mmHg, or the use of antihypertensive medication.

### **Blood sample collection and preparation**

Fasting blood samples were collected between 0700 and 1100 h from the antecubital vein using sterile winged infusion sets and syringes. Blood samples intended for PAI-1<sub>act</sub> analysis were collected in 3.2% sodium citrate tubes. For serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides,  $\gamma$ -glutamyl transferase (GGT) and C-reactive protein (CRP), blood samples were collected in tubes that did not contain anticoagulants. Samples were centrifuged at 2000g for 15 min within 30 min of collection. Serum and plasma fractions were removed followed by the removal of the buffy coat for DNA isolation and stored at -80°C.

### **Biochemical analysis**

Plasma PAI-1 activity (PAI-1<sub>act</sub>) was determined by an indirect enzymatic method (Spectrolyze PAI-1; Trinity Biotech, Bray, Ireland). Serum CRP, GGT, triglycerides, TC and HDL-C were determined using the Konelab20i auto-analyser (Thermo Fisher Scientific, Vantaa, Finland). Low-density lipoprotein cholesterol (LDL-C) was calculated according to the formula described by Sathiyakumar *et al.* [36].

QIAGEN Flexigene DNA extraction kits (QIAGEN, Valencia, California, USA) were used for the isolation of genomic DNA from buffy coat fractions of blood samples collected in 2005. The DNA quality was monitored and its concentration determined using a NanoDrop spectrophotometer (ND-1000, Wilmington, Delaware, USA). In cases wherein the output of the NanoDrop was not satisfactory, isolations were repeated using Maxwell blood DNA purification kits (Promega Corporation, Madison, Wisconsin, USA). Genotyping of the 4G/5G polymorphism in the promoter region of the PAI-1 (*SERPINE1*) gene was performed [37] with TaqMan-based assays (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as previously described [38] using a MyIQ5 BioRad real-time PCR thermal cycler (Bio-Rad Laboratories Inc., Hercules, California, USA). The 4G/5G polymorphism adhered to the

assumptions of Hardy-Weinberg equilibrium and were within the 95% confidence interval ranges (chi-square=0.9,  $P=0.268$ ).

HIV testing was performed on whole blood samples using a First Response HIV 1-2-0 test (Premier Medical Corporation Ltd, Daman, India). In case of positive results, confirmatory testing was done using the Pareeshak card test (BHAT Bio-tech, Bengaluru, Karnataka, India), the SD BIOLINE HIV 1/2 3.0 test (Standard Diagnostics Inc., Gyeonggi-do, Korea) and the ABON HIV test (Biopharm Co. Ltd., Hangzhou, China) in 2005, 2010 and 2015, respectively.

### **Statistical analysis**

Only participants with available data for the main variables and/or covariates used in statistical models were included in statistical analyses ( $n=1697$ ). Statistical analyses were performed in the total group and also split by sex as sex-specific biology for PAI-1 has been suggested [10, 39] and because of the known sex difference in body composition of the PURE participants [40].

Sample bSBP, bPP, PAI-1<sub>act</sub>, waist circumference and hypertension status in 2005, 2010 and 2015 were reported using medians and interquartile ranges, and by counts and percentages. cSBP and cPP were measured in 2015 only. The change in continuous outcomes over the observational period was evaluated using a generalized linear model of the log-transformed outcome and the year as covariate. A logistic model was used for hypertension status. The p-trend reported is given by the statistical test applied to the slope of the outcome over the three time points. A statistical comparison by sex was also reported at the three time points (**Table 1**).

The cross-sectional and prospective associations between PAI-1<sub>act</sub>, blood pressure measures and waist circumference at the different time points were determined by Spearman correlation coefficients, firstly adjusted by age, sex and rural/urban status, and then supplementary adjusted by alcohol and tobacco use, HDL-C, LDL-C, triglycerides, GGT, CRP, HIV status, anti-hypertensive medication use, waist circumference (PAI-1 and blood pressure measures only) and contraceptive use (women only) (**Table 2**).

The associations of the 4G/5G polymorphism with PAI-1<sub>act</sub> and waist circumference were evaluated using a mixed model with rural/urban status as a random factor (**Table 3** and **Supplement, Table 3**). The associations of the 4G/5G polymorphism with cSBP and cPP in 2015 and with bSBP and bDBP were performed using a fixed model with the logarithms of the blood pressures as outcomes and the 4G/5G polymorphism as a covariate (**Table 4** and **Supplement, Tables 2, 4 and 5**). Residual confounding was evaluated by sequential

modelling applied to three nested sets of covariates (**Tables 3-4** and **Supplement, Tables 2-5**). The first model was adjusted for age and sex with rural/urban status as a random factor (**Table 4** and **Supplement, Tables 2, 4 and 5**). Elsewhere, rural/urban status was considered as a basic adjusting factor in fixed models (**Table 3** and **Supplement, Table 3**). A second model was supplementary adjusted for HDL-C, LDL-C, triglycerides, CRP, GGT, HIV status and anti-hypertensive medication use (when BP was the outcome). Finally, a third model was supplementary adjusted for waist circumference. The least square means of exponentially retro-transformed variables, along with its 95% confidence limit, were provided. The p-value for difference was performed by means of Tukey-Kramer adjustment method to consider the unequal sizes of the 4G/5G polymorphism groups [41].

Finally, the associations of PAI-1<sub>act</sub> and the 4G/5G polymorphism with brachial blood pressure were further determined by performing four different random effect logistic regressions where PAI-1<sub>act</sub>, the 4G/5G polymorphism and waist circumference were sequentially added to a mixed logistic regression with hypertension status as the outcome (**Table 5**). All of these models had rural/urban status as a random factor and were adjusted for age, alcohol and tobacco use, HDL-C, LDL-C, triglycerides, CRP, GGT and HIV status. All analyses conducted in women were additionally adjusted for contraceptive use. The SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA) was used to perform statistical analyses. All statistical tests were two-tailed and type-I error rate was set to 5% ( $\alpha=0.05$ ).

## RESULTS

### *Study population profile*

We performed data collection in 2005 (n=2010), 2010 (n=1288) and 2015 (n=926), where men represented the minority, comprising 37, 34 and 30% at the three time points, respectively. Participants were equally represented in terms of geographical area, being n=1006 rural and n=1004 urban participants at baseline. The baseline median age was 48.3 (41.9-56.4) years. The baseline characteristics of the study participants are given in **Supplement Table 1**. Hypertension status, bPP and waist circumference increased over the 10 years in both men and women, whilst bSBP increased only in men. At each time point, brachial blood pressure measures were higher in men than in women. Conversely, PAI-1<sub>act</sub> and waist circumference were consistently higher in women (**Table 1**).

### *Associations between plasminogen activator inhibitor-1<sub>act</sub> and blood pressure*

The associations between PAI-1<sub>act</sub>, blood pressure and waist circumference are reported in **Table 2**. In the basic model in the total group, PAI-1<sub>act</sub> correlated with many of the measured blood pressure measures both cross-sectionally and prospectively. After full adjustment, most of the cross-sectional correlations remained significant, whilst the only prospective associations that remained were between PAI-1<sub>act</sub> in 2005 and bSBP in 2010 ( $r=0.0815$ ,  $p<0.05$ ) and bPP in 2015 ( $r=0.0832$ ,  $p<0.05$ ). Sensitivity analysis performed only in individuals not using anti-hypertensive medication did not significantly alter the results ( $r=0.0906$  and  $r=0.1076$ , respectively).

In men (adjusted for age and rural/urban status), we found correlations between PAI-1<sub>act</sub> and brachial, but not central, blood pressure, whereas in women prospective associations with cSBP and cPP were also found. After full adjustment of these models, bSBP in 2005 correlated prospectively with PAI-1<sub>act</sub> in 2015 in men ( $r=0.1709$ ,  $p<0.05$ ), whereas in women, the only prospective association that remained was between PAI-1<sub>act</sub> in 2005 and cPP in 2015 ( $r=0.1125$ ,  $p<0.05$ ). Sensitivity analysis revealed that separate, individual adjustment for waist circumference nullified the prospective association between PAI-1<sub>act</sub> and cSBP ( $p=0.14$ ).

### *Association between plasminogen activator inhibitor-1<sub>act</sub> and waist circumference*

PAI-1<sub>act</sub> was strongly associated with waist circumference, with a clear prospective association observed in men in whom baseline PAI-1<sub>act</sub> correlated with follow-up waist circumference measurements (**Table 2**).

**Table 1.** Number of hypertensive participants, PAI-1<sub>act</sub>, brachial and central blood pressures and waist circumference in the PURE study participants during the period 2005-2015

| <b>Total group n = 2010</b>  | <b>2005</b>          | <b>2010</b>          | <b>2015</b>           | <b>p-value</b> <small>trend</small> |
|------------------------------|----------------------|----------------------|-----------------------|-------------------------------------|
| Hypertensive n (%)           | 884 (44.0)           | 1,072 (53.3)         | 1,163 (57.9)          | < 0.0001                            |
| PAI-1 <sub>act</sub> (IU/mL) | 4.25 (1.27, 7.91)    | 4.65 (2.17, 7.85)    | 2.79 (0.00, 7.72)     | 0.0396                              |
| Brachial SBP (mmHg)          | 132.0 (118.0, 147.0) | 130.0 (116.0, 147.0) | 130.0 (116.0, 148.0)  | 0.3211                              |
| Central SBP (mmHg)           | -                    | -                    | 125.0 (113.0, 138.0)  | -                                   |
| Brachial PP (mmHg)           | 43.0 (35.0, 53.0)    | 44.0 (36.0, 55.0)    | 46.0 (36.0, 57.0)     | < 0.0001                            |
| Central PP (mmHg)            | -                    | -                    | 40.0 (32.0, 50.0)     | -                                   |
| Waist circumference (cm)     | 77.5 (70.2, 87.6)    | 79.6 (71.4, 90.4)    | 87.5 (76.2, 99.0)     | < 0.0001                            |
| <b>Men n = 747</b>           | <b>2005</b>          | <b>2010</b>          | <b>2015</b>           | <b>p-value</b> <small>trend</small> |
| Hypertensive n (%)           | 318 (42.6)           | 383 (51.3)*          | 420 (56.2)*           | < 0.0001                            |
| PAI-1 <sub>act</sub> (IU/mL) | 2.23 (0.00, 5.68)*** | 3.62 (0.95, 6.93)*** | 1.04 (0.00, 4.69)***  | 0.0038                              |
| Brachial SBP (mmHg)          | 133 (119, 149)**     | 136 (122, 153)**     | 136 (121, 152)**      | 0.0219                              |
| Central SBP (mmHg)           | -                    | -                    | 127.0 (114.0, 138.0)  | -                                   |
| Brachial PP (mmHg)           | 46.0 (39.0, 55.0)*** | 48.0 (39.0, 58.0)*** | 49.0 (40.0, 61.0)***  | 0.0006                              |
| Central PP (mmHg)            | -                    | -                    | 39.0 (32.0, 50.0)     | -                                   |
| Waist circumference (cm)     | 74.4 (69.8, 81.2)*** | 75.5 (69.9, 83.5)*** | 78.9 (72.1, 90.5)***  | < 0.0001                            |
| <b>Women n = 1263</b>        | <b>2005</b>          | <b>2010</b>          | <b>2015</b>           | <b>p-value</b> <small>trend</small> |
| Hypertensive n (%)           | 566 (44.8)           | 689 (54.6)*          | 743 (58.8)*           | < 0.0001                            |
| PAI-1 <sub>act</sub> (IU/mL) | 5.30 (2.49, 8.89)*** | 5.06 (2.73, 8.35)*** | 3.57 (0.00, 8.43)***  | 0.0413                              |
| Brachial SBP (mmHg)          | 130 (116, 146)**     | 128 (115, 146)**     | 128 (114, 146)**      | 0.6882                              |
| Central SBP (mmHg)           | -                    | -                    | 124.0 (113.0, 138.0)  | -                                   |
| Brachial PP (mmHg)           | 41.0 (33.0, 51.0)*** | 42.0 (34.0, 53.0)*** | 44.0 (35.0, 55.0)***  | 0.0001                              |
| Central PP (mmHg)            | -                    | -                    | 40.0 (32.0, 51.0)     | -                                   |
| Waist circumference (cm)     | 81.0 (70.6, 91.4)*** | 83.0 (73.1, 93.5)*** | 91.3 (80.2, 102.0)*** | < 0.0001                            |

**Notes:** Continuous variables were reported as median and interquartile range; categorical variables were reported as count and percentages. Abbreviations: SBP, systolic blood pressure; PP, pulse pressure.

\*p-value sex comparison within year < 0.05, \*\*p-value sex comparison within year < 0.001, \*\*\*p-value sex comparison within year < 0.0001.

**Table 2.** Spearman partial correlation coefficients of PAI-1<sub>act</sub> with brachial and central blood pressure and waist circumference in 2005, 2010 and 2015

|                     | Total group <sup>a</sup> |            |            | Total group <sup>c</sup> |            |            |
|---------------------|--------------------------|------------|------------|--------------------------|------------|------------|
|                     | PAI-1 2005               | PAI-1 2010 | PAI-1 2015 | PAI-1 2005               | PAI-1 2010 | PAI-1 2015 |
| Brachial SBP (2005) | 0.1333**                 | 0.0516     | 0.0880*    | 0.1287**                 | -0.0222    | -0.0330    |
| Brachial SBP (2010) | 0.0971*                  | 0.0001     | 0.0649     | 0.0815*                  | -0.0364    | -0.0545    |
| Brachial SBP (2015) | 0.0620                   | -0.0051    | 0.1060*    | -0.0113                  | -0.0736    | 0.0986*    |
| Central SBP (2015)  | 0.0934*                  | 0.0531     | 0.1356*    | 0.0221                   | -0.0031    | 0.0829*    |
| Brachial PP (2005)  | 0.0519                   | -0.0027    | 0.0237     | -0.0297                  | -0.0786    | 0.0148     |
| Brachial PP (2010)  | 0.0604                   | -0.0680    | 0.0264     | -0.0289                  | -0.1471**  | 0.0680     |
| Brachial PP (2015)  | 0.1111**                 | 0.0066     | 0.0960*    | 0.0832*                  | -0.0820*   | -0.0302    |
| Central PP (2015)   | 0.1114**                 | 0.0700     | 0.0757*    | 0.0266                   | -0.0173    | 0.0061     |
| Waist (2005)        | 0.3297***                | 0.2320***  | 0.2095***  | 0.2503***                | 0.1833***  | 0.1705***  |
| Waist (2010)        | 0.2938***                | 0.2364***  | 0.2200***  | 0.2438***                | 0.2069***  | 0.2204***  |
| Waist (2015)        | 0.2472***                | 0.2147***  | 0.2297***  | 0.2303***                | 0.2079***  | 0.2673***  |
|                     | Men <sup>b</sup>         |            |            | Women <sup>b</sup>       |            |            |
|                     | PAI-1 2005               | PAI-1 2010 | PAI-1 2015 | PAI-1 2005               | PAI-1 2010 | PAI-1 2015 |
| Brachial SBP (2005) | 0.1678*                  | -0.0280    | 0.2102***  | 0.1327**                 | 0.0870     | 0.0449     |
| Brachial SBP (2010) | 0.1458*                  | -0.0362    | 0.0846     | 0.0698                   | -0.0046    | 0.0527     |
| Brachial SBP (2015) | 0.0421                   | -0.1823**  | 0.0904     | 0.0757                   | 0.0626     | 0.1113*    |
| Central SBP (2015)  | 0.0138                   | -0.0966    | 0.0982     | 0.1259**                 | 0.1174*    | 0.1596**   |
| Brachial PP (2005)  | 0.1106                   | -0.0758    | 0.1524**   | 0.0272                   | 0.0245     | -0.0324    |
| Brachial PP (2010)  | 0.0979                   | -0.0538    | 0.0258     | 0.0341                   | -0.0840    | 0.0192     |
| Brachial PP (2015)  | 0.1120                   | -0.0955    | 0.1264     | 0.1132**                 | 0.0420     | 0.0798     |
| Central PP (2015)   | 0.0326                   | 0.0521     | 0.0576     | 0.1476**                 | 0.0918*    | 0.0886     |
| Waist (2005)        | 0.3949***                | 0.2255***  | 0.2559***  | 0.2844***                | 0.2267***  | 0.1980***  |
| Waist (2010)        | 0.4299***                | 0.2766***  | 0.2773***  | 0.2028***                | 0.2054***  | 0.1943***  |
| Waist (2015)        | 0.3791***                | 0.2702***  | 0.3031***  | 0.1699***                | 0.1906**   | 0.2071***  |
|                     | Men <sup>c</sup>         |            |            | Women <sup>c</sup>       |            |            |
|                     | PAI-1 2005               | PAI-1 2010 | PAI-1 2015 | PAI-1 2005               | PAI-1 2010 | PAI-1 2015 |
| Brachial SBP (2005) | 0.0976                   | -0.1054    | 0.1709*    | 0.0515                   | -0.0056    | -0.0664    |
| Brachial SBP (2010) | 0.1177                   | -0.1059    | 0.0162     | 0.0672                   | -0.0048    | -0.0294    |
| Brachial SBP (2015) | 0.0315                   | -0.1329*   | 0.0092     | 0.0057                   | -0.0031    | 0.0315     |
| Central SBP (2015)  | -0.0193                  | -0.0536    | 0.0167     | 0.0382                   | 0.0333     | 0.0705     |
| Brachial PP (2005)  | 0.0613                   | -0.1173    | 0.0871     | 0.0064                   | -0.0363    | -0.0556    |
| Brachial PP (2010)  | 0.0565                   | -0.0581    | -0.0142    | 0.0151                   | -0.1505*   | -0.0211    |
| Brachial PP (2015)  | 0.0740                   | -0.0818    | 0.0115     | 0.0640                   | -0.0097    | 0.0076     |
| Central PP (2015)   | -0.0029                  | 0.0640     | 0.0978*    | 0.1125*                  | 0.0183     | -0.0330    |

**Notes:** <sup>a</sup> adjusted for sex, age and rural/urban status; <sup>b</sup> adjusted for age and rural/urban status; <sup>c</sup> adjusted for sex, age, rural/urban status, alcohol use, tobacco use, high density lipoprotein, low density lipoprotein, triglycerides,  $\gamma$ -glutamyl transferase, C-reactive protein, HIV status, waist circumference and anti-hypertensive medication use. Spearman partial correlation coefficients in women were supplementary adjusted for contraceptive use. \*p-value < 0.05, \*\*p-value < 0.001, \*\*\*p-value < 0.0001. Abbreviations: SBP, systolic blood pressure; PP, pulse pressure; WC, waist circumference.

***The contribution of plasminogen activator inhibitor-1<sub>act</sub> and the 4G/5G polymorphism to central blood pressure measures and the development of hypertension***

In the total group, PAI-1<sub>act</sub> differed across the three genotypes, with the mean PAI-1<sub>act</sub> levels being consistently higher in 4G/4G than in 5G/5G carriers. When split for sex, this result was confirmed in women only (**Table 3**). Likewise, cSBP was associated with the 4G/5G polymorphism. In particular, we observed a significantly higher cSBP in 4G/4G than in 5G/5G carriers in the total group in all three models. When split for sex, 4G/4G carriers again displayed higher cSBP in both men and women separately with difference reaching significance in men ( $p_{\text{omnibus}}=0.048$ ) only (**Table 4**). A similar association between the 4G/5G polymorphism and cPP was observed in both sexes, and between the 4G/5G polymorphism and bSBP and bDBP in women, albeit nonsignificantly (**Supplement, Tables 2, 4 and 5**). Notably, these results were confirmed in the fully adjusted models, hence the relation observed has to be considered as independent with respect to the adjusting factors considered. No significant differences in waist circumference were observed across the three genotypes in either men or women (**Supplement, Table 3**). Finally, we considered the effect of a 2-unit increase in PAI-1<sub>act</sub> on hypertension risk (**Table 5**). This resulted in a 4% increased risk of developing hypertension in the total group (Model 1). When split for sex, significance remained in women only with a 2-unit increase in PAI-1<sub>act</sub> increasing the odds of developing hypertension by 5%. Carriers of the 4G/4G genotype had an 82% increased risk of developing hypertension when compared with 5G/5G carriers in the total group (Model 2). In Model 3, the addition of PAI-1<sub>act</sub> to the model decreased the odds of developing hypertension from 82 to 30% compared with the 5G/5G genotype. In this model, a 2-unit increase in PAI-1<sub>act</sub> still resulted in a 4% increased odds to develop hypertension. When split for sex, the 4G/5G polymorphism was not associated with increased odds of developing hypertension in either men or women, whilst in women, the odds remained at 5% for a 2-unit increase in PAI-1<sub>act</sub>. In Model 4, which was supplementary adjusted for waist circumference, PAI-1<sub>act</sub> levels no longer increased the odds of developing hypertension in any group, whilst the 4G/4G genotype increased the odds of hypertension by 85% when compared with the 5G/5G genotype. This was observed in the total group but not when split for sex. A 10-cm increase in waist circumference, however, significantly increased the odds of developing hypertension with 55% in the total group, 58% in the men and 52% in the women.

**Table 3.** PAI-1 4G/5G polymorphism vs. PAI-1<sub>act</sub>

| <b>Total</b> | <b>PAI-1</b> polymorphism | <b>PAI-1<sub>act</sub></b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
|--------------|---------------------------|---------------------------------------------|-------------------------------|----------------------------|
| Model 1      | 4G/4G (n=43)              | 4.30 (3.31, 5.53)                           | 0.0290                        | 0.0020                     |
|              | 4G/5G (n=422)             | 3.48 (3.19, 3.79)                           | 0.0211                        |                            |
|              | 5G/5G (n=1232)            | 3.04 (2.88, 3.20)                           | Ref.                          |                            |
| Model 2      | 4G/4G                     | 4.46 (3.47, 5.67)                           | 0.0110                        | 0.0003                     |
|              | 4G/5G                     | 3.55 (3.27, 3.86)                           | 0.0058                        |                            |
|              | 5G/5G                     | 3.05 (2.90, 3.21)                           | Ref.                          |                            |
| Model 3      | 4G/4G                     | 4.40 (3.44, 5.57)                           | 0.0163                        | 0.0001                     |
|              | 4G/5G                     | 3.64 (3.36, 3.94)                           | 0.0018                        |                            |
|              | 5G/5G                     | 3.09 (2.94, 3.25)                           | Ref.                          |                            |
| <b>Men</b>   | <b>PAI-1</b> polymorphism | <b>PAI-1<sub>act</sub></b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
| Model 1      | 4G/4G (n=10)              | 1.95 (0.83, 3.77)                           | 0.8976                        | 0.4383                     |
|              | 4G/5G (n=167)             | 2.56 (2.19, 2.97)                           | 0.4695                        |                            |
|              | 5G/5G (n=466)             | 2.29 (2.08, 2.52)                           | Ref.                          |                            |
| Model 2      | 4G/4G                     | 2.18 (1.01, 4.03)                           | 0.9907                        | 0.2489                     |
|              | 4G/5G                     | 2.64 (2.27, 3.04)                           | 0.2248                        |                            |
|              | 5G/5G                     | 2.28 (2.08, 2.49)                           | Ref.                          |                            |
| Model 3      | 4G/4G                     | 2.16 (1.02, 3.94)                           | 0.9905                        | 0.1159                     |
|              | 4G/5G                     | 2.69 (2.33, 3.09)                           | 0.0988                        |                            |
|              | 5G/5G                     | 2.26 (2.06, 2.46)                           | Ref.                          |                            |
| <b>Women</b> | <b>PAI-1</b> polymorphism | <b>PAI-1<sub>act</sub></b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
| Model 1      | 4G/4G (n=33)              | 6.28 (4.81, 8.13)                           | 0.0034                        | 0.0004                     |
|              | 4G/5G (n=255)             | 4.62 (4.17, 5.10)                           | 0.0299                        |                            |
|              | 5G/5G (n=766)             | 3.97 (3.74, 4.21)                           | Ref.                          |                            |
| Model 2      | 4G/4G                     | 6.31 (4.87, 8.11)                           | 0.0025                        | 0.0004                     |
|              | 4G/5G                     | 4.62 (4.19, 5.09)                           | 0.0324                        |                            |
|              | 5G/5G                     | 4.00 (3.78, 4.23)                           | Ref.                          |                            |
| Model 3      | 4G/4G                     | 6.17 (4.78, 7.88)                           | 0.0038                        | 0.0003                     |
|              | 4G/5G                     | 4.67 (4.25, 5.14)                           | 0.0174                        |                            |
|              | 5G/5G                     | 4.00 (3.79, 4.23)                           | Ref.                          |                            |

**Notes:** model 1: age and sex adjusted with rural/urban status as random factor; model 2 is model 1 supplementary adjusted for high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, C-reactive protein,  $\gamma$ -glutamyl transferase and HIV status; model 3 is model 2 supplementary adjusted for waist circumference. All analyses conducted in women were supplementary adjusted for contraceptive use.  $p_{\text{difference}}$  is the p-value comparing outcome LS mean by PAI-1 polymorphism;  $p_{\text{omnibus}}$  is the Type-III test for the overall effect of PAI-1 polymorphism.

**Table 4.** PAI-1 4G/5G polymorphism vs. central SBP

| <b>Total</b> | <b>PAI-1</b> polymorphism | <b>cSBP</b> LS mean (95% CI) | <b>p</b> <sub>difference</sub> | <b>p</b> <sub>omnibus</sub> |
|--------------|---------------------------|------------------------------|--------------------------------|-----------------------------|
| Model 1      | 4G/4G (n=17)              | 137.2 (128.0, 146.5)         | 0.0796                         | 0.0374                      |
|              | 4G/5G (n=189)             | 125.0 (122.2, 127.8)         | 0.4481                         |                             |
|              | 5G/5G (n=547)             | 127.0 (125.2, 128.7)         | Ref.                           |                             |
| Model 2      | 4G/4G                     | 134.6 (125.4, 144.6)         | 0.1231                         | 0.0470                      |
|              | 4G/5G                     | 124.2 (121.6, 126.9)         | 0.7953                         |                             |
|              | 5G/5G                     | 125.2 (123.7, 126.8)         | Ref.                           |                             |
| Model 3      | 4G/4G                     | 133.8 (124.7, 143.7)         | 0.1667                         | 0.0450                      |
|              | 4G/5G                     | 124.3 (121.7, 127.0)         | 0.8199                         |                             |
|              | 5G/5G                     | 125.2 (123.7, 126.8)         | Ref.                           |                             |
| <b>Men</b>   | <b>PAI-1</b> polymorphism | <b>cSBP</b> LS mean (95% CI) | <b>p</b> <sub>difference</sub> | <b>p</b> <sub>omnibus</sub> |
| Model 1      | 4G/4G (n=2)               | 143.2 (118.6, 167.8)         | 0.4652                         | 0.0399                      |
|              | 4G/5G (n=67)              | 122.7 (118.5, 127.0)         | 0.0745                         |                             |
|              | 5G/5G (n=164)             | 128.3 (125.6, 131.0)         | Ref.                           |                             |
| Model 2      | 4G/4G                     | 139.3 (114.5, 169.4)         | 0.4875                         | 0.0478                      |
|              | 4G/5G                     | 122.0 (117.9, 126.3)         | 0.0712                         |                             |
|              | 5G/5G                     | 126.9 (124.2, 129.6)         | Ref.                           |                             |
| Model 3      | 4G/4G                     | 139.3 (114.5, 169.4)         | 0.5263                         | 0.0477                      |
|              | 4G/5G                     | 122.0 (117.9, 126.3)         | 0.0638                         |                             |
|              | 5G/5G                     | 126.9 (124.2, 129.6)         | Ref.                           |                             |
| <b>Women</b> | <b>PAI-1</b> polymorphism | <b>cSBP</b> LS mean (95% CI) | <b>p</b> <sub>difference</sub> | <b>p</b> <sub>omnibus</sub> |
| Model 1      | 4G/4G (n=15)              | 136.3 (126.2, 146.4)         | 0.1351                         | 0.1573                      |
|              | 4G/5G (n=122)             | 126.3 (122.7, 129.8)         | 0.9998                         |                             |
|              | 5G/5G (n=383)             | 126.3 (124.3, 128.3)         | Ref.                           |                             |
| Model 2      | 4G/4G                     | 134.2 (124.1, 145.1)         | 0.1572                         | 0.1734                      |
|              | 4G/5G                     | 125.4 (122.0, 128.9)         | 0.9027                         |                             |
|              | 5G/5G                     | 124.5 (122.6, 126.5)         | Ref.                           |                             |
| Model 3      | 4G/4G                     | 133.4 (123.5, 144.2)         | 0.2029                         | 0.1775                      |
|              | 4G/5G                     | 125.5 (122.1, 129.0)         | 0.8886                         |                             |
|              | 5G/5G                     | 124.6 (122.7, 126.5)         | Ref.                           |                             |

**Notes:** model 1: age, rural/urban status and sex adjusted; model 2 is model 1 supplementary adjusted for high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, C-reactive protein,  $\gamma$ -glutamyl transferase, HIV status and anti-hypertensive medication use; model 3 is model 2 supplementary adjusted for waist circumference. All analyses conducted in women were supplementary adjusted for contraceptive use.  $p_{\text{difference}}$  is the p-value comparing outcome LS mean by PAI-1 polymorphism;  $p_{\text{omnibus}}$  is the Type-III test for the overall effect of PAI-1 polymorphism.

**Table 5.** PAI-1 4G/5G polymorphism, PAI-1<sub>act</sub> and waist circumference vs. hypertension risk

| <b>Total</b>                     | <b>Model 1 OR (95% CI)</b> | <b>Model 2 OR (95% CI)</b> | <b>Model 3 OR (95% CI)</b> | <b>Model 4 OR (95% CI)</b> |
|----------------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
| PAI-1 <sub>2-unit increase</sub> | 1.04 (1.01, 1.08)          | -                          | 1.04 (1.01, 1.07)          | 1.00 (0.98, 1.04)          |
| PAI-1 <sub>polymorphism</sub>    |                            |                            |                            |                            |
| 4G/4G vs. 5G/5G                  | -                          | 1.82 (1.07, 3.12)          | 1.30 (1.03, 3.28)          | 1.85 (1.03, 3.32)          |
| 4G/5G vs. 5G/5G                  | -                          | 0.91 (0.76, 1.09)          | 1.05 (0.76, 1.12)          | 0.97 (0.80, 1.18)          |
| WC <sub>10 cm increase</sub>     | -                          | -                          | -                          | 1.55 (1.42, 1.68)          |
| <b>Men</b>                       | <b>Model 1 OR (95% CI)</b> | <b>Model 2 OR (95% CI)</b> | <b>Model 3 OR (95% CI)</b> | <b>Model 4 OR (95% CI)</b> |
| PAI-1 <sub>2-unit increase</sub> | 1.00 (0.96, 1.05)          | -                          | 1.00 (0.95, 1.04)          | 0.98 (0.93, 1.03)          |
| PAI-1 <sub>polymorphism</sub>    |                            |                            |                            |                            |
| 4G/4G vs. 5G/5G                  | -                          | 2.18 (0.66, 7.12)          | 3.05 (0.84, 13.0)          | 3.17 (0.80, 12.5)          |
| 4G/5G vs. 5G/5G                  | -                          | 0.88 (0.66, 1.19)          | 1.09 (0.65, 1.22)          | 0.93 (0.68, 1.27)          |
| WC <sub>10 cm increase</sub>     | -                          | -                          | -                          | 1.58 (1.31, 1.90)          |
| <b>Women</b>                     | <b>Model 1 OR (95% CI)</b> | <b>Model 2 OR (95% CI)</b> | <b>Model 3 OR (95% CI)</b> | <b>Model 4 OR (95% CI)</b> |
| PAI-1 <sub>2-unit increase</sub> | 1.05 (1.02, 1.08)          | -                          | 1.05 (1.02, 1.09)          | 1.02 (0.99, 1.06)          |
| PAI-1 <sub>polymorphism</sub>    |                            |                            |                            |                            |
| 4G/4G vs. 5G/5G                  | -                          | 1.73 (0.94, 3.19)          | 1.64 (0.84, 3.12)          | 1.64 (0.85, 3.20)          |
| 4G/5G vs. 5G/5G                  | -                          | 0.96 (0.76, 1.20)          | 1.10 (0.75, 1.24)          | 1.02 (0.79, 1.31)          |
| WC <sub>10 cm increase</sub>     | -                          | -                          | -                          | 1.52 (1.38, 1.67)          |

**Notes:** model 1 includes PAI-1<sub>act</sub> only; model 2 includes PAI-1 polymorphism only; model 3 includes both PAI-1<sub>act</sub> and PAI-1 polymorphism; model 4 includes PAI-1<sub>act</sub>, PAI-1 polymorphism and waist circumference. All models had rural/urban status as a random factor and were adjusted for age, alcohol use, tobacco use, high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, C-reactive protein,  $\gamma$ -glutamyl transferase and HIV status. All analyses conducted in women were supplementary adjusted for contraceptive use. Abbreviations: WC, waist circumference.

## DISCUSSION

The aim of this study was to investigate the 10-year prospective associations of PAI-1 and the 4G/5G PAI-1 polymorphism with hypertensive status and blood pressure measures. In our sample of black South Africans, we found that the 4G/4G (compared with the 5G/5G) genotype and PAI-1<sub>act</sub> levels increased the odds of developing hypertension. Furthermore, PAI-1<sub>act</sub> was prospectively associated with brachial blood pressure in the total group and with central blood pressure in women. This adds supporting prospective, population-based evidence to the hypothesis that the 4G/5G polymorphism and PAI-1 may contribute to hypertension development. The association between PAI-1<sub>act</sub> and blood pressure was mediated in part by central obesity.

### *Plasminogen activator inhibitor-1<sub>act</sub> and blood pressure*

The prospective association of PAI-1 with blood pressure is reflected in the observation that PAI-1<sub>act</sub> increased the odds for developing hypertension in the total group and in analysis split for sex, in women specifically (**Table 5**). PAI-1<sub>act</sub> also associated prospectively with brachial SBP and PP in the total group and central PP in women (**Table 2**). On the basis of these findings, it appears that the observations in the total group are largely due to associations found in women. The seemingly contradicting findings that PAI-1<sub>act</sub> increased hypertension odds in women but did not associate prospectively with brachial blood pressure can potentially be explained by hypertension status being defined not only by brachial blood pressure measurements but also by the use of anti-hypertensive medication. As such, the use of anti-hypertensive medication may have decreased brachial blood pressure, obscuring its association with PAI-1<sub>act</sub>. The association of PAI-1<sub>act</sub> with central PP was still evident in women despite the use of anti-hypertensive medication, possibly due to anti-hypertensive medications' divergent effects on peripheral (muscular) and central (elastic) arteries [42]. The observed associations of PAI-1<sub>act</sub> with blood pressure appear to be mediated in part by central adiposity, as the significance of PAI-1<sub>act</sub> was lost upon addition of waist circumference to the statistical models. Nevertheless, our results suggest that PAI-1 levels are already elevated before the onset of hypertension, as has also been suggested by other studies [3, 16, 17], as opposed to solely being a consequence of hypertension [14, 15]. PAI-1's involvement in endothelial dysfunction [6] and structural vascular alterations [7] are possible mechanisms by which PAI-1 contributes to vasculopathy, and consequently hypertension development. It should be mentioned that, despite the number of prospective correlations of PAI-1<sub>act</sub> with blood pressure that existed (**Table 2**), this was not consistent at all time points and the relationships were comparatively weaker than the relationships between PAI-1<sub>act</sub> and waist circumference. A single prospective correlation of bSBP in 2005

with PAI-1<sub>act</sub> in 2015 in men also supports the concept that elevated PAI-1 levels result from raised blood pressure [14, 15].

***Plasminogen activator inhibitor-1<sub>act</sub>, the 4G/5G polymorphism and blood pressure***

The 4G/5G polymorphism was significantly associated with PAI-1<sub>act</sub> levels in women, not in men, with 4G/4G carriers presenting with higher PAI-1<sub>act</sub> levels (**Table 3**). This is in agreement with the literature wherein the 4G/4G genotype is associated with higher PAI-1 levels [43, 44]. The 5G-allele, which contains an additional guanosine, creates a binding site for a transcriptional repressor that decreases basal PAI-1 transcription [45]. Our observation that the 4G/5G polymorphism was associated with PAI-1<sub>act</sub> levels in women only is in agreement with findings from other studies [12, 46]. The 4G/5G polymorphism, being a response polymorphism [47], implies that the extent of its effect on gene expression depends on the concurrent presence of other environmental and/or disease factors that influence PAI-1 expression (such as the presence of central adiposity). In our study population, this sex difference may therefore be explained by the higher degree of central adiposity in the women (**Table 1**), with adipose tissue being one of the main sites of PAI-1 production [33] and PAI-1 release from adipose tissue increasing in obesity [48]. In support of this hypothesis, Sartori *et al.* [49] reported associations between the 4G/5G polymorphism and PAI-1<sub>ag</sub> levels in participants with central obesity. Furthermore, in conjunction with the central obesity in the women, baseline triglyceride levels were also higher in women than men [1.35 (1.31, 1.40) vs. 1.21 (1.15, 1.26) mmol/l,  $p < 0.0001$ ]. Triglyceride levels are thought to influence PAI-1 levels [50] via a very low-density lipoprotein (VLDL)-response element in the PAI-1 promoter area [51]. This relationship has also been shown to be modulated by the 4G/5G polymorphism, adjacent to and upstream of the VLDL-response element [12, 37, 52]. Regardless of the above, however, significance remained even after adjustment for waist circumference and triglycerides, suggesting that sex-specific factors presumably also play a role.

In the total group, the 4G/4G (compared with the 5G/5G) genotype increased the odds of developing hypertension (**Table 5**) in fully adjusted models. The addition of PAI-1<sub>act</sub> to the model decreased the significance of the 4G/5G polymorphism, indicating that the contribution of the polymorphism to hypertension is partly mediated by PAI-1<sub>act</sub> levels. Furthermore, the observation that the 4G/4G genotype still contributed to hypertension to such a large extent despite the addition of PAI-1<sub>act</sub> to the model suggests that the polymorphism also contributes to hypertension in ways other than via PAI-1<sub>act</sub> levels. A similar hypothesis was suggested by Martínez-Calatrava *et al.* [11], who also found an association between the 4G/4G genotype and hypertension risk in a Spanish population, irrespective of plasma PAI-1<sub>ag</sub> levels.

Apart from the 4G/5G polymorphism's association with hypertension status (**Table 5**) and brachial blood pressures in women (**Supplement, Tables 4 and 5**), the polymorphism was also associated with central blood pressure measures (**Table 4** and **Supplement, Table 2**), with 4G/4G carriers consistently presenting with higher central blood pressure. The observation that PAI-1<sub>act</sub> was prospectively associated with central PP in women (**Table 2**) provides rationale for the 4G/4G genotype (responsible for higher PAI-1<sub>act</sub> levels in women) also being associated with higher central blood pressure in women, albeit nonsignificantly (**Table 4** and **Supplement, Table 2**). The latter associations not reaching statistical significance may be explained by the small sample size of the 4G/4G genotype, and alternatively by sex-specific influences as well as the higher degree of central adiposity and higher triglyceride levels in women. The latter factors may contribute to central blood pressure to such an extent that the direct association of the 4G/5G polymorphism with central blood pressure in women is partially obscured. Furthermore, the observation that the 4G/5G polymorphism (**Table 4**), but not PAI-1<sub>act</sub> (**Table 2**), is associated with cSBP in men suggests that the 4G/5G polymorphism may also contribute to central blood pressure via pathways other than PAI-1<sub>act</sub> levels in men.

***The influence of central adiposity on the association of plasminogen activator inhibitor-1 with blood pressure***

The strong correlations observed between PAI-1<sub>act</sub> and waist circumference (**Table 2**) are in agreement with PAI-1's known association with central obesity [33]. Visceral adipose tissue is a major source of PAI-1 production [33], but studies have also suggested PAI-1 to contribute to adiposity [53, 54], likely by influencing adipocyte differentiation [55]. Our results support the latter hypothesis, showing a prospective association of PAI-1<sub>act</sub> with waist circumference in men (**Table 2**). No clear pattern was observed in women, likely due to sex-specific factors that also contribute to female central adiposity, thereby obscuring PAI-1's contribution. Despite PAI-1's prospective association with waist circumference in men, the 4G/5G polymorphism showed no associations with waist circumference (**Supplement, Table 3**).

Our results show that both PAI-1<sub>act</sub> and the 4G/4G genotype increased the odds of developing hypertension (**Table 5**), but that the contribution of PAI-1<sub>act</sub> was obscured by waist circumference (Model 4). This emphasizes the comparatively large contribution of central adiposity to hypertension, or suggests that the contribution of PAI-1 to hypertension is mediated via central adiposity. Given that PAI-1 [56], central adiposity and raised blood pressure [57] are recognized components of the metabolic syndrome, the possibility exists that the observed associations may be driven by the combined cardiometabolic derangements associated with the metabolic syndrome rather than solely by waist

circumference. Although the contribution of PAI-1<sub>act</sub> to hypertension development was influenced by waist circumference, the contribution of the 4G/4G genotype was not.

Given obesity's link to both PAI-1 [33] and hypertension [32], the sex differences observed in our study may be explained by the higher degree of (central) obesity amongst black South African women as compared with men. However, other studies that reported significant associations of the 4G/5G polymorphism with central blood pressure [10] or PAI-1 [12] in women only were conducted in groups wherein women and men had similar BMIs (unfortunately no indices of central adiposity were reported). This suggests that the sex differences observed in our study population cannot be explained solely by differences in central adiposity, but that other sex-specific factors may also play a role.

### ***Strengths and limitations***

A major strength of the study was its prospective design, and the results obtained strengthen the currently limited data on the possible contribution of PAI-1 to hypertension development. Despite the prospective associations found, causality cannot be established. One of the main findings was that the 4G/4G genotype predisposes to hypertension development. Although this may seem trivial due to the low prevalence of the 4G/4G genotype in this sample from the black South African population (2.5%; 4G allele frequency=0.15), the 4G allele is much more common in other ethnic groups [43, 44, 58]. Limitations of our study include the small sample size of the 4G/4G genotype group (which may have induced potential type II errors), and the availability of central blood pressure measures for one timepoint only. Our results are limited to black South Africans and extrapolation to other populations should be done with caution due to possible ethnic, lifestyle and environmental differences.

In conclusion, this study adds to the currently limited number of prospective studies investigating the association of PAI-1 with blood pressure. The 4G/4G (vs. the 5G/5G) genotype contributed to the development of hypertension, irrespective of the role of central obesity. PAI-1<sub>act</sub> also increased the odds for hypertension development and was prospectively associated with brachial and central blood pressure in the total group and women, respectively. These associations were mediated in part by central obesity. Our results strengthen the argument that elevations in PAI-1 may contribute to hypertension rather than solely being a consequence thereof. The sex differences observed in our study can, partly, be attributed to the high prevalence of central obesity in the women, but other sex-specific factors presumably also play a role.

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## SUPPLEMENTARY MATERIAL

**Supplement Table 1.** Baseline characteristics of the PURE study participants

|                                | <b>*Total group</b><br>n = 1697 |
|--------------------------------|---------------------------------|
| Age (years)                    | 48.3 (41.9, 56.4)               |
| Sex n (%)                      |                                 |
| Men                            | 643 (37.9)                      |
| Women                          | 1,054 (62.1)                    |
| Rural/urban status n (%)       |                                 |
| Rural                          | 870 (51.3)                      |
| Urban                          | 827 (48.7)                      |
| Tobacco use n (%)              |                                 |
| Never                          | 897 (52.9)                      |
| Current                        | 737 (43.4)                      |
| Former                         | 63 (3.7)                        |
| Alcohol use n (%)              |                                 |
| Never                          | 937 (55.2)                      |
| Current                        | 686 (40.4)                      |
| Former                         | 74 (4.4)                        |
| Waist circumference (cm)       | 77.0 (70.2, 87.6)               |
| PAI-1 <sub>act</sub> (IU/mL)   | 4.25 (1.27, 7.91)               |
| PAI-1 4G/5G polymorphism n (%) |                                 |
| 5G/5G                          | 1,232 (72.6)                    |
| 4G/5G                          | 422 (24.9)                      |
| 4G/4G                          | 43 (2.5)                        |
| HIV positive n (%)             | 276 (16.3)                      |
| HDL-C (mmol/L)                 | 1.42 (1.06, 1.88)               |
| LDL-C (mmol/L)                 | 2.78 (2.07, 3.63)               |
| Triglycerides (mmol/L)         | 1.08 (0.82, 1.55)               |
| CRP (mg/L)                     | 3.25 (0.95, 9.29)               |
| GGT (U/L)                      | 46.0 (29.5, 87.0)               |
| Female contraceptive use n (%) | 370 (35.1)                      |

**Notes:** \*Total group after exclusion of participants with missing values for the covariates used in the statistical models. Abbreviations: HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; GGT,  $\gamma$ -glutamyl transferase.

**Supplement Table 2.** PAI-1 4G/5G polymorphism vs. central pulse pressure (cPP)

| <b>Total</b> | <b>PAI-1</b> polymorphism | <b>cPP</b> LS mean (95% CI) | <b>p</b> <sub>difference</sub> | <b>p</b> <sub>omnibus</sub> |
|--------------|---------------------------|-----------------------------|--------------------------------|-----------------------------|
| Model 1      | 4G/4G (n=17)              | 46.93 (39.76, 54.09)        | 0.2645                         | 0.2845                      |
|              | 4G/5G (n=189)             | 40.98 (38.82, 43.14)        | 0.9847                         |                             |
|              | 5G/5G (n=547)             | 41.19 (39.85, 42.52)        | Ref.                           |                             |
| Model 2      | 4G/4G                     | 44.36 (38.89, 50.60)        | 0.4503                         | 0.2492                      |
|              | 4G/5G                     | 40.56 (38.99, 42.20)        | 0.9459                         |                             |
|              | 5G/5G                     | 40.87 (39.93, 41.82)        | Ref.                           |                             |
| Model 3      | 4G/4G                     | 43.84 (38.48, 49.95)        | 0.5507                         | 0.2418                      |
|              | 4G/5G                     | 40.60 (39.04, 42.22)        | 0.9564                         |                             |
|              | 5G/5G                     | 40.87 (39.94, 41.81)        | Ref.                           |                             |
| <b>Men</b>   | <b>PAI-1</b> polymorphism | <b>cPP</b> LS mean (95% CI) | <b>p</b> <sub>difference</sub> | <b>p</b> <sub>omnibus</sub> |
| Model 1      | 4G/4G (n=2)               | 46.65 (27.14, 66.17)        | 0.8758                         | 0.3595                      |
|              | 4G/5G (n=66)              | 39.09 (35.72, 42.46)        | 0.3880                         |                             |
|              | 5G/5G (n=164)             | 41.76 (39.61, 43.92)        | Ref.                           |                             |
| Model 2      | 4G/4G                     | 41.22 (27.75, 61.23)        | 0.9947                         | 0.4242                      |
|              | 4G/5G                     | 39.14 (36.52, 41.95)        | 0.7259                         |                             |
|              | 5G/5G                     | 40.41 (38.69, 42.21)        | Ref.                           |                             |
| Model 3      | 4G/4G                     | 41.26 (27.87, 61.07)        | 0.9942                         | 0.4178                      |
|              | 4G/5G                     | 39.12 (36.52, 41.90)        | 0.7111                         |                             |
|              | 5G/5G                     | 40.42 (38.72, 42.20)        | Ref.                           |                             |
| <b>Women</b> | <b>PAI-1</b> polymorphism | <b>cPP</b> LS mean (95% CI) | <b>p</b> <sub>difference</sub> | <b>p</b> <sub>omnibus</sub> |
| Model 1      | 4G/4G (n=15)              | 47.22 (39.43, 55.01)        | 0.2924                         | 0.2829                      |
|              | 4G/5G (n=123)             | 42.24 (39.51, 44.97)        | 0.7784                         |                             |
|              | 5G/5G (n=383)             | 41.17 (39.62, 42.71)        | Ref.                           |                             |
| Model 2      | 4G/4G                     | 44.98 (39.12, 51.71)        | 0.4206                         | 0.4042                      |
|              | 4G/5G                     | 41.32 (39.35, 43.39)        | 0.9751                         |                             |
|              | 5G/5G                     | 41.07 (39.95, 42.21)        | Ref.                           |                             |
| Model 3      | 4G/4G                     | 44.40 (38.67, 50.98)        | 0.5223                         | 0.3959                      |
|              | 4G/5G                     | 41.37 (39.42, 43.42)        | 0.9644                         |                             |
|              | 5G/5G                     | 41.07 (39.97, 42.20)        | Ref.                           |                             |

**Notes:** model 1: age, rural/urban status and sex adjusted; model 2 is model 1 supplementary adjusted for high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, C-reactive protein,  $\gamma$ -glutamyl transferase, HIV status and anti-hypertensive medication use; model 3 is model 2 supplementary adjusted for waist circumference. All analyses conducted in women were supplementary adjusted for contraceptive use.  $p_{\text{difference}}$  is the p-value comparing outcome LS mean by PAI-1 polymorphism;  $p_{\text{omnibus}}$  is the Type-III test for the overall effect of PAI-1 polymorphism.

**Supplement Table 3.** PAI-1 4G/5G polymorphism vs. waist circumference (WC)

| <b>Total</b> | <b>PAI-1</b> polymorphism | <b>WC</b> LS mean (95% CI) | <b>p</b> difference | <b>p</b> omnibus |
|--------------|---------------------------|----------------------------|---------------------|------------------|
| Model 1      | 4G/4G (n=43)              | 81.53 (78.62, 84.43)       | 0.8629              | 0.1398           |
|              | 4G/5G (n=422)             | 79.74 (78.81, 80.67)       | 0.1494              |                  |
|              | 5G/5G (n=1232)            | 80.75 (80.21, 81.29)       | Ref.                |                  |
| Model 2      | 4G/4G                     | 81.22 (78.50, 83.94)       | 0.9831              | 0.1928           |
|              | 4G/5G                     | 80.07 (79.19, 80.94)       | 0.1752              |                  |
|              | 5G/5G                     | 80.97 (80.47, 81.48)       | Ref.                |                  |
| <b>Men</b>   | <b>PAI-1</b> polymorphism | <b>WC</b> LS mean (95% CI) | <b>p</b> difference | <b>p</b> omnibus |
| Model 1      | 4G/4G (n=10)              | 78.11 (72.85, 83.38)       | 0.9935              | 0.1657           |
|              | 4G/5G (n=167)             | 76.53 (75.37, 77.69)       | 0.1438              |                  |
|              | 5G/5G (n=466)             | 77.82 (77.14, 78.50)       | Ref.                |                  |
| Model 2      | 4G/4G                     | 78.90 (74.10, 83.70)       | 0.8908              | 0.2790           |
|              | 4G/5G                     | 76.82 (75.76, 77.89)       | 0.2917              |                  |
|              | 5G/5G                     | 77.77 (77.15, 78.39)       | Ref.                |                  |
| <b>Women</b> | <b>PAI-1</b> polymorphism | <b>WC</b> LS mean (95% CI) | <b>p</b> difference | <b>p</b> omnibus |
| Model 1      | 4G/4G (n=33)              | 84.67 (81.15, 88.19)       | 0.8844              | 0.3769           |
|              | 4G/5G (n=255)             | 82.86 (81.57, 84.15)       | 0.4164              |                  |
|              | 5G/5G (n=766)             | 83.81 (83.10, 84.52)       | Ref.                |                  |
| Model 2      | 4G/4G                     | 83.73 (80.45, 87.01)       | 0.9911              | 0.2977           |
|              | 4G/5G                     | 82.85 (81.65, 84.06)       | 0.2647              |                  |
|              | 5G/5G                     | 83.95 (83.28, 84.61)       | Ref.                |                  |

**Notes:** model 1: age and sex adjusted with rural/urban status as random factor; model 2 is model 1 supplementary adjusted for high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, C-reactive protein,  $\gamma$ -glutamyl transferase and HIV status. All analyses conducted in women were supplementary adjusted for contraceptive use.  $p_{\text{difference}}$  is the p-value comparing outcome LS mean by PAI-1 polymorphism;  $p_{\text{omnibus}}$  is the Type-III test for the overall effect of PAI-1 polymorphism.

**Supplement Table 4.** PAI-1 4G/5G polymorphism vs. brachial systolic blood pressure (bSBP)

| <b>Total</b> | <b>PAI-1</b> polymorphism | <b>bSBP</b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
|--------------|---------------------------|------------------------------|-------------------------------|----------------------------|
| Model 1      | 4G/4G (n=43)              | 140.7 (133.9, 147.8)         | 0.0735                        | 0.1152                     |
|              | 4G/5G (n=422)             | 133.3 (131.0, 135.6)         | 0.3758                        |                            |
|              | 5G/5G (n=1232)            | 134.4 (132.7, 136.1)         | Ref.                          |                            |
| Model 2      | 4G/4G                     | 136.9 (130.6, 143.5)         | 0.0741                        | 0.1829                     |
|              | 4G/5G                     | 130.7 (128.8, 132.7)         | 0.7744                        |                            |
|              | 5G/5G                     | 131.1 (129.9, 132.2)         | Ref.                          |                            |
| Model 3      | 4G/4G                     | 136.6 (130.4, 143.1)         | 0.0855                        | 0.2114                     |
|              | 4G/5G                     | 130.8 (128.9, 132.7)         | 0.8200                        |                            |
|              | 5G/5G                     | 131.1 (129.9, 132.2)         | Ref.                          |                            |
| <b>Men</b>   | <b>PAI-1</b> polymorphism | <b>bSBP</b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
| Model 1      | 4G/4G (n=10)              | 136.3 (123.0, 150.9)         | 0.9832                        | 0.8148                     |
|              | 4G/5G (n=167)             | 134.8 (131.2, 138.5)         | 0.5248                        |                            |
|              | 5G/5G (n=466)             | 136.1 (133.5, 138.7)         | Ref.                          |                            |
| Model 2      | 4G/4G                     | 133.3 (121.0, 146.8)         | 0.9556                        | 0.8635                     |
|              | 4G/5G                     | 132.6 (129.5, 135.8)         | 0.5882                        |                            |
|              | 5G/5G                     | 133.6 (131.8, 135.5)         | Ref.                          |                            |
| Model 3      | 4G/4G                     | 133.3 (121.1, 146.8)         | 0.9690                        | 0.9025                     |
|              | 4G/5G                     | 132.8 (129.7, 135.9)         | 0.6632                        |                            |
|              | 5G/5G                     | 133.6 (131.7, 135.5)         | Ref.                          |                            |
| <b>Women</b> | <b>PAI-1</b> polymorphism | <b>bSBP</b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
| Model 1      | 4G/4G (n=33)              | 142.0 (134.2, 150.3)         | 0.0217                        | 0.0509                     |
|              | 4G/5G (n=255)             | 132.0 (129.1, 134.9)         | 0.5587                        |                            |
|              | 5G/5G (n=766)             | 132.9 (130.8, 135.0)         | Ref.                          |                            |
| Model 2      | 4G/4G                     | 137.4 (130.2, 145.0)         | 0.0358                        | 0.1098                     |
|              | 4G/5G                     | 129.7 (127.2, 132.2)         | 0.9080                        |                            |
|              | 5G/5G                     | 129.5 (128.1, 131.0)         | Ref.                          |                            |
| Model 3      | 4G/4G                     | 137.1 (130.0, 144.6)         | 0.0378                        | 0.1151                     |
|              | 4G/5G                     | 129.7 (127.1, 132.3)         | 0.8946                        |                            |
|              | 5G/5G                     | 129.5 (127.9, 131.1)         | Ref.                          |                            |

**Notes:** model 1: age, rural/urban status and sex adjusted; model 2 is model 1 supplementary adjusted for high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, C-reactive protein,  $\gamma$ -glutamyl transferase, HIV status and anti-hypertensive medication use; model 3 is model 2 supplementary adjusted for waist circumference. All analyses conducted in women were supplementary adjusted for contraceptive use.  $p_{\text{difference}}$  is the p-value comparing outcome LS mean by PAI-1 polymorphism;  $p_{\text{omnibus}}$  is the Type-III test for the overall effect of PAI-1 polymorphism.

**Supplement Table 5.** PAI-1 4G/5G polymorphism vs. brachial diastolic blood pressure (bDBP)

| <b>Total</b> | <b>PAI-1</b> polymorphism | <b>bDBP</b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
|--------------|---------------------------|------------------------------|-------------------------------|----------------------------|
| Model 1      | 4G/4G (n=43)              | 90.7 (86.3, 95.3)            | 0.1453                        | 0.3433                     |
|              | 4G/5G (n=422)             | 87.3 (85.8, 88.8)            | 0.1589                        |                            |
|              | 5G/5G (n=1232)            | 87.5 (86.4, 88.6)            | Ref.                          |                            |
| Model 2      | 4G/4G                     | 88.5 (84.4, 92.8)            | 0.4772                        | 0.4884                     |
|              | 4G/5G                     | 86.3 (85.1, 87.7)            | 0.9147                        |                            |
|              | 5G/5G                     | 86.0 (85.3, 86.8)            | Ref.                          |                            |
| Model 3      | 4G/4G                     | 88.3 (84.3, 92.5)            | 0.2817                        | 0.5204                     |
|              | 4G/5G                     | 86.4 (85.1, 87.7)            | 0.6301                        |                            |
|              | 5G/5G                     | 86.0 (85.3, 86.8)            | Ref.                          |                            |
| <b>Men</b>   | <b>PAI-1</b> polymorphism | <b>bDBP</b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
| Model 1      | 4G/4G (n=10)              | 84.0 (75.5, 93.5)            | 0.5739                        | 0.8278                     |
|              | 4G/5G (n=167)             | 87.0 (84.6, 89.4)            | 0.8376                        |                            |
|              | 5G/5G (n=466)             | 86.7 (85.0, 88.4)            | Ref.                          |                            |
| Model 2      | 4G/4G                     | 82.8 (74.8, 91.6)            | 0.5692                        | 0.7999                     |
|              | 4G/5G                     | 85.6 (83.5, 87.8)            | 0.7604                        |                            |
|              | 5G/5G                     | 85.3 (84.0, 86.5)            | Ref.                          |                            |
| Model 3      | 4G/4G                     | 82.8 (74.9, 91.6)            | 0.5779                        | 0.7704                     |
|              | 4G/5G                     | 85.7 (83.7, 87.9)            | 0.6774                        |                            |
|              | 5G/5G                     | 85.2 (84.0, 86.5)            | Ref.                          |                            |
| <b>Women</b> | <b>PAI-1</b> polymorphism | <b>bDBP</b> LS mean (95% CI) | <b>p<sub>difference</sub></b> | <b>p<sub>omnibus</sub></b> |
| Model 1      | 4G/4G (n=33)              | 92.7 (87.7, 97.9)            | 0.0584                        | 0.1378                     |
|              | 4G/5G (n=255)             | 87.4 (85.5, 89.3)            | 0.6743                        |                            |
|              | 5G/5G (n=766)             | 87.8 (86.5, 89.1)            | Ref.                          |                            |
| Model 2      | 4G/4G                     | 90.5 (85.8, 95.5)            | 0.1030                        | 0.2640                     |
|              | 4G/5G                     | 86.8 (85.1, 88.4)            | 0.8175                        |                            |
|              | 5G/5G                     | 86.5 (85.6, 87.5)            | Ref.                          |                            |
| Model 3      | 4G/4G                     | 90.4 (85.8, 95.2)            | 0.1095                        | 0.2764                     |
|              | 4G/5G                     | 86.8 (85.1, 88.5)            | 0.7993                        |                            |
|              | 5G/5G                     | 86.5 (85.5, 87.6)            | Ref.                          |                            |

**Notes:** model 1: age, rural/urban status and sex adjusted; model 2 is model 1 supplementary adjusted for high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides, C-reactive protein,  $\gamma$ -glutamyl transferase, HIV status and anti-hypertensive medication use; model 3 is model 2 supplementary adjusted for waist circumference. All analyses conducted in women were supplementary adjusted for contraceptive use.  $p_{\text{difference}}$  is the p-value comparing outcome LS mean by PAI-1 polymorphism;  $p_{\text{omnibus}}$  is the Type-III test for the overall effect of PAI-1 polymorphism.

# **CHAPTER 6**

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## **Summary of main findings and conclusion**

## 6.1. Introduction

In this chapter, the main findings of the three research articles are summarised and compared with the relevant existing literature and our initial hypotheses (as outlined in Chapter 1). The chapter also includes the strengths and limitations of our study, recommendations for future research and final conclusions.

## 6.2. Main findings and comparison with the relevant literature

This study aimed to elucidate the associations of blood pressure and retinal microvascular function with PAI-1 in Black South Africans, and to investigate the influences of selected modifiable and non-modifiable factors on these associations. Certain findings from our study confirm results from previous studies, whereas numerous novel findings contribute to the existing literature. The main findings of the three research articles are summarised below.

### 6.2.1. *Article 1: Retinal vasodilatory responses are inversely associated with plasminogen activator inhibitor-1: The African-PREDICT study (Chapter 3)*

Aim and hypotheses: We investigated whether retinal vasodilatory responses to flicker light provocation (a measure of microvascular function) are associated with plasma PAI-1 in young (aged 20–30 years) and healthy Black and White women and men. We hypothesised that maximal retinal arteriolar and venular dilation in response to flicker light would associate negatively with PAI-1. We furthermore hypothesised that the associations would likely differ in men and women and that it would be ethnic-specific.

Findings: Upon adjustment for multiple potential confounders, we found an independent negative association of maximal retinal venular dilation with PAI-1<sub>act</sub> in the total group. Upon exploratory analysis, this association remained in certain subgroups, i.e. White women, those of younger age, those with lower blood pressure, greater central adiposity, higher low-density lipoprotein cholesterol and inflammation, and in non-smokers. A weak independent negative association of maximal retinal arteriolar dilation with PAI-1<sub>act</sub> was also found in White women.

Comparison with previous studies: To our knowledge this is the first study to investigate the associations of retinal vessel function with PAI-1. Two previous studies, conducted in patients with hypertension (1) and normoglycemic individuals with and without a family history of diabetes (2), found PAI-1 to be associated with impaired coronary (1) and cutaneous microvascular function (2), which is in line with our findings in the retinal microcirculation. In exploratory subgroup analyses, the negative association of maximal

retinal venular dilation with PAI-1<sub>act</sub> persisted in White women only, and a weak negative association of maximal retinal arteriolar dilation with PAI-1<sub>act</sub> was found in the same group. Given the strong negative maximal retinal venular dilation-PAI-1<sub>act</sub> association in the total group (n=518), we speculate that this association may also be present in White men and Black individuals, but that statistical significance was likely affected by the small sample sizes upon stratification. However, differences in retinal microvascular function between men and women (3) and between different ethnicities (4) have previously been demonstrated, supporting the possibility that our observed associations may be sex- and ethnic-specific.

Acceptance/rejection of hypotheses: Our initial hypotheses are accepted, since maximal retinal venular and arteriolar dilation associated negatively with PAI-1<sub>act</sub>, although the latter association was weak and in White women only. The negative associations found in White women only upon subgroup analysis is in agreement with our second hypothesis, i.e. that the associations will likely differ between women and men and will be ethnic-specific.

#### *6.2.2. Article 2: The association of PAI-1 with 24 h blood pressure in young healthy adults is influenced by smoking and alcohol use: The African-PREDICT study (Chapter 4)*

Aim and hypotheses: We investigated whether an association between 24 h ambulatory blood pressure and PAI-1 already exists in young (aged 20–30 years) Black and White women and men in the absence of overt hypertension, and whether this association is influenced by smoking and alcohol consumption. We hypothesised that 24 h blood pressure would associate positively with PAI-1, and that the association would be influenced by smoking and alcohol consumption. We furthermore hypothesised that the association would likely differ in men and women and that it would be ethnic-specific.

Findings: In age-adjusted regression analyses stratified by ethnicity and sex, we found positive associations of 24 h blood pressure with PAI-1<sub>act</sub> in most groups. After multivariate adjustment, positive associations of 24 h blood pressure with PAI-1<sub>act</sub> persisted only in Black men who smoked and consumed alcohol.

Comparison with previous studies: To our knowledge, no previous studies have investigated the association of 24 h ambulatory blood pressure with PAI-1 in young and healthy individuals. Our findings do, however, confirm previous findings of office brachial and central blood pressure being positively associated with PAI-1 (5-11). The independent 24 h blood pressure-PAI-1<sub>act</sub> association was found in Black men only, who are known generally to display less favourable cardiovascular profiles compared with their White counterparts (12-17). The Black men in our study also had higher monocyte chemoattractant protein-1 (MCP-1) and von Willebrand factor antigen (vWF<sub>ag</sub>) compared with their White counterparts,

reflecting a degree of endothelial dysfunction and vascular inflammation (18, 19). Their unique cardiovascular profile, in addition to known sex differences in blood pressure regulation (20) and PAI-1 biology (21, 22), may explain our findings in this subgroup only. Our finding that 24 h blood pressure associated with PAI-1<sub>act</sub> only in Black men who smoked and consumed alcohol relates to a previous study conducted in Japanese hypertensive patients (23). This study showed that both smoking and high plasma PAI-1 antigen (PAI-1<sub>ag</sub>) increased the risk of cardiovascular events, and more importantly, that the combination of smoking and high PAI-1<sub>ag</sub> resulted in an even greater risk (23).

Acceptance/rejection of hypotheses: The hypotheses formulated for this research article are accepted. As hypothesised, 24 h blood pressure associated positively with PAI-1, and this association differed between women and men and was also ethnic-specific (the independent association was found in Black men only). Furthermore, smoking and alcohol consumption mediated this association, since the positive association between 24 h blood pressure and PAI-1<sub>act</sub> was found only in Black men who smoked and consumed alcohol.

### *6.2.3. Article 3: Plasminogen activator inhibitor-1 activity and the 4G/5G polymorphism are prospectively associated with blood pressure and hypertension status (Chapter 5)*

Aim and hypotheses: We investigated the associations of blood pressure and hypertension status with PAI-1 and the 4G/5G polymorphism in prospective analysis over a 10-year period in an older cohort (aged 30 years and older at baseline) of Black South Africans. The influence of central obesity on the investigated associations was also determined. We hypothesised that brachial and central blood pressure would be positively associated with PAI-1, and that elevations in PAI-1 would precede elevations in blood pressure and would increase the odds of developing hypertension in prospective analysis. Furthermore, we hypothesised that blood pressure and hypertension status would be associated with the 4G/5G polymorphism, with the 4G/4G genotype being associated with higher blood pressure and increased odds of developing hypertension. We also hypothesised that the associations of blood pressure with PAI-1 and the 4G/5G polymorphism would be influenced by central obesity.

Findings and comparison with previous studies: We found positive associations of brachial and central blood pressure with PAI-1<sub>act</sub> at most time points in cross-sectional analysis, confirming results from previous studies (5-7, 10). In multivariate analysis, PAI-1<sub>act</sub> associated independently and prospectively with brachial SBP and PP in the total group, and with central PP in women. In the total group, higher PAI-1<sub>act</sub> was also associated with increased odds of developing hypertension. This is in agreement with the currently limited

prospective data on these associations (8, 9, 11) and supports the notion that elevated PAI-1 may contribute to hypertension development rather than solely being a consequence thereof. We found that blood pressure and hypertension status were associated with the 4G/5G polymorphism, with 4G/4G (vs 5G/5G) carriers presenting with higher blood pressure (especially central SBP) and increased odds of developing hypertension in the total group, which is in agreement with reports from previous studies (22, 24). In our study, the contribution of the 4G/4G genotype to hypertension development was partially mediated by increased PAI-1<sub>act</sub> levels. The associations of blood pressure and hypertension status with PAI-1<sub>act</sub> were mediated, at least in part, by central obesity, since the addition of waist circumference to statistical models either decreased or nullified the significance of the associations. This finding was not unexpected given obesity's known link to both PAI-1 (25) and hypertension (26), and adipose tissue, especially visceral adipose tissue, being a major source of PAI-1 production (25). The contribution of the 4G/4G genotype to hypertension development was, however, not diminished by the presence of central obesity.

Acceptance/rejection of hypotheses: Our initial hypotheses are accepted, since both brachial and central blood pressure associated positively with PAI-1. Furthermore, PAI-1 was prospectively associated with blood pressure and hypertension status, i.e. elevations in PAI-1 preceded elevations in blood pressure and increased the odds of developing hypertension. Both blood pressure and hypertension status were associated with the 4G/5G polymorphism, with 4G/4G carriers presenting with higher blood pressure and increased odds of developing hypertension, which is also in agreement with our initial hypotheses. As hypothesised, the presence of central obesity influenced (diminished or nullified) the associations of blood pressure and hypertension status with PAI-1. Although the presence of central obesity also influenced the associations of the 4G/5G polymorphism with blood pressure and hypertension status, it did not affect its statistical significance.

### 6.3. Summary of main findings and overall contribution of this PhD study

Our study, aimed at elucidating the associations of retinal microvascular function and blood pressure with PAI-1 in Black South Africans across the life course, confirmed findings from previous studies in addition to introducing numerous novel findings (summarised in **Figure 1**).

*PAI-1 and its association with the microvasculature:* The inverse association of maximal retinal venular dilation in response to flicker light with PAI-1 in young healthy adults suggests that even at an early stage of an individual's life, PAI-1 is already associated with subclinical microvascular dysfunction.

*PAI-1 and its association with blood pressure:* Apart from its association with microvascular function, we found PAI-1 to be positively associated with blood pressure (24 h, brachial and central) in cross-sectional analysis in both young and older adults. PAI-1 was also prospectively associated with blood pressure, with elevations in PAI-1 preceding elevations in blood pressure and also increasing the odds of developing hypertension. This adds to the currently limited data on the controversial direction of the PAI-1–blood pressure association, suggesting that PAI-1 may contribute to hypertension development rather than solely being a consequence thereof. On a genetic level, the *SERPINE1* 4G/4G (vs the 5G/5G) genotype was also associated with higher blood pressure and increased odds of developing hypertension, the latter being partly mediated by increased PAI-1 levels.

*Sex and ethnic differences:* The associations of retinal microvascular function and blood pressure with PAI-1 differed between women and men and also between Black and White individuals.

*PAI-1 and the importance of lifestyle risk factors:* Modifiable lifestyle factors such as central obesity, smoking and alcohol consumption mediated the associations of blood pressure with PAI-1. The inverse association of maximal retinal venular dilation with PAI-1 was also more prominent in the presence of greater central adiposity, higher low-density lipoprotein cholesterol and inflammation. This highlights the significance of these lifestyle risk factors in modifying PAI-1 levels as well as their association with microvascular function and blood pressure. Interventions aimed at reducing these lifestyle risk factors, some of which currently present a major health concern according to the South African Demographic and Health Survey (27), may lead to a reduction in PAI-1 levels and consequently a decrease in the accompanying cardiovascular risk.

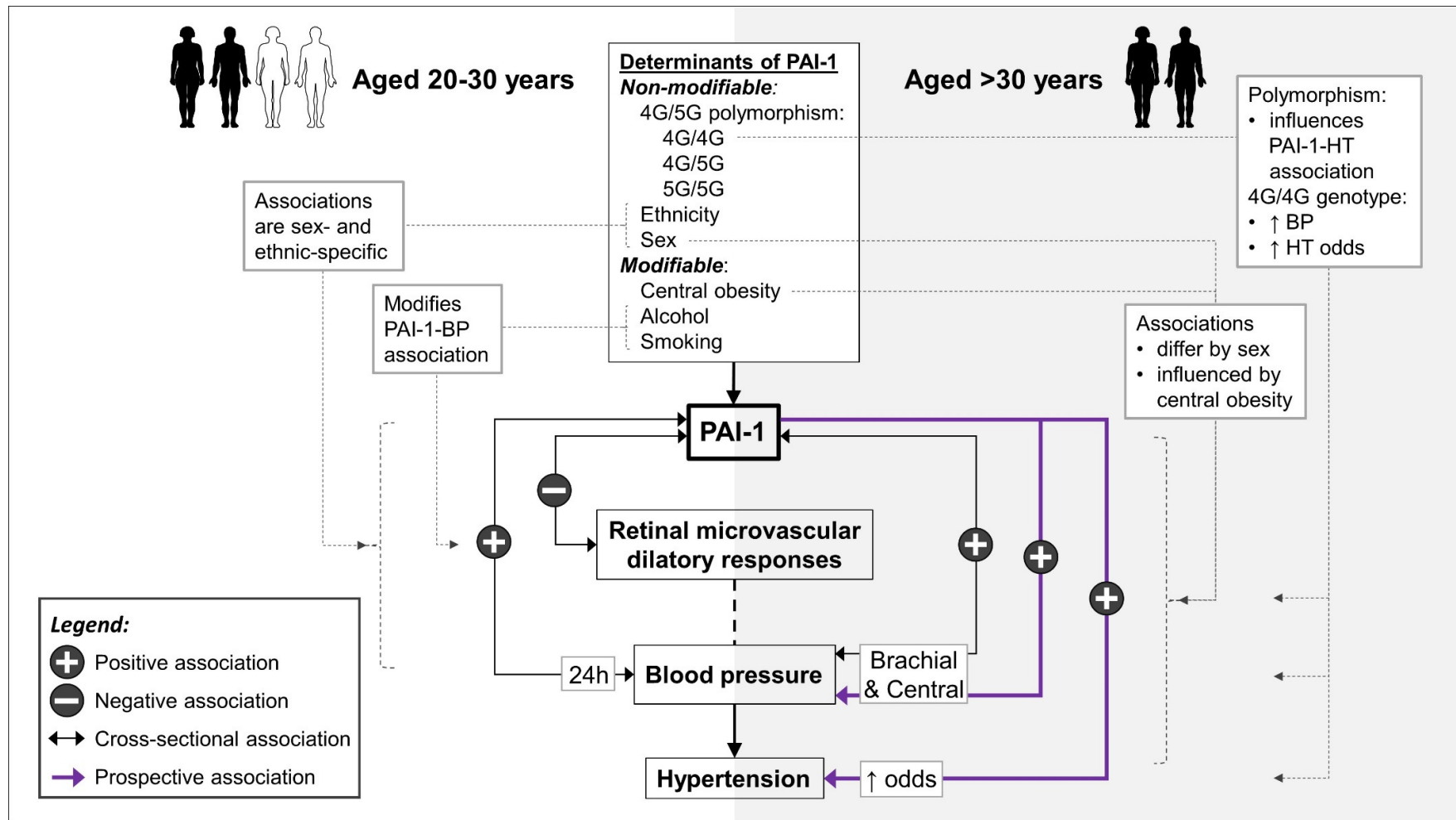


**Figure 1.** Summary of the main findings of this PhD study as investigated in the African-PREDICT and PURE-SA study cohorts (individuals aged 20–30 years, and older than 30 years, respectively)

#### 6.4. Strengths and limitations of this study

The findings from our study should be interpreted within the context of its strengths and limitations. To our knowledge, this is the first study to investigate the association of retinal microvascular function with PAI-1, as well as the association of 24 h blood pressure with PAI-1 in young healthy individuals in the absence of overt hypertension. The blood pressure–PAI-1 association was also investigated in a cohort of older Black South Africans. Thus, major strengths of this PhD study include the investigation of the associations of PAI-1 with both micro- and macrovascular measures across the life course and in different stages of hypertension development (ranging from young healthy individuals to older individuals already presenting with hypertension). Further strengths include the use of genetic data to investigate the association of the 4G/5G polymorphism with blood pressure and hypertension status, which has not previously been investigated in South Africans. This thesis furthermore investigated the association of PAI-1 not only with conventional brachial blood pressure measures, but also with 24 h ambulatory and central blood pressure.

Although several novel cross-sectional and prospective associations of PAI-1 with microvascular function and blood pressure were found, the study design does not lend itself to causality being established. Although potential confounders were taken into consideration in multivariate analyses, the possibility of residual confounding cannot be excluded (e.g. dietary data not being taken into account). Data on smoking, alcohol consumption and hormonal contraceptive use were obtained using questionnaires, and the reliability of these data is dependent on the honesty with which these were reported by participants (whose reports may not always be a true reflection of their lifestyle habits). Lastly, participants of the African-PREDICT study were not randomly selected and inclusion was based on certain eligibility criteria, i.e. aged 20–30 years and being apparently healthy. Furthermore, although participants of the PURE-SA study were randomly selected, this was performed within selected rural and urban communities in South Africa. Hence, the findings from our study cannot necessarily be extrapolated to the larger population owing to possible ethnic, lifestyle and environmental differences.

##### *Strengths and limitations of the African-PREDICT study:*

The African-PREDICT study, in which some of our research questions were addressed, has numerous strengths. These include being a well phenotyped cohort with relatively large sample size, its bi-ethnic design, providing an opportunity for comparison of the investigated associations between Black and White individuals, and the inclusion of young (aged 20–30 years) apparently healthy individuals, presenting the ideal study population for studies investigating the early stages of hypertension development. Furthermore, rigorous measurement methods and sophisticated techniques were used for data collection under

highly controlled conditions. Limitations include data on the 4G/5G polymorphism not being available, and data on PAI-1, microvascular function and blood pressure currently being available only for cross-sectional analysis (there is, however, potential for future prospective studies once follow-up data have been collected). Retinal microvascular function data were available for only approximately half of the African-PREDICT study's participants and, although this still greatly exceeds the size of previous studies that investigated the association of microvascular function with PAI-1, full retinal microvascular data on all African-PREDICT participants would have increased the study's statistical power.

*Strengths and limitations of the PURE-SA study:*

Our other research questions were addressed using data from the South African arm of the PURE study. A major strength of this study, apart from its large sample size, is its prospective design, with data being available for three time points over a period of 10 years. Limitations of this study include the large loss to follow-up (attrition rate of  $n=1084$  over the 10-year period), central blood pressure measures being available for one timepoint only, and the small sample size of the 4G/4G genotype group, which may have induced potential type II errors. One also needs to take into consideration that much of the study's data collection took place in rural areas where temporary field research units had to be set up. Hence, data acquisition using very sensitive, normally stationary, instrumentation (e.g. a retinal vessel analyser for assessment of the retinal microvasculature) was not feasible.

## **6.5. Recommendations and perspectives for future studies**

- Although we investigated the prospective association of PAI-1 with blood pressure and hypertension status, longitudinal data on this association are still very limited (8, 9, 11) and investigations in the African-PREDICT study follow-up cohort and other study populations are encouraged.
- Studies such as these may provide insight into the controversial direction of the association of PAI-1 with blood pressure, although causality cannot be established. Experimental studies on this association are limited (28, 29) and hence additional experimental studies (by using e.g. PAI-1 inhibitors or knockout animal models) are recommended. Furthermore, Mendelian randomisation can be used to infer causality in observational studies (30) and hence studies making use of this approach are recommended to elucidate the potential causal role of PAI-1 in hypertension development.

- We investigated the prospective associations of PAI-1 with brachial blood pressure and hypertension status (based on brachial blood pressure measures) over a period of 10 years. However, central blood pressure measures were available for one time point only. The investigation of the prospective association of PAI-1 with central blood pressure is encouraged, since central blood pressure has also been regarded as a strong predictor of cardiovascular risk (31).
- It has been proposed that microvascular dysfunction not only accompanies, but also possibly precedes, macrovascular dysfunction (32), yet limited studies are available on the association of microvascular function with PAI-1 (1, 2). Hence, studies on PAI-1's association with both micro- and macrovascular structure and function across the entire life course are encouraged. Furthermore, studies investigating PAI-1's predictive role in cardiovascular events (e.g. stroke) and outcome in African populations are recommended.
- A potential limitation of our study is that dietary data were not included in statistical models and inclusion thereof is recommended for future research, since dietary factors have been shown to influence PAI-1 levels (33, 34).
- We speculated that PAI-1's inhibitory action on tissue-type plasminogen activator (t-PA) may explain the inverse association of maximal retinal vasodilatory responses with PAI-1, since t-PA has been shown to promote the vasodilatory response associated with neurovascular coupling (35, 36). Alternatively, this finding could be explained by PAI-1's inhibitory action on endothelial nitric oxide synthase (eNOS) (37), thereby reducing synthesis of the vasodilator nitric oxide. Either way, we conclude that measurement of PAI-1 alone is not sufficient to decipher the potential mechanisms involved, and future studies measuring t-PA and markers of nitric oxide bioavailability in conjunction with PAI-1 are recommended.

## 6.6. Final conclusion

Our data provide evidence that PAI-1 is already independently associated with microvascular function and blood pressure during the early stages of hypertension development, and that it is prospectively associated with blood pressure and hypertension later in life. These associations seem to be sex- and ethnic-specific, and are influenced by lifestyle risk factors such as central obesity, smoking and alcohol consumption and genetic factors such as the 4G/5G polymorphism. Furthermore, the 4G/4G genotype is associated with higher blood pressure and increased hypertension odds, the latter being partly mediated by elevated PAI-1 levels. Whether or not PAI-1 plays a causal role in impaired microvascular function and hypertension development is yet to be established.

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# **APPENDIX A**

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**NWU Health Research Ethics Committee  
approval of the African-PREDICT study**

**(NWU-00001-12-A1)**



NORTH-WEST UNIVERSITY  
YUNIBESITHI YA BOKONE-BOPHIRIMA  
NOORDWES-UNIVERSITEIT

Private Bag X6001, Potchefstroom  
South Africa 2520

Tel: (018) 299-4900  
Faks: (018) 299-4910  
Web: <http://www.nwu.ac.za>

Prof A Schutte

**Ethics Committee**  
Tel +27 18 299 4850  
Fax +27 18 293 5329  
Email [Ethics@nwu.ac.za](mailto:Ethics@nwu.ac.za)

2012/07/31

## ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

|                                                                                                                                                                 |             |   |   |   |                |   |   |   |   |                         |   |        |   |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|---|---|---|----------------|---|---|---|---|-------------------------|---|--------|---|-----|
| <b>Project title :</b> African Prospective study for the Early Detection and Identification of Cardiovascular disease and hyperTension. (African-PREDICT study) |             |   |   |   |                |   |   |   |   |                         |   |        |   |     |
| <b>Project Leader:</b> Prof A Schutte                                                                                                                           |             |   |   |   |                |   |   |   |   |                         |   |        |   |     |
| <b>Ethics number:</b>                                                                                                                                           | N           | W | U | - | 0              | 0 | 0 | 0 | 1 | -                       | 1 | 2      | - | A 1 |
|                                                                                                                                                                 | Institution |   |   |   | Project Number |   |   |   |   | Year                    |   | Status |   |     |
| Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation                                                                     |             |   |   |   |                |   |   |   |   |                         |   |        |   |     |
| Approval date: 2012/04/12                                                                                                                                       |             |   |   |   |                |   |   |   |   | Expiry date: 2017/04/11 |   |        |   |     |

Special conditions of the approval (if any): None

### General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The project leader (principle investigator) must report in the prescribed format to the NWU-EC:
  - annually (or as otherwise requested) on the progress of the project,
  - without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.
- The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviation from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.
- In the interest of ethical responsibility the NWU-EC retains the right to:
  - request access to any information or data at any time during the course or after completion of the project;
  - withdraw or postpone approval if:
    - any unethical principles or practices of the project are revealed or suspected,
    - it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,
    - the required annual report and reporting of adverse events was not done timely and accurately,
    - new institutional rules, national legislation or international conventions deem it necessary.

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof Amanda Lourens  
(chair NWU Ethics Committee)



Prof A Schutte  
HART

Private Bag X6001, Potchefstroom  
South Africa 2520

Tel: +2718 299-1111/2222  
Web: <http://www.nwu.ac.za>

**Faculty of Health Sciences Ethics  
Office for Research, Training  
and Support**

Tel: 018 299 2092  
Email: [minrie.greeff@nwu.ac.za](mailto:minrie.greeff@nwu.ac.za)

30 September 2019

Dear Prof Schutte

## FEEDBACK ON NWU-HREC ANNUAL MONITORING REPORT: NWU-00001-12-A1

We would like to thank you for submitting the annual monitoring report for your project entitled, "*African-PREDICT study (African Prospective study for the Early Detection and Identification of Cardiovascular disease and hypertension)*", to the North-West University Health Research Ethics Committee (NWU-HREC) in a timely manner. Please find below the decision of the NWU-HREC regarding the continuation of your project.

| Classification                       | Mark with<br>X | Comment                         |               |
|--------------------------------------|----------------|---------------------------------|---------------|
| <i>Clarification</i>                 |                |                                 |               |
| <i>Completion<br/>(Final report)</i> |                |                                 |               |
| <i>Suspended</i>                     |                |                                 |               |
| <i>Continuation</i>                  | X              | Date of next monitoring report: | 31 March 2020 |
| <i>Termination</i>                   |                |                                 |               |

Should you have any further queries, please feel free to contact Mr Buti Majola at your earliest convenience (E-mail: [Ethics-HRECMonitoring@nwu.ac.za](mailto:Ethics-HRECMonitoring@nwu.ac.za); Tel: 018 299 2266). We wish you well in your future endeavours.

Yours sincerely

Digitally signed by Wayne  
Towers  
Date: 2019.10.16  
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**Prof Wayne Towers**  
Chairperson: NWU-HREC

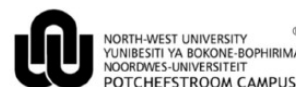
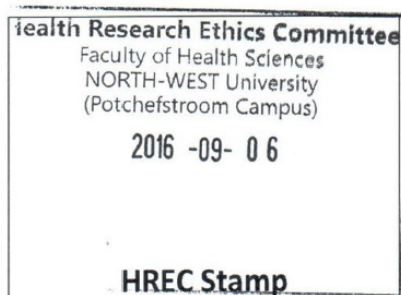
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Greeff  
Date: 2019.10.17  
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**Prof Minrie Greeff**  
Head of Health Sciences Ethics  
Office for Research, Training and  
Support

# **APPENDIX B**

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**African-PREDICT study informed consent form**



## INFORMED CONSENT FORM FOR THE African-PREDICT STUDY (RESEARCH PHASE):

**TITLE OF THE RESEARCH PROJECT:** African PRospective study on the Early Detection and Identification of Cardiovascular disease and HyperTension (African-PREDICT)

**ETHICS REFERENCE NUMBER:** NWU-00001-12-A1

**PRINCIPAL INVESTIGATOR:** Prof. Alta Schutte (PhD Physiology)

*Prof. Schutte and the research team have the expertise and interest in Cardiovascular Physiology, namely to understand the biological processes in humans when high blood pressure and heart disease develop.*

**ADDRESS:** NORTH-WEST UNIVERSITY (Potchefstroom Campus); Hypertension in Africa Research Team (HART); Hypertension Research and Training Clinic Building F11, Office 101.

**CONTACT NUMBERS:** 018 299 2444 / 018 285 2466 / 018 299 2780

You are invited to take part in the African-PREDICT research study. Please take some time to read the information presented here, which will explain the details of this study. Please ask the researcher or person explaining the research to you any questions about any part of this study that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research is about and how you might be involved. Also, your participation is **entirely voluntary** and you are free to say no to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part now.

This study has been approved by the Health Research Ethics Committee of the Faculty of Health Sciences of the North-West University (NWU-00001-12-A1) and will be conducted according to the ethical guidelines and principles of Ethics in Health Research: Principles, Processes and Structures (DoH, 2015) and other international ethical guidelines applicable to this study. It might be necessary for the research ethics committee members or other relevant people to inspect the research records.



### What is this research study all about?

You will know already from taking part in the screening phase of the study that heart disease and especially high blood pressure (or hypertension) is a big problem in South Africa. Also, many people are unaware of it, as it has no symptoms. High blood pressure is a very important risk factor which may result in heart disease, kidney disease and stroke. (*When blood stops flowing to the heart, this can cause a heart attack and part of the heart dies. A stroke is when there is a problem with the blood supply to the brain and a part of the brain is damaged.*) That is why many people in South Africa suffer from these diseases resulting in death.

## Informed Consent Form – Research Version Aug 2016

Since heart disease is mostly seen in older people, the purpose of this study is to include and focus on young healthy people to understand how high blood pressure and heart disease develop. It is believed that our lifestyle (e.g. what we eat, drink, and do) may have an impact on whether we will develop high blood pressure and heart disease. Also, it is not well known whether there are perhaps certain measurements (e.g. in your blood or urine) that may predict whether you will develop heart disease when you are older.

The aim of this study is therefore to determine how high blood pressure and heart disease develop in a group of 1200 healthy young South Africans living in and around Potchefstroom, by tracking everyone over 5-20 years. It is therefore of great importance that we take detailed measurements of your lifestyle, and your current health (e.g. heart, blood vessels, eyes, blood and urine). These measurements will be made at the beginning of the study, but it will be most important to repeat these measurements in following visits every 5 years, to see how these health measurements have changed. We expect that some participants will remain healthy with normal blood pressures, and other will develop high blood pressure. Only by tracking the changes in blood pressures and other detailed measurements will be able to understand the influences of e.g. lifestyle on changes in blood pressure.

If your results show that a certain measurement predicts that high blood pressure will develop later in life, this information could help doctors and nurses to prevent more people in the local community having strokes and heart attacks in the future.



#### Why have you been invited to participate?

Your screening tests show that you are healthy and suitable to take part in this study. You are also in the most important age group of 20 to 30 years. As we would like to follow you over time it is ideal that you have indicated that you intend to stay in or around or visit Potchefstroom for the next 5 years at least.

It will also be very important for us to be able to keep in touch with you. We kindly ask that you **tell us immediately about any changes of your contact details** (address, telephone number, email address etc.).

Once we have performed all of the measurements as described below, we will have a much better understanding of your health status. If we are not able to obtain important measurements (such as 24-hour blood pressure measurements, or if we are unable to obtain a blood sample, or if we detect a serious health abnormality), you will most likely not be able to take further part in the research project. Once we have completed the measurements, we will discuss your results with you and the way forward.



#### What will be expected of you?

The research team will make an appointment with you, and if necessary, transport will be provided to bring you to the Hypertension Clinic (Building F12) on the Potchefstroom Campus of the North-West University. Such an appointment will be made for early in the morning, as the measurements will start at approximately 08:00, and in total will take about 5 hours to complete.

To make sure that your results are valid and useful, it is important to take note of the following:

1. The **evening before** – Do not eat or drink anything except water after 10pm or before you come to the clinic in the morning.
2. **On the study day**, please wear comfortable clothing such as trousers and a top that can be easily removed for the tests (please avoid wearing skirts, dresses or tights as we will need to access your bare foot and put a blood pressure cuff around your thigh over your trousers).
3. **Please bring with you:**
  - All medication you currently are taking
  - Your ID document & clinic card/book

## Informed Consent Form – Research Version Aug 2016

- Some good quality sunglasses to protect your eyes after the measurements
4. Let us know if transport should be arranged for you.

If you are happy to participate, we will ask you to sign this consent form stating that you are volunteering to participate in this study and that you understand all the procedures that will be performed. You are free to contact us with any questions should there be any uncertainty about any of the information provided. Then we will take the measures listed in the table below. Tests will be done in the Hypertension Clinic and we will provide you with a meal during the day. You will not be able to bath or shower for 24 hours after your clinic appointment due to the equipment you will be wearing when you leave the clinic.

**WHAT TESTS WILL BE DONE?**

- **Body composition:** we will measure your height, weight, waist, hip and neck circumference in a private room, while you are wearing your underwear. In another room, while you are clothed and lying down on a bed, we will also measure your body fat percentage by using a device that connects with sensors on your hand and on your foot. This is a completely painless procedure. (the measurements should take about 20 minutes to complete)
- **Biological samples:** early in the morning while you are lying down on a bed, a research nurse will take a blood sample from a vein in your arm by using standard clinical procedures. (10-20 min) We will also ask you to provide a urine sample in the morning, in a private restroom. At the end of the day, we will kindly request that you collect your urine over the next 24 hours (we will give you the containers and detailed instructions for this). These urine and blood samples will be used to test for genetic and a detailed range of biochemical markers (biomarkers) related to high blood pressure, heart disease and diabetes, such as glucose, cholesterol and markers of inflammation. You are more likely to have high blood pressure if one of your parents or a close family member has high blood pressure. This is because high blood pressure can be caused by differences in our genes. Our genes are like a very complicated "manual" in each of our cells that tells the body how to work properly. When there are changes in the genes, it changes the "manual" and the body then does not work as well as it should for example causing high blood pressure. We share our genes with our family because half of the gene "manual" comes from your mother and half from your father. Therefore, if they have high blood pressure due to differences in their genes then it is likely that you will get the same changes in your genes and develop high blood pressure. We would like to find out what these differences are in order to better understand how they cause high blood pressure so that we can find ways to stop it happening. Take note that some of your samples may be stored for many years in freezers before we will analyse the samples. We may also need to ship some of your to other local or international expert laboratories for analyses.
- **Blood pressure:** while you are sitting down in a private room, we will measure blood pressure twice on both arms, by placing a cuff around your upper arm. (20 min) Another blood pressure measurement will also be done by placing a small blood pressure cuff around your finger, and upper arm, while you are lying on a bed. We will then test your blood pressure responses when you do a colour word reading test and when you place your hand in cold water for 1 minute. (30 min) At the end of the measurement day, we will fit a portable blood pressure monitor to you which will assess your blood pressure over the next 24 hours, thus over a day and when you are sleeping at night. It is important that the device is not removed during this time to ensure a reliable measurement.
- **Blood vessel & heart health:** in a private room we will again ask you to lie down comfortably on a bed. We will first test your blood pressure at your upper arm, with a device that will also measure the blood pressure at your heart. We will then test how stiff your blood vessels are by using a small pen-like device rested on your neck to register the pulse in your neck on a computer. At the same time another blood pressure cuff will be placed around your thigh. (15 min) Afterwards, in a

## Informed Consent Form – Research Version Aug 2016

semi-dark room we will use a sonar device (usually used during pregnancy) to take some sonar pictures and video clips of the blood vessels in your neck and of your heart on the bare chest. We will provide a blanket or gown for cover. (20 min)

- **ECG (Electrocardiography test) for heart health:** while you are lying down on a bed in a private room, we will test the natural electrical activity of your heart by placing several stickers with sensors on your chest. We will take care to ensure your privacy. (10 min)
- **Eye Pressure:** a research nurse will put some eye drops in both eyes and then she will measure the pressure in your eyes with a device that rests lightly on your eye. (10 min) This test will inform us whether you have a condition called glaucoma, which means that the pressure within your eyes are quite high. If so, we will advise you and refer you for necessary treatment. If the pressure is normal, we will continue with the next eye test as described below.
- **Testing the small vessels of the eye:** a research nurse will put an eye drop in one eye, and a researcher will ask you to look into a special camera, named a fundoscope. This is the same device used by ophthalmologists (eye doctors). This camera will shine a light into your eye and we will take some pictures of the small blood vessels at the back of your eye (there will be a camera-like flash). We will also check how well your small blood vessels respond to light flickering, by doing a light-flicker test with this special camera. (20-30 min)
- **Physical activity:** at the end of the measurement day, a researcher will place a small monitor on your chest that will record your activity and movement levels for 7 days. No pain or discomfort is associated with this device, and you are kindly requested not to remove the device before the 7 day measurements were completed.
- **HIV test:** As this test was done during the screening phase, we will not test again for HIV. However, with each follow-up visit every 5 years, we would like to perform this test again.
- **Questionnaires:** during the course of the morning, you will be asked to complete several questionnaires with the help of a researcher. These include a general health questionnaire (with questions about your age, family history of disease, education, occupation, lifestyle habits, 15 min), Berlin sleep questionnaire (asking questions about how well you sleep, 5 min), physical activity questionnaire (to report on how active your lifestyle is, 5 min), dietary questionnaire (with the help of a dietician you will be asked what you ate during the past day (30 min). Within the next week the dietician will contact you again on two occasions to complete the questionnaire again. This should give us the best reflection on your eating habits). Finally, a trained psychologist will help you to complete a number of questionnaires on your personal well-being (including questions on stress and how well you cope with stress, 30-45 min).



#### Will you gain anything from taking part in this research?

- You will receive direct feedback during each advanced measurement on your health status. All of these advanced clinic tests are provided to you at no cost (worth ±R3 000).
- Should any abnormalities be detected, we will refer you to doctors, clinics or hospitals for further tests or treatment and the test results may assist your doctor in making decisions about further treatment.
- Apart from this personal benefit, your research data will help biomedical health researchers to gain a better understanding on how high blood pressure and heart disease develops, and may help us to develop better programmes to prevent or treat these diseases in our community and elsewhere. The data may also be used to advise the Ministry of Health on changes to the health system that may benefit the broader South Africa.



#### Are there risks involved in you taking part in this research and what will be done to prevent them?

To help you with a better understanding of the potential risks, and what we are doing to prevent these, please refer to the table below:

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| Risks                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | Precautions                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"> <li>• Taking a blood sample at a vein in the upper arm, may cause some pain and discomfort;</li> <li>• Applying an eye drop may cause a slight burning sensation;</li> <li>• Performing the eye pressure test is slightly uncomfortable;</li> <li>• Performing a light flicker test may also be slightly uncomfortable.</li> <li>• After the eye measurement some discomfort may be experienced (similar to a visit to an eye doctor) while waiting for the pupil to constrict.</li> <li>• Placing the hand in an ice water bucket for 1 minute may cause some pain in your hand.</li> <li>• You may experience some discomfort when having to undress for the body measurements or heart sonar measurements.</li> <li>• When you complete the psychological questionnaires you may feel uncomfortable when giving personal information, such as feeling depressed or stressed.</li> <li>• All health measurements may cause some anxiety when you are worried about the results of the tests.</li> <li>• If a health abnormality is identified, others may become aware of this private information, e.g. diabetes.</li> </ul> | <ul style="list-style-type: none"> <li>• A trained registered research nurse perform all blood sampling and regularly undergo training on clinical measurements.</li> <li>• She also performs the eye pressure test and apply the eye drop. To ensure correct procedures and minimum participant discomfort she has undergone training at an eye doctor to ensure that she use the safest techniques to make the measurement quickly and correctly. The light flicker test may cause discomfort but the researcher is highly experienced and ensures that the measurement is done quickly and accurately. It does not cause any long term harm and is comparable to standard eye doctor measures. Afterwards, when the pupil is dilated, the eye is sensitive to light. Therefore an eye patch is provided and all lights of the clinic turned off when these assessments start (at the end of the day's measurements). You are also encouraged to bring sunglasses for when you leave the clinic. We also provide transport to you after we are finished as you are not encouraged to drive if your eye has not yet returned to normal.</li> <li>• Placing the hand in ice water causes some pain due to the very cold water. The time is only for 1 minute to reduce discomfort to a minimum, and a small electric blanket or hot water bottle is provided afterwards to heat up the hand and ensure comfort.</li> <li>• All measurements are done in private temperature controlled rooms. For sensitive measurements a female scientist is trained to perform measurements to ensure especially comfort of female participants. All staff are also trained in these aspects to be highly professional and discreet and to ensure maximum comfort and to avoid any embarrassment. For heart sonars, an expert clinical technologist has vast experience in performing the sonars in a semi-dark room and also provides a blanket should you require this.</li> <li>• For psychological questionnaires a psychologist is well trained to complete the questionnaires in a private area. All necessary aspects are adhered to to make sure it is done in a professional and comfortable manner. If any abnormality is detected, the psychologist informs the research nurse, who will then privately discuss the results with you.</li> <li>• For other health measurements, such blood pressure, the results may be stressful. We will therefore provide you with the information privately and if we note something abnormal, we will ensure that you are referred appropriately for further tests or treatment.</li> <li>• If any health abnormalities was identified, you will meet individually with the research nurse in a private room for a feedback session. She will explain your results to you and provide you with a letter of referral for further testing or treatment. This will also be placed in a</li> </ul> |

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|                                                                                                                                                                                                                            |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"> <li>As measurements take place during the working week you may suffer from a loss of income, or may get into trouble for not being at work due to time spent in the project.</li> </ul> | <p>sealed envelope.</p> <ul style="list-style-type: none"> <li>If you will lose wages due to your participation in the study, you need to inform the research nurse, who will make sure that communication is taken up with your employer. We will normally discuss your participation with your employer beforehand to make sure there won't be any loss in income. Once your employer agrees that you can attend the study during normal working hours without having to take leave or lose any wages, you can join the study.</li> </ul> |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

There are more gains for you in joining this study than there are risks.



#### How will we protect your confidentiality and who will see your findings?

Anonymity of your findings will be protected by all of the researchers involved. A number, and not your name, will be assigned to your research results, and all scientists using your data will only note this number, and not your name. Your privacy will be respected by making sure that all the measurements are taken in private rooms and performed by well-trained scientists. Your results will be kept confidential by storing hard copies of your documentation in a locked cupboard within the Hypertension Clinic, and only the Principal Investigator, Head of the Hypertension Clinic and Data Manager having direct access. Electronic files with data are stored and handled by the Data Manager in a password protected online database using the University web-network (with firewall and security features), as well as some backup files on external password protected harddrives. Only the researchers, their postgraduate students and local and international collaborators will be able to look at your findings – however, all findings will be anonymised using your unique participant number. As this is a long term project, your data will be stored for 20 years or longer.



#### What will happen with the findings or samples?

As indicated above, your research results are safely stored on electronic files, with some results on hard copies, and in the form of blood or urine samples in biofreezers. We will store your data and your blood and urine samples for at least 30 years. Over time the research team will make sure that all of this information is analysed in the utmost detail to create new knowledge on how high blood pressure, heart disease, and related diseases develop over time. It is important to store the data and samples for a long period, as new scientific discoveries on markers of high blood pressure will be made by other scientists or ourselves in the future. It will then allow us to test if these markers are also useful in your (the South African) samples, and whether these can be used throughout South Africa in the future.

Some of your biological samples (from urine and blood) will be analysed immediately, but others will be stored for many years before analyses are performed. Please note that we will perform the biochemical analyses in our laboratories on the Potchefstroom Campus. But we may need to ship some of your samples to other laboratories in South Africa or internationally, when we do not have the funds, skills or the equipment to perform the analyses locally. Samples will be shipped using courier services approved for handling biological samples, to ensure the safekeeping and protection of the samples during transit. We will also ensure that the appropriate approvals from the South African Department of Health (export permit) and the Health Research Ethics Committee are obtained prior to shipping the samples.

Apart from your samples, your anonymised data may also be shared with other national or international collaborators. It is therefore possible that your anonymised results will be reported as stand alone data as part of the African-PREDICT study, or your data may be pooled into other datasets from the province, country or

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globally in further research studies on high blood pressure and related health status. Your data will therefore be used to analyse your original state of blood pressure and health – in South Africa and in comparison to other local and international populations – and to analyse how your health status changes over time.

If we were to share your anonymised data or samples with external groups, the external groups will sign confidentiality and data or material transfer agreements with us. This process is overseen by the Legal Services of the North-West University. This will ensure that your information is adequately handled and protected, and that your data is only used for the intended purpose as described in the agreement.

It is also possible that your data may be useful for other purposes apart from the aim of the present study. When the data is to be used for such purposes, new applications will be submitted to the Health Research Ethics Committee, where the Committee will stand in on your behalf.

Findings from the study will be published in scientific journals, and discussed locally and internationally with scientific experts and the Department of Health.



#### **How will you know about the results of this research?**

During the course of the day you will receive direct feedback from each research station on your health status and findings. As described earlier, if any abnormalities are detected, a detailed report within a referral letter will be compiled by the research nurse and you will be directed to the appropriate healthcare provider. If at any stage (also after you have visited the clinic) you wish to know any of your research results, you are welcome to contact the researchers at the Hypertension Clinic.

The research team also intends to publish the research findings of the larger study in scientific literature, but also in local media, and perhaps also national media. This will not include you as an individual, but the collective findings of all the research participants. Furthermore, as this is a longitudinal study, the research team may provide you with further results of the study when you return to the clinic during follow-up measurements. As the research team will contact you annually to ensure that your contact details are still correct, we will inform you if any important research findings became apparent that you need to take note of.



#### **Will you be paid to take part in this study and are there any costs for you?**

No, you will not be paid to take part in the study, but the research team will provide you with a R50 gift voucher as a token of appreciation for your participation. We hope that the results of the measurements will be useful to you to understand your own health status.

We will provide transport to all participants, and a meal will be served during the course of the morning after you have given a blood sample.

There will thus be no costs involved for you, if you do take part in this study.

To cover all of the research expenses, this study is funded by several local and international funding bodies, including the Department of Science and Technology (National Research Foundation), Medical Research Council of South Africa and the Medical Research Council of the United Kingdom, as well as scientific grants from industry (GlaxoSmithKline, Pfizer, Boehringer-Ingelheim, Medi-Clinic Hospital Group).



#### **Note\* What happens after the study day?**

At the end of the study, you may have one eye covered so it is advisable not to drive until you see that your eye has recovered, due to a possible loss of depth perception. You will know that the eye is fully recovered when the black part of the treated eye (pupil) has been reduced to a similar size as the pupil of the untreated eye. In the week following the study day, we will make three short appointments with you to collect the blood

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pressure monitor, your urine collection and the activity monitor and to do two more short interviews (20-30 minutes) about your diet. We will give you a diary sheet so you can keep track of these appointments and they will be arranged to suit your schedule.

**Is there anything else that you should know or do?**

- You can contact Sr. Adele Burger (or Prof. Alta Schutte) at 018 285 2261/2446 if you have any further questions or have any problems.
- You can also contact the Health Research Ethics Committee via Mrs Carolien van Zyl at 018 299 1206 or [carolien.vanzyl@nwu.ac.za](mailto:carolien.vanzyl@nwu.ac.za) if you have any concerns that were not answered about the research or if you have complaints about the research.
- You will receive a copy of this information and consent form for your own purposes.

Address: Building F11, Potchefstroom Campus, North-West University, Potchefstroom 2520  
Tel: 018-285 2261 (Office hours Mon-Fri) Fax: 018-285 2260; Email: [adele.burger@nwu.ac.za](mailto:adele.burger@nwu.ac.za)

## Informed Consent Form – Research Version Aug 2016

**Declaration by participant**

By signing below, I ..... agree to take part in the research study titled: The African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT).

I declare that:

- I have read this information/it was explained to me by a trusted person in a language with which I am fluent and comfortable.
- The research was clearly explained to me.
- I have had a chance to ask questions to both the person getting the consent from me, as well as the researcher and all my questions have been answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be handled in a negative way if I do so.
- I may be asked to leave the study before it has finished, if the researcher feels it is in the best interest, or if I do not follow the study plan, as agreed to.

|                                                                                                                                                                                                         |     |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|----|
| I agree that my blood or urine samples may be sent to laboratories in South Africa or in other countries for analyses (with my personal details removed, and only identifiable by an anonymous number). | Yes | No |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|----|

Signed at (*place*) ..... on (*date*) ..... 20....

\_\_\_\_\_  
Signature of participant

\_\_\_\_\_  
Signature of witness

**Declaration by person obtaining consent**

I (*name*) ..... declare that:

- I clearly and in detail explained the information in this document to .....
- I did not use an interpreter.
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above.
- I gave him/her time to discuss it with others if he/she wished to do so.

Signed at (*place*) ..... on (*date*) ..... 20....

\_\_\_\_\_  
Signature of person obtaining consent

\_\_\_\_\_  
Signature of witness

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Informed Consent Form – Research Version Aug 2016

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**Declaration by researcher**

I, *Aletta E. Schutte*, declare that:

- I explained the information in this document to the Head of the Hypertension Clinic, Head of Screening, and research assistants.
- I did not use an interpreter.
- I encouraged them to ask questions and took adequate time to answer them.

And that I was available should they want to ask any further questions.

- The informed consent was obtained by an independent person.
- I am satisfied that she adequately understands all aspects of the research, as described above.
- I am satisfied that she had time to discuss it with others if she wished to do so.

Signed at (*place*) ..... on (*date*) ..... 20....

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Signature of researcher

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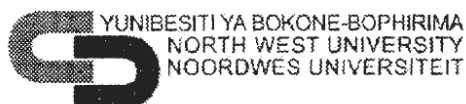
Signature of witness

# **APPENDIX C**

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**NWU Health Research Ethics Committee approval of  
the PURE-SA study for the time period 2005 to 2009**

**(04M10)**

**Etiëkkomitee**

Tel (018) 299 2558  
 Faks (018) 297 5308  
 E-Pos dnveair@puk.ac.za

2 September 2004

Dr A Kruger  
 Bussie 594  
 Noordwes-Universiteit  
 (Potchefstroomkampus)

Geagte dr Kruger

**GOEDKEURING VIR EKSPERIMENTERING MET MENSE**

Hiermee wens ek u in kennis te stel dat u projek getiteld "*PURE study (Prospective Urban and Rural Epidemiology study)*" deur die Etiëkkomitee goedgekeur is met nommer 04M10.

Gebruik asseblief die nommer genoem in paragraaf 1 in alle korrespondensie rakende bogenoemde projek en let daarop dat daar van projekteleers verwag word om jaarliks in Junie aan die Etiëkkomitee verslag te doen insake etiese aspekte van hulle projekte asook van publikasies wat daaruit voortgespruit het. U sal in Mei 2005 die dokumentasie hieroor ontvang.

Goedkeuring van die Etiëkkomitee is vir 'n termyn van hoogstens 5 jaar geldig (volgens Senaatsbesluit van 4 November 1992, art 9.13.2). Vir die voortsetting van projekte na verstryking van hierdie tydperk moet opnuut goedkeuring verkry word.

Die Etiëkkomitee wens u alle voorspoed met u werk toe.

Vriendelike groete

**ESTELLE LE ROUX**  
**NAMENS SEKRETARIAAT**



**POTCHEFSTROOMKAMPUS**  
 Privaatsak X6001, Potchefstroom, Suid-Afrika, 2520  
 Tel: (018) 299-1111 • Faks: (018) 299-2799  
 Internet: <http://www.nwu.ac.za>



# **APPENDIX D**

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**NWU Health Research Ethics Committee  
approval of the PURE-SA study for the time  
period 2010 to 2015 and 2015 to 2020  
(NWU-00016-10-A1)**



NORTH-WEST UNIVERSITY  
YUNIBESITHI YA BOKONE-BOPHIRIMA  
NOORDWES-UNIVERSITEIT

Private Bag X6001, Potchefstroom  
South Africa 2520

Tel: (018) 299-4900  
Faks: (018) 299-4910  
Web: <http://www.nwu.ac.za>

**Ethics Committee**  
Tel: +27 18 299 4850  
Fax: +27 18 293 5329  
Email: [Ethics@nwu.ac.za](mailto:Ethics@nwu.ac.za)

2010-02-23

# ETHICS APPROVAL OF PROJECT

This is to certify that the next project was approved by the NWU Ethics Committee:

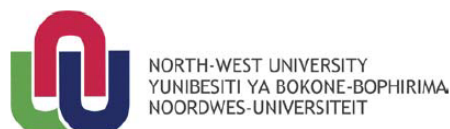
|                                                                                    |                 |
|------------------------------------------------------------------------------------|-----------------|
| Project title : <i>PURE study (Prospective Urban and Rural Epidemiology study)</i> |                 |
| Project leader / Student : Prof Annamarie Kruger                                   |                 |
| Ethics number:                                                                     | NWU-00016-10-A1 |
| Expiry date: 20/01/2015                                                            |                 |

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

The formal Ethics approval certificate will be sent to you as soon as possible.

Yours sincerely

Me. Marietjie Hargryn  
NWU Ethics Secretariate



Private Bag X6001, Potchefstroom  
South Africa 2520

Tel: (018) 299-4900  
Faks: (018) 299-4910  
Web: <http://www.nwu.ac.za>

#### Ethics Committee

Tel +27 18 299 4849  
Email [Ethics@nwu.ac.za](mailto:Ethics@nwu.ac.za)

### ETHICS APPROVAL OF PROJECT

The North-West University Research Ethics Regulatory Committee (NWU-RERC) hereby approves your project as indicated below. This implies that the NWU-RERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

|                                                                                                            |  |             |   |   |                |   |   |   |   |                         |      |   |        |   |   |   |
|------------------------------------------------------------------------------------------------------------|--|-------------|---|---|----------------|---|---|---|---|-------------------------|------|---|--------|---|---|---|
| <b>Project title: PROSPECTIVE URBAN AND RURAL EPIDEMIOLOGY STUDY (PURE STUDY)</b>                          |  |             |   |   |                |   |   |   |   |                         |      |   |        |   |   |   |
| <b>Project Leader: Prof A Kruger</b>                                                                       |  |             |   |   |                |   |   |   |   |                         |      |   |        |   |   |   |
| <b>Ethics number:</b>                                                                                      |  | N           | W | U | -              | 0 | 0 | 0 | 1 | 6                       | -    | 1 | 0      | - | A | 1 |
|                                                                                                            |  | Institution |   |   | Project Number |   |   |   |   |                         | Year |   | Status |   |   |   |
| <small>Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation</small> |  |             |   |   |                |   |   |   |   |                         |      |   |        |   |   |   |
| Approval date: 2015-01-20                                                                                  |  |             |   |   |                |   |   |   |   | Expiry date: 2020-01-20 |      |   |        |   |   |   |

Special conditions of the approval (if any): None

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>General conditions:</p> <p>While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:</p> <ul style="list-style-type: none"> <li>The project leader (principle investigator) must report in the prescribed format to the NWU-RERC:             <ul style="list-style-type: none"> <li>annually (or as otherwise requested) on the progress of the project,</li> <li>without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.</li> </ul> </li> <li>The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-RERC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.</li> <li>The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-RERC and new approval received before or on the expiry date.</li> <li>In the interest of ethical responsibility the NWU-RERC retains the right to:             <ul style="list-style-type: none"> <li>request access to any information or data at any time during the course or after completion of the project;</li> <li>withdraw or postpone approval if:                 <ul style="list-style-type: none"> <li>any unethical principles or practices of the project are revealed or suspected,</li> <li>it becomes apparent that any relevant information was withheld from the NWU-RERC or that information has been false or misrepresented,</li> <li>the required annual report and reporting of adverse events was not done timely and accurately,</li> <li>new institutional rules, national legislation or international conventions deem it necessary.</li> </ul> </li> </ul> </li> </ul> |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

**Linda du Plessis**  
Digitally signed by Linda du Plessis  
 DN: cn=Linda du Plessis, o=NWU,  
 Vaal Triangle Campus, ou=Vice-  
 Rector: Academic,  
 email=linda.duplessis@nwu.ac.za,  
 c=US  
 Date: 2014.12.02 18:42:40 +02'00'

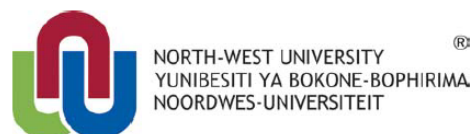
**Prof Linda du Plessis**  
 Chair NWU Research Ethics Regulatory Committee (RERC)

# **APPENDIX E**

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**NWU Health Research Ethics Committee approval of the  
PURE-affiliated study titled “Characterisation of the  
haemostatic profile of a black South African population”**

**(NWU-00034-17-A1)**



Private Bag X6001, Potchefstroom,  
South Africa, 2520

Tel: (018) 299-4900

Faks: (018) 299-4910

Web: <http://www.nwu.ac.za>

**Institutional Research Ethics Regulatory Committee**

Tel: +27 18 299 4849

Email: [Ethics@nwu.ac.za](mailto:Ethics@nwu.ac.za)

## ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **Health Research Ethics Committee (HREC)** on **18/05/2017** after being reviewed at the meeting held on **19/04/2017**, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

|                                                                                                                                                                                |   |   |              |   |   |   |   |      |   |   |        |   |   |                |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---|---|--------------|---|---|---|---|------|---|---|--------|---|---|----------------|
| <b>Study title:</b> Characterisation of the haemostatic profile of a black South African population.                                                                           |   |   |              |   |   |   |   |      |   |   |        |   |   |                |
| <b>Study Leader/Supervisor:</b> Prof M Pieters                                                                                                                                 |   |   |              |   |   |   |   |      |   |   |        |   |   |                |
| <b>Student:</b> -                                                                                                                                                              |   |   |              |   |   |   |   |      |   |   |        |   |   |                |
| <b>Ethics number:</b>                                                                                                                                                          |   |   |              |   |   |   |   |      |   |   |        |   |   |                |
| N                                                                                                                                                                              | W | U | -            | 0 | 0 | 0 | 3 | 4    | - | 1 | 7      | - | A | 1              |
| Institution                                                                                                                                                                    |   |   | Study Number |   |   |   |   | Year |   |   | Status |   |   |                |
| Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation                                                                                    |   |   |              |   |   |   |   |      |   |   |        |   |   |                |
| <b>Application Type:</b> Larger Study                                                                                                                                          |   |   |              |   |   |   |   |      |   |   |        |   |   |                |
| <b>Commencement date:</b> 18/05/2017                                                                                                                                           |   |   |              |   |   |   |   |      |   |   |        |   |   |                |
| <b>Risk:</b>                                                                                                                                                                   |   |   |              |   |   |   |   |      |   |   |        |   |   | <b>Minimal</b> |
| <b>Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.</b> |   |   |              |   |   |   |   |      |   |   |        |   |   |                |

### Special conditions of the approval (if applicable):

- Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC (if applicable).
- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

### General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via HREC:
  - annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study
  - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.
- Annually a number of studies may be randomly selected for an external audit.
- The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the HREC, prior to implementation. Would there be deviated from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility the NWU-IRERC and HREC retains the right to:
  - request access to any information or data at any time during the course or after completion of the study;
  - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.
  - withdraw or postpone approval if:
    - any unethical principles or practices of the study are revealed or suspected,
    - it becomes apparent that any relevant information was withheld from the HREC or that information has been false or misrepresented,
    - the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,
    - new institutional rules, national legislation or international conventions deem it necessary.
- HREC can be contacted for further information or any report templates via [Ethics-HRECAppl@nwu.ac.za](mailto:Ethics-HRECAppl@nwu.ac.za) or 018 299 1206.

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the IRERC or HREC for any further enquiries or requests for assistance.

Yours sincerely

Prof LA Du Plessis  
Digitally signed by  
Prof LA Du Plessis  
Date: 2017.05.26  
10:13:35 +02'00'

Prof Linda du Plessis

Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

# **APPENDIX F**

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**PURE-SA study informed consent form  
2005 and 2010**



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

## PURE-SA Project (Prospective Urban and Rural Epidemiology)

### INFORMED CONSENT FORM (including the PRIMER-study)

I, the undersigned .....(full names and surname)

read / listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project. I understand that I have the right to withdraw at any time from the study.

|                                      |     |    |
|--------------------------------------|-----|----|
| I agree to be tested for HIV .....   | Yes | No |
| I want to know my HIV-status .....   | Yes | No |
| I agree to give a blood sample ..... | Yes | No |

I hereby also declare that I am aware that:

1. This blood sample will be used for the purpose of
  - a. Isolating DNA to look at genetic factors that are currently associated with Type 2 Diabetes (i.e. the Calpain10, Adiponectin, Leptin and Leptin Receptor genes), or genetic factors that may be associated with Non Communicable diseases in the future. We give the assurance that all genetic tests and experiments will only focus on genotypes suspected to contribute to an increased risk of non communicable diseases of lifestyle.
  - b. DNA damage due to environmental factors such as cooking methods and smoke will be tested for.
  - c. Testing for liver function by determining liver enzymes such as AST, GGT,
  - d. Analyses of other than genetic parameters for Diabetes Mellitus such as HbA<sub>1c</sub>, Blood glucose and Insulin
  - e. Analyses of the clotting profile and hypertension markers
  - f. Analyses of bone health, iron and nutrition status

And may be stored until such time as the above measurements/analyses will be done.
2. Body measurements such as height, weight, skin fold thicknesses, arm and leg circumferences will be taken
3. Vascular sonar will be done
4. Blood pressure to be taken
5. Pulse wave velocity measurements will be made
6. A Spirometer test to be performed to determine lung function
7. Bone density will be determined with an osteometer to detect possible osteoporosis

.....  
(Signature of the subject)

Signed at ... Potchefstroom / Ganyesa ... (delete not applicable option) on ...../...../ 2010

#### Witnesses

1. .... 2. ....

Signed at ... Potchefstroom / Ganyesa ... (delete not applicable option) on ...../...../ 2010

## PART 1

1. Research Unit and Faculty:  
Africa Unit for transdisciplinary Health research (AUTHeR); Faculty of Health Sciences, North-West University

2. Title of project/trial:  
PURE: Prospective Urban and Rural Epidemiological study

3. Full names, surname and qualifications of project leader:  
Prof. Annamarie Kruger, Ph.D. (Nutrition)

4. Rank/position of project leader:  
Research Director and project manager

5. Aim of this project  
PURE's aim is that understanding the different lifestyle and health transitions of individuals in response to societal changes will elucidate societal and individual adaptive strategies that could diminish the adverse health effects of industrialization and urbanization on health, while retaining its benefits.

6. Explanation of the nature of all procedures, including identification of new procedures:  
Each participant will have to fill in a number of questionnaires (Adult questionnaire, Physical activity questionnaire, Food frequency questionnaire, Health questionnaire, psychology questionnaire) with the help of field workers. A blood sample will be taken. Physical measures will be performed, including anthropometric measures (such as weight, height, and waist circumference), blood pressure, lung capacity and lung volume and bone density will be performed.

6. Description of the nature of discomfort or hazards of probable permanent consequences for the subjects which may be associated with the project: (Including possible side-effects of and interactions between drugs or radio-active isotopes which may be used.)  
It will take each participant quite a while (about 6 (six) hours) to complete all the tests and questionnaires. Discomfort may be experienced with the taking of blood samples. No measures will have permanent damage or consequences for the participants.

8. Precautions taken to protect the subjects:  
The research nurse will be present at all times, and will be responsible for the blood sampling. She is very experienced and has performed these procedures numerous times in previous studies.

9. Description of the benefits which may be expected from this project:  
When measures with immediate results are taken, such as blood glucose, haemoglobin, HIV status and blood pressure, the information will be communicated to the individual to seek professional help during an individual feedback session. Since this study is a longitudinal study, subjects that are high at risk will be identified over time and personal feedback and counselling will be given.

On the research site each participant will receive a cooked meal, a fruit and fruit juice. Also an amount of R50 will be paid to each participant to cover transport costs and enable each person to go for follow-up at a clinic of choice.

10. Alternative procedures which may be beneficial to the subjects:  
There will be tested for HIV/AIDS, therefore pre-test counselling will be given. If the subject wants to know his/her status and whether tests positive, post counselling will also be given.

## PART 2

## To the subject signing the consent:

You are invited to participate in a research project. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. Participation in this project is voluntary.
2. It is possible that you personally will not derive any benefit from participation in this project, although the health knowledge obtained from the results may be beneficial to you and other people.
3. You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.
4. The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.
5. We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.
6. The University staff will use standardised procedures and take all possible precaution to protect the subject from risks.
7. All information will be kept CONFIDENTIAL and no personal information will be published without my consent.

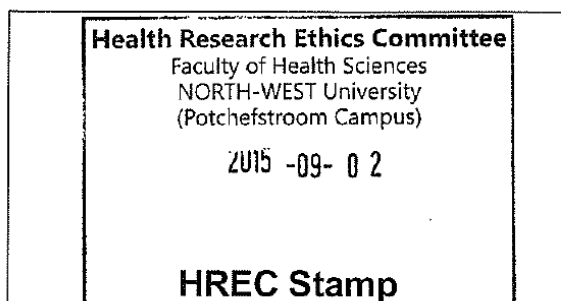
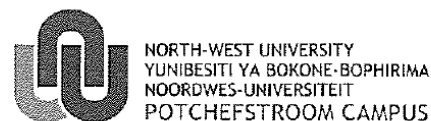
Prof ANNAMARIE KRUGER

Contact details: 082 771 5778 / 018 299 2095(Office)

# **APPENDIX G**

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**PURE-SA study informed consent form 2015**



## INFORMATION LEAFLET AND WRITTEN INFORMED CONSENT FORM FOR PARTICIPANTS OF THE PURE STUDY

**TITLE OF THE RESEARCH PROJECT:** Prospective Urban and Rural Epidemiology  
(PURE) study

**REFERENCE NUMBERS:** NWU-00016-10-A1

**PRINCIPAL INVESTIGATORS:** Prof A Kruger and Dr IM Kruger

**ADDRESS:** North-West University (Potchefstroom Campus), Africa Unit for  
Transdisciplinary Health Research (AUTHeR), Building E8, Office G03.

**Dear participant**

You have been part of the PURE research since it started in 2005. Whether you took part in the research every year or even only some of the years, we would like to invite you once more to be part of this research.

This information document will provide you with all the information that you need to know about the research. A fieldworker who lives within your community will discuss all of the information in this document with you in order for you to understand why this research is done and why you are invited to be part of this research.

**What is this research about?**

This research has already started in 2005 and will finish in December 2015. This research study wants to look at different risks to your health and how these risks can develop over time.

What are some of the risks to your health?

- Being too fat (overweight or obese) or too thin (underweight),
- Having high blood pressure (hypertension) or too low blood pressure (hypotension),
- Having too high blood sugar levels (hyperglycaemia) or too low blood sugar levels (hypoglycaemia),

What might cause you to develop some of these risks?

- The type of foods you eat, for example too much salt or too much fat;
- If you don't do any exercise;
- Smoking or using tobacco products;
- Drinking alcohol;
- Some factors (genetic characteristics) that you inherit from your parents or even your grandparents,

**Why have you been invited to take part in the study?**

You have been invited to be part of the study because:

- In 2005:
  - you were between the ages of 35 and 70 years
  - you were a healthy man or woman

- you did not have any chronic disease or illness
- you permanently lived in a household within the community

Even though you might have developed some illness since 2005, you can still be part of the study. If you are part of the study now we want to see how you are doing.

### **Do you have to take part in the study?**

You are not forced to take part in this research. It is important that you understand that you don't have to be part of this research study. You are invited, and therefore you can choose if you want to be part or not. You can agree to be part of the research now and change your mind later. If you do not want to be part of the research anymore, you will not be treated differently and you will not be punished or judged for not wanting to be part of the research.

This research was approved by a group of people (known as a committee) from the North-West University. These people looked at all the procedures of the research to make sure that if you decide to be a part of the research, you will not be hurt or harmed in any way. These people are known as the Health Research Ethics Committee of the Faculty of Health Sciences of the North-West University on the Potchefstroom campus. It might happen that during the research one or more of these committee members may come to check that the researchers are keeping to the procedures that was explained to you and no unnecessary procedures are performed on you.

### **What will you have to do if you decide to be part of the study?**

If you decide to be part of the study the following will be done:

- A fieldworker that lives within your community will visit you at your house and he / she will explain everything to you and let you ask any questions if you don't understand anything. You will then be given 7 days to think about whether you want to continue to be part of the research.
- If you, after 7 days, agree to continue to be part of the research and you understand everything, you will sign a form, known as a written informed consent form, with the fieldworker.

- Once you have signed the written informed consent form an appointment will be made with you for you to come to the research facility. The date that will be scheduled with you, will be one that suits you the best. All of the arrangements will be made with you to bring you to the place where the research will take place.
- On the day of the appointment one of the taxis involved in the research will pick you up from a place that was arranged with you.
- When you arrive at the place where the research will take place the following will be done with your permission / consent:
  - A small amount of blood (33 ml) will be drawn from the inside of your arm.
  - Your blood pressure will be measured.
  - Your heart function will be checked (electrocardiogram). For us to be able to do this test, you will be asked to take off your shirt or blouse to reveal your chest. If you are a women, you are allowed to keep on your underwear. Little round stickers will be placed on your skin all around the area of your heart.
  - The speed that the blood travels through your blood vessels will be measured (pulse wave velocity). This will be done by placing a thing that looks like a pen on the skin of your neck while placing another one on the skin near your pubic area. For us to be able to do this test, the researcher will gently pull down your pants or skirt to reveal just a small area between the inside of your leg and your private area (pubic area) where the pen-like device will be placed.
  - Your blood vessels in your neck will be checked for any blockages or deposits (intima-media thickness) by placing some liquid gel on the skin of your neck and then by using a thing that looks like a torch to take a picture (sonar) of your blood vessels.
  - You will be weighed and your body length will be measured. You will be asked to remove your shoes and all of your extra clothing. You will be allowed to keep on your pants / skirt and your shirt / blouse.
  - The amount of body fat and amount of muscles you have will be measured. You will be asked to remove just your shirt / blouse. If you are a women, you may keep your underwear on. The thickness of your skin on

your stomach, arms and back will be measured using a tong-like instrument. The researcher will pinch the skin between their fingers and place the tong over the skin to measure how thick the skin fold is. A measuring tape will also be used to measure the circumference of your hip, waist and upper arm.

- The amount of fat and muscle will also be measured using a special machine called the BodPod. You will be asked to climb into the machine and sit on a little bench. A see-through lid will close around you. You will be asked to sit very still and make no movements at all. The machine will blow some air around you and this will then measure the amount of fat and muscle in your body. You will need to wear a tight pants and tight shirt. We will also give you a swimming cap to put on your head while you are in the machine.
- A photo (X-ray) will be taken of your forearm to see if the bones are still hard and strong (bone mineral density).
- Your lung function will be measured. A little plastic pipe will be placed between your lips. Then you have to take a deep breath and blow out the air as hard and as fast as you can through the little pipe.
- You will perform a test to see how strong you're muscles in your hands / forearm (hand grip strength) and legs are (chair stand / rise and walk test). To test how strong your muscles in your hand is you will be asked to squeeze your hand very tight around an instrument with a handle. To test the strength of your muscles in your legs you will have to sit down on a chair and then stand up without using your hands and arms. You will also be asked to walk on a straight line for 6 metres without any help.
- You will complete several questionnaires.
- You will be asked information regarding all the medicines that you currently use.
- You will be asked to give a little bit of urine in a small bottle.
- If you agree and give permission, a HIV test will be performed (If you do not want to be tested for HIV, you can still be part of the study). A needle will be used to prick one of your fingers to get a small drop of blood. This drop of blood will be used to do the HIV test.
- If you are newly diagnosed with HIV, or if you are currently HIV positive we will test your CD4 count. This test will show us how many CD4 cells

there are in your body. These cells keep your body healthy and prevent you from getting sick. If the number of cells is too low then you will need medicine (anti-retroviral therapy (ART)). This test is done by a small prick on one of your fingers to get a drop of blood. The drop of blood will be used to count the number of CD4 cells in your body.

- If you are HIV positive, and you agree and give permission, personal information will be collected from your personal files that are kept at the hospital or clinic that you attend. This will provide us with information to better understand how HIV and the treatment you receive for HIV influence your health.

#### **What do you have to do before coming to the study?**

- We ask that you do not eat or drink anything (except for water) from the previous night 22:00 (ten o'clock). This will help to see if your blood sugar levels are normal.
- Please bring along all the medication that you are currently drinking
- Please bring along your South African ID document
- Please bring along your clinic book or medicine sheet that contains all your medication that is prescribed to you

#### **Will you benefit from taking part in this research?**

When you are finished for the day, some of the results will be given back to you. This will include:

- Blood pressure
- Blood sugar levels
- Heart function (electrocardiogram)
- Lung function (spirometry)
- How hard the bone in your forearm is (bone mineral density)
- How strong your muscles in your hand / forearm (hand grip strength) and legs (chair stand / rise and walk test) are
- Your height and weight
- Your HIV status and most recent CD4 count – if you gave permission for the test to be done and you want to know your status

If you participate you will also help us to understand what the risks are to your health. If we know what the risks are we can help the government to set up policies that will address this health risks in order to help other South Africans, like you, to have better health.

**Are there any dangers involved in you taking part in this research?**

Most of the measurements that will be performed won't hurt or harm you in any way, but you might experience the following:

1. If you give permission to a blood sample, you might feel uncomfortable or scared. We want to make sure that you are not hurt in any way and therefore we will use a qualified nurse that has a lot of experience. She will talk to you and explain to you everything that she is going to do in order for you to feel at ease. Some of this blood will be used (tested) to help us to understand what the reason(s) are that some people get non-infectious (non-communicable) diseases (such as heart attack and stroke) as well as high blood sugar (Type 2 Diabetes) and high body weight (obesity) and others may not. All the blood tests and experiments will only look at the reasons that change your chance of getting non-communicable diseases of lifestyle.
2. Some of this blood will also be used to get genetic material (DNA and RNA) to look at genetic factors. Genetic factors (inherited from your parents) are like a book that tells your body how to work. Sometimes there are mistakes (genetic alterations) that cause an increase in your chance of getting non-communicable diseases (these are diseases of the heart and blood vessels (such as heart attack and stroke) as well as high blood sugar (Type 2 Diabetes) and high body weight (obesity). We want to look at these genetic mistakes to better understand how these diseases develop. We promise that all genetic tests and experiments will only focus on genetic factors that contribute to your chance of getting non-communicable diseases of lifestyle.
3. During some of the measurements (weight and height, body composition, ECG, and pulse wave velocity) you will be asked to remove some of your clothes keeping only your underwear or light clothing on. This might make you feel uncomfortable or shy. To help you feel less shy and uncomfortable the area where these measurements will be done will be private and closed off. This

means that no one will be able to see you and that only the person that will take the measurements and someone to help him / her will be with you.

4. You will be asked to do a test (strength test) in order to see how strong the muscles in your hand / forearm and legs are. Although this is not a very hard test to do, and it does not take very long (only a few minutes) you might get tired. The researcher will ask you to rise / stand up from a chair as well as to walk 6 meters. If you feel that you cannot do the test, you will not be forced to do it, and you can move on to the next station.
5. You will be asked not to eat or drink anything from 22:00 (ten o'clock) the night before you come to the study. You will only be allowed to drink water. You should also not eat any breakfast on the morning of the study and not drink coffee, tea, juice or cold drink. Not eating or drinking anything might make you feel uncomfortable or light headed (dizzy or faint). As soon as you arrive at the research study, we will take a blood sample (if you give us permission) and then we will give you a light breakfast to make you feel better.
6. Doing all of the measurements on the day of the study, will take the whole day. This might make you feel very tired. We will give you a lunch as well as tea / coffee / juice / water throughout the duration of the day at the seating / waiting area.
7. Being part of such a big study can be frightening and overwhelming. To prevent us from wasting your time and to make sure that you know where to go and what to do, there will be people available at all times to help you and show you where you have to go every time.
8. HIV testing will also be performed on the day of the study (with your permission). Being newly diagnosed with a long-lasting disease such as HIV might cause you to worry. To help reduce the worry, there will be a professional person (counsellor) that will come and talk to the whole group about HIV and the test they will do. They will explain everything to you. The counsellor will then take you alone to a private room where nobody can hear you when you talk to the counsellor. When they do the test, a small needle is used to prick your finger and

a little drop of blood is used to test for HIV. You can decide if you want to know your status. Whether the test is positive or negative, the counsellor will talk to you alone and give you all of the information that you need to know. If you are positive and you want to know your status, you will receive a letter that you can take to your clinic or hospital and they will help you with treatment.

There are more benefits than dangers / risks when you partake in the study.

### **Who will see my personal information (data)**

When you arrive at the study, you will be given a unique number, and only this number will be used when you provide us with any personal information or when we do the measurements. No one, except the supervisor of this research will be able to identify you. All your information will be kept safe and secret and will be locked away. No one will have access to your information except the supervisors of this study. All of the information will be stored for 5 years after the study has finished.

The PURE study also takes place in other countries. The main country that is supervising this study is Canada. Because we have a contract with them, we have to share the data with them. Only the information you provide us within the questionnaires and the body measurements will be shared with them. We won't share your information from your HIV status or DNA with them. They will also keep your information safe and secret. They will not share your information with anyone else.

### **Will you be paid to take part in this study and are there any costs involved?**

You will be provided with a breakfast when you arrive at the study. Furthermore, the measurements will take about a whole day, and therefore we will also provide you with lunch. Water / juice / coffee / or tea will also be provided.

There will not be any travel costs for you, since we will arrange for transport for you to the research facility and back to the point where you were picked-up. You will also receive R50.00 (fifty rand) for any expenses due to your attendance at the study. If you are referred to the hospital or clinic you can use this money for any expenses.

If you are fulltime employed we will make special arrangements with your employer for you to be able to take part in the study. If you lose your work income for the day you will be compensated for the day (one day of work).

**Is there anything else that you should know or do?**

- If you have any questions that the fieldworkers cannot answer you can ask that the researcher explains it to you. You can contact the researcher, Dr Lanthé Kruger at 018 299 2093 or [lanthe.kruger@nwu.ac.za](mailto:lanthe.kruger@nwu.ac.za). You can also contact the researcher if you have any problems with the research.
- You can contact the Health Research Ethics Committee via Mrs Carolien van Zyl at 018 299 2089 or [carolien.vanzyl@nwu.ac.za](mailto:carolien.vanzyl@nwu.ac.za) if you have any concerns or complaints that have not been fully addressed by the researcher.
- You will receive a copy of this information and consent form for you to keep with you.

**How will you know about the findings?**

Results that are immediately available will be given to you in private and in person on the day of the study. Other results of the research will be shared with you as a group (everyone that was a part of this study) and with your community leaders verbally during a personal visit from the lead researchers. When we share the results with the community leaders and with the group we will never share personal information. This means that people will not be able to identify you, and you will be safe from any harm such as stigmatization.

**Storage of blood and urine samples**

The blood and urine samples that we collect from you will be stored in a special freezer that will protect it. Only we will have access to your blood and urine samples, and no one else. The samples will be kept in these freezers for an unlimited period of time until we have money available to test (analyse) them. All of the tests (analyses) will be related to this study (PURE) and what you give permission for. Some of your blood samples may be sent to laboratories outside of South Africa for analysis if it cannot be done here. If we are finished with the tests of the blood, we will destroy it so that no one else can use it.

**Declaration by participant**

By signing below, I ..... agree to take part in a research study entitled:

**Prospective Urban and Rural Epidemiology study (PURE)**

I declare / confirm that:

- I have read this information and consent form and it is written in a language that I understand and is comfortable with.
- I have had a chance to ask questions and all my questions have been answered to my satisfaction.
- I understand that taking part in this study is completely my choice and I have not been forced to take part.
- I may choose to leave the study at any time and will not be penalised or judged in any way.
- I may be asked to leave the study before it has finished, if the researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

**If you want to get tested for HIV you have to give permission here below:**

**Yes, I want to get tested for HIV**

☐

**No, I don't want to get tested for HIV**

☐

**If you want to know your HIV status you have to indicate it to us here below:**

**Yes, I want to know my HIV status**

☐

**No, I don't want to know my HIV status**

☐

**Do you give permission that we may have access to your medical records at the hospital or clinic?**

**Yes, I give permission**

☐

**No, I don't give permission**

☐

**Do you give permission that we may collect your genetic material from your blood sample?**

**Yes, I give permission**

☐

**No, I don't give permission**

☐

**Do you give permission that your genetic samples may be analysed outside of South Africa if it cannot be done here?**

**Yes, I give permission**

☐

**No, I don't give permission**

☐

**Do you give permission that some of your blood samples may be analysed outside of South Africa if it cannot be done here?**

**Yes, I give permission**

☐

**No, I don't give permission**

☐

**Do you want to be contacted when there are any NEW research studies that will take place within your area?**

**Yes, I want to be contacted**

☐

**No, I don't want to be contacted**

☐

Signed at (*place*) ..... on (*date*) ..... 20....

.....  
**Signature of participant**

.....  
**Signature of witness**

#### **Declaration by person obtaining consent**

I (*name*) ..... declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter.

Signed at (*place*) ..... on (*date*) ..... 20....

.....  
**Signature of person obtaining consent**

.....  
**Signature of witness**

**Declaration by researcher**

I (*name*) ..... declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter.

Signed at (*place*) ..... on (*date*) ..... 20....

.....  
**Signature of researcher**

.....  
**Signature of witness**

## ADDENDUM A

### SUMMARY FOR DATA COLLECTION (2005 – 2015) AND INTENDEND FUTURE USE FOR THE PROSPECTIVE URBAN AND RURAL EPIDEMIOLOGIC (PURE) STUDY

| WHAT WAS COLLECTED?                        | WHAT INFORMATION DOES THIS GIVE US?                                                                                                                                                                                                                                                                                        |
|--------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1. Blood sample                            | 1.1 DNA damage due to environmental factors<br>1.2 Genetic factors related to non-communicable diseases<br>1.3 Indication of liver function<br>1.4 Clotting factors related to non-communicable<br>1.5 Risk markers for non-communicable diseases<br>1.6 Indication of bone health<br>1.7 Indication of nutritional status |
| 2. Urine sample                            | 2.1 Indication of kidney function<br>2.2 Indication of salt intake<br>2.3 Hypertension markers                                                                                                                                                                                                                             |
| 3. Hair sample                             | 3.1 Presence of toxins (fumonisin mycotoxins)                                                                                                                                                                                                                                                                              |
| 4. Body composition (Anthropometrics)      | 4.1 Height<br>4.2 Weight<br>4.3 Amount of fat in the body<br>4.4 Amount of muscles in the body<br>4.5 Amount of water in the body<br>4.6 Waist circumference<br>4.7 Hip circumference<br>4.8 Head circumference<br>4.9 Arm circumference<br>4.10 Leg circumference<br>4.11 Skinfolds                                       |
| 5. Blood pressure                          | 5.1 To see if the blood pressure is too high or too low.                                                                                                                                                                                                                                                                   |
| 6. Pulse wave velocity                     | 6.1 Indication of the flexibility of the arteries.                                                                                                                                                                                                                                                                         |
| 7. Electrocardiograph                      | 7.1 Indication of heart function.                                                                                                                                                                                                                                                                                          |
| 8. Vascular sonar (intima-media thickness) | 8.1 Indication of blockages in the blood vessels.                                                                                                                                                                                                                                                                          |
| 9. Bone mineral density                    | 9.1 Indication of the strength of the bones in the arm.                                                                                                                                                                                                                                                                    |
| 10. Lung function test (spirometry)        | 10.1 Indication of lung function.                                                                                                                                                                                                                                                                                          |
| 11. Hand grip strength                     | 11.1 Indication of the strength of the muscles in the hand.                                                                                                                                                                                                                                                                |
| 12. Chair rise/stand test                  | 12.1 Indication of the strength of the muscles in the legs.                                                                                                                                                                                                                                                                |
| 13. 6 metre walk test                      | 13.1 Indication of the strength of the muscles in the legs.                                                                                                                                                                                                                                                                |
| 14. HIV test and CD4 count                 | 14.1 Test for HIV and to test for the levels of CD4 cells in the body.                                                                                                                                                                                                                                                     |
| 15. PURE Adult questionnaire               | 15.1 Provides demographic information.<br>15.2 Provides information regarding tobacco and alcohol use.                                                                                                                                                                                                                     |

|                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
|------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                                                  | <p>15.3 Provides information regarding the use of medication.</p> <p>15.4 Provides information regarding your health.</p> <p>15.5 Provides information regarding violence and injuries.</p> <p>15.6 Provides information on major personal events.</p> <p>15.7 Provides information on female health.</p> <p>15.8 Provides information on support systems used.</p> <p>15.9 Provides information on consumer habits.</p> <p>15.10 Provides information on work-, financial-, and home related stress.</p> <p>15.11 Provides information regarding your neighbourhood.</p> <p>15.12 Provides non-personal information regarding people living close to you who have HIV/AIDS.</p> |
| 16. PURE Family census questionnaire                             | 16.1 Provides demographic- and health information on family members currently living within the household.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| 17. PURE Physical activity questionnaire                         | 17.1 Provides information on the type, duration and frequency of physical activity.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| 18. International Physical Activity Questionnaire (IPAQ)         | 18.1 Provides additional information on the type, duration and frequency of physical activity.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 19. PURE Health Services questionnaire                           | 19.1 Provides information on health services, health related expenditures, medication use and health knowledge.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| 20. PURE Annual contact form                                     | 20.1 Provides mortality and morbidity information regarding participant and members of household.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| 21. PURE Event reports                                           | 21.1 Provides information regarding new diagnoses of non-communicable disease(s).                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| 22. PURE Verbal Autopsy questionnaire                            | 22.1 Provides information regarding death of participants.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| 23. PURE: EPOCH 1: Environmental Profile of a community's health | 23.1 Provides information regarding community.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 24. PURE: EPOCH 2: Environmental Profile of a community's health | 24.1 Provides information regarding community.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 25. PURE Community profile questionnaire                         | 25.1 Provides information regarding community.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 26. Assessing neighbourhood walkability                          | 26.1 Provides information regarding community.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 27. Mental health questionnaire: MHC-SF                          | 27.1 Provides information on emotional-, social-, and psychological wellbeing.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| 28. Mental health questionnaire: SWLS                            | 28.1 Provides information regarding satisfaction with life.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| 29. Mental health questionnaire: AFM                             | 29.1 Affectometer provides information regarding positive mental health.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| 30. Mental health questionnaire: MLQ                             | 30.1 Measures to what extent respondents feel their lives are of meaning. Measures how engaged and motivated respondents are in efforts to find meaning or deepen their understanding of meaning in their lives.                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| 31. Mental health questionnaire: PHQ-9                           | 31.1 Provides information on possible mental health disorders.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |

|                                                      |                                                                                                                                                                                                    |
|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 32. Mental health questionnaire: K6                  | 32.1 Provides information psychological distress (depressive and anxiety symptoms).                                                                                                                |
| 33. Mental health questionnaire: MRG                 | 33.1 Provides information on the most meaningful things, most important relationships and most important goals.                                                                                    |
| 34. Mental health questionnaire: MMSE                | 34.1 Provides information on positive impairment.                                                                                                                                                  |
| 35. Bone health questionnaire                        | 35.1 Provides information regarding risk factors for bone health.<br>35.2 Provides information regarding bone fractures.                                                                           |
| 36. HIV and stigma questionnaire                     | 36.1 Provides information regarding attitudes and stigma on HIV/AIDS.                                                                                                                              |
| 37. Medicine use questionnaire                       | 37.1 Provides information regarding use of chronic medication.                                                                                                                                     |
| 38. Quantitative Food Frequency Questionnaire (QFFQ) | 38.1 Provides quantitative information regarding frequency of dietary intake.<br>38.2 Provides quantitative nutrient information.<br>38.3 Provides quantitative information on nutritional status. |
| 39. 24-hour recall dietary intake                    | 39.1 Provides information of dietary intake over the past 24 hours.                                                                                                                                |

### Declaration by participant

By signing below, I ....., ID number: ..... give permission that all of my data (information obtained from questionnaires, personal communication and interviews, physical measurements, and biological samples) that was voluntarily collected from me during the entire duration of the **Prospective Urban and Rural Epidemiology study (PURE) study** (between January 2005 - December 2015) may be used for any further analyses (primary and secondary), however, with the understanding that any analyses and its related results will only be relevant to the scope of the **Prospective Urban and Rural Epidemiology study (PURE) study**, and for which I gave permission (written informed consent).

I further agree to the secure storage and archiving of all of my data on the premises of the North-West University, Potchefstroom Campus. I understand that all of my data that was collected between January 2005 and December 2015, will be stored for an unspecified period until such time is suitable for appropriate further analyses (primary and secondary), and that it will be kept anonymously to everyone except the principle investigator.

Signed at (*place*) ..... on (*date*) ..... 20....

.....  
**Signature of participant**

.....  
**Signature of witness**

.....  
**Signature of researcher**

.....  
**Signature of witness**

**ADDENDUM B****AMENDED PROCEDURE ON PULSE WAVE VELOCITY MEASUREMENT WITHIN  
THE PROSPECTIVE URBAN AND RURAL EPIDEMIOLOGIC (PURE) STUDY**

Dear participant,

I would like you to read the following letter. If you don't understand, please ask us to explain this to you. In your letter where you give permission to be part of the research, all of the tests (measurements) that you will do, are explained to you. On page number 2 of the permission letter, it is explained that we will measure how fast the blood travels through your blood vessels.

The words in the block below was used in your permission letter:

The speed that the blood travels through your blood vessels will be measured (pulse wave velocity). This will be done by placing a thing that looks like a pen on the skin of your neck while placing another one on the skin near your pubic area. For us to be able to do this test, the researcher will gently pull down your pants or skirt to reveal just a small area between the inside of your leg and your private area (pubic area) where the pen-like device will be placed.

But, the way we will do this test, will be different now:

You will be asked to lie down on a bed. You don't have to take off all your clothes. A strap (cuff) will be placed around your upper arm. This strap (cuff) is the same as the one your blood pressure is measured with. Another strap (cuff) will be placed around your upper leg. If you are wearing pants, the strap (cuff) on your leg will be placed over your pants. If you are wearing a skirt or dress, the researcher will gently move your skirt or dress upwards to place the strap (cuff) on the skin of your leg. The researcher will place a thing that looks like a pen and put it on the skin of your neck. You then need to lie very still while the test is done.

**Declaration by participant**

By signing below, I ....., ID  
 number: ..... I  
 admit that I have been told that the test to measure the speed that the blood travels  
 through the blood vessels has changed. The new way of using a cuff and a pen like  
 object was explained to me and I understand how the new test will be done.

Signed at (*place*) ..... on (*date*) ..... 20....

.....  
**Signature of participant**

.....  
**Signature of witness**

.....  
**Signature of independent person**

.....  
**Signature of witness**

.....  
**Signature of researcher**

.....  
**Signature of witness**

# **APPENDIX H**

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**NWU Health Research Ethics Committee  
approval of this PhD study  
(NWU-00105-17-A1)**



NORTH-WEST UNIVERSITY  
YUNIBESITHI YA BOKONE-BOPHIRIMA  
NOORDWES-UNIVERSITEIT

Private Bag X6001, Potchefstroom,  
South Africa, 2520

Tel: (018) 299-4900

Faks: (018) 299-4910

Web: <http://www.nwu.ac.za>

**Institutional Research Ethics Regulatory Committee**

Tel: +27 18 299 4849

Email: [Ethics@nwu.ac.za](mailto:Ethics@nwu.ac.za)

## ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by Health Research Ethics Committee (HREC) on 30/10/2017, the North-West University Research Ethics Regulatory Committee (NWU-RERC) hereby approves your study as indicated below. This implies that the NWU-RERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

**Study title:** Endothelial function and vascular health of black South Africans: the significance of plasminogen activator inhibitor-1

**Study Leader/Supervisor:** Prof AE Schutte

**Student:** A Jacobs-13062778

**Ethics number:**

|             |   |   |   |              |   |   |   |   |   |      |   |        |   |   |
|-------------|---|---|---|--------------|---|---|---|---|---|------|---|--------|---|---|
| N           | W | U | - | 0            | 0 | 1 | 0 | 5 | - | 1    | 7 | -      | A | 1 |
| Institution |   |   |   | Study Number |   |   |   |   |   | Year |   | Status |   |   |

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

**Application Type:** Single study

**Commencement date:** 30/10/2017

**Risk:**

**Minimal**

**Approval of the study is initially provided for a year, after which continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.**

### General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The study leader (principle investigator) must report in the prescribed format to the NWU-RERC via HREC:
  - annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study
  - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.
- Annually a number of studies may be randomly selected for an external audit.
- The approval applies strictly to the proposal as stipulated in the application form. Should any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility the NWU-RERC and HREC retains the right to:
  - request access to any information or data at any time during the course or after completion of the study;
  - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.
  - withdraw or postpone approval if:
    - any unethical principles or practices of the study are revealed or suspected,
    - it becomes apparent that any relevant information was withheld from the HREC or that information has been false or misrepresented,
    - the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,
    - new institutional rules, national legislation or international conventions deem it necessary.
- HREC can be contacted for further information or any report templates via [Ethics-HRECAApply@nwu.ac.za](mailto:Ethics-HRECAApply@nwu.ac.za) or 018 299 1206.

The RERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the RERC or HREC for any further enquiries or requests for assistance.

Yours sincerely

**Prof Refilwe Phaswana-Mafuya**

Chair NWU Institutional Research Ethics Regulatory Committee (RERC)

# **APPENDIX I**

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**Proof of language editing**

Mary Hoffman  
55 May Avenue  
ARCON PARK  
1939  
Tel: 016 428 1577  
Cell: 073 147 8764  
e-mail: maryhoffman@telkomsa.net

---

8 December 2020

**To Whom It May Concern**

This certifies that the following PhD thesis has been edited for English language correctness and fluency. I trust that the corrections made have been applied after due consideration by the author of the document:

***Endothelial function and vascular health of black South Africans: The significance of plasminogen activator inhibitor-1***

by

Adriaan Jacobs

Thesis submitted in fulfilment of the requirements of the degree Doctor of Philosophy in Science (Physiology) in the faculty of Health Sciences at the North-West University



**Mary Hoffman**

(SATI Registration: 1001632)

# **APPENDIX J**

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**Turnitin receipt**



## Digital Receipt

This receipt acknowledges that **Turnitin** received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

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 File name: c4-45e0-8bc4-e71e9d1b2f08\_Adria...  
 File size: 2.46M  
 Page count: 114  
 Word count: 32,064  
 Character count: 191,884  
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